EPITHELIAL SODIUM TRANSPORT AND ITS CONTROL BY ALDOSTERONE: THE STORY OF OUR INTERNAL ENVIRONMENT REVISITED

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I. INTRODUCTION

A. Homer Smith Legacy

Homer W. Smith (1895–1962) was a PhD, renal physiologist who spent most of his academic career at NYU School of Medicine as Chairman of the Department of Physiology (Figure 1). As stated by Giebisch (122) “. . . Homer Smith was, for three decades, from the 1930s until his death in 1962, one of the leaders in the field of renal physiology. His contributions were many: he played a major role in introducing and popularizing renal clearance methods, introduced non-invasive methods for the measurement of glomerular filtration rate, of renal blood flow and tubular transport capacity, and provided novel insights into the mechanisms of excretion of water and electrolytes. Homer Smith’s contributions went far beyond his personal investigations. He was a superb writer of several inspiring textbooks of renal physiology that exerted great and lasting influence on the development of renal physiology. Smith’s intellectual insights and ability for critical analysis of data allowed him to create broad concepts that defined the functional properties of glomeruli, tubules and the renal circulation.” As described by Fishman (99) not only was Homer Smith a superb physiologist establishing precise methods to measure reliably glomerular filtration rate, tubular excretion and reabsorption, and renal blood flow, but he provided novel insights in the evolution of the kidney in the light of comparative physiology and geology (99). His observations of lungfish activity in Africa during the wet and dry season as well as his observations of freshwater shark and rays in Thailand and Malaya or his collection of circus camel urine trying to understand how these animals could concentrate so much their urine were remarkable and novel (99), and the basis of his famous essay “From Fish to Philosopher, The Story of our Internal Environment” (300). In the first page of his introduction Smith noted “. . . Nearly a century has elapsed since Claude Bernard . . . first pointed out that the true medium in which we live is neither air nor water but the plasma or the liquid part of the blood that bathes all the tissue elements. This internal environment . . . is so isolated that atmospheric disturbances cannot alter it or penetrate beyond it. It was Bernard’s view that we achieve a free and independent life, physically and mentally, because of the constancy of the composition of our internal environment . . .”. In his book, Smith beautifully
demonstrates the importance of the evolution of kidney from basal vertebrates to mammals through cartilaginous fishes, bony fishes, lungfishes, amphibians, reptiles, and birds to explain how the control of the extracellular fluid ionic composition could be achieved with such extraordinary precision. The main take home message was that high cognitive functions of primates could not have developed without the appearance during evolution of one critical homeostatic process, the precise control of the extracellular sodium concentration (i.e., osmolarity) appearing with fish and then maintained throughout the evolution of vertebrates. In 1953, when “From Fish to Philosopher” was first published, the structure of DNA was just published (342) opening the way to the development of molecular genetics and molecular evolution. In 1953 Simpson and Tait had just described the isolation of a novel steroid hormone secreted by the adrenal cortex (295) that was the most potent salt-retaining hormone that turned out to play the most important and critical role in achieving sodium balance and the control of the osmolality of the extracellular fluid. It is thus not surprising that aldosterone was not mentioned in Smith’s book and obviously the importance of molecular genetics and phylogenetic trees could not yet be recognized.

B. Scope of This Review

Dramatic advances have been made during the last 60 years in the understanding of our genome due to a wealth of information concerning the evolution of genomes from unicellular to multicellular organisms. Of special interest for the present discussion are the mechanistic approaches to the study of evolution coined as the “functional synthesis.” As reviewed by Harms and Thornton (143), the combination of molecular biology (manipulative molecular experiments) and evolutionary genetics (statistical analyses of gene sequences) can provide better clues on the evolutionary history of proteins than were possible in the past. This has been proven useful to reveal how biochemical processes were altered by ancient mutations and how they led to novel phenotypes (143). As a result, our understanding of the molecular mechanisms of aldosterone synthesis, secretion, and actions on epithelial and nonepithelial tissues has recently significantly increased. Indeed, the functional synthesis approach has been used to study the evolution of aldosterone and its receptor (57).

In this context we review on the molecular and functional evolution of key players in the control of sodium balance, i.e., some important components the aldosterone-dependent signaling pathway: the mineralocorticoid receptor (MR), 11β-HSD2, and its main effectors epithelial sodium channel (ENaC) and Na⁺-K⁺-ATPase.

II. EVOLUTION OF THE INTRA- VERSUS EXTRACELLULAR MILIEU

A. Prebiotic

Cellular life started around 3.5 billion years ago, as suggested by the presence of stromatolites (fossil structures made by microbial organisms) (3). There are three main scenarios for
the origin of life: “Primordial Soup,” “RNA World,” and “Metabolism First.” The “Primordial Soup” was hypothesized by Alexander Oparin in 1924 (243) and experimentally supported by Stanley Miller and Harold Urey in their famous experiment in which they put electric impulse in a mixture of basic chemical components (222). They spontaneously obtained more complex components, such as amino acids. They proposed that over long periods of geological time, generation of amino acids would accumulate in warm ponds and thus lead spontaneously to protein formation, and accumulation of lipids in the form of droplets, similar to cell membranes. The “RNA World” is similar to the primordial soup, except that RNA molecules occurred first and, thanks to their catalytic properties, were able to perform the basic enzymatic reactions. These reactions were ultimately replaced by proteins. As discussed recently by Robertson and Joyce (276), there is evidence that an “RNA World” did exist in the very beginning of life on the Earth before DNA- and protein-based life. RNAs were working as catalytic units and were able to autoreplicate, ensuring the maintenance of a genetic content. It is still difficult to understand how the replication and translational machineries of today’s organisms would have evolved from an RNA world first. Ribozymes have specific requirement for magnesium (or strontium or calcium) but not potassium (52), suggesting that in the RNA world, potassium concentration did not have to be high. Recently, however, potassium was shown to modulate a G-quadruplex-ribozyme activity (23). Finally, “Metabolism First” hypothesized that enzymatic simple reactions occurred first, using mineral reactors, like the porous surface in rocks. Biochemical enzymes and genetic content to encode these reactions appeared later (298).

Just as it is not clear on how life emerged, it also is not clear where on earth life emerged. Prebiotic chemistry is concerned by the question of the ionic composition required for protein and nuclear acid synthesis. It was assumed that the high sodium chloride content found in the primordial ocean was a prerequisite for these reactions. It was however difficult to understand how the prebiotic soup could have been high in sodium and low in potassium, since the cytosolic composition from bacteria to multicellular organisms is high in potassium and low in sodium. This question is still very controversial, but the idea that the original soup was in fact high in potassium and low in sodium has recently emerged from different laboratories (114, 225). Along the same line of thought, Dubina et al. (77) have recently shown that potassium is more effective in L-glutamic acid oligomerization in aqueous solutions than the same concentration of sodium. The authors suggested that the first peptides could have been assembled with potassium as the driving force, and not sodium as commonly believed.

We present here two opposing hypotheses, the “hydrothermal vents in deep ocean” hypothesis and the “inland pond of fresh water” hypothesis. The hypothesis that life emerged close to hydrothermal vents in the deep ocean is supported by the energy provided by proton gradients. Using the phylogenomic framework and protein structure of metalloproteins, Dupont et al. (79) established the development of increased ratio before the translation machinery. They conclude that Fe, Mn, and Mo were first selected before the increase of oxygen (~2.3 Gya; Ref. 25), while Cu and Zn were selected after. A main problem of the origin of life in oceans is the preference of cells to potassium over sodium, whereas oceans are sodium rich and potassium poor. The problem of the need for high potassium content may be solved by the freshwater pond hypothesis. Mulikidjanian et al. (225) suggested that life originated in inland ponds of freshwater (anoxic geothermal fields), with high potassium content, zinc, manganese, and phosphate ions. If peptides were formed in the primordial soup by auto-oligomerization, there is strong interest for the proto-cell to keep the same concentration levels. This suggests that the high K+/Na+ ratio was present in the environment of the proto-cell. High potassium concentration in the intracellular milieu (>100 mmol) is an extremely well-conserved feature that is achieved by the work of P-ATPases (K-ATPases in unicellular organisms, Na+-K+-ATPases in animals).

Whatever the correct hypothesis, an important step was the formation of membranes (presumably phospholipids) separating the protocell from the external milieu to create a common ancestor of all life. The ancestral protocells must have developed various sets of pumps to use this gradient to provide energy to the cell (226, 227). Thus the understanding of membrane bioenergetics is crucial to understand how energy (ATP) is generated and how it is provided to the different machineries inside the cell (65, 190, 303, 306). A common view is that all modern organisms can trace their ancestry to a single common ancestor (21). The Last Universal Common Ancestor (LUCA) is not the only common ancestor of all the three domains of life (Bacteria, Archaea, and Eukaryota), but rather the most recent one (21). The common ancestor of all life featured universal homologies found in present organisms, i.e., 1) core cellular components [genetic material (DNA or RNA) with their genetic letter code, a cell envelope (lipoprotein membranes) and proteins]; 2) cellular complexes (transcription and translation machinery); and 3) membrane transport proteins (exchangers, channels, pumps) to control the internal environment of the cell and allowing to create electrochemical gradient between the intracellular compartment and the external milieu.

B. Unicellular and Intracellular Milieu

1. Importance of potassium for replication and translation: another chicken-or-egg dilemma

Life, by definition, needs replication machineries to reproduce and evolve. The present replication and translational machineries are a highly complex assembly of proteins and
DNA (replication) or proteins and RNA (translation). Similar to auto-oligomerization of peptides, these machines have an absolute requirement for high potassium, low sodium concentrations that can only be achieved by membrane transporters raising a classic chicken-or-egg dilemma. The structures of the ribosomes machinery of both prokaryotes and eukaryotes have recently been solved with a high resolution but do not seem to provide an easy explanation for the potassium requirement. On the other hand, Foucher et al. (100) have shown that a unique family of bacterial GTPases (EngA) interact with the bacterial ribosome and are directly involved in its biogenesis. The authors showed that GTPase activity was uniquely activated in presence of high potassium (but not sodium) and thereby play the role of GAD proteins that usually are required for GTPase activity. This is another evidence for the importance of a high potassium concentration for the biogenesis and assembly of the ribosome.

2. Control of K concentration in bacteria and archaea by Kdp ATPase and K channels

As reviewed by Epstein (89) and by Ballal et al. (18), potassium is the major intracellular cation in bacteria. To maintain the physiological concentration of internal potassium, bacteria possess a battery of different transport systems, including the uptake (Kdp) and efflux potassium systems (Trk or Ktr) that vary in kinetics, energy coupling, and regulation (FIGURE 2A). The Kdp system is prevalent among bacteria and is an inducible high-affinity K⁺ transporter that is synthesized under stress conditions, such as severe K⁺ limitation or osmotic upshift. This Kdp ATPase is encoded by the Kdp FABC operon and works as a chimera of ion pumps and ion channels, based on homologies with potassium channels and ABC transporter gene families (131). This Kdp system also exists in archaea, such as the halophilic archaeon *Halobacterium salinarum*, but is regulated by different pathways (182). As reviewed by Kuo et al. (187), the conservation of important parts of potassium channels, such as K⁺ filter, the gate, and some of the gate's regulatory domains, is pervasive in nearly all free-living unicellular organisms (bacteria, archaea, and eukaryotes). The comparative genomics of prokaryotic channels and structural studies have allowed the acquisition of novel insights on animal potassium channels. As the life’s diversity is mainly composed by such free-living unicellular organisms, the variety of potassium channels is much greater than in the sole animal clade (187).

3. Control of K concentration in unicellular eukaryotes and fungi

Like bacteria and archaea, unicellular eukaryotes (protist, yeast) control the ionic composition of their cytosolic com-

![Diagram](http://physrev.physiology.org/DownloadedFrom/10.220.32.247)
partment (high potassium/low sodium) by similar transport systems as discussed above and in particular different members of the P-ATPase family (47, 316). As reviewed by Arino et al. (8), yeast plasma membrane expresses (FIGURE 2B) many transport systems that serve to 1) fulfill the need of potassium to the cells; 2) maintain the intracellular potassium concentration; 3) eliminate toxic cations such as sodium or lithium; 4) preserve the electrostatic potential of the membrane; 5) regulate the intracellular pH; 6) keep the intracellular osmotic pressure at positive levels, to accommodate cell division and cell wall/plasma membrane expansion; and 7) manage osmotic stress. The most important systems are a proton V-ATPase (Pma1) and a sodium P-ATPase (Ena1-5) that create an electrochemical gradient that will favor the accumulation of potassium by a potassium channel of the Trk family (Trk1,2). A sodium hydrogen exchanger (Nha1) will also allow the extrusion of sodium thanks to the proton gradient generated by the proton ATPase (8). The ouabain-insensitive sodium ATPase (ENA) belong to the P-type ATPase gene family and plays the main role in maintaining high cytosolic potassium in fungi and plants. Interestingly, this transport system is also expressed in parasitic protists (160).

C. Origin of Multicellularity and Extracellular Milieu

O’Malley et al. (239) brilliantly describe the diversity, classification, and evolutionary importance of the protists (unicellular eukaryotic microorganisms) and how an evolutionary understanding of protists is crucial for understanding eukaryotes in general (FIGURE 3A). The authors focus on three crucial episodes of this history: the origins of multicellularity, the origin of sex, and the origin of the eukaryotic cell (239). As far as the problem of the origin of multicellularity is concerned, many scenarios have been proposed among them: 1) increased size of a multicellular organism would prevent ingestion by unicellular predators; 2) cell-to-cell interactions would mean better cooperation to access shared resources; and 3) more efficient absorption and storage of nutrient, thus building a buffering capacity against environmental changes. Multicellularity appears to have evolved many times (69, 170), and as many as 20 separate attempts at forming plants, animals and fungi have been described (344). As discussed by Rokas et al. (277), a common trend observed in the emergence of multicellularity (i.e., the origin of animal) in different lineages that it is frequently accompanied by an increase in genes involved in cell-cell communication, adhesion, and cell differentiation. These genes represent a few hundred genes from a few dozen gene families (277).

As presented by Ruiz-Trillo (284), a phylogenomic multitaxon genome-sequencing initiative (UNICellular Opisthokont Research iNitiative = UNICORN) aimed to gain knowledge into how multicellularity first evolved in the opisthokont lineage, of which the common ancestor was a single cell that expanded into several unicellular and multicellular lineages, such as in metazoan (animals) and fungi. Of interest in the context of the present review is the finding that choanoflagellates are the closest free-living relatives to animals (45, 285). Choanoflagellates have the ability to form colonies and the capacity for cell-to-cell communication (63). Multicellularity arising from the division of a single cell and from its offspring sticking together must be distinguished from multicellularity arising from aggregation of several solitary cells as seen in bacterial colonies or in yeast (185). Interestingly, as recently shown by Farcough et al. (94), multicellularity in choanoflagellates may not arise from a mere aggregation process (as seen in bacteria or archaea) but rather to be driven by cell division as found in metazoa. One important difference may rely on cell signaling and adhesion proteins. Thus King et al. (179–181) identified many genes in choanoflagellates that have not previously been isolated from nonmetazoa. Among them are a high number of genes involved in adhesion and cell signaling, such as C-type lectins, cadherins, and genes involved in posttranslation modifications (i.e., tyrosine kinase signaling pathway components). This suggests that these genes were acquired before the emergence of metazoa and were later co-opted for multicellularity regulation. One should emphasize the absolute necessity of epithelial development as the only way biology knows to separate two compartments and to allow a much better control of the cellular environment.

