J. Membrane Biol. 90, 193–205 (1986)

The Journal of
Membrane Biology

Topical Review

Mechanisms of Aldosterone Action in Tight Epithelia

Haim Garty
Department of Membrane Research, The Weizmann Institute of Science, Rehovot 76100, Israel

I. Introduction

Since the discovery of aldosterone in the early 1950's (Simpson & Tait, 1952) this adrenocorticosteroid has been recognized as a potent regulator of electrolyte metabolism in all vertebrates (Forman & Mulrow, 1975). In mammals the target tissues for the hormonal action are: the distal segments of the renal tubules, the urinary bladder, the descending colon mucosa, and the salivary and sweat glands. In all of them an aldosterone-induced increase of Na^+ reabsorption was observed (Crabbé, 1963*b*; Sharp & Leaf, 1973; Taylor & Palmer, 1982). Most of the present knowledge on the natriferic action of this hormone came, however, from studies on amphibian tight epithelia and in particular the toad urinary bladder. This classical model system for the mammalian distal nephron was chosen by many workers because of its relative histological simplicity and the ease with which its Na^+ transport can be monitored by short-circuit current recordings (for review *see* Macknight, DiBona & Leaf, 1980). In biochemical studies, on the other hand, rat and rabbit kidney segments, which provide larger amounts of starting material, were often preferred.

An *in vitro* aldosterone-induced stimulation of Na^+ transport in toad bladder was first reported by Crabbé (1961, 1963*a*). Shortly afterwards it was shown that the hormonal effect involves the induction of protein synthesis and also depends on the availability of metabolic substrates (Edelman, Bogoroch & Porter, 1963; Porter, Bogoroch & Edelman, 1964; Porter & Edelman, 1964; Crabbé & DeWeer, 1964; Sharp & Leaf, 1964*b*). Since then research has been carried out in four main areas:

1) Characterization of the cytoplasmic and nuclear aldosterone binding sites.
2) Identification of the hormone-induced RNA and proteins.
3) Study of aldosterone-induced biochemical changes which presumably mediate the physiological responses.
4) Electrophysiological measurements of the hormonal effects on Na^+ transport and identification of the transporting units influenced by it.

It is generally accepted that the interaction of aldosterone with target cells brings about alteration of gene expression and enhanced synthesis of a group of proteins termed the aldosterone-induced proteins (AIP). These proteins induce a number of biochemical responses, e.g. activation of mitochondrial enzymes and increased lipid metabolism, which eventually lead to an increased transepithelial transport rate. Unclear yet is the nature of the AIP, the molecular mechanisms involved in the intracellular and plasma membrane hormone-induced changes, and the relevance of the biochemical events identified, to the physiological responses.

This review attempts to summarize the current data concerning the action of aldosterone in tight epithelia, point out some gaps in our knowledge, and suggest working hypotheses for the molecular mechanisms by which the hormone regulates Na^+ transport. The discussion of data accumulated so far will be limited to that which presumably are relevant to the changes in Na^+ transport, with special emphasis on studies using tight amphibian epithelia. Additional functions of the hormone, e.g. promotion of K^+ excretion or H^+ secretion, will not be discussed in this article.

II. Aldosterone Effects on Tight Amphibian Epithelia—General Considerations

Na^+ transport across the toad urinary bladder and other related tight epithelia is a two-step process as

Key Words aldosterone · mineralocorticoids · Na^+ channels · Na^+/K^+ ATPase · Na^+ transport · tight epithelia · toad bladder

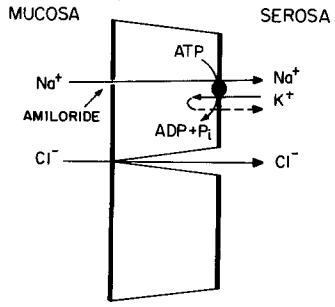

Fig. 1. Mechanisms of ion transport across tight amphibian epithelia. Na^+ enters the cell from the lumen through an amiloride-blockable channel and is pumped to the serosal side by the (Na^+/K^+)ATPase. K^+ accumulated in the cell leaks back to the serosa and the transepithelial flow of Na^+ is accompanied by a passive movement of Cl^-. Cl^- flows either through the paracellular shunt or mitochondria rich cells

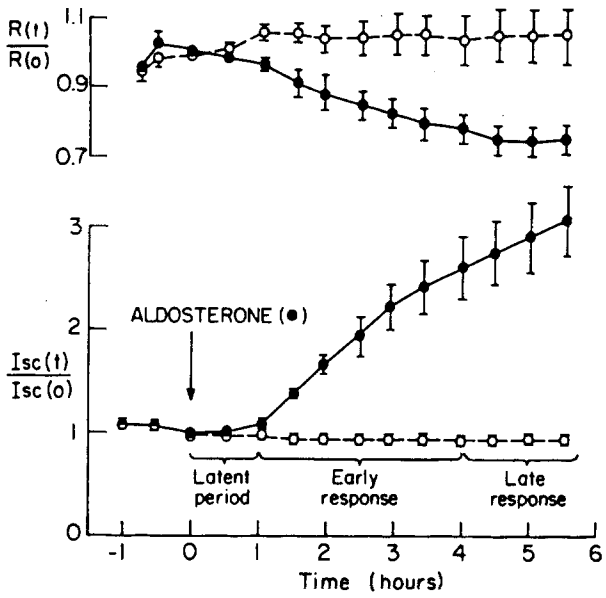

Fig. 2. A characteristic response of toad urinary bladder to aldosterone. The hormone evokes more than threefold increase of the short-circuit current (I_{sc}) and about 30% decrease of the transepithelial resistance (R). The time course of the fractional current ($I_{sc}(t)/I_{sc}(o)$) and resistance ($R(t)/R(o)$) changes can be divided into three phases: the "latent period," the "early response" and the "late response." (Replotted from data by Spooner and Edelman (1975) with permission of Elsevier Biomedical Press, B.V.)