D. Early Animal Differentiation and Development of Epithelial Polarity

As reported by Philippe et al. (256), the origin of many of the defining features of eumetazoan (“true animal”) body plans, such as symmetry, nervous system, and the mesoderm, remains uncertain regarding the order of emergence order of the early branching taxa: sponges, placozoa, ctenophores, cnidarians, and bilaterians. The authors built a phylogenetic tree which yields to significant conclusions that the sponges (Porifera) are actually a monophyletic lineage, and that both sponges and placozoa (Trichoplax) are the most basal organisms of Metazoan. They also suggest that Ctenophora is together with Cnidaria, but this view has been challenged by the recent sequencing of the ctenophore Mnemiopsis leidyi. In this study, Ryan et al. (286) suggest that Ctenophora are actually the most basal organism of all animals. More work would be needed to reach a definitive conclusion.

This phylogenetic tree (FIGURE 3B) shows the unique position of Trichoplax adhaerens as the simplest multicellular free-living organism made of a highly differentiated epithelial layer. This epithelial layer is made of at least five cell types delineating an internal milieu (extracellular compartment) populated by contractile cells. This organism appears...
A: relationship of representative organisms presented in this review. The emergence of multicellularity in animals is indicated by an arrow. B: relationship of representative chordates presented in this review. The terrestrial adaptation of tetrapods during late Devonian (~400 Mya) is indicated by an arrow. Three rounds of genome duplication are indicated by red dots (1R, 2R, 3R).
to be arrested at a stage defined as blastula in eumetazoans. The ionic composition of the internal milieu is not known, but it could be speculated that it is rich in sodium and low in potassium.

As discussed by Fayeh and Degnan (93), epithelial organization is not restricted to eumetazoan (i.e., ctenophores, cnidarians, insects, or vertebrates) since a similar organization has been identified in a demosponge (*Amphimedon queenslandica*). While very few or no orthologs of genes involved in basal lamina, septe junction or tight junction have been identified in the Amphimedon genome, many other genes have been identified as orthologs to bilaterian genes involved in adherens junction and epithelial polarity. Many of these orthologs seem to originate as the basis of metazoan (or eumetazoan), with the exception of Discs large and Par-1, which apparently appeared prior to the choanoflagellate-metazoan split. In general, the multidomain architectures of these different proteins have been formed at the very beginning of metazoan history, as the domain composition is very similar among orthologs from contemporary demosponges, placozoans, cnidarians, and other bilaterians (93).

1. Components of epithelial polarity

As pointed out by Bryant and Mostow (39), polarized cells can group together in a concerted spatiotemporal arrangement to form a variety of different tissue structures. Desmosomes, tight junctions, adherens junctions, and gap junctions are key components of epithelia allowing the asymmetric distribution of specific components (proteins, membrane lipids) in the apical versus the basolateral membrane of the cell (FIGURE 4). Specific extracellular matrix proteins are secreted at the apical (glycocalix) and the basolateral membrane (basement membrane) and contribute to the maintenance of epithelial polarity. The asymmetric distribution of these specific components is an absolute requirement for vectorial reabsorption or secretion of ions, water, and macromolecules (proteins, RNAs). In eumetazoan, the primary driving force in most cells is a member of the P-ATPase family (Na\(^+\)-K\(^-\)-ATPase) and in few specialized cells a V-ATPase. The energy cost to maintain this asymmetry is very high [up to 20% of the ATP produced by the cell and up to 90% in organs (kidney) specialized in transporting ion and water] to achieve the constancy of the extracellular fluid compartment.

**FIGURE 4.** Components of epithelial polarity: apical versus basolateral membrane, junctional complexes and extracellular matrix. Tight junctions separate the apical from the basolateral membrane characterized by specific lipid and protein composition. Extracellular matrix proteins are secreted at the apical membrane (glycocalix) and at the basolateral membrane (basement membrane or matrix). Schematic drawing of the epithelial junctional complex showing tight junctions as well as the two adhesive epithelial junctions: adherens junctions and desmosomes (307). The yellow arrows indicate the paracellular route for the diffusion of ions and hydrophilic solutes. Diffusion is not restricted until the level of the tight junction, and this represents a regulated, semi-permeable diffusion barrier that is ion and size selective. Diffusion across the tight junction is a passive process that occurs along concentration gradients. The orange arrow indicates the transcellular route either absorption or secretion.
As stated by Franke (104), the key genes necessary for the formation and organization of tissues in metazoans have a much earlier origin. For example, the attachment between a cell and another cell, or a cell and the extracellular matrix, is mediated by integrins, which are transmembrane receptors. As proposed by Sebé-Pedros et al. (291), it appears that core components of the integrin adhesion complex predate the divergence of Opisthokonta. Furthermore, Sebé-Pedros et al. (291) suggest that the main components of this system have been lost independently in fungi and choanoflagellates. Cell junctions in vertebrates can be classified into four main types (FIGURE 4):

1) Desmosomes (or maculae adherentes) connect epithelial and some other cell types.

2) Adherens junctions (or zonula adherentes) are formed by glycoproteins, such as cadherins and catenins, which initiate and maintain cell-cell contacts (144). As pointed out by Magie and Martindale (207), these contacts, both between the cell and extracellular matrix and between the cell and the extracellular matrix, are critical for the three-dimensional formation of tissues and organs. Zhao et al. (358) recently presented a study on the history of the catenin family. They proposed that the last common ancestor of metazoans had three catenin subfamilies (alpha2 catenin, beta catenin, and delta2/ARVCF). Similarly, Nichols et al. (236) estimated that cadherins predate the divergence of choanoflagellates and metazoan lineages, as they found homologs of leftyrin, coherin, and hedgling in the genome of Capsaspora owczarzaki which diverged before the choanoflagellates-metazoan split. Recently, Dickinson et al. (70) have proposed that multicellularity in social amoebae and multicellularity in animals may share a common history, especially as the nonmetazoan Dictyostelium discoideum can assemble in a polarized epithelium. Such polarized epithelium is a necessary request for the multicellular development in animals. A striking finding is that, despite the absence of a cadherin homolog, an alpha-catenin ortholog that binds a beta-catenin-related protein was identified in this social amoebae (71). This suggests that the multicellularity in animals is maybe more ancient than previously thought, and that catenins are more important in cell polarity than the cadherins and the Wnt signaling pathway, which evolved later in metazoans. Dickinson et al. (70) speculate that the common ancestor of all unikonts (social amoebae + fungi + animals) spent a part of its life cycle in a multicellular state. This ancestor would have all the necessary toolkit for forming an epithelial tissue. Some descendants (i.e., animals) kept multicellularity, while others shifted back to unicellularity (i.e., amoeba).

3) Tight junctions (TJs; or zonulae occludentes) are formed by different proteins, mainly claudins and occludins, but also cadherins or catenins (70, 97, 236, 307, 358). As described by Anderson and Van Itallie (6), the paracellular barrier is made of two components: a first pathway of charge-selective small pores influenced by claudin expression patterns and a second pathway lacking charge or size discrimination controlled by different proteins and signals. Eckert and Fleming (80) emphasize the importance of studying TJ formation during early development in mammalian and amphibian models. TJ formation and biogenesis occur during cleavage of the egg and the formation of the first epithelium. TJs are also involved in signaling, for instance, by binding transcription factors (such as ZONAB) that regulate the switch between proliferation and differentiation of epithelial cells (200). In addition, the process is controlled by cell-cell interactions, gap junction communications, and Na+ -K+ -ATPase activity (80). Expression of claudin in teleost fishes has become a field of intense investigation (19, 202). For instance, Loh et al. (202) identified 21 additional claudin genes in the whole genome of Takifugu rubripes, a teleost fish, compared with mammals. These paralogs may have been generated during the fish-specific whole-genome duplication, suggesting a contribution to the distinct physiology of fishes and mammals (202).

4) Gap junctions (GJs) are formed by connexons (or hemichannels), a hydrophilic channel made of six connexins. Connexins are tetraspan proteins, similar but not homologous to pannexins, as reviewed by Scemes et al. (290). Connexins are restricted to chordates, whilst pannexins (named innexins in nonchordates) are present in all eumetazoans, with the exception of echinoderms, as revealed by Abascal and Zardoya (1). As with TJs, they are also arranged head to head, allowing intercellular exchange of small molecules. Homomeric contacts in conserved extracellular residues in the occludins and claudins could play a similar role (153). With the assumption that they evolve from a common ancestral gene, the absence of conservation between the four different families suggests that they have diverged considerably to perform different new functions (153).

III. EVOLUTION OF THE ALDOSTERONE SIGNALING PATHWAY

A. Sodium in Body Fluid Compartments and Sodium Balance in Humans

The total body sodium content is ~60 mmol/kg body wt in male adults, that is, ~4,200 mmol (or ~100 g) in a 70 kg man (see p107 of Ref. 34). About 29% of bodily sodium exist, chemically bound, in the bones (145), in a form that is thought to be physiologically unavailable for rapid exchange with sodium in the extracellular fluid (ECF). More recently, Machnik et al. (206) proposed that sodium can also be stored on proteoglycans in interstitial sites, where it becomes osmotically inactive. The remainder (~70% of the total body pool) is called exchangeable because of its ability to equilibrate rapidly with injected radioactive sodium (30,
Most of the exchangeable sodium resides in the ECF compartment, at a concentration of 140 mM within the vascular and interstitial-lymph spaces. Only a very small amount of exchangeable sodium (<3%) occurs in the intracellular fluid (ICF) compartment. Sodium is unequally distributed between ECF (~140 mM) and ICF (~15 mM) due to the activity of the Na\(^{+}\)-K\(^{+}\)-ATPase pump, which constantly pumps 3 Na\(^{+}\) ions out the cells in exchange for 2 K\(^{+}\) ions into the cells.

The cerebrospinal fluid (CSF) is a small extracellular compartment (~150 ml in human) providing to the brain a highly stable ionic (sodium, potassium, and pH) environment slightly hyperosmolar with respect to plasma (263). Of special importance is to maintain the sodium and potassium gradient across the plasma membrane of neurons as constant as possible to allow the transmission of the action potential along the dendrites and axons. Equally important is keeping the potassium concentration low (around 3 mM) and as constant as possible despite release of potassium during neuronal activity. Potassium can be efficiently buffered by Na\(^{+}\)-K\(^{+}\)-ATPase expressed in the abluminal membrane of the choroid plexus epithelium and in astrocytes that surround tightly each neuron in the central nervous system (208, 347). The CSF is thus a “secondary” internal milieu allowing the development of all remarkable cognitive performances associated with the human brain justifying Homer Smith’s remarks. It is interesting to note that an even more restricted extracellular compartment, i.e., the endolymph of the cochlea (a few hundred microliters) with a unique ionic composition (high potassium, very low sodium) that is of critical importance for the mechanoelectric transduction that takes place in hair cells (195, 196). The development of audition required the evolution of a specific extracellular ionic composition just opposite to what is required in the brain. In the mouse, the switch between a high sodium/low potassium to a low sodium/high potassium takes place during embryonic development (E16,5 in mouse) and is linked to the expression of pendrin (196). Interestingly, three components of the aldosterone signaling cascade (MR, ENaC, and Na\(^{+}\)-K\(^{+}\)-ATPase) are expressed in both the choroid plexus and in the cochlea, but their physiological roles are not yet fully understood.

The balance of sodium is achieved when sodium intake each day exactly matches sodium output including the obligatory losses. The obligatory sodium losses refer to the uncontrollable losses of sodium from the body in sweat, feces, and urine (62). In the absence of diarrhea and profuse sweating, the total obligatory sodium losses are very small, no more than 180 mg/day, equivalent to 8 mmol/day. Obligatory losses of sodium in the urine are very tiny as little as 1 mmol/day, a value that may be assumed to represent obligatory urinary loss of sodium (242). Obligatory losses of sodium from skin have been reported to average <1.1 mmol (1). They may be assumed to result from unnoticed sweat, insensible perspiration, epithelial cell desquamation, and sebaceous secretions. Under normal conditions, the only avenue for sodium intake is ingestion of food and beverage, whereas sodium output occurs through the urine, sweat, and feces. Sodium balance ensures a constant amount of sodium in the body, which is crucial for the maintenance of the ECF volume, and thus blood pressure.

1. Regulation of sodium balance

In principle, sodium balance can be regulated by altering either sodium intake or sodium output. However, intake is dependent on dietary preferences and usually is excessive. Therefore, regulation of sodium balance is achieved primarily by regulating sodium output. Because elimination of sodium through respiration, sweating, and stools is low and not easily regulated, sodium output is primarily regulated by the kidneys, which constantly adjust the amount of urinary sodium excretion relative to the daily intake of salt. The kidney is extremely adaptive to extremes of sodium intake and can maintain sodium balance in face of large changes in sodium intake; kidneys are capable of reducing sodium excretion during periods of extreme sodium restriction. Sodium balance is dependent on circadian oscillations of sodium transport in the distal nephron and in the colon followed by day-night blood pressure variations. The circadian clock can be involved in generation of these rhythms through external circadian time cues [e.g., the renin-angiotensin-aldosterone system (RAAS)] or through the intrinsic renal circadian clock. Rakova et al. (271) observed in humans that, at constant salt intake, daily sodium excretion exhibited weekly (circaseptan) or longer infradian rhythm periods (about monthly or longer) defining a new paradigm of rhythmic sodium excretion and retention independent of blood pressure or body water. In summary, sodium balance is regulated by mechanisms involving short-term (hours and days) and long-term (weeks and months) regulation. The molecular mechanisms of short-term regulation are the best understood and involve aldosterone as the main hormonal factor as a critical component of the (RAAS) (FIGURE 5).

Essential genes of the RAAS pathway are ACE1, ACE2, angiotensinogen, angiotensin II receptor type 1, renin receptor, MAS1 oncogene [a GPCR which binds the angiotensin-II metabolite angiotensin-(1–7)], mineralocorticoid receptor, and renin. Fournier et al. (101) deciphered the emergence of these genes and the order using phylogenetic methods. Apart from the MAS receptor which appeared later in the tetrapod lineage, all major components were already present in the vertebrate ancestor, and even important parts were present in the ancestral chordates (as they are also present in basal chordates such as cephalochordates and tunicates). Fournier et al. (101) found that angiotensinogen made its appearance in gnathostomes, according to its presence in cartilaginous fish. The presence of several genes in organisms that lack all the components of RAAS suggests that these genes had other ancestral functions outside of their current role (101).
B. Aldosterone Signaling Pathway in the Aldosterone-Sensitive Distal Nephron

Aldosterone plays a key role in controlling sodium reabsorption in aldosterone-sensitive distal nephron (ASDN), thereby maintaining blood volume and blood pressure within physiological margins (281, 282, 330). It is indeed the most potent sodium-retaining factor in mammals. The mineralocorticoid aldosterone is secreted by the zona glomerulosa, whereas glucocorticoids (cortisol or corticosterone) are produced by the zona fasciculata of the adrenal cortex. Aldosterone differs from cortisol by the presence of an aldehyde group in position 18 on the D ring. This minimal change in the structure between aldosterone and cortisol confers a strikingly different biological activity of both steroids. The two major physiological stimuli of aldosterone secretion are 1) concentration of extracellular K⁺ ions: small increases in blood levels of K⁺ strongly stimulate aldosterone secretion; and 2) angiotensin II: activation of the renin-angiotensin-aldosterone system RAAS in response to decreased vascular volume results in release of angiotensin II, which stimulates aldosterone secretion. Important progress in understanding the physiological role of aldosterone was provided by human pathology. Addison’s disease,
or adrenal insufficiency, is characterized by a salt-losing syndrome, hyperkalemia, metabolic acidosis, hypotension, weight loss, fatigue, muscle weakness, and increased skin pigmentation. This disease is lethal if not treated. Primary hyperaldosteronism is characterized by hypokalemia, metabolic alkalosis, and salt-sensitive hypertension that can be cured completely by the surgical removal of the adrenal tumor or treated by mineralocorticoid (MR) antagonists (spironolactone). Adrenal insufficiency and aldosteronism are thus strong pathophysiological evidence for the critical role of aldosterone in achieving sodium balance and survival. Aldosterone acts primarily as a circulating hormone. The major target of aldosterone is the distal segment of the renal tubule, called the ASDN (201) (FIGURE 6A). Here, aldosterone stimulates exchange of sodium and potassium, and this has three primary physiological effects. It causes the kidney 1) to increase sodium reabsorption back into the bloodstream, thereby sodium loss in urine is decreased, and 2) to increase water reabsorption back into the bloodstream. This action is an osmotic effect directly related to increased resorption of sodium. Water conservation results in the expansion of extracellular fluid volume, and 3) to increase renal excretion of potassium. Therefore, the effect of aldosterone in the ASDN is critical in the regulation of sodium homeostasis, volume regulation, and thus systemic blood pressure. The main target cells for aldosterone are the connecting cell and the principal cell of the connecting tubule and the collecting duct, respectively (FIGURE 6B). This is consistent with Guyton’s hypothesis stating that long-term blood pressure control is critically dependent on vascular tone and fluid handling by the proximal (357) and distal nephron (282). Warnock et al. (339) recently outlined the emerging evidence that describes the central role of amiloride-sensitive sodium channels expressed both in vessels (235) and in epithelia (278), two arms of this homeostatic system (339).