originally suggested by Koefoed-Johnsen and Ussing (1958) for frog skin. According to this well-established scheme, Na^+ ions passively diffuse from the lumen into the cell through an amiloride-blockable apical pathway, and are later pumped to the interstitial space by the basolateral (Na^+/K^+) ATPase (Fig. 1). The K^+ ions accumulated in the cell by this process leak back to the interstitial space. The transepithelial movement of Na^+ is also accompanied by a mucosal to serosal flow of Cl^-, presumably through the paracellular pathway and/or the mitochondria-rich cells (Voute & Meier, 1978; Macknight et al., 1980; Larsen & Rasmussen, 1982; Taylor & Palmer, 1982). Transport measurements in this tissue are usually done by recording the transepithelial short-circuit current (I_{sc}), which for identical mucosal and serosal solutions is equal to the net Na^+ flow.

Analyses of the amiloride-induced fluctuations in I_{sc} provided direct evidence that the apical Na^+ entry is mediated by a Na^+-specific channel, which under physiological conditions conducts 10^6–10^7 ions/sec (Lindemann & Van Driessche, 1977; Palmer, Li, Lindemann & Edelman, 1982; Lindemann, 1984). The current voltage relationships of these channels are in good agreement with the Goldman-Hodgkin-Katz equation (Hodgkin & Katz, 1949) over a wide range of potentials and Na^+ concentrations (Fuchs, Hviid-Larsen & Lindemann, 1977; Palmer, Edelman & Lindemann, 1980; Thomas, Suzuki, Thompson & Schultz, 1983; DeLong & Civan, 1984; Lindemann, 1984). Thus, the translocation of Na^+ ions across the apical membrane can be described as a simple diffusion in a constant electric field; voltage gating phenomena or ion-ion interactions are probably not involved.

The characteristic response of a mounted toad bladder to exogenous aldosterone is composed of two distinct phases (Fig. 2). (i) A latent period of 30–90 min during which the hormone induces mRNA, protein, and lipid synthesis but I_{sc} is unaffected. (ii) A natriferic response of up to 8 hr during which I_{sc} increases by two to fourfold, the transepithelial electrical resistance decreases, and other intracellular processes take place. It was suggested that the current changes during this period can be divided into 'early' and 'late' responses which reflect different hormonal actions on the apical and basolateral membranes, respectively (Spooner & Edelman, 1975; Geering et al., 1982; Truscello, Geering, Gaggeler & Rossier, 1983). Removing the hormone from the bathing solutions of an activated bladder reverses the current increase with a $t_{1/2}$ of about 7 hr (Lahav, Dietz & Edelman, 1973).

The scheme of Fig. 1 suggests three possible target sites for the natriferic action of aldosterone:

1) Facilitation of Na^+ entry by an increase in the number of apical channels or in their "turnover" rate.
2) Enhancement of Na^+ exit by an increase in the number of basolateral pumps or in their affinity to Na^+.

3) Elevation of the ATP/ADP ratio that drives the Na^+/K^+ exchange by activating mitochondrial oxidative phosphorylation.

The exact site of hormonal action was subject to a long debate, and evidence in favor of each of the above possibilities were presented. It is quite clear today that aldosterone acts on all the above target processes, i.e., it increases the apical Na^+ permeability, enhances the activity of several mitochondrial enzymes, and induces the synthesis of (Na^+/K^+)ATPase. Thus, at least the long term enhancement of transport can be viewed as the result of a multiple hormonal action at several sites rather than the modulation of one pathway only. Such a coordinated hormonal action, proposed by Lipton and Edelman (1971), may have two advantages: (i) it enables the elevation of transepithelial Na^+ transport without inducing an acute change in the cytoplasmic Na^+ activity (Schultz, 1981). (ii) It prevents a situation in which a step not effected by aldosterone will become rate limiting and tend to diminish the overall stimulation of transport.

III. Initial Events in the Hormonal Action

A. The Aldosterone Receptors

The accepted scheme for the initial stages of aldosterone-target cell interaction is that the hormone enters cells by mere diffusion, binds to a cytoplasmic receptor, and is translocated into the nucleus in a temperature-sensitive process. In the nucleus the hormone-receptor complex binds to acceptor sites on chromatin and induces RNA and protein synthesis (Edelman, 1978, 1979; Edelman & Marver, 1980). This mechanism, which appears to be a general mode of action of steroid hormones, is based on the identification and isolation of aldosterone binding sites from both cytosolic and nuclear fractions of target cells and their specific binding to chromatin (Fanestil & Edelman, 1966; Herman, Fimognari & Edelman, 1968; Ludens & Fanestil, 1971; Marver, Goodman & Edelman, 1972). The significance of the cytoplasmic binding sites was questioned recently, and the possibility was raised that these are in fact nuclear components which leak to the cytoplasmic fraction during the separation (Bonvalet, Manillier & Farman, 1984). Both the cytoplasmic and nuclear aldosterone-binding macromolecules were identified as proteins. ^{3}H-aldosterone could be dissociated from these sites by proteases and sulfhydryl reagents but not by nucleases or lipases (Fanestil & Edelman, 1966; Herman et al., 1968).