C. Genetic Evidence for the Limiting Steps

Rossier et al. (282) reviewed genetic evidence supporting Guyton’s hypothesis. Thanks to the study of Mendelian forms of hypertension and their corresponding transgenic mouse models, the main molecular components of the aldosterone- and angiotensin-dependent signaling pathways were identified over the past 20 years. Nineteen genes cause Mendelian hypertension (salt retaining) or hypotension (salt losing) in humans. Strikingly they all map to two components of RAAS either the adrenal gland (5 genes expressed in the adrenal cortex) or the kidney (14 genes expressed in the distal nephron and collecting duct). This underscores the importance of RAAS in the control of blood pressure in humans. For this review we have selected eight genes that when mutated or deleted cause the most severe phenotypes in humans (FIGURE 6B), indicating that they are coding for proteins that are limiting factors in the aldosterone signaling pathway and critical in establishing vectorial reabsorption of sodium in ASDN. These genes code for 1) the MR (156, 158, 235); 2) 11β-HSD2 (48, 240), which protects the MR from illicit occupancy by glucocorticoids (cortisol or corticosterone); 3) ENaC α-, β-, or γ-subunit (174, 278); and 4) Na+/K+-ATPase α-, β-, and γ-subunit (118, 119). Mutations of the regulatory subunit (γ or FYXD2) are compatible with survival (115) unlike loss-of-function mutations on αβ, which have not been observed in humans because they are likely to be embryonic lethal according to gene inactivation in transgenic mouse models (20).

Interestingly, some of these genes are MR target genes notably Na+/K+-ATPase α- and β-subunits, FYXD4, as well as ENaC β- and γ-subunit (see TABLE 1). Transcriptional analysis in kidney cell lines (35, 274, 325, 360) has identified a large number of MR-dependent up- and downregulated genes, but very few have been validated. In TABLE 1, we have listed the genes that have been partially validated by in vitro and/or in vivo approaches, including transgenic mouse models.

D. Aldosterone, MR, Glucocorticoid Receptor, and 11β-HSD2

1. The problem of mineralocorticoid specificity

In mammals, aldosterone, the main mineralocorticoid hormone, is synthesized in the glomerulosa of the adrenal cortex. The biosynthesis pathway is well established and conserved between rodents and humans (FIGURE 7A) with the notable differences that rodents (and the toad Bufo marinus) produce corticosterone, whereas humans produce cortisol as the main glucocorticoid. The main limiting steps in the synthesis pathway of aldosterone are HSD3B1 and CYP11B2 (aldosterone synthase), which are specifically expressed in the glomerulosa, whereas HSD3B2 and CYP17 are expressed in the fasciculata where cortisol is produced (73, 279). Aldosterone and cortisol have almost identical structures with the exception of the aldehyde group that gave the name to the hormone aldosterone (FIGURE 7B). Although aldosterone is the physiological mineralocorticoid for human MR, 11-deoxycorticosterone (DOC), corticosterone, cortisol, 11-deoxycorticisol, and progesterone also have a similar high affinity for the MR (186). In cell culture, cortisol and corticosterone are anywhere from equivalent to aldosterone to two orders of magnitude less potent as transcriptional activators of human MR (9, 121, 254), despite having about the same affinity for the MR. In the kidney, aldosterone and DOC are physiological mineralocorticoids, while progesterone is an antagonist. In the kidney (ASDN) and other epithelia responding to aldosterone (colon, sweat glands), the target cells coexpress the MR and the glucocorticoid receptor (GR). In vitro the affinity for aldosterone or cortisol for MR or GR is similar. In vivo, the free plasma concentrations of cortisol (10–100
nM) are 100–1,000 times higher than that of aldosterone (10–100 pM), raising the question of how, at physiological concentrations, aldosterone can occupy the MR and why cortisol does not “illicitly” occupy the MR (81). Part of this apparent paradox is explained by the coexpression of 11β-HSD2 (HSD11B2), a very active enzyme that converts cortisol (active) into cortisone (inactive) due to its low affinity for the MR and GR (83, 110).

The MR, GR, progesterone receptor (PR), androgen receptor (AR), and estrogen receptor (ER) belong to the nuclear receptor family, a diverse group of transcription factors that arose in multicellular animals (28, 37, 156). Sequence analyses indicate that in chordates, the first divergence separated the common ancestor of ERs from the common ancestor of the AR, MR, GR, and PR (14). The ER first appears in amphioxus, a protochordate in the line leading to vertebrates. Sequence analyses indicate that the MR and GR are descended from a common ancestor (15, 36), a corticoid receptor (CR), found in lampreys and hagfish, cyclostomes (jawless fish), which evolved at the base of the vertebrate line (246, 301) (FIGURE 8). The ancestral CR sequence is closer to the MR than to the GR. Distinct orthologs of the MR and GR first appear in skates and sharks, which are Chondrichthyes (cartilaginous fishes). Further sequence divergence of the MR and GR occurred in lobe-finned fish, such as lungfish and coelacanth, forerunners of terrestrial vertebrates, as well as in terrestrial vertebrates and teleosts (ray-finned fish) (16).

Mineralocorticoid specificity in rodents and humans depends on three main limiting factors: 1) the proper synthesis of aldosterone; 2) the activity of the MR; 3) the activity of the Na-K-ATPase; 4) the expression of the ENaC. Four limiting steps are indicated (yellow): 1) 11β-HSD2, 2) MR, 3) ENaC, 4) Na-K-ATPase.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>MR Specific Transcriptional Response</th>
<th>Experimental Model</th>
<th>Organ or Cell</th>
<th>Comments</th>
<th>Reference Nos.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transporters</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ATP1A1</td>
<td>Na(^{+})-K(^{-})-ATPase α1-subunit</td>
<td>Early (15 min)</td>
<td>In vitro</td>
<td>A6 kidney cell</td>
<td>Twofold increase rate of transcription</td>
<td>331</td>
</tr>
<tr>
<td>ATP1B1</td>
<td>Na(^{+})-K(^{-})-ATPase β1-subunit</td>
<td>Early (30 min)</td>
<td>In vitro</td>
<td>A6 kidney cell</td>
<td>Twofold increase rate of transcription</td>
<td>331</td>
</tr>
<tr>
<td>ATP1G1 (FXYD2)</td>
<td>Na(^{+})-K(^{-})-ATPase γ-subunit</td>
<td>?</td>
<td>In vitro</td>
<td>A6 kidney cell</td>
<td>No evidence for regulation by aldo</td>
<td>115, 314</td>
</tr>
<tr>
<td>FXYD4</td>
<td>CHIF channel inducing factor</td>
<td>Within 3 h</td>
<td>In vivo</td>
<td>Rat kidney/colon</td>
<td>Differential regulation of mRNA level in colon and MCD</td>
<td>44, 127</td>
</tr>
<tr>
<td>SCNN1A</td>
<td>ENaC α-subunit</td>
<td>Within 3 h</td>
<td>In vivo</td>
<td>Kidney/colon</td>
<td>No evidence for transcriptional control in vivo</td>
<td>24</td>
</tr>
<tr>
<td>SCNN1B</td>
<td>ENaC β-subunit</td>
<td>Within 3 h</td>
<td>In vivo</td>
<td>Rat colon</td>
<td>Evidence for MR-regulated transcription</td>
<td>88, 106</td>
</tr>
<tr>
<td>SCNN1G</td>
<td>ENaC γ-subunit</td>
<td>Within 3 h</td>
<td>In vivo</td>
<td>Rat colon</td>
<td>Evidence for MR-regulated transcription</td>
<td>88, 106</td>
</tr>
<tr>
<td><strong>Signaling</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>SGK1</td>
<td>Serum- and glucorticoid-induced kinase 1</td>
<td>Early (30 min)</td>
<td>In vivo</td>
<td>Rat kidney/colon A6 kidney cell</td>
<td>Early and strongly induced gene, initiating the main MR-dependent signaling cascade</td>
<td>49, 96, 154, 253</td>
</tr>
<tr>
<td>KS-WNK1</td>
<td>Kidney specific WNK1 isoform</td>
<td>Early (30 min)</td>
<td>In vitro</td>
<td>M1-MR+ cell</td>
<td>Physiological relevance to Na/K transport supported by in vitro and in vivo data</td>
<td>231</td>
</tr>
<tr>
<td>KRAS2</td>
<td>K-ras 2</td>
<td>Within 60 min</td>
<td>In vitro</td>
<td>A6 kidney cell</td>
<td>Relevance to Na/K transport in vivo?</td>
<td>27, 87, 136</td>
</tr>
<tr>
<td>NDRG2</td>
<td>N-Myc downstream regulated gene 2</td>
<td>Early (45 min)</td>
<td>In vivo</td>
<td>Rat kidney</td>
<td>Relevance to Na/K transport in vivo?</td>
<td>35</td>
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<tr>
<td>USP2-45</td>
<td>Deubiquitylating enzyme</td>
<td>Early (30 min)</td>
<td>In vivo</td>
<td>Rat kidney</td>
<td>Relevance to Na/K transport in vivo?</td>
<td>95, 262, 329</td>
</tr>
<tr>
<td>TSCD22</td>
<td>GILZ</td>
<td>Within 3 h</td>
<td>In vivo</td>
<td>Rat kidney</td>
<td>Relevance to Na/K transport in vivo?</td>
<td>228, 313</td>
</tr>
<tr>
<td>CNKSR3</td>
<td>KSR scaffold protein</td>
<td>Early (60 min)</td>
<td>In vitro</td>
<td>M1 cells Microdissected tubules</td>
<td>Relevance to Na/K transport in vivo?</td>
<td>360</td>
</tr>
<tr>
<td><strong>Ex Vivo</strong></td>
<td></td>
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</tbody>
</table>

MR, mineralocorticoid receptor; ENaC, epithelial sodium channel; MCD, medullary collecting duct.
and secretion of aldosterone in the glomerulosa, 2) the proper expression of MR and GR in target epithelia, and 3) the proper expression of 11β-HSD2. When any of these proteins undergoes pathogenic gain-of-function or loss-of-function mutations, severe salt-sensitive (hypertensive) or salt-losing (hypotensive) syndromes may occur. This raises the question how such a trio consisting of a ligand (aldosterone), a receptor (MR), and a protective enzyme (11β-HSD2) evolved. We use an evolutionary perspective to review the physiological actions of aldosterone and other corticosteroids in transcriptional activation of the human MR in “traditional” target epithelia (kidney, colon, sweat gland, and salivary gland), as well as in “nontraditional” organs (brain, vessels, and heart), and the close relationship between mineralocorticoids and glucocorticoids and their receptors. Here, we focus on MR in “traditional” target epithelia through an analysis of the sequences of cyclostome CRs in elasmobranches, lobe-finned fish, ray-finned fish, and terrestrial vertebrates and the crystal structures of human MR and GR and a three-dimensional model of lamprey CR (16, 17).

FIGURE 8. Schematic view of the corticoid receptor evolution. MR, mineralocorticoid receptor; GR, glucocorticoid receptor; AR, androgen receptor; PR, progesterone receptor; CR, corticoid receptor. The first duplication separating AncMR/GR/CR/PR and AncAR/PR/CR/PR is likely to be due the second whole-genome duplication (2R-WGD), which occurred in vertebrates, prior to the separation between cyclostomes and gnathostomes (85). Previous reports indicated that the CR in cyclostomes was close to MR/GR than to AR/PR (318). However, recent reports, as well as our own analysis, showed that both CR and PR are closer to AR/PR than to MR/GR (85). It is possible that the ancestral AncGR/MR in cyclostomes was lost after the split cyclostomes/gnathostomes. The ancestral AncGR/MR in gnathostomes has been duplicated to give rise to modern MR and GR. In the other side, the split between cyclostomes/gnathostomes gave rise to AncCR/PR and AncAR/PR. In cyclostomes, a duplication produced the modern CR and PR in sea lampreys and hagfishes. In gnathostomes, a duplication produced AR and PR.

2. Late evolution of the ligand with respect to its receptor: aldosterone evolved in lobe-finned fish and is the main terrestrial mineralocorticoid

Aldosterone is the physiological mineralocorticoid in terrestrial vertebrates. Aldosterone first appears in lobe-finned fish (169). Interestingly, aldosterone is not found in ray-finned fish (41, 266), in which cortisol appears to be the transcriptional regulator of the MR and GR. This indicates that regulation of transcriptional response to the MR by aldosterone evolved after the divergence of ray-finned and lobe-finned fish, which suggests a role for aldosterone and the MR in the evolution of terrestrial vertebrates. “Late” evolution of aldosterone in the line leading to terrestrial vertebrates is intriguing, and it raises some questions regarding the evolution of transcriptional activation by corticosteroids of the MR in humans, as well as in jawless fishes, cartilaginous fishes, and ray-finned fishes.

FIGURE 7. A: corticosteroid synthesis in adrenal cortex in rodents and in humans. The genes encoding the proteins responsible for the major enzymatic steps are shown. The specific expression of HSD3B1 (human) or HSD3B6 (rodents) and of CYP11B2 (aldosterone synthase) in the zona glomerulosa determines the synthesis of aldosterone, whereas the synthesis of cortisol (humans) or corticosterone (rodents) is restricted to the zona fasciculata of the adrenal cortex. The target cell conversion of corticosterone (rodents) or cortisol (humans) by β-HSD2 (HSD11B2) into inactive metabolites is shown. [From (279).] B: structures of mineralo- and glucocorticoids. Aldosterone (Aldo) and deoxycorticosterone (DOC) are the main physiological mineralocorticoids in vertebrates. Cortisol (C) and corticosterone (B) are the main physiological glucocorticoids in vertebrates. 1α-Hydroxycorticosterone (1α-OH-B) is found in sharks. 11-Deoxycortisol (S) is a mineralocorticoid and glucocorticoid in lamprey CR (46). Progesterone (Prog) is an anti-mineralocorticoid for human MR. In mammals, 11-dehydrocorticosterone (A) and cortisone (E) are inactive metabolites of B and F, respectively.
Which steroids are physiological mineralocorticoids in lamprey, cartilaginous fishes, and ray-finned fish, and are some of these steroids also glucocorticoids?