Further studies led to the identification of two distinct classes of binding sites, with low and high affinity to the hormone, in rat kidney (Funder et al., 1973) and toad bladder (Farman, Kusch & Edelman, 1978; Kusch, Farman & Edelman, 1978; Claire et al., 1985). In toad bladder the high affinity sites (type I) are characterized by a K_d value of 2.7×10^{-9} M and a concentration of 1.5×10^{-13} mole/mg DNA. The low affinity sites (type II) have a K_d value and a concentration of 4.6×10^{-7} M and 1.5×10^{-12} mole/mg DNA, respectively (Farman et al., 1978).

Four lines of evidence indicate that the high affinity sites are the receptors which mediate the mineralocorticoid effects: (i) A correlation was established between the occupancy of these sites and the transport changes (Farman et al., 1978; Rossier et al., 1980, 1983). Geering et al. (1985), however, suggested that only the early increase in I_{sc} correlates with the occupancy of type I sites, while the latter induction of (Na^+/K^+)ATPase requires binding to both the high and low affinity receptors. (ii) The affinity of various steroid agonists to type I sites correlates with their mineralocorticoid potency. Type II sites, on the other hand, also bind steroids with no, or very little, mineralocorticoid activity (Rousseau et al., 1972; Funder et al., 1973; Kusch et al., 1978). (iii) Antimineralocorticoids such as spirolactones displace ^{3}H-aldosterone from its binding sites and form inactive complexes which do not interact with chromatin (Marver et al., 1974; Claire et al., 1979; Rossier et al., 1980, 1983). (iv) Perfusing adrenalectomized rats, with aldosterone and other mineralocorticoids, downregulates the number of type I, but not type II, sites (Claire et al., 1981; Rafestin-Oblin et al., 1984). These data and the high affinity of type II sites to glucocorticoids led to the suggestion that the high affinity sites are the mineralocorticoid receptors and the low affinity sites are presumably glucocorticoid receptors (Rousseau et al., 1972; Farman et al., 1978; Kusch et al., 1978). According to this interpretation, an important criterion for identifying an aldosterone-induced event as a process related to its mineralocorticoid action, is either to demonstrate its induction by low doses of the hormone that will occupy the high affinity site only, or to study the relative potency of both mineralo- and gluco-corticoids.

Two nuclear steroid binding sites were also found in cultured toad kidney (A6) and toad bladder (TB6c) cells (Handler et al., 1981; Watlington, Perkins, Munson & Handler, 1982; Pratt & Johnson, 1984). However, in the cultured epithelia the enhancement of Na^+ transport was correlated with the occupancy of the lower affinity site and corticosterone was more potent in stimulating I_{sc} than aldosterone. This difference may result from mutations in the cultured cells which may have led to the re-

Table. Aldosterone-induced proteins from toad bladder and A6 cells[a]

Additions		Resolving method		AIP		Reference
Treated cells	Control cells		Fraction	Molecular weight	Iso-electric point	
Aldosterone (10^{-6} M)	Diluent	SDS gel electrophoresis	Total protein extract	12,000		Benjamin & Singer, 1974
(−) Dexamethasone (10^{-6} M)	Diluent					
Aldosterone (2×10^{-8} M)	Diluent	A combination of methods	Cytosol	17,000–38,000	4.5	Scott & Sapirstein, 1975
(−) Corticosterone (2×10^{-8} M)	Diluent					
Aldosterone (7×10^{-8} M)	Diluent	SDS gel electrophoresis	Membrane cytosol	12,000, 85,000, 170,000 6,000, 12,000, 36,000		Scott et al., 1978
Aldosterone (7×10^{-8} M)	Aldosterone + cycloheximide	SDS gel electrophoresis	Membrane cytosol	85,000, 110,000, 170,000 6,000, 36,000		Reich et al., 1981
Aldosterone (1.8×10^{-7} M)	Diluent	SDS gel electrophoresis	Membrane cytosol	15,000 70,000		Geheb et al., 1981*a*
Aldosterone (1.4×10^{-7} M)	Diluent	2-DG				Geheb et al., 1981*b*
Aldosterone	Aldosterone + actinomycin D		Membrane cytosol	70,000–80,000 70,000	5.8–6.4 ~6.0	
(−) Cortisol (5×10^{-8} M)	Diluent					
Aldosterone (10^{-7} M)	Diluent	2-DG	Membrane	70,000–80,000	5.6–6	Blazer-Yost et al., 1982
Aldosterone (1.4×10^{-7} M)	Aldosterone + spironolactone	2-DG	Membrane cytosol	70,000–80,000 70,000	5.8–6.4 ~6.0	Geheb et al., 1983

[a] Summary of the aldosterone-induced proteins identified by comparing the incorporation of radioactively labeled amino acids into proteins in steroid-stimulated (treated) and control tissues. The study by Blazer-Yost et al. (second to last row) was done on A6 cultured cells; all the other data come from measurements in toad bladder. The (−) sign refers to a treatment that failed to induce the proteins specified, and 2-DG is an abbreviation for a two-dimensional gel electrophoresis.

placement of one receptor by the other. It is, however, unlikely that both the A6 and TB6c lines, established from different organs of different toad species, had developed the same mutation. An alternative possibility is that the stimulation of Na^+ transport in cultured cells is mediated by a different chain of events than in the naturally occurring epithelia.

B. The Induction of RNA

An aldosterone-induced synthesis of a specific class of nonmethylated RNA sedimenting at 9–12 S was first reported by Rossier, Wilce and Edelman (1974). These findings were extended by Wilce, Rossier & Edelman (1976*a*), who demonstrated an aldosterone-induced incorporation of ^3H uridine and ^3H adenosine into 7S, 12S and 18S poly(A) (+) RNA isolated from the cytoplasmic fraction of toad bladder cells. Both studies implied that the aldosterone-induced RNA is mRNA, and in both cases enhanced labeling of RNA was observed during the first 30 min of the hormonal action, i.e., before the increase in short-circuit current.

Three lines of evidence suggest that the induction of this putative mRNA by aldosterone does initiate its natriferic action. First, the incorporation of radioactive precursors into the RNA species was blocked by spironolactone (SC 9420), and it was not elicited by the glucocorticoid cortisol, or the inactive isomere 7-α-isoaldosterone (Rossier et al., 1974). Second, a linear relationship between the fractional change in Na current and the labeling of 12S cytoplasmic mRNA was established (Rossier, Wilce & Edelman, 1977*a*). Third, inhibiting RNA synthesis with actinomycin D or 3′-deoxyadenosine (cordycepin) also inhibited the aldosterone-induced increase in Na^+ transport (Chu & Edelman, 1972; Lahav et al., 1973). On the basis of the differential effects of these inhibitors on the incorporation of ^3H uridine into poly (A) (+) RNA and the increase of I_{sc}, Rossier, Gaggeler and Rossier (1978) concluded that synthesis of both poly (A)(+) and poly (A)(−) RNA is required for the natriferic action.

Aldosterone was also found to promote the synthesis of nuclear and cytoplasmic rRNA (Wilce, Rossier & Edelman, 1976*b*). However, the role of this process in the physiological response to the hormone is not clear; inhibiting the incorporation of labeled uridine into rRNA by 3′-deoxycytidine had no effect on the stimulation of Na^+ transport (Rossier et al., 1977*b*).

C. Aldosterone-Induced Proteins

In several studies, summarized in the Table, proteins whose synthesis is stimulated by aldosterone were identified. The strategy used in these studies was to compare the incorporation of labeled amino acids into polypeptides in control and hormone-stimulated cells. In other cases that will be discussed separately, enzymatic assays and immunoprecipitations were employed to test for the possible induction of specific proteins (Law & Edelman, 1978; Geering et al., 1982). Characterization of the AIP by means of one-dimensional gel electrophoresis required pooling of tissue from a large number of animals and gave variable results (Scott & Sapirstein, 1975; Scott, Reich, Brown & Young, 1978; Reich, Skipski & Scott, 1981). Much higher sensitivity and better reproducibility were achieved by applying the two-dimensional gel electrophoresis (Geheb, Huber, Hercker & Cox, 1981*b*; Geheb, Alvis, Hercker & Cox, 1983). The polypeptides identified by this method were quite different from those reported before and were composed of a cluster of four to six membrane-bound proteins (M.W. 70,000–80,000, PI ~ 5.8–6.4) and a single cytosolic protein (M.W. ~ 70,000, PI ~ 6.0). Similar membrane proteins were also found in cultured toad kidney cells (Blazer-Yost et al., 1982). Synthesis of these AIP could be induced by submaximal doses of aldosterone, it was inhibited by spironolactone, and could not be induced by non-natriferic concentrations of cortisol (Geheb et al., 1981*b*, 1983). In addition, blocking Na^+ transport by amiloride or enhancing it by ADH and theophylline had no effect on protein induction. These data prove that the AIP identified by the two-dimensional gel electrophoresis are indeed mineralocorticoid specific, and their synthesis is not secondary to changes in Na^+ transport.

The identification and physical characterization of mineralocorticoid-induced proteins has not yet provided any information on their cellular functions or possible role in the natriferic response. Although it is clear that the stimulation of Na^+ transport depends on *de novo* protein synthesis, one may not exclude the possibility that the specific proteins identified in the above studies are involved in other functions of the hormone (e.g. H^+ or K^+ transport). The time course of the AIP synthesis or their subcellular location (apical, basolateral or inner membrane) are not known, and a correlation between the labeling of proteins and transport changes has not yet been established. It is feasible, however, that with the aid of the recently available procedures for quantitating AIP synthesis (Cox & Geheb, 1984), and fractionating toad bladder plasma membrane (Chase & Al-Awqati, 1981), it will soon be possible to shed more light on the role of these proteins in the natriferic response.