What were the sequence and structural changes that led to the evolution of specificity of aldosterone and DOC for the MR and cortisol and corticosterone for the GR?

In which species did these evolutionary events occur?

What is the role of the common ancestry of the MR and GR in their overlapping responses to cortisol and corticosterone?

How does the evolution of 11β-HSD2 relate to the evolution of aldosterone as a transcriptional regulator of the MR in the kidney and colon, which depends on the presence of 11β-HSD2 to inactivate glucocorticoids, such as cortisol and corticosterone, which contain an 11β-hydroxyl group (11, 12, 75, 83, 110, 233, 240, 292)?

3. Sequence analysis of CRs

The sequences of lamprey and hagfish CRs and elasmobranch, coelacanth, ray-finned fish, and terrestrial vertebrate MRs and GRs provide a resource for investigating the transition in the responses to different corticosteroids by the CR in cyclostomes and the MR and GR in elasmobranches, coelacanths, ray-finned fish, and terrestrial vertebrates. We used these sequences to construct an alignment of the steroid binding domain on the CR, MR, and GR from various vertebrates and a phylogenetic tree which indicates that the MR is closer than the GR to CR. Interestingly, lamprey and hagfish CR cluster with the PR which appears to be closer to the root of 3-keto-steroid receptors. Several amino acids that have been shown to be important for the response of human MR and GR to corticosteroids will be discussed later in this review.

4. 11-Deoxycortisol is both a mineralocorticoid and glucocorticoid in Atlantic Sea lamprey

The corticosteroids that regulate CR responses in cyclostomes are still being elucidated. An important advance came from Close et al. (54), who used antibodies to cortisol and corticosterone as well as HPLC and mass spectrometry to investigate which corticosteroids are present in male and female lamprey serum. Interestingly, they found that lamprey serum contains DOC and 11-deoxycortisol, but no cortisol or corticosterone. Bridgham et al. (37) found no appreciable levels of aldosterone in lamprey serum. DOC is a precursor of corticosterone, which is a precursor of aldosterone. 11-Deoxycortisol is a precursor of cortisol (FIGURE 7B). Thus DOC and 11-deoxycortisol are the beginning of the pathway for synthesis of aldosterone and cortisol, respectively. Both DOC and 11-deoxycortisol lack an 11β-hydroxyl, and thus these two steroids are inert to metabolism by 11β-HSD2, which is an important mechanism for excluding cortisol and corticosterone from mammalian MR in epithelia (11, 12, 75, 83, 110, 233, 240, 292). This suggests that this mechanism for restricting access of 11β-hydroxy-corticosteroids to the MR is not present in lamprey, which is supported by a BLAST search, which did not find an ortholog of human 11β-HSD2 in the lamprey genome.

Close et al. (54) found that 11-[3H]deoxycortisol binds with high affinity (Kd ~2.7 nM) to the CR in lamprey gill cytosol. Interestingly, DOC had an affinity of ~5% of that of 11-deoxycortisol for the CR. Moreover, aldosterone, cortisol, corticosterone, and progesterone did not inhibit binding of 11-[3H]deoxycortisol to the CR. Highest levels of specific binding of 11-[3H]deoxycortisol were found in gill, intestine, and testis, with much lower levels in liver and kidney.

Close et al. (54) studied the effects in lampreys of 11-deoxycortisol implants on levels of gill Na+-K+-ATPase and of testosterone and estradiol in serum, and of the effects of stress on levels of 11-deoxycortisol and DOC in serum. Close et al. (54) measured Na+-K+-ATPase in gills in male and female lampreys containing 11-deoxycortisol implants because gills regulate ion balance in lampreys and fish (178, 327, 328). These implants upregulated gill Na+-K+-ATPase, an enzyme involved in sodium transfer, which suggests that 11-deoxycortisol is a mineralocorticoid in lamprey.

11-Deoxycortisol implants also reduced serum levels of testosterone and estradiol, suggesting that 11-deoxycortisol may have roles in lamprey that are associated with glucocorticoid action in terrestrial vertebrates. 11-Deoxycortisol implants also increased DOC levels in male and female lampreys.

Synthesis of 11-deoxycortisol in both male and female lampreys was upregulated after an exposure to stress for 1 h. After 24 h, 11-deoxycortisol levels were similar to that of unstressed lampreys. Interestingly, after 4 h, there was a 10% increase in DOC. Together, these in vivo studies indicate that the CR functions as both an MR and GR in lamprey. Two recent studies in Pacific lamprey (269) and in sea lamprey (275) indicate that stress rapidly induces the secretion of 11-deoxycortisol via the hypothalamic-pituitary-adrenal axis (HPA) (269, 275) and via additional pathways (275). The study of vertebrate stress physiology in fish may help to understand the evolution of the corticosteroid signaling within the vertebrate lineage (269).

The specificity of 11-deoxycortisol for the CR in lamprey gill cytosol contrasts with studies of the transcriptional activation by corticosteroids of the ligand binding domain of lamprey CR cloned into a GAL4-DNA-binding domain expression vector, which was then transfected into Chinese hamster ovary (CHO-K1) cells (36). In these assays, lamprey CR is promiscuous for corticosteroids. Indeed, aldosterone, cortisol, corti-
corticosterone, DOC, and 11-deoxycortisol stimulate CR-mediated gene transcription in mammalian cells (36). The EC\textsubscript{50} of 11-deoxycortisol for lamprey CR is \(~80\) nM in these assays, which is considerably higher than the \(K_d\) of \(~3\) nM for 11-deoxycortisol binding to lamprey gill cytosol.

These differences between the studies of Close et al. (54) and Bridgham and co-workers (36, 38) raise an important caveat in interpreting data from transcriptional activation of steroid receptors in heterologous systems. Ideally, transcriptional activation of lamprey CR by 11-deoxycortisol and other corticosteroids should be studied in lamprey cells. Similar considerations hold for assays of the response of GR and MR in skates, coelacanth, and ray-finned fish. Assays using mammalian cells to study the transcriptional response to corticosteroids of the MR from skates, coelacanth, and ray-finned fish may be misleading.

Other factors may account for the differences between the binding of \(^3\)H-S to the CR in lamprey gill cytosol and transcriptional activation of lamprey CR by 11-deoxycortisol and other corticosteroids in CHO-K1 cells. First, different CRs were used in the two studies. Close et al. (54) studied the native CR containing a DNA-binding domain and an NH\textsubscript{2}-terminal domain, while Bridgham et al. (36) studied the ligand-binding domain of lamprey CR cloned into a GAL4-DNA binding domain expression vector. Second, binding of 11-deoxycortisol to the CR in lamprey gill cytosol was done at 0°C. At this temperature, the CR may form complexes with one or more heat shock proteins, which could influence CR conformation and its specificity for corticosteroids. Third, in lamprey gills, the CR may have undergone phosphorylation or some other posttranslational modification that influenced its specificity for corticosteroids. Transcriptional activation was done in CHO-K1 cells at 37°C with the ligand-binding domain of lamprey CR (36). In these assays, the CR lacked the NH\textsubscript{2}-terminal domain, which has been shown to influence transcriptional activation of mammalian and teleost MR by corticosteroids (109, 260). Coactivators, corepressors, and other coregulators (146, 217, 351) in mammals and lampreys may differ in their sequences and in their effects on the conformation of lamprey CR. Lamprey also may contain novel coregulators. Each or both of these differences in coregulators could provide specificity for CR-mediated physiological responses to 11-deoxycortisol and not to other corticosteroids. Finally, interactions between nuclear receptors and chromatin regulate gene transcription (134, 168). The interactions of the CR with chromatin in lamprey cells may differ from that in CHO-K1 cells, leading to differences in transcriptional activation by corticosteroids.

Studies in lamprey exposed to implants of cortisone, corticosterone, aldosterone, and DOC on gill Na\textsuperscript{+}-K\textsuperscript{-}-ATPase synthesis and on levels of \(E_2\) and T would provide stronger evidence for the selectivity of lamprey CR for 11-deoxycortisol and the absence of mineralocorticoid activity of other corticosteroids. The presence of DOC in lamprey serum is intriguing and suggests a physiological role for DOC in lamprey. There may be conditions in some lamprey cells in which the CR mediates a response to DOC. Alternatively, if DOC does not regulate a CR response in lamprey, then DOC may be a transcriptional regulator of lamprey PR because DOC is 21-hydroxyprogesterone and may have high affinity for lamprey PR, similar to the high affinity of DOC for the chick oviduct PR (299).

5. DOC, corticosterone, and 1\alpha-hydroxycorticosterone are potential mineralocorticoids in elasmobranches

Elasmobranches contain separate orthologs of the MR and GR. It is not clear, however, which steroids regulate mineralocorticoid and glucocorticoid responses. 1\alpha-Hydroxycorticosterone (7, 90) appears to be unique to sharks, skates, and rays and is considered to be the main physiological corticosteroid in elasmobranches. Lesser concentrations of corticosterone, DOC, and 11-dehydrocorticosterone are found in elasmobranch serum. Neither aldosterone, cortisol, nor 11-deoxycortisol has been reported to be in elasmobranch serum (173, 211, 302).

Carroll et al. (46) provided important data on corticosteroid activation of skate MR. They used the ligand-binding domain of skate MR, cloned into a GAL4-DNA binding domain expression vector, to investigate transcriptional activity of various corticosteroids. Using this assay, Carroll et al. (46) found that skate MR is transcriptionally activated by DOC (EC\textsubscript{50} = 0.03 nM), aldosterone (EC\textsubscript{50} = 0.07 nM), corticosterone (EC\textsubscript{50} = 0.09 nM), cortisol (EC\textsubscript{50} = 1 nM), 1\alpha-hydroxycorticosterone (EC\textsubscript{50} = 3.8 nM), 11-dehydrocorticosterone (EC\textsubscript{50} = 9 nM), and 11-deoxycortisol (EC\textsubscript{50} = 22 nM) (46). Thus skate MR has broad specificity for transcriptional activation by 3-keto-steroids. In these assays, the EC\textsubscript{50} for transcriptional activation of skate MR by DOC and corticosterone are 110- and 42-fold, respectively, lower than that of 1\alpha-hydroxycorticosterone. This leaves open that possibility that, even at low concentrations, DOC and cortisosterone could be physiological mineralocorticoids in skates. Transcriptional activity of 11-dehydrocorticosterone is unexpected because like cortisone, 11-dehydrocorticosterone has a C11-ketone and cortisone does not activate human MR. The levels of 1\alpha-hydroxycorticosterone in stingray (Dasyatis sabina) serum vary from 8–77 nM (90), which is sufficient for activation of elasmobranch MR. Moreover, incubation of interrenal cells from stingray with angiotensin II resulted in synthesis of 1\alpha-hydroxycorticosterone (90), supporting a role for this steroid in mineralocorticoid action. The data of Carroll et al. (46) on corticosteroid activation of skate GR are puzzling. The most potent steroid is aldosterone (EC\textsubscript{50} = 11 nM), while corticosterone (EC\textsubscript{50} = 58 nM), cortisol (EC\textsubscript{50} = 139 nM), DOC (EC\textsubscript{50} = 306 nM), and
1α-hydroxycorticosterone (EC$_{50} = 947$ nM) are much weaker. There may be another, as yet untested, physiological glucocorticoid in skates (16). A complication in evaluating which corticosteroids activate either the MR or GR is the binding of corticosteroids to serum proteins. That is, corticosteroid activity of corticosterone and 1α-hydroxy-corticosterone may be influenced by differences in binding to serum proteins in elasmobranchs, as has been found for cortisol, corticosterone, DOC, and aldosterone in mammals (186).

There is a need for studies of in vivo exposure of skates and other elasmobranchs to various corticosteroids, to determine if 1α-hydroxycorticosterone is much less active than either DOC or corticosterone in activating skate MR. Similar considerations apply to determining activation of skate GR by corticosteroids.

6. Origin in elasmobranchs of the 11β-HSD2 mechanism for regulating glucocorticoid access to the MR

In epithelial cells, where the MR regulates electrolyte transport, 11β-HSD2 acts as a gatekeeper to control access of glucocorticoids to the MR (48, 240). The origins of this mechanism are not fully understood (11, 12). The evidence that lamprey serum contains 11-deoxycortisol and DOC, which lack an 11β-hydroxyl, but do not contain either cortisol or corticosterone, which have an 11β-hydroxyl, is consistent with the results of our BLAST search of the lamprey genome, which did not find an ortholog of 11β-HSD2. This suggests a later origin for the 11β-HSD2 mechanism (FIGURE 9A). Elasmobranches, however, contain corticosterone and 1α-OH-corticosterone, which have an 11β-hydroxyl and, thus, could be metabolized to 11-keto steroids by 11β-HSD2. To investigate if elasmobranchs contain an ortholog of 11β-HSD2, we performed a BLAST search of the skate base server [http://skatebase.org] and the elephant shark server [http://esharkgenome.imcb.a-star.edu.sg/], and we found an 11β-HSD2 ortholog in skate and elephant shark. FIGURE 9B shows the alignment of human, skate, and elephant shark 11β-HSD2. Selective expression in MR-containing tissues of skate 11β-HSD2 could regulate access of corticosterone to skate MR because its EC$_{50}$ for corticosterone is 0.09 nM and for 11-dehydrocorticosterone is 9 nM (46), a 100-fold reduction in transcriptional potency of 11-dehydrocorticosterone for skate MR.

A partner in regulating levels of glucocorticoids in terrestrial vertebrates is 11β-HSD1, which catalyzes the conversion of 11-dehydrocorticosterone and cortisol to cortisone and cortisol, respectively. A BLAST search of skatebase and the elephant shark server with human 11β-HSD1 retrieved orthologs of 11β-HSD1 in skate and elephant shark. Thus elasmobranches contain homologs of the two enzymes required for the interconversion of corticosterone and 11-dehydrocorticosterone, and possibly 1α-hydroxy-corticosterone and its 11-keto-metabolite.

7. Cortisol is the main mineralocorticoid in ray-finned fish

Ray-finned fish do not synthesize aldosterone, which is the physiological mineralocorticoid in terrestrial vertebrates (41, 266). Cortisol appears to be the transcriptional activator of fish MR. The EC$_{50}$ values for cortisol activation of the MR in rainbow trout (Oncorhynchus mykiss) (312), cichlid (Haplochromis burtoni) (130), midshipman fish (Porichthys notatus) (10), carp (Cyprinus carpio) (309), and zebrafish (Danio rerio) (259) are 1, 0.02, 0.2, 4. and 0.22 nM, respectively (10, 41, 215, 216, 266). In addition, DOC may function as a mineralocorticoid in fish (10, 13, 15, 41, 124, 266, 312). However, binding and transcriptional activation assays show that aldosterone has higher affinity and transcriptional activity for fish MR (130, 259, 309, 312).

8. Mineralocorticoid signaling pathways

As shown in FIGURE 10, despite notable differences in ligand-receptor interactions among different species, the mineralocorticoid signaling pathways target gills and kidney in lamprey (FIGURE 10A), skates (FIGURE 10B), zebrafish (FIGURE 10C), and lungfish (FIGURE 10D). It also targets rectal glands in skates (FIGURE 10B) and rectum in lungfish (FIGURE 10D). In tetrapods, it targets skin, kidney, bladder, and colon in toad (FIGURE 10E) as well as kidney, distal colon, and sweat glands in humans (FIGURE 10F). In all cases, Na$^+$/K$^+$-ATPase activity is upregulated by the mineralocorticoid signaling pathways contributing to salt reabsorption by the kidney and/or salt secretion by gills or rectal glands. The expression of ENaC (see below) and aldosterone in lungfish (FIGURE 10D) suggests that an aldosterone signaling pathway controlling the activity of ENaC and Na$^+$/K$^+$-ATPase evolved before the emergence of tetrapods.