IV. Biochemical Changes Elicited by Aldosterone

A number of aldosterone-induced biochemical processes which presumably mediate the natriferic action of the hormone were reported and explored. These processes are: Activation of mitochondrial enzymes (Kirsten, Kirsten, Leaf & Sharp, 1968; Kirsten, Kirsten & Sharp, 1970), changes in lipid metabolism and membrane phospholipid composition (Goodman, Allen & Rasmussen, 1971; Goodman, Wong & Rasmussen, 1975; Lien, Goodman & Rasmussen, 1975), and dephosphorylation of cytoplasmic and membrane-bound proteins (Liu & Greengard, 1974, 1976). In principle a biochemical reaction that mediates the physiological action of the hormone should fulfill three criteria:

1) It has to be mineralocorticoid specific, i.e., the ability of various steroids to induce it should correlate with their potency as stimulators of Na^+ transport.
2) Its activation should precede or coincide with the transport changes, and a positive correlation has to exist between the amount of the "biochemical product" and the increase of I_{sc}.
3) Specific blocking of this process should prevent the natriferic action of aldosterone. On the other hand, inhibiting the transport itself (by amiloride, ouabain, or Na^+-free solutions) should not influence it.

Not all of these criteria were thoroughly examined in each of the above studies. Nevertheless, the current data indicate that both activation of mitochondrial enzymes and changes in lipid metabolism mediate the aldosterone-induced increase in Na^+ transport. In addition, protein phosphorylation/dephosphorylation reactions might be involved, as well.

A. Aldosterone Effects on Energy Metabolism

Kirsten et al. (1968) reported that aldosterone increases the enzymatic activity of several mitochondrial enzymes, and in particular citrate synthase. This process could be blocked by actinomycin D or puromycin and was insensitive to the removal of mucosal Na^+. Aldosterone effects on mitochondrial enzymes had the same time course as the stimula-

tion of Na^+ transport, and a positive correlation was established between the aldosterone-induced fractional change in citrate synthase activity and I_{sc}. In a later work (Kirsten et al., 1970) the activation of the TCA cycle enzymes was shown to be mineralocorticoid specific, and insensitive to the stimulation of Na^+ transport by vasopressin or cAMP. An aldosterone-induced activation of citrate synthase has also been shown in rat kidney (Kirsten & Kirsten, 1972) and isolated rabbit cortical nephrons (Marver & Schwartz, 1980). Law and Edelman (1978) isolated citrate synthase from rat kidney and reported that aldosterone added either *in vivo* or *in vitro* increased the incorporation of [^{35}S]-methionine into the enzyme by 30–55%. In cultured toad bladder and toad kidney cells, on the other hand, aldosterone did not influence citrate synthase activity (Johnson & Green, 1981; Handler et al., 1981). This observation led Johnson and Green to suggest that the activation of this enzyme, even though it takes place in a number of target tissues, is not essential for the natriferic response. The difference between these cell lines and the natural epithelia may, however, stem from the fact that in the cultured cells the augmentation of Na^+ transport is induced by the occupancy of a "glucocorticoid-like" receptor (Watlington et al., 1982; Pratt & Johnson, 1984).

Other evidence for enhanced energy metabolism associated with the action of aldosterone on toad bladder are the observed increases in the tissue ATP/ADP/P_i ratio (Cortas et al., 1984), and the thermodynamic affinity of the metabolic reaction coupled to Na^+ transport (Saito, Essig & Caplan, 1973; Beauwens, Beaujean & Crabbé, 1982). It was also shown that an adequate supply of metabolic substrates is a prerequisite for the hormone-induced stimulation of I_{sc} (Edelman et al., 1963; Sharp & Leaf, 1964*b*, 1965, 1966; Fimognari, Porter & Edelman, 1967). Accordingly, the application of aldosterone to bladders maintained in substrate-free media failed to enhance Na^+ transport. A later addition of glucose or pyruvate to such substrate depleted tissues, evoked an immediate increase of I_{sc} which was large in aldosterone-treated bladders and much smaller in the control, aldosterone depleted, bladders. In the past it was assumed that the only energy-dependent step involved in the action of aldosterone is the active extrusion of Na^+ ions across the basolateral membrane. Recent studies revealed that the apical Na^+ permeability is coupled to cell metabolism, too (Palmer et al, 1980; Garty, Edelman & Lindemann, 1983). Accordingly, the amiloride-blockable apical conductance could be strongly reduced by metabolic inhibitors such as 2-deoxyglucose or oxythiamine, and restored upon the removal of the inhibitors and the addition of glucose or pyruvate. The inhibitory effect of 2-deoxyglucose and oxythiamine was demonstrated in ouabain-blocked bladders maintained in Na^+-free solutions (Garty et al., 1983). Therefore, the observed changes in apical conductance are not secondary to the inhibition of the (Na^+/K^+)ATPase and elevation of the cytoplasmic Na^+ activity. These findings raise the possibility that metabolic energy is required for the facilitation of passive Na^+ entry, as well as for its active extrusion. An aldosterone-induced enhancement of energy metabolism may therefore be essential for the primary natriferic response.

B. Aldosterone Effects on Phospholipid Metabolism

Goodman et al. (1971, 1975) studied effects of aldosterone on lipid metabolism in toad bladder epithelium, by analyzing the incorporation of radioactivity from ^{14}C glucose, ^{14}C pyruvate, and several lipogenic precursors into various lipid fractions. The hormone was shown to stimulate both fatty acid synthesis and phospholipid deacylation/reacylation. As much as a 75% increase in the incorporation of ^{14}C from glucose into diglycerides was observed 20 min after the application of aldosterone, suggesting that the activation of lipid metabolism is one of the earliest aldosterone-induced events. At longer times (e.g. 6 hr) the hormone increased the weight percentage of several polyunsaturated phospholipid fatty acids. These effects could be blocked by inhibitors of RNA and protein synthesis (Lien et al., 1976). Evidence that the alterations in membrane phospholipid composition are involved in the natriferic action of aldosterone was provided by demonstrating that 2-methyl-2-[-*p*-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy] propionic acid (TPIA), an acetyl-CoA carboxylase inhibitor, prevents both the hormone-induced changes in lipid metabolism and the increase in Na^+ transport (Lien et al., 1975). A possible role of the ongoing phospholipid synthesis in the stimulation of Na^+ transport had been suggested by Scott, Reich and Goodman (1979) who noted that TPIA inhibited the aldosterone-induced labeling of membrane proteins. These authors proposed that the alteration in membrane lipids either accelerates the insertion of, or decreases the exit of, AIP into or out of the membrane. Since no information is currently available on the subcellular site of the altered phospholipids (apical, basolateral, or inner membrane) it is hard to speculate at this stage how the above events are related to transport changes.

C. Aldosterone-Induced Protein Dephosphorylation

Liu and Greengard (1974) reported that incubating toad bladder slices with aldosterone increased the endogenous dephosphorylation of a specific protein (M.W. 49,000), present both in the membrane and cytosol fractions. Half-maximal dephosphorylation of this protein, designated protein D, was achieved with 20–40 nM aldosterone, a concentration similar to the amount required to produce a 50% increase of I_{sc}. The hormonal effect on protein dephosphorylation was inhibited by spironolactone, actinomycin D, cycloheximide and puromycin, i.e., it was mediated by the occupancy of the aldosterone receptor and dependent on the induction of mRNA and protein synthesis. Since aldosterone increased the rate of *in vitro* dephosphorylation of protein D but had no effect on its phosphorylation (by externally added [^{32}P]-ATP and endogenous kinase), it had been concluded that the hormone induces the synthesis of protein phosphatase rather than affecting the amount of protein D or the membrane-bound kinase. Removal of ^{32}P from protein D could also be induced by other stimulators of Na^+ transport such as cAMP and vasopressin (DeLorenzo, Walton, Curran & Greengard, 1973; Walton, DeLorenzo, Curran & Greengard, 1975). However, Ferguson and Twite (1974) reported that the dephosphorylation of protein D is induced only by vasopressin concentrations which are sufficiently high to evoke the hydroosmotic response and therefore much higher than those required to produce maximal stimulation of Na^+ transport.

A similar protein was found in the cytosolic fraction of several mammalian steroid target organs, and in each tissue its endogenous dephosphorylation could be induced by *in vitro* administration of the respective steroid (Liu & Greengard, 1976). In mammals, unlike amphibian epithelia, steroid hormones appear to regulate the amount of this protein or its ability to become phosphorylated. This steroid-regulated protein, common to various target organs, was tentatively identified as the regulatory subunit of type II cAMP-dependent protein kinase, and it was suggested that its autophosphorylation is a mediating step in the physiological action of steroid hormones (Schwartz et al., 1979; Liu, Walter & Greengard, 1981). In toad bladder the type II cAMP-dependent kinase is mostly cytosolic and has about 80% of the cell cAMP binding capacity (Schlondorff & Franki, 1980). The relevance of the steroid-induced protein dephosphorylation to the natriferic action of aldosterone is not clear and no direct link has been made yet between dephosphorylation and transport changes. Nevertheless, the fact that in toad bladder this process is mineralocorticoid specific makes it a potential mediating event.

V. The Hormonal Stimulation of Na^+ Transport

A. Aldosterone Effects on the Apical Na^+ Permeability

Evidence for aldosterone effects on the apical Na^+ permeability was provided by both chemical and electrophysiological measurements. Crabbé (1963*a*) and Sharp and Leaf (1964*a*) were the first to suggest that the mucosal surface is the main action site of aldosterone. This hypothesis was based on the observation that the hormonal treatment increased the size of the "Na^+ transport pool," i.e., the intracellular pool of Na^+ ions which could readily be labeled by $^{22}Na^+$ added to mucosal compartment. The same observation was made in additional studies (Crabbé & DeWeer, 1965, 1969; Handler, Preston & Orloff, 1972; Leaf & Macknight, 1972), and an aldosterone-induced elevation of the cellular Na^+ activity was measured by other methods as well (Eaton, 1981; Palmer et al., 1982). An increase of the steady-state intracellular Na^+ activity upon the augmentation of its transcellular flux is, of course, indicative of an enhanced mucosal entry rate. Moreover, the data indicate that under the experimental conditions of the above studies, the increase of apical permeability is the primary event and not secondary to hormonal effects on the basolateral pump which releases channels from a Na^+ feedback inhibition (Lewis, Eaton & Diamond, 1976; Frizzell & Schultz, 1978). Additional evidence for mucosal effects of aldosterone is the lack of natriferic response after shunting the apical membrane with amphotericin B (Crabbé, 1967) and the failure to enhance the short-circuit current of an aldosterone-treated colon by a mucosal addition of amphotericin B (Frizzell & Schultz, 1978).