9. Binding and transcriptional regulation of human MR by aldosterone, DOC, cortisol, corticosterone, 11-deoxycortisol, and progesterone

Our survey of physiological mineralocorticoids in lampreys, elasmobranchs, lobe-finned fish, ray-finned fish, and mammals finds important changes in the endogenous corticosteroids that mediate the mineralocorticoid response. 11-Deoxycortisol is a mineralocorticoid in lamprey (FIGURE 10A), and DOC, corticosterone, and 1α-OH-B may be mineralocorticoids in elasmobranchs (FIGURE 10B). Cortisol and possibly DOC are the transcriptional activators of the MR in ray-finned fish (FIGURE 10C), while aldosterone and DOC regulate electrolyte homeostasis in lungfish (FIGURE 10D), toads (FIGURE 10E), and mammals (FIGURE 10F). Cortisol and corticosterone regulate physiological responses mediated by mammalian MR in cells that lack 11β-HSD2. In humans and other mammals, aldosterone, DOC,
corticosterone, cortisol, 11-deoxycortisol, and progesterone have a high affinity for the MR in vitro (16, 121, 186). In vitro, aldosterone, DOC, corticosterone, cortisol, and 11-deoxycortisol are mineralocorticoids, while progesterone is an antimineralocorticoid. Interestingly, a variety of steroids, with or without hydroxyl groups at different positions, are mineralocorticoids. Thus, as shown in Figure 7B, DOC and 11-deoxycortisol lack an 11β-hydroxyl; DOC, corticosterone, and aldosterone lack an 17α-hydroxyl, and cortisol and 11-deoxycortisol contain a 17α-hydroxyl, while progesterone, which lacks an 11β-hydroxyl, 21-hydroxyl, and 17α-hydroxyl, is a mineralocorticoid antagonist (16, 121, 186). Although the basis for transcriptional activation of the MR and GR by these diverse corticosteroids in different species is still not fully understood, structural and sequence differences between the steroid-binding domain in the MR and GR are likely to play important roles (33, 197), which we discuss next.

10. Structural and sequence analysis of three key sites that influence steroid specificity in the MR

Research from several laboratories (32, 33, 36, 121, 158) provided insights into the structural basis for changes in the response of the MR and GR to corticosteroids. In particular, three sites in vertebrate CR, MR, and GR provide clues to the evolution of the MR and GR that led to their divergence in their specificity for corticosteroids.

**Figure 9.** A: evolution of 11β-HSD2. Orthologs of 11β-HSD2 first appear in sharks and skates, two members of the elasmobranch subclass. B: alignment of human 11β-HSD2 with the proposed skate 11β-HSD2. Out of 290 residues there are 141/290 (49%) identical and 181/290 (62%) including conservative replacements. The probability of finding this similarity by chance is $2 \times 10^{-10}$. 

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**Table:**

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**Figure 9A:** Evolution of 11β-HSD2. Orthologs of 11β-HSD2 first appear in sharks and skates, two members of the elasmobranch subclass. B: Alignment of human 11β-HSD2 with the proposed skate 11β-HSD2. Out of 290 residues there are 141/290 (49%) identical and 181/290 (62%) including conservative replacements. The probability of finding this similarity by chance is $2 \times 10^{-10}$. 

---

**Figure 9B:** Alignment of human 11β-HSD2 with the proposed skate 11β-HSD2. Out of 290 residues there are 141/290 (49%) identical and 181/290 (62%) including conservative replacements. The probability of finding this similarity by chance is $2 \times 10^{-10}$. 

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**Figure 9C:** Alignment of human 11β-HSD2 with the proposed skate 11β-HSD2. Out of 290 residues there are 141/290 (49%) identical and 181/290 (62%) including conservative replacements. The probability of finding this similarity by chance is $2 \times 10^{-10}$. 

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**Figure 9D:** Alignment of human 11β-HSD2 with the proposed skate 11β-HSD2. Out of 290 residues there are 141/290 (49%) identical and 181/290 (62%) including conservative replacements. The probability of finding this similarity by chance is $2 \times 10^{-10}$. 

---

**Figure 9E:** Alignment of human 11β-HSD2 with the proposed skate 11β-HSD2. Out of 290 residues there are 141/290 (49%) identical and 181/290 (62%) including conservative replacements. The probability of finding this similarity by chance is $2 \times 10^{-10}$. 

---

**Figure 9F:** Alignment of human 11β-HSD2 with the proposed skate 11β-HSD2. Out of 290 residues there are 141/290 (49%) identical and 181/290 (62%) including conservative replacements. The probability of finding this similarity by chance is $2 \times 10^{-10}$.
A  Lamprey (*Petromyzon marinus*)

- 11-deoxycorticosterone
- Deoxycorticosterone
- CR (MR + GR)
- Cell specific transcriptome
- Mineralo- and glucocorticoid effects

**Target:** Gill, kidney (filtration, tubular secretion and reabsorption).
No countercurrent mechanism

**Physiological effects:** Increased sodium reabsorption and osmoregulation

**Mechanism:** Increased Na,K-ATPase activity

---

B  Skates (*Leucoraja erinacea*)

- **Aldo? In vitro**
- Corticosterone? Unknown ligand?
- 11β-OH-corticosterone
- DOC Corticosterone?
- 11β-HSD2
- MR
- Cell specific transcriptome
- Glucocorticoid effects
- Mineralocorticoid effects

**Target:** Gill, kidney (filtration, tubular secretion and reabsorption).
Presence of countercurrent mechanism (urea concentration).
Rectal gland

**Physiological effects:** Increased sodium reabsorption and osmoregulation

**Mechanism:** Increased Na,K-ATPase activity

---

C  Zebrafish (*Danio rerio*)

- Deoxycorticosterone
- Aldosterone (in vitro)
- Cortisol
- GR
- MR
- Cell specific transcriptome
- Glucocorticoid effects
- Mineralocorticoid effects

**Target:** Gill, kidney (filtration, tubular secretion and reabsorption).
Presence of countercurrent mechanism (urea concentration)

**Physiological effects:** Increased sodium reabsorption and osmoregulation

**Mechanism:** Increased Na,K-ATPase activity

---

D  Lungfish (*Neoceratodus forsteri*)

- Corticosterone
- Aldosterone
- 11-dehydrocorticosterone
- 11β-HSD2
- GR
- MR
- Cell specific transcriptome
- Glucocorticoid effects
- Mineralocorticoid effects

**Target:** Gill, kidney, rectum
No countercurrent mechanism

**Physiological effects:** Increased sodium reabsorption

**Mechanism:** Increased ENaC and Na,K-ATPase activity

---

E  Toad *Bufo marinus* (*Rhinella marina*)

- Corticosterone
- Aldosterone
- 11-dehydrocorticosterone
- 11β-HSD2
- GR
- MR
- Cell specific transcriptome
- Glucocorticoid effects
- Mineralocorticoid effects

**Target:** Skin, kidney, bladder, colon

**Physiological effects:** Increased sodium reabsorption and proton secretion

**Mechanism:** Increased ENaC and Na,K-ATPase activity

---

F  Human (*Homo sapiens*)

- Corticosterone
- Aldosterone
- Cortisol
- GR
- MR
- Cell specific transcriptome
- Glucocorticoid effects
- Mineralocorticoid effects

**Target:** Kidney (ASDN), colon, sweat glands

**Physiological effects:** Increased sodium reabsorption, increased proton and potassium secretion

**Mechanism:** Increased ENaC and Na,K-ATPase activity

**Non epithelial target:** Vessels (endothelial and SMVC)
11. Novel responses to steroids by the Ser810Leu mutant human MR

A major discovery, with important clinical and evolutionary implications, was the report by Geller et al. (121) that a mutant human MR, in which Ser-810 was replaced by leucine, had novel responses to various 3-keto-steroids. This mutation caused early-onset hypertension that was markedly exacerbated in pregnancy (preeclampsia). This mutation results in constitutive MR activity by altering receptor specificity. Progesterone, normally an MR antagonist, becomes a potent agonist. Geller et al. (121) found that progesterone was a transcriptional activator of Leu-810 MR. Indeed, progesterone had an EC\textsubscript{50} of ~1 nM for transcriptional activation of the Leu-810 MR, which is unexpected because progesterone is an antagonist for wild-type human MR. At nanomolar concentrations, 19-nor-progesterone, cortisone, which binds poorly to human MR (9), is an agonist for the Leu-810 MR (158), which is further evidence of the unexpected effects of replacing Ser-810 with leucine.

Geller et al. (121) constructed a three-dimensional model of the mutant MR, which predicted a van der Waals contact between Ala-773 on helix 3 and Leu-810 on helix 5 (121) (Figure 11A, top panel). This prediction was confirmed in the crystal structures of Leu-810 MR with progesterone (158). In contrast, in wild-type human MR complexed with aldosterone or progesterone (32) (Figure 11A, bottom panel), there are no van der Waals contacts between Ala-773 and Ser-810. There are other important stabilizing contacts between helices 3 and 5 in Leu-810 MR crystalized with progesterone. Leu-810 contacts Gln-776, Met-777, and Val-780 on helix 3. Geller et al. (121) found that the Ser810Met mutant human MR was activated by 19-nor-progesterone. They proposed that the longer side chain of methionine forms a van der Waals contact with Ala-773, providing a stabilizing contact between helix 3 and helix 5, as was found for the Leu-810 mutant MR. Replacement of Ala-773 with glycine, which would lead to the loss of the van der Waals contact with Leu-810, only led to a small reduction in activation of Leu-810 MR. This supports the role of stabilizing contacts between Met-810 on helix 5 and Met-777 and Val-780 on helix 3 in the response to 19-nor-progesterone and possibly progesterone. Overall, these data emphasize the role of the helix 3-helix 5 contact in providing specificity in the MR response to 19-nor-progesterone, progesterone, cortisone, and other steroids.

In human GR complexed with dexamethasone (Dex), Met-604 in helix 5 and Gly-567 in helix 3, which correspond to Ser-810 and Ala-773 in human MR, do not have a van der Waals contact (33). Zhang et al. (354) mutated Met-604 in helix 5 to leucine in human GR to provide a van der Waals contact with Gly-567 on helix 3. This mutant GR had an increased affinity for glucocorticoids.

These data with human MR and GR indicate that the presence or absence of contacts between helix 3 and helix 5 are important in their response to different 3-keto-steroids. Helices 3–5 and helix 12 form the coactivator binding groove on the MR, GR, and other steroid receptors (16, 109, 251, 349). Stabilization of the helix 3-helix 6 contact in the Leu-810 mutant MR converts progesterone from an antagonist to an agonist (121) and cortisone from an inactive steroid to an agonist (268). It appears that a point mutation in the MR (and GR) can alter the response to steroids by altering the configuration of the coactivator binding groove.

**Figure 10.** Evolution of the mineralocorticoid signaling pathway. Representative examples of the mineralocorticoid signaling pathways in fishes, amphibians, and mammals. A: lamprey. 11-Deoxycorticisol is the main corticosteroid activating a corticosteroid receptor (CR) presumably triggering both mineralo- and glucocorticoid effects. No evidence for the presence of 11\(\beta\)-HSD2. Osmoregulation is achieved by the gills and the kidney. 11-Deoxycorticisol is regulated by the hypothalamus-pituitary axis and responds to acute stress. 11-Deoxycorticisol upregulates gill Na\(^{+}\)-K\(^{-}\)-ATPase (see Ref. 54). B: skate. 1\(\alpha\)-Hydroxy-corticosterone is the main mineralocorticoid hormone that activates MR triggering mineralocorticoid effects on gills, rectal gland, and kidney. MR may be protected from illicit occupation by glucocorticoids thanks to the expression of 11\(\beta\)-HSD2. Osmoregulation involves a countercurrent mechanism allowing urea concentration and high plasma urea. Environmental salinity controls the activity of Na\(^{+}\)-K\(^{-}\)-ATPase in gills and rectal glands of the Atlantic stingray (Dasyatis sabina) (see Ref. 257). C: zebrafish. Cortisol is presumably the main mineralocorticoid hormone that activates MR triggering mineralocorticoid effects on gills and kidney. The role of 11\(\beta\)-HSD2 remains to be determined since glucocorticoid receptor, but not mineralocorticoid receptor, have recently been proposed to mediate cortisol regulation of epidermal ionocyte development and ion transport (61). Osmoregulation is mainly ensured by gill ionocytes (159). Soft water acclimation increases the activity of Na\(^{+}\)-K\(^{-}\)-ATPase in gills (58). D: lungfish. Aldosterone is proposed to be the main mineralocorticoid hormone (169) that activates MR triggering the mineralocorticoid effect on gills, kidney, and rectum. MR may be protected from illicit occupation by glucocorticoids thanks to the expression of 11\(\beta\)-HSD2. ENaC is expressed in gills, kidney, and rectum suggesting an ENaC-dependent, aldosterone-controlled sodium reabsorption before the conquest of land by tetrapods (324). E: toad. Aldosterone is the main mineralocorticoid hormone that activates MR triggering the mineralocorticoid effect in skin, kidney, and colon. MR is fully protected from illicit occupation by corticosterone thanks to high expression of 11\(\beta\)-HSD2 in the target cell. ENaC and Na\(^{+}\)-K\(^{-}\)-ATPase are tightly regulated by aldosterone (81, 112, 113). F: human. Aldosterone is the main mineralocorticoid hormone that activates MR triggering the mineralocorticoid effect in kidney, sweat glands, trachea, and colon. MR is fully protected from illicit occupation by corticosteroids thanks to high expression of 11\(\beta\)-HSD2 in the target cell. ENaC and Na\(^{+}\)-K\(^{-}\)-ATPase are tightly regulated at the transcriptional, translational, and posttranscriptional level by aldosterone.
An analysis of the site corresponding to Ser-810 in vertebrate CR, MR, and GR provides an insight into the evolution of this regulatory mechanism. Lamprey and hagfish CR and skate MR contain a methionine at the position corresponding to Ser-810 in human MR. These CRs also contain a cysteine corresponding to Ala-773 in human MR. Interestingly, lamprey PR also contains this methionine and cysteine (16), indicating that this Met-Cys pair was present in the 3-ketosteroid ancestor of the CR and PR. As shown by Baker et al. (17), a three-dimensional model of lamprey CR with 11-deoxycortisol (FIGURE 11B, top panel) reveals the presence of a van der Waals contact between Met-264 and Cys-227, which correspond to Ser-810 and Ala-773, respectively, in human MR. Together these data suggest that both human MR and human GR have lost a key contact between helix 3 and helix 5 that was present in the CR and skate MR, and this evolutionary event was important in the regulation of specificity and responses to steroids in vertebrate MR and GR.

Examination of the sequence of coelacanth MR reveals that it contains an alanine and serine corresponding to Ala-773 and Ser-810 in human MR. It is not known if there is a van der Waals contact between Ala-132 and Ser-169 in coelacanth MR. Nevertheless, the evolution of these residues in helix 3-helix 5 in an MR in a lobe-finned fish coincides with the first evidence for the synthesis of aldosterone in lungfish (169). This suggests that coelacanth MR marks an important transition to terrestrial MRs.
Regarding the divergence of the GR from the MR, skate GR has a glycine corresponding to Gly-567 in human GR, which indicates that an important step in the divergence of the GR from its common ancestor with the MR occurred in elasmobranchs. However, the low affinity of skate GR for cortisol and other glucocorticoids indicates that other differences between skate GR and human GR are important in the evolution of transcriptional activation of the GR by corticosteroids.