Cuthbert and Shum (1975) measured high affinity binding of ^{14}C-amiloride to isolated toad bladder cells, and reported that aldosterone doubles the number of amiloride-binding sites without affecting their affinity to the diuretic. It is well established that, at low concentrations, amiloride inhibits the transepithelial transport by a specific binding to the apical Na^+ channels (Benos, 1982). Accordingly, an increase in the number of amiloride-binding sites implies that aldosterone induces apical Na^+ transporters. However, a later study by these authors (Cuthbert & Shum, 1977) showed that the number of amiloride-binding sites in frog skin is reduced when the pump is blocked by ouabain. Thus,

changes in amiloride binding seem to be secondary to variations of the intracellular Na^+ or membrane potential, irrespective of the mechanism by which these variations were induced.

Another strong indication for apical effects of aldosterone is the hormone-induced decrease of transcellular, and in particular apical, resistance (Civan & Hoffman, 1971; Saito & Essig, 1973; Spooner & Edelman, 1975; Siegel & Civan, 1976; Nagel & Crabbé, 1980; Eaton, 1981). Moreover, in two studies a correlation between the fractional changes in I_{sc} and the apical Na^+-specific conductance (or permeability) could be established (Palmer et al., 1982; Garty et al., 1983). The most likely interpretation of aldosterone effects on the apical resistance is the activation of a Na^+-specific rheogenic pathway, i.e., the amiloride blockable channels.

The first insight into the molecular mechanism by which aldosterone augments the apical Na^+ permeability was provided by Palmer et al. (1982), who measured and analyzed the amiloride-induced fluctuations in I_{sc}. These authors reported that incubating toad bladder for 4–6 hr with aldosterone evokes a parallel increase of I_{sc} and the apical density of Na^+ conducting channels, but has no effect on the single-channel current. The fact that the hormone modulates the number of Na^+ transporting units rather than the conductance of individual transporters, suggests two general schemes for the nature of its natriferic effect: (i) Induction of the synthesis and/or insertion of new channels in the apical membrane. (ii) Activation of pre-existing nonconductive apical channels, or increasing the lifetime of the channel open state. Since aldosterone mediates its action by *de novo* protein synthesis, the first, simpler, hypothesis seemed a more likely one. However, in three recent studies evidence in favor of the second scheme was provided (Palmer & Edelman, 1981; Garty & Edelman, 1983; Kipnowski, Park & Fanestil, 1983). In these studies the base-line I_{sc} was partly inhibited by treating the mucosal surface of mounted toad bladders with trypsin or protein-modifying reagents, and the response of these partly blocked preparations to aldosterone was examined. In all three cases it was found that the irreversible blockage of base-line channels impaired, at least to the same extent, the ability of aldosterone to augment apical Na^+ transport. Thus, the response to aldosterone appears to involve proteins, presumably channel precursors, which are accessible to impermeable reagents present in the mucosal bathing solution, i.e., resident in the apical membrane prior to the hormonal stimulation. Since none of the reagents used in the above studies is specific to the channel protein, the possibility remains that the inhibition observed reflects some nonspecific damage to the apical surface, which impaired its ability to accept new aldosterone-induced components. This possibility seems unlikely at least for the inhibition of I_{sc} by apical trypsinization (Garty & Edelman, 1983). In this study it was shown that amiloride (30 μM) can protect both the base-line and aldosterone-induced I_{sc} from irreversible inhibition by proteolytic digestion, i.e., the apical protein whose cleavage inhibits the ability of aldosterone to augment Na^+ transport is an amiloride-binding protein. The possibility that aldosterone functions by activating pre-existing membranal pool of nonconductive channels is also supported by the metabolic effects discussed in section IV.A. The ability of glucose and pyruvate to induce fast activation of channels in substrate-depleted aldosterone-treated (but not control) bladders, indicates the existence of an energy-requiring step, following the synthesis of AIP, which is essential for the expression of hormone-induced permeability changes.

Little information is available at the moment on the mechanism by which Na^+ channels are activated. Sariban-Sohraby et al. (1984*a*) has recently reported that the amiloride-blockable $^{22}Na^+$ uptake into apical vesicles derived from A6 cells is doubled after 30 min incubation with S-adenosylmethionine. This effect could be prevented by methylation inhibitors such as 3-deazaadenosine and S-adenosylhomocysteine. Vesicles isolated from aldosterone-treated cells exhibited enhanced rates of tracer uptake which could not be further stimulated by the methyl donor. Thus, the methylation of membrane-bound proteins or lipids is a possible mechanism for the activation of channels. The role of aldosterone in such mechanism could be to activate (induce?) a membrane-bound methyltransferase or to increase the amount of S-adenosylmethionine available to this enzyme. However, the differences in the interaction of aldosterone with A6 cells and toad bladder or mammalian kidney discussed in sections III.A and IV.A, make the generalization of this scheme to other epithelia questionable.