12. Differences in the specificity of the MR and GR for the 17α-hydroxyl on cortisol

Despite the nanomolar affinity of cortisol for mammalian MR, in some cell cultures cortisol is a weak transcriptional activator of human MR (9, 147). Insights into the structural basis for the poor transcriptional activation of the MR by cortisol have come from analyses of human GR ligand binding domain cocrystallized with dexamethasone (33) and human MR ligand binding domain cocrystallized with corticosterone. On the basis of a comparison of these structures, two amino acid differences between human MR and GR (Ser-843 and Leu-848 in human MR and Pro-637 and Gln-642 on the GR) (FIGURE 11B, bottom panel) have been proposed to explain transcriptional activation of human GR in cells by cortisol, dexamethasone, and other glucocorticoids that contain a 17α-hydroxyl group and the poor response of human MR in cells to these steroids (33, 36, 197, 245, 268).

These two amino acid differences between human MR and GR result in a different configuration for helices 6 and 7. As noted by Li et al. (197), this binding pocket has very different conformations in the GR and MR, which is clearly seen when the GR and MR are superimposed (FIGURE 11C, top panel). Ser-843 in the MR is displaced by 4.7 Å from Pro-637 in the GR and Leu-848 is 5.2 Å from Gln-642 (197). In human GR, Pro-637 opens up a pocket for the 17α-hydroxyl on cortisol and dexamethasone (33), and Gln-642 has a hydrogen bond with the 17α-hydroxyl (33). In the MR, the hydrophobic side chain on Leu-848 would clash with the 17α-hydroxyl of cortisol or dexamethasone, but not with aldosterone and DOC, which lack a 17α-hydroxyl. Indeed, experiments with transcriptional activation of mutant human MR and GR by cortisol and corticosterone (197) and of an ancestral CR by aldosterone and cortisol (36) indicate that amino acids corresponding to Ser-843 and Leu-848 in human MR and Pro-637 and Gln-642 on human GR are important in their specificity for steroids with a 17α-hydroxyl.

However, this appears to be an incomplete explanation for specificity of the GR for 17α-hydroxysteroids because cortisol has an EC50 of 1 nM for skate MR (46), and cortisol has an EC50 for transcriptional activation of the MR in cichlid, midshipman fish, trout, carp, and zebrafish of 0.02 nM (130), 0.2 nM (10), 1 nM (312), 4 nM (309), and 0.22 nM (259), respectively. Both skate MR and ray-finned fish MR contain a serine and leucine that align with Ser-843 and Leu-848 in human MR. Also, lamprey CR, which contains a similar serine and leucine, responds to S, which contains a 17α-hydroxyl. Finally, when dexamethasone is modeled in human MR, Leu-848 does not have a steric clash with the 17α-hydroxyl on dexamethasone (16). Thus, although the transition from serine and leucine in the MR to proline and glutamine, respectively, in the GR is important in the selective response of human GR and MR to the presence or absence, respectively, of a 17α-hydroxyl on steroids, other sites on the MR and GR are important in their transcriptional response to cortisol and other corticosteroids with a 17α-hydroxyl group.

We also note that although skate GR, like skate MR, contains a serine and leucine corresponding to Ser-843 and Leu-848 in human MR, skate GR has a weak response to cortisol (EC50 = 139 nM), unlike skate MR (EC50 = 1 nM). This further supports the hypothesis that amino acids other than Pro-637 and Gln-642 on the GR contribute to selectivity for cortisol and other corticosteroids with a 17α-hydroxyl group.

Examination of sequences of vertebrate MR and GR reveals that the Pro-637 and Gln-642 characteristic of the GR in ray-finned fish, coelacanth, and terrestrial vertebrates first appear in an ancestral Euteleostomi (bony vertebrates) GR. Analysis of transcriptional activation by corticosteroids of coelacanth GR and MR would provide insights into the transition to terrestrial vertebrate GRs and MRs.

13. Ser-949 in the loop connecting helix 11 and helix 12 in human MR

The loop connecting helix 11 and helix 12 in human MR is important in positioning the activation function 2 (AF-2) segment in helix 12 into the coactivator binding groove (32, 92, 148, 197). Like other nuclear receptors, binding of the MR and GR to coactivators is part of the mechanism for specificity for ligands (109, 157). In helix 12 in human MR and GR, Glu-962 and Glu755, respectively, have key stabilizing contacts with coactivators (33, 157). Human GR contains a deletion at the site corresponding to Ser-949 in human MR, which alters the conformation of the loop between helix 11 and 12 (16). O62 on Glu-962 in human MR and Glu-755 on human GR are displaced by 3.5 Å (FIGURE 11C, bottom panel), which would be expected to change the interaction of some coactivators with the MR and GR.

Analysis of the evolution of this serine and its deletion in the GR provides insights into the divergence of the GR and MR from their common ancestor. A serine corresponding to Ser-949 is conserved in hagfish CR and all vertebrate MRs (16). Lamprey CR contains a threonine, a conservative replacement for serine. Interestingly, lamprey PR also contains this serine, indicating that the common ancestor of the
CR and PR contained this serine. Moreover, human PR and AR contain this serine, another indication of the strong conservation of this serine in 3-ketosteroid receptors. Skate GR also has this serine but not coelacanth and teleost GRs which contain the serine deletion, indicating that an important transition occurred in the GRs in coelacanths and ray-finned fishes (16).

E. Na\(^+\)-K\(^+\)-ATPase

The sodium pump or Na\(^+\)-K\(^+\)-ATPase has the unique property of exchanging three sodium against two potassium ions across the plasma membrane of animal cells. In addition to playing a critical role in maintaining intracellular potassium and sodium low, it is the primary active pump required to control the ionic composition of the extracellular fluid. Many recent reviews deal with various aspects of the structural and functional maturation of the enzyme (117, 118, 151, 224, 321).

1. Biochemical and biophysical properties

Na\(^+\)-K\(^+\)-ATPase belongs to the large family of P-ATPases which is phosphorylated during the functional cycle: the γ-phosphate of ATP is transferred to an aspartate residue that belongs to a phosphorylation site motif DKTG highly conserved in the whole family (151). Na\(^+\)-K\(^+\)-ATPase is observed in two conformations: the E1P and E2P conformations that differ in affinity for Na\(^+\) and K\(^+\), sensitivity to ADP and ATP, sensitivity to proteolysis, and intrinsic fluorescence (151). As discussed by Gadsby (111), Na\(^+\)-K\(^+\)-ATPase uses an alternating opening/closing gating system (two gates) to allow the passage of one atom at a time (either sodium or potassium). This opening/closing mechanism exports three Na\(^+\) and import two K\(^+\) per cycle, and there can be up to 100 cycles/s. The result is a measurable current defining the Na\(^+\)-K\(^+\)-ATPase as electrogenic (111). Na\(^+\)-K\(^+\)-ATPase is inhibited by ouabain with large differences in affinity (nmol to mmol) depending on the species and/or the tissue/cell considered.

2. Primary and secondary structure

Na\(^+\)-K\(^+\)-ATPase is a heteromeric protein consisting of one α, one β, and one γ (FXYD) subunit (FIGURE 12A). The α-subunit is the catalytic subunit with ~1,000 residues and carries out the main function of the enzyme. It has 10 transmembrane domains (M1-M10), with short nonglycosylated extracellular loops and large cytoplasmic domain that express the ATP binding and phosphorylation site.

The β-subunit has a crucial role as chaperone in the structural and functional maturation of the enzyme (117, 118, 151, 224). The β-subunit has one transmembrane domain with a large extracellular heavily glycosylated domain. As discussed below, the Na\(^+\)-K\(^+\)-ATPase β-subunit through homotypic interactions plays an important role in cell adhesion, a critical step in the evolution of multicellularity.

The γ-subunit belongs to a protein family (FXYD proteins) characterized by a common consensus amino acid sequence (FXYD). FXYD1, FXYD2, FXYD3, FXYD4, and FXYD7 can associate with αβ-subunits and play a regulatory role in a tissue- and isoform-specific way (115, 119).

In humans there are four α (α1, α2, α3, and α4), three β (β1, β2, and β3) and five γ (FXYD1-4 and 7) isoforms allowing the tissue and cell specific expression of a large number of variants with distinct transport properties (118, 119). FXYD proteins interact specifically with Na\(^+\)-K\(^+\)-ATPase and change the apparent affinities for sodium, potassium, and ATP as well as V\(_{\text{max}}\). The kinetic effects are small but could be of physiological importance for instance along the distal segments of the nephron (115). Mishra et al. (223) reported that FXYD1, FXYD2, and FXYD4 can specifically associate with α to form complexes that protect and stabilize Na\(^+\)-K\(^+\)-ATPase activity by specific phosphatidylinositol-protein interactions (223).

3. Tertiary and quaternary structure

Despite the many technical challenges and methodological difficulties to crystallize the heteromer, high-resolution crystal structures of Na\(^+\)-K\(^+\)-ATPase (α1β1γ) were obtained in E2P state (224, 241, 293), and recently, a molecular mechanism for the Na/K exchange has been proposed based on the crystal structure of the E1P state (172, 237).

4. Na\(^+\)-K\(^+\)-ATPase and P-ATPase gene family and history of α-subunit

Many extensive phylogenetic analyses of the P-type ATPases have been published (47, 64, 287). Schematically, P-ATPases can be divided five groups: 1) group I made of group la not present in human genome (for example, bacterial KDPB subunit) whereas group Ib is present as cation pumps able to transport unidirectionally Ag\(^+\), Cd\(^2+\), and Cu\(^2+\); 2) group II found in human genome made of three subgroups: Ila [i.e., sarcoplasmic endoplasmic reticulum calcium ATPase (SERCA)]; Iib [plasma membrane calcium ATPase (PMCA)]; Iic (the Na\(^+\)-K\(^+\)- and H\(^+\)-K\(^+\)-ATPase). Na\(^+\)-K\(^+\)- and H\(^+\)-K\(^+\)-ATPases are unique in their ability to couple directly sodium/potassium or potassium/proton exchange and their absolute requirement of α-subunit for transport to and function at the plasma membrane; 3) group III (as proton or magnesium ATPase found in yeast and plants but not in Metazoan); 4) group IV, a large group of genes found in human genome and protozoa; 5) group V, a large group of genes found in human genome and protozoa [of special interest an aminophospholipid (APL) transporter or “flip-pase” (317)]. Consistent with previous observations, Studer et al. (311) found homologs of Na\(^+\)-K\(^+\)-ATPase α-subunit in all three domains of life, so it is likely to have already
existed in the last universal common ancestor (LUCA). They are detected in many bacterial clades, in a few Archaea, and, as expected, in most eukaryotic lineages, with the interesting exception of plants. The \(\text{Na}^+\)-subunit is a homolog of the bacterial potassium-transporting ATPase (KdpB) discussed above. In vertebrates, two rounds of whole genome duplication (2R WGD) at the origin of this clade (149, 267) associated with consecutive single gene duplication have generated at least six paralogous copies of the \(\text{Na}^+\)-subunit: four paralogs of Na\(^+\)-K\(^+\)-ATPase and the two paralogs of H\(^+\)-K\(^+\)-ATPase, the gastric and the nongastric subunit. In other animals, the insect genes mostly form a monophyletic group. We also found orthologs of the \(\text{Na}^+\)-subunit in basal animals (sponge (Amphimedon), placozoan (Trichoplax)) and in the unicellulars such as the choanoflagellate Monsosiga (the sister clade to animals), Dictyostelium purpureum, and Naegleria gruberi, one of the most distant eukaryotic species from animals (311).

5. \(\beta\)-Subunit

In previous studies (89, 294) it was hypothesized that the \(\beta\)-subunit is a homolog of the potassium-transporting ATPase C chain (KdpC) in bacteria. Studer et al. (311) did not confirm this theory and put the origin of the \(\beta\)-subunit at the origin of metazoans (311). Due to the structural constraints acting upon membrane proteins, it is not surprising to find some similarities between the \(\beta\)-subunit and KdpC (345). The \(\beta\)-subunit is therefore more recent,
as the distant organism found to date is the choanoflagellate *Monosiga brevicolis* (311). So it likely emerged in the last common ancestor of Filozoa, the monophyletic group that encompasses Metazoan and choanoflagellate. As Monosiga can form colonies, it would be interesting to determine if that β-subunit has a direct role in polarizing cells within these colonies. The ancestor of metazoans could have greatly benefited from this function to establish its first internal milieu.

6. γ-Subunit

The γ-subunit is specific to vertebrates and belongs to the phospholemman family, a family of at least seven paralogs, as it possesses the short motif FXYD (119). This family evolved relatively recently as the most distant phospholemman family, a family of at least seven paralogs, (311). The γ-subunit (also called FXYD2) appeared later in tetrapods, interestingly at the same time as aldosterone, which controls the expression of Na⁺-K⁺-ATPase in kidney.

An interesting finding was that the genes FXYD6 and FXYD8 arose from a gene duplication at the origin in primates (311). FXYD6 is expressed in the inner ear (66, 67), and some studies found a relationship between FXYD6 and schizophrenia (51, 359), while others have not (161, 353). FXYD8 is expressed in the inner ear (66, 67), and co-workers (296, 297) have measured intracellular and extracellular concentrations of sodium and potassium in gastrula/blastodeum embryos of *Xenopus laevis* (FIGURE 2C). Upon fertilization, the egg undergoes 12 rapid synchronous cleavages (morula = a ball of cells) followed by a period of slower asynchronous divisions more typical of somatic cells at the so-called mid-blastula transition (213). In *Xenopus laevis*, blastula is reached around 8–10 h after fertilization and is characterized by the formation of the blastocoele cavity filled with a primordial extracellular fluid. This means that the first differentiation is the formation of epithelial cells with formation of tight junctions able to create a vectorial absorption of fluids from the cell compartment to the cavity, creating the first “internal milieu” of the organism. Slack and co-workers (296, 297) have measured intracellular and extracellular concentrations of sodium and potassium in gastrular embryos of *Xenopus laevis*. The intracellular potassium concentration is close to 100 mM. The intercellular fluid contains 100 mM sodium and 1 mM potassium. As no net uptake of sodium or potassium from the external milieu occurs before gastrulation, these cations must have been transported from the cells to the blastocoele (296, 297). Gillespie et al. (123) have confirmed these findings: the free intracellular Na⁺ (~20 mM) and potassium concentrations (~90 mM) remain approximately constant. The extracellular potassium concentration falls steadily from 7 mM at the mid-blastula stage to 2 mM at the end of neurulation, whereas sodium concentration remains constant (~90 mM). Geering et al. (120) and Burgener-Kairuz et al. (40) have shown that these ion distributions changes occurring during early development may be linked to the assembly of Na⁺-K⁺-ATPase αβ1-heteromers, their transport to the plasma membrane and the activity of Na⁺-K⁺-ATPase at the cell surface. During oocyte growth (from stage I to stage VI) α1-, but not β1-mRNAs accumulate. β1-mRNAs are sequestered in an untranslated pool in fully-grown oocytes (stage VI). In fully grown *Xenopus* oocytes (stage VI), the synthesis of Na⁺-K⁺-ATPase β-subunits is limiting for the formation of functional Na⁺-K⁺-ATPase αβ1 heteromers (120). As summarized by Burgener-Kairuz et al. (40), “from fertilization to morula-
tion, the total pools of α1- and β1-mRNAs vary little. Whereas polyadenylated α1-mRNAs did not change significantly, polyadenylated β1-mRNA abundance increased three to fourfold at morulation, accompanied by a parallel increase in β1-protein synthesis. The abundance of polyadenylated β1-mRNA is rate-limiting during embryonic development for the assembly of α1/β1-heterodimers, shown to be involved in the vectorial transport of sodium in kidney cells. In addition the polyadenylation of β1-mRNA is a rate-limiting factor during morulation for the synthesis and assembly of new sodium pumps at the time of blastocoele fluid formation.” The constitution of the first extracellular fluid (high sodium/low potassium) seems to be controlled by the developmental program controlling Na⁺-K⁺-ATPase gene expression both at the transcriptional and post-transcriptional level. Not surprisingly, embryonic lethality for α1 gene inactivation in mouse was reported (162). Before fertilization (mature oocyte stage VI), it appears that ENaC is not expressed at any significant level as shown by the absence of any significant amiloride-sensitive conductance (248), allowing the identification of ENaC by expression cloning (42). It is not known when ENaC is expressed for the first time during early development of Xenopus laevis.

In mammals, the blastocyst is composed of the outer epithelial trophectoderm surrounding a large fluid-filled cavity and the inner cell mass, progenitors of all embryonic cell lineages. A fully differentiated trophoeoderm is a prerequisite for a normal blastocyst formation followed by its implantation in the uterus endometrium. This step requires a normal Na⁺-K⁺-ATPase activity (252) as shown by gene deletion of α-subunit (20), and Na⁺-K⁺-ATPase activity in turn regulates TJ formation by affecting the distribution of ZO-1 and occludin (332). During brain development, the Na⁺-K⁺-ATPase β2-subunit expressed on glial cells (also called AMOG = adhesion molecule on glia; Ref. 126) plays a critical role in calcium-independent neuronal-glial cell interaction as evidenced by severe neurodegeneration observed in β2 null mice (209). Na⁺-K⁺-ATPase (α2/β2) dependent potassium uptake by glial cell allows an efficient buffering of extracellular potassium that may rise by a few millimoles during neuronal activity (183, 208).

In summary, Na⁺-K⁺-ATPase plays a pivotal role controlling the ionic composition of the blastocoele fluid during early development, of plasma and interstitial fluid (kidney), CSF (choroid plexus), and finally interstitial fluid in the brain (glial-neuron interstitium) in adult animals.

8. Dual role of Na⁺-K⁺-ATPase β-subunit as chaperone and cell adhesion molecule is conserved in fruit flies

Of special interest is the dual role of the β-subunit as a chaperone allowing the functional expression of the enzyme at the plasma membrane and as a cell adhesion molecule, a limiting step in the formation of a multicellular organism (270). As pointed out by Vagin et al. (326), the integrity of epithelial junctions is critical to maintain the gradient requested for the efficiency of Na⁺-K⁺-ATPase transport (FIGURE 12B).

As shown by Tokhtaeva et al. (319), epithelial junctions depend on intercellular trans-interactions between Na⁺-K⁺-ATPase β1-subunit. The homotypic β1/β1 interaction is mediated by N-glycan, regulating stability and tightness of intercellular junctions (319) and the 10 residues participating in this contact interface have been identified (320) (FIGURE 12C). In fruit flies, Paul et al. (252) demonstrated that the junctional activity at the septate junction (the equivalent of tight junction in vertebrates) of Drosophila was mediated by noncatalytic activity of a specific α-isoform associated with a specific β-isoform. Remarkably, the phenotype of the null Drosophila allele could be fully restored by the rat α-isoform. This represents strong genetic evidence for a sodium pump-independent function of Na⁺-K⁺-ATPase (252).

F. ENaC

The amiloride-sensitive ENaC belongs to the ENaC/DEG (degenerins) family of ion channels implicated in a variety of physiological functions. The specific tissue and species expression patterns of the channels allow us to classify them in five subfamilies: 1) α (or δ), β, and γ ENaC subunits, mainly expressed in epithelia and other tissues (see below); 2) MDEG1, MDEG2, ASIC, and DRASIC genes, identified in the nervous system of mammals involved in pain sensation; 3) FaNaCh involved in synaptic transmission in snail; 4) MEC-4, MEC-10, DEG-1 (degenerins), and UNC105 of the Caeorohabditis elegans nematode expressed in sensory neurons and muscles, respectively; and 5) the pickpocket gene family in Drosophila.

1. Biophysical properties

The amiloride-sensitive ENaC is highly selective for Na⁺ over K⁺, exhibits a low Na⁺ single-channel conductance (5 pS), and has a high sensitivity to amiloride block (Kᵢ <0.1 μM) (177; see review by Verrey et al., Ref. 330).

2. Primary and secondary structure

The primary structure of the low-conductance (5 pS) highly Na⁺-selective and amiloride-sensitive Na⁺ channel (138, 249) has been difficult to establish because of the low abundance of the active channel protein in the different aldosterone target epithelia. ENaC was molecularly identified from rat distal colon by functional expression cloning in Xenopus oocytes (43). The channel is composed of three homologous subunits denoted α-, β-, and γ-ENaC (FIGURE 13A). A fourth subunit denoted δ-ENaC was identified in human...
tissues (334). The δ-ENaC subunit shares 37% protein sequence identity with the α-ENaC subunit and functionally can substitute for the α-ENaC to make channels in nonepithelial tissues (brain) that are not involved in transepithelial sodium transport. The distinct functional and pharmacological properties of heteromeric channels comprising αδ-subunit has been recently reviewed (125). Each subunit has two transmembrane domains (M1 and M2), yielding a protein with a large extracellular (50 kDa) hydrophilic loop (between M1 and M2) and short hydrophilic cytoplasmic NH2 and COOH termini (9 and 10 kDa) (177). The most notable feature of the extracellular loop is the presence of two highly conserved cysteine-rich domains (CRD) covering 50% of the sequence. The cysteines present in these CRD may participate in the formation of cysteine bridges (98) also conserved among the genes expressed in mammalian nervous system (MDEG 1 and 2, ASIC, and DRASIC) as well as among the degenerins and FaNaCh.

3. Tertiary and quaternary structure

There are no data about the three-dimensional crystallographic structure of ENaC. On the basis of high-resolution crystallographic structures of ASIC1 from Gouaux’s laboratory (128, 163), homology models have been proposed by Kashlan and Kleyman (174). Accordingly, ENaC is proposed to be a heterotrimer (αβγ) contrasting with the heterotetrameric structure αβαγ proposed earlier based on four indirect, but independent methods (5, 26, 177, 184). As discussed in a recent review (278), a high-resolution crystallographic structure of active (“open”) ENaC channels is needed to solve the architecture of the amiloride binding sites, the pore and the selectivity filter as well as the detailed understanding of the proteolytic-dependent gating mechanisms.

 FIGURE 13. Heteromeric structure of ENaC and conservation of cysteine-rich domain (CRD). A: ENaC is made of three homologous subunits (α, β, γ) sharing around 30–40% identity at the protein level. Each subunit has short cytoplasmic NH2 and COOH termini and two transmembrane domains (M1 and M2) with a very large (60 kDa) extracellular loop characterized by two cysteine-rich domains (CRD) and 6–12 glycosylation sites (177). B: conserved domains and their localization in ENaC/DEG family members: linear representation of the primary structure shows the conserved regions found in each of the main subfamilies. [Adapted from Kellenberger and Schild (177).] C: αC133-aC305 loop is a site of ENaC activation by serine proteases (283). αC133 makes a disulfide bridge with αC305 of human ENaC that is critical for proper folding and channel expression at the plasma membrane (98).
4. ENaC/degenerin gene family history

Phylogenetic analysis of the ENaC/Degenerin family showed that it was restricted to bilaterians (177). Giraldez et al. (125) studied the evolution of the ENaC subunit genes (SCNN1A, SCNN1B, SCNN1G, and SCNN1D) and found them in tetrapods as well as in coelacanth (Latimeria chalumnae). Recently, Uchiyama et al. (324) identified and functionally characterized α, β-, and γ- (but not δ-) ENaC subunits in the Australian lungfish Neoceratodus forsteri, a member of the subclass Dipnoi, which are close to tetrapods (324). The lungfish subunits are closely related to the corresponding amphibian subunits and were highly expressed in the gills, kidney, and rectum, strongly suggesting a role in regulating sodium transport of the lungfish, which apparently has a renin-angiotensin-mineralocorticoid system (324). One can speculate that there may have been an ENaC sodium absorption system controlled by mineralocorticoid before the conquest of land by vertebrates (324). In vertebrates, the four paralogous subunits ENaC α/β/γ/δ are likely to have evolved during the two rounds of whole genome duplications (311).

Studer et al. (311) found that ENaC/degenerin exists in all metazoans screened including nonbilaterians such as the sponge Amphimedon, the Trichoplax, and other cnidarians. This suggests its presence in ancestors of Metazoa, so its origin seemed to occur at the base of multicellular animals. By analogy of the well-defined functions of degenerins in C. elegans (29) as mechanotransducers [MEC-2 (355), MEC-4 (238) and MEC-10 (155), acid or gustatory sensors(258)], they could play similar functions in nonbilaterian animals, but more experimental analyses are needed to characterize these genes in basal metazoans.

While ENaC/degenerin seems exclusively restricted to metazoans, Studer et al. (311) identified putative ENaC homologs in Naegleria gruberi, a protozoan. This presence was unexpected, as they are the only homologs found in an organism outside metazoans. Two possible scenarios can explain that surprising finding: either there was one common ancestor at the dawn of eukaryotes, and was lost in other lineages, or it was due to a lateral gene transfer between the ancestors of Nagleria and metazoans. Again, the function of these genes in Naegleria remains to be elucidated. However, sequence comparisons identify highly conserved residues that are the most conserved signature of the entire gene family. Of special interest for the present discussion is the presence of a putative disulfide bridge in position 440 and 937 corresponding to Cys133-Cys305 in α-hENaC discussed above, important for proper protein folding and involved in one case of PHA-1 (FIGURE 13A). This cysteine belongs to the FPxxTxC motif (127–133 in α-hENaC). However, we do not know if cysteines are making cysteine bridge, as there are no structural or biochemical data for these Naegleria sequences.

Naegleria gruberi, among other Naegleria species, possesses the ability in fresh water to shift rapidly from an amoebaean to a flagellate form, involving the formation of new organelles (72, 105). This transformation can be stopped by increasing the electrolyte or osmolyte concentration (i.e., sodium or potassium chloride) in the external medium (164). One hypothesis would be that these ENaC homologs in N. gruberi could act as sodium or osmolar sensors and have the ability to detect these ionic changes, as these ion channels in animals are usually highly selective for sodium (311). Naegleria gruberi is not a human pathogen. In contrast, Naegleria fowleri is a free living, thermophilic protist that can cause primary amoebic meningoencephalitis (PAM), a fatal disease in 95% of the cases (102). At present, there is no treatment available, and one can speculate that the ENaC homologs expressed in this microbe could be a drug target for amiloride. Clearly the function of these proteins as potential amiloride-sensitive channels should be studied in detail and the sensitivity of the cell cycle of this organism to amiloride tested in vitro. FIGURE 14 summarizes the evolution of the two main effectors of the aldosterone signaling pathways as discussed in this review.

IV. PERSPECTIVES AND CONCLUSIONS

A. Phylogeny of Hominoids and Other Primates

As shown by Perelman et al. (255), based on a wealth of new genomic data, comparative genomic analyses of primates allow resolving the primate phylogeny with a much greater accuracy. The origin of anthropoids (monkey, apes, human) predates the well-known phylogenetic split between chimpanzee and human lineage that occurs 6–8 million year ago (343). Recent data by Langergraber et al. (191) confirmed the divergence time of modern humans-Neanderthals of 400,000–800,000 years and the divergence time of the human-chimpanzee to at least 7–8 million years (191). As shown by the study of higher primates and the increasing dense fossil record of the earliest anthropoid radiations, several key adaptive change characteristics of the human lineage (body mass, diet, locomotion, eye and colored vision, olfaction) take their origin much before the split between chimpanzee and human lineages (343).

Within this context, the emergence of Homo sapiens should be taken as an “accidental species” (116) and not as the product of linear evolution (with “missing links”) leading from monkey to apes and then “culminating” by the appearance of Homo as the organism of the highest complexity in nature (116). The earliest remains attributable to Homo sapiens are around 200,000 years old and were found in eastern Africa. In 1987 Allan C. Wilson published his seminal paper supporting the Out-of-Africa hypothesis stating that ancient modern humans evolved in Africa, they
spread throughout the world displacing other hominids already established in Europe (i.e., Neanderthal) in opposition to the “multiregional continuity stating that Homo sapiens evolved several times independently from various locations across the world” (116). Genomic data of today human populations together with that of ancient DNA (Neanderthal, Denisovan) together with still a very partial fossil records (specially in Africa) indicate a much more complex picture of human evolution (107, 221, 265, 288). The study of gene flow between Neanderthals and Denisovans (265) is inconsistent with a simple model in which the entire Neanderthal and Denisovan genomes come from the same source population but more consistent with the existence of highly diverged unknown archaic population (see discussion in Ref. 31). These recent findings imply the existence of many waves of immigration out of Africa with hominid populations colonizing the planet in many occasions and doing so undergoing climate and environmental changes from hot and humid in Africa to dry and cold conditions in the north of Europe and Asia. In addition, the cultural changes (discussed below) may have represented too fast of changes for natural adaptation to operate.

B. Evolution of RAAS: Implication for the Pandemia of Hypertension

Acute infectious diseases such as plague, smallpox, and influenza have decimated human populations and influenced world history, but present human populations are now threatened by worldwide epidemic of chronic diseases: obesity, diabetes, and hypertension (188, 189, 289). RAAS has important implications for the emergence of hypertension as a major health problem worldwide. The present analysis and that of Fournier et al. (101) underscore the utility of sequence comparisons in the study of evolution of specific homeostatic functions, for instance, the control of body fluid ionic composition, the control of blood volume, and blood pressure. As stated by Fournier et al. (101), “such analyses may provide new hypotheses as to how and why in today’s population an increased activity of the RAAS frequently leads to faulty salt and volume regulation, hypertension, and cardiovascular diseases, opening up new and clinically important research areas for evolutionary medicine.” The physiological importance of RAAS is underscored by its pharmacological relevance for the treatment of...
hypertension and the prevention of cardiovascular diseases (333).

Hypertension has a very high incidence in human populations. According to the World Health Organization (WHO) website (http://www.who.int/cardiovascular_diseases/publications/global_brief_hypertension/en/), worldwide hypertension is estimated to affect more than one in three adults aged 25 and over, or about one billion people. Hypertension is a major risk factor to cardiovascular diseases such as myocardial infarction, stroke, and chronic kidney failure, which together make up the world’s number one cause of premature death and disability. In countries having access to proper treatments, not surprisingly hypertension represents the most expensive public health problem. The etiology of the disease is known in only ~10% of the cases (secondary hypertension), but in 90% of the cases, no specific cause can be identified (primary or essential hypertension). As proposed by Guyton and co-workers (59, 135), however, the handling of body fluid by the kidney is a critical factor in the long-term control of blood volume and blood pressure and, when faulty, in causing hypertension (55, 56). Decreased glomerular filtration [for instance, by nephron mass reduction (205)] and/or increased tubular reabsorption of sodium are the pathophysiological mechanisms leading to abnormal pressure-natriuresis relationships. Hypertension is a multifactorial disease involving genetic and environmental factors together with risk-conferring behaviors. In this context, blood pressure is a complex trait, sensitive to a large number of individual variables (rest vs. effort, morning vs. afternoon, stress vs. no stress, etc.). A reliable and standardized measurement of blood pressure in human populations is notoriously challenging, explaining the small trait variability that can be accounted for by genome-wide association studies (see below).

1. Genetic factors

Genetic factors determine an important fraction of the variability of the trait of either the systolic and/or the diastolic blood pressure (152). In a cohort of elderly twins reared apart or together allowing to distinguish between the importance of shared rearing environments and genetic effects, Hong et al. (150) found a heritability of 44 and 34% of systolic and diastolic blood pressure, respectively, but a substantial influence of shared family effect was revealed accounting up to 27% of the variation. Despite this strong heritability of the trait, it has been difficult to identify the genes responsible for the hypertensive phenotype. The only and dramatic exception to that are rare forms of familial hypertension with Mendelian transmission as reviewed by Lifton and co-workers (198, 199). As of today, 20 genes (the great majority of them belonging to the RAAS) have been identified as genes causing a salt-losing (hypotensive) or a salt-retaining (hypertensive) phenotype (282) (FIGURE 15). These genes are either expressed in the glomerulosa of the adrenal cortex, where aldosterone is synthesized, or in the kidney. The disease-causing genes have been instrumental in identifying major regulatory pathways controlling kidney development (renal tubular dysgenesis) and the maintenance of blood pressure and renal blood flow during fetal life (132, 133), sodium/potassium balance, and blood pressure in newborns and adults but, so far, of little significance to explain the phenotype of a complex genetic disease such as essential hypertension. As discussed by Ehret (84), three main features of the disease-associated allelic variant must be considered: 1) the frequency of the variant in the population, 2) the effect size of the variant on the phenotype, and 3) the number of genetic variants determining the phenotype (84). We have just discussed above the variants with rare allelic frequency (<0.001) and large phenotypic effect size (3–50) (212) (FIGURE 15). We will now discuss the three other cases: 1) common allelic frequency (>0.05) and a small size effect (1.1 to 1.5), 2) low frequency (0.005–0.05) and intermediate size (1.5 to 3.0), and 3) common allele frequency (>0.05) with a large effect size (3–50) (212). Finally, rare variants with small effect size are obviously difficult to identify and of less physiological importance.

2. Common polymorphism with small phenotypic effect

Genetic variants (mutations) fall in different categories: 1) single nucleotide polymorphisms (SNPs, also called substitutions) in coding or noncoding regions of the genome, functional or neutral (310); and 2) insertion-deletion (indels) duplication, inversions, and copy-number variations. Historically, in 1992, Jeunemaitre et al. (166) were the first to obtain evidence of genetic linkage between the angiotensin gene (AGT) (on the RAAS pathway) and hypertension (165). Significant differences in plasma concentrations of angiotensin among hypertensive subjects with different AGT genotypes were observed, suggesting that the variants were functional. Dufour et al. (78) performed a molecular screening of the chimpanzee angiotensinogen (AGT), renin (REN), angiotensin I-converting enzyme (DCP-1), and the angiotensin II type 1 receptor (AGTR-1) genes, coding for the main molecules of the renin angiotensin pathway. They analyzed these genes in three to six chimpanzees. The authors observed that these genes, with the exception of the AGT gene, are under negative selection, as indicated by the contrast in the high diversity at the synonymous sites and the low diversity at nonsynonymous sites. They also identified that 119 sites in chimpanzee are different in humans (62 coding sites, with 17 at nonsynonymous sites). Thus the analysis of polymorphism within species and divergence between species shed light on the evolutionary constraints on these genes. Nakajima et al. (229) started from the observation that the frequency of the G(-6) variant over A(-6) in the promoter of AGT is higher in non-African populations than in African populations. The A(-6) is generally associated with higher plasma angiotensinogen levels (and increased risk of essential hypertension). This suggest that the G(-6) promoter has been selec-
Fortunately advantageous outside Africa, and thus favored by natural selection (229). More recently, Watkins et al. (341) stressed the importance of analyzing haplotypes in addition to single genotypes in association studies.

So far, genome-wide association studies (GWAS) have looked at SNPs using the HapMap genome map. GWAS have identified loci in or near genes that generally were not expected to be associated with blood pressure or essential hypertension (84). A summary of the main loci identified as of 2012 can be found in Yang et al. (348) who compared the first genome-wide gene-based association scan for hypertension in a Han Chinese population with that previously conducted in other populations worldwide (2, 50, 103, 175, 176, 192–194, 234, 244, 338) (see TABLE 1 of Ref. 348).

Overall, it appears that relatively few loci are replicated across the different GWAS studies depending on the ancestry of the population studied (Asian, European, African-American, Amish, Caucasian). Second, the overall effect on blood pressure is small and may account only 1 or 2% of the trait variability. Clearly we are still at the beginning of this kind of approach, but significant improvement in methods will allow to examine the role of other variants (indels, duplication, inversions, and copy-number variations) that have not yet been studied in any detail as far as the hypertension is concerned.

3. Common polymorphism with strong phenotypic effect

This is a rather unusual situation described for only few genes: uromodulin (247, 273, 322) with a common noncoding variant within the GRE of the promoter and corin (76, 272, 335–337, 346) with a SNP in a coding region of the
frizzle domain (R539C) found in 12% of African-Americans with hypertension. Common variants of APOL1 that confer resistance to trypanosomal infections in many regions of Africa are linked to the development of CKD (thus indirectly to hypertension) in African-American populations (250, 340).

A) UROMODULIN. As reviewed by Rampoldi et al. (273), uromodulin is the most abundant protein excreted in the urine under physiological conditions. It is exclusively produced as a membrane-bound protein in the thick ascending limb and secreted into the urine by proteolytic cleavage. Strong genetic evidence associates UMOD risk variants with disease susceptibility in the general population (247), but the underlying mechanism remained elusive until the recent work of Trudu et al. (322). These authors clearly established experimental criteria to link genetic susceptibility to hypertension to the level of uromodulin expression and uromodulin’s effect on salt reabsorption in the kidney. Consistent with these findings, Graham et al. (129) found that systolic blood pressure was significantly lower in Umod knockout versus wild-type mice under standard salt diet. Umod knockout mice blood pressure were resistant to the administration of saline, whereas the wild type were salt sensitive.

B) CORIN. As reviewed by Wu et al. (346), atrial natriuretic peptide (ANP) is produced in heart atrium and is an important regulator of salt and body-fluid balance. In heart cells, ANP is made as a precursor form (pro-ANP) that is converted in a sequence-specific manner to active form (ANP) by the ANP-converting enzyme corin, a transmembrane serine protease. Dries et al. (76) identified in the corin gene 2 nonsynonymous, nonconservative single nucleotide polymorphisms in near-complete linkage disequilibrium, thus defining a single minor corin gene allele. This allele was present in the heterozygote state in 12% of African-Americans, but was extremely rare in Caucasians. The minor allele was associated with higher blood pressure and an increased risk for prevalent hypertension and was an independent predictor of left ventricular mass (76). As suggested by Rame et al. (272), the corin polymorphism was associated with impaired corin zymogen activation as also shown by Wang et al. (336) and Dong et al. (74). Supporting the role of corin in the control of blood pressure, corin KO mice became hypertensive on a high-salt diet, whereas the littermate control remained normotensive (335).

4. Rare independent alleles with strong phenotypic effect on blood pressure variations

As discussed by Matullo et al. (214), the allelic frequency spectrum emerging from several Next Generation Sequencing (NGS) projects allows identification of new low-frequency and rare variants. The main limitation of this approach is that it requires very large sample sizes to achieve sufficient statistical power. For the control of blood pressure, whether risk alleles comprise a small number of common variants or many rare independent mutations at trait loci is still largely unknown. To address this question, Ji et al. (167) screened members of the Framingham Heart Study for variation in three genes (SLC12A3, SLC12A1, and KCNJ1) causing rare recessive salt-losing syndromes with low blood pressure (167). The authors identified subjects with functional mutations producing significant blood pressure reduction and protecting the general population from development of hypertension (167). The frequency of carrier for some mutations in SLC12A3 coding for the sodium chloride cotransporter NCC (the receptor to thiazide diuretics) is estimated to be as high as 1% in the population. These mutations may provide to the heterozygote subject a selective advantage by lowering blood pressure compared with the controls (60).

5. Environmental factors and risk-confering behaviors

The major risk factors for the development of hypertension are 1) excess weight and obesity, 2) lack of physical training, 3) drugs (i.e., contraceptive pill), 4) smoking, and 5) salt intake. In the context of this review, salt intake is of special interest. Epidemiological data among human populations around the world support a strong relationship between the average daily intake of sodium chloride and the incidence of hypertension (218–220). As described by Oliver et al. (242), the Yanomami Indians from northern Brazil and southern Venezuela do not use salt in their diet (“no salt culture”) presenting an unusual opportunity to study the hormonal regulation of sodium metabolism with parallel observations on blood pressure. Urinary excretion of sodium averaged only 1 mmol/day, and blood pressure remained normal during the entire life. Sodium balance was achieved by a strong stimulation of RAAS with high plasma renin activities and high urinary secretion of aldosterone (242). Aldosterone excretion rate in the Yanomami Indians (Salt-Scarce World: <0.5 g/day) is >25-fold higher than that of individuals (today) who consume a salt-replete diet (>10 g/day). As pointed out by the authors, these elevated levels of aldosterone and renin were probably the norm for Homo sapiens during much of human evolution and suggested that the values observed in controls from developed countries are depressed by an excessive salt intake in contemporary diets (86, 242).

Luft and co-workers (203, 204) studied the effect of increasing salt intake from 0.25 to 28 g/day on young healthy human volunteers. About 30% were “salt-sensitive” and became overtly hypertensive, whereas 60% were normotensive and “salt-resistant.” Similar studies performed on hypertensive patient showed that up to 50% are sensitive to salt intake (203, 204). African-American populations have a blunted natriuretic response upon salt loading, suggesting salt sensitivity and corresponding to a higher prevalence of hypertension in African-Americans than in Caucasians.
(203, 204). Together, these studies suggest that susceptibility genes in the human populations may determine salt sensitivity or salt resistance. Since long-term studies of high salt intake in human are not ethically acceptable, Denton et al. (68) studied salt sensitivity in a cohort of chimpanzees, the closest species to human. After 18 mo of increased salt intake (15 g/day), there was a highly significant increase in blood pressure in 60% of the animals, that was reversible upon return to a low-sodium high-potassium diet. On a chronic Western diet, a colony of chimpanzees raised in the United States became severely hypertensive with age. This study suggests the presence of susceptibility genes in chimpanzees as also proposed for humans. The topic has been extensively reviewed by Meneton et al. (219), and many additional references will be found supporting their conclusion that “hypertension and cardiovascular diseases in human populations are causally correlated to high salt intake.”

C. Possible Origin of the Pandemia of Hypertension: A Gene-Culture-Environment Mismatch?

In 1962, James Neel (232) proposed his “Thrifty gene hypothesis” stating that “genes that predispose to diabetes were selected in human populations of hunter-gatherers, who were undergoing rapid change in the amount of food available.” These naturally selected genes would allow individuals to efficiently absorb food and transform it into fat during period of food abundance. This period of (relative) obesity would allow the hunter-gatherer to survive better during periods of famine. Natural selection would have selected these “thrifty” genes the paleolithic (Stone Age) (-3 million year to -12,000 years, beginning of the neolithic). Since the neolithic with the appearance of agriculture, famine became less frequent and food abundance reached the high levels we know today. This would thus explain the recent pandemia of obesity and diabetes. Neel’s hypothesis of positive selection has been contested. The debate is still ongoing and alternative hypothesis (“genetic drift”) was proposed (264, 304).

Could Neel’s hypothesis be applied to the control of sodium homeostasis and be valid to explain the pandemia of hypertension? The idea that “salt” genes have been naturally selected during the early history of hominids is plausible. Indeed, these populations lived in a hot and tropical environment. Homo sapiens developed an efficient mechanism to control the body temperature with the ability to lose heat through sweat glands. But this was not achieved without some obligatory loss of sodium and chloride. On the other hand, these populations were living in a scarce or “no salt” world (very low sodium/high potassium diet) like some populations of hunter-gatherers still surviving in tropical climate in Africa or Amazonia. One could speculate that salt retaining genes were critical for survival during the life cycle of hunter-gatherers, for example, during neonatal development. Bartter syndrome and PHA-1 are caused by loss-of-function mutations of two sodium transport proteins expressed in the nephron (NKCC2 and ENaC respectively) and characterized by perinatal lethality. Likewise, without a strong RAAS, diarrheas, a common disease, in newborn babies and infants may cause very high lethality without an efficient RAAS. Before the immigration of Homo sapiens out of Africa, it is likely that the main genes of the RAAS were positively selected for retaining salt. During the neolithic period, the development of food preservation by salting made food available in all seasons. Going rapidly (in term of evolution) from a daily intake of 0.5 g salt to an average of 10 g/day in most Westernized countries did not allow adaptation by selection of “salt-losing” genes with as consequence a pandemic of hypertension. Moreover, the selective pressure may even be minimal because hypertension becomes predominant after reproduction and does not seem to affect fertility to any significant extent. Acute infectious diseases are increasingly considered as they key drivers of genetic selection. Interactions between salt handling and susceptibility to pathogens could reconcile these two potential mechanisms as discussed above for CKD (340).

Overall, the “salt-retaining” gene hypothesis is appealing but not yet fully supported by available evidence. Many questions remain unsolved. First, the genetic evidence reviewed above is still quite preliminary and incomplete. Second, as reviewed by Turner et al. (323), we still have little objective information about the “paleolithic” diet of our hunter-gatherer ancestors. Moreover, as stated by the authors, “human eating habits are learned primarily through behavioral, social and physiological mechanisms; thus adaptations that appear to be strongly genetic may reflect Neolithic rather than Paleolithic adaptations” (323).

D. Concluding Remarks

This review is an attempt to put the RAAS into an evolutionary perspective. Clearly some important aspects could not be discussed here. The question of the selection and adaptation in the human genome is becoming a key question for further investigation (108). The detection of adaptive evolution from genomic analyses identifies genes potentially subject to positive selection either at the protein or regulatory level (108). Natural selection acts on the human genome across many timescales: long-term selection in primate (−20 million to −1 million years), hominin-specific selection (−1 million to 100,000 years), and finally recent human selection (−100,000 year to present) (108). The detection of adaptive evolution from interspecific divergence allows the identification of genes potentially subject to positive selection either at the protein or regulatory level (108). A major research effort is also to detect adaptive evolution from intraspecific polymorphisms. In a few in-
stances, *Homo sapiens* genes undergoing adaptive evolution have been identified: lactase persistence by the expression of the lactase (LCT) gene was correlated with the ability to digest dairy products throughout the adulthood (261, 315). Another case is that of a gene, *EPAS1* or *HIF2α* (coding for a transcription factor involved in hemoglobin concentration), conferring resistance to hypoxia to populations living at high elevation (Tibetan, Andean) (22, 350).

We have reviewed the possible importance of common polymorphisms in a few genes coding for proteins of RAAS (angiotensinogen, corin, uromodulin). Interestingly, they code for regulatory proteins, whereas the final renal effectors, i.e., sodium transporters (NKCC2, NCC, ENaC), have not been reproducibly identified in GWAS or other association studies. Further haplotype analysis in *Homo sapiens* and ancient hominids may bring new information. Alternative models of adaptive evolution based on selection on standing variations (i.e., acting simultaneously on multiple independent loci) (108, 139, 140, 142) may be highly relevant to the field of hypertension. Evolutionary medicine is an emerging field with a great potential for the identification of novel diagnostic tests, drug targets, and therapies.

**ACKNOWLEDGMENTS**

We thank David Warnock, Larry Palmer, Olivier Devuyst, Qais Al-Awqati, Nicolette Farman, and Gabriel Markov for their thoughtful comments and suggestions.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**REFERENCES**


EVOLUTION AND ALDOSTERONE REGULATION OF SODIUM TRANSPORT