An alternative possibility is that the hormonal induced activation of channels is mediated by changes in the cytoplasmic Ca^{2+} activity. Much circumstantial evidence suggests that an increase of the cytoplasmic Ca^{2+} activity downregulates Na^+ transport (for review *see* Taylor & Windhager, 1979; Chase, 1984). Ca^{2+} effects on the amiloride-blockable Na^+ fluxes were recently investigated in toad bladder apical vesicles, and evidence was presented for two different mechanisms: (i) A direct, presumably noncovalent, Ca^{2+}-channel interaction which can be demonstrated by measuring Na^+ transport in isolated membranes in the presence and

absence of Ca^{2+} ions (Chase & Al-Awqati, 1983; Garty, 1984). (ii) A Ca^{2+}-dependent process which takes place in whole cells only but induces a stable, presumably covalent, modification of the apical surface preserved by the isolated membrane in the absence of Ca^{2+} ions (Garty & Asher, 1985). Thus, an aldosterone-induced decrease of the cytoplasmic Ca^{2+} activity could activate channels by releasing them from a Ca^{2+}-dependent downregulation. Such a decrease has not been reported yet, but it is a plausible outcome of the well-documented hormone-dependent activation of mitochondrial enzymes. The downregulation of channels by cytoplasmic Ca^{2+} may also account for the metabolic dependence of apical permeability (e.g. energy-dependent Ca^{2+} transport across the basolateral, mitochondrial, or endoplasmic reticulum membrane).

In addition to aldosterone, the apical channels can also be activated by antidiuretic hormones or cAMP (Li, Palmer, Edelman & Lindemann, 1982; Helman, Cox & Van Driessche, 1983). This process, however, seems to be independent of the natriferic action of aldosterone for two reasons: (i) aldosterone and antidiuretic hormones have additive effects on I_{sc} (Sharp & Leaf, 1966). (ii) Trypsinization of the mucosal surface impairs the response to aldosterone but not to vasopressin (Garty & Edelman, 1983).

In summary, considerable evidence indicates that at least part of the aldosterone-induced augmentation of Na^+ transport is the result of an increase in the apical Na^+ permeability. This increase is apparently due to the modification of pre-existing apical channels by mechanisms which have not been resolved yet. Additional, possibly slower, aldosterone effects on *de novo* channel synthesis cannot be excluded.

B. Aldosterone Effects on the Basolateral (Na^+/K^+)ATPase

An aldosterone-induced increase of the apical permeability alone, can result in a proportionate change of I_{sc} only if the basolateral pump operates far away from equilibrium and in particular is not saturated by the increased cellular Na activity. Thus, a large and long-term augmentation of transepithelial Na^+ flow may require, in addition to the activation of channels, an increase of the capacity to extrude Na^+ from the cell. Early attempts to demonstrate *in vitro* effects of aldosterone on the (Na^+/K^+)ATPase in toad bladder were disappointing, and mineralocorticoid-induced activation of the enzyme could not be observed (Snart, 1972; Hill, Cortas & Walser, 1973; Park & Edelman, 1984). In several other studies *in vivo* effects of aldosterone and other corticosteroids on the (Na^+/K^+)ATPase activity of rat and rabbit kidney were reported (Jorgensen, 1969; Garg, Knepper & Burg, 1981; Rodriguez, Sinha, Starling & Klahr, 1981; Mujais et al., 1984; O'Neil & Dubinsky, 1984). These effects, however, developed over a time period of days, were induced only by high doses of aldosterone, and were not always mineralocorticoid specific. In recent, *in vitro*, studies using defined segments of rat and rabbit nephron, a mineralocorticoid-specific activation of the (Na^+/K^+)ATPase in a time scale of a few hours could be demonstrated (Horster, Schmid & Schmid, 1980; Petty, Kokko & Marver, 1981; El Mernissi & Doucet, 1983; Rayson & Lowther, 1984). The mineralocorticoid effect reported in these studies was confined to the medullary and cortical-collecting tubules, while the thick ascending limb of the loop of Henle seemed to be controlled mainly by glucocorticoids (El Mernissi & Doucet, 1983; Rayson & Lowther, 1984). Interesting enough, at least in one case, the increase in enzymatic activity was prevented by pretreatment with amiloride, suggesting that it is secondary to the elevated apical transport (Petty et al., 1981).

Geering et al. (1982) studied the *in vitro* biosynthesis of (Na^+/K^+)ATPase in toad bladder by immunoprecipitating its α and β subunits with polyclonal antibodies. Aldosterone was found to increase by 2.8-fold the incorporation of [^{35}S]-methionine into the α subunit, and by 2.4-fold the labeling of a 42,000-dalton protein recognized by the anti β-serum (presumably a precursor of the β subunit). Maximal enhancement of the (Na^+/K^+)-ATPase synthesis was achieved with only 20 nM aldosterone, it was blocked by spironolactone, and was not inhibited by amiloride. The observed induction of (Na^+/K^+)ATPase considerably lagged behind the initial rise of I_{sc} and appeared to correlate in time with the "late response," a period of 3–4 hours during which the current changes are not accompanied by a decrease of the transepithelial resistance (Spooner & Edelman, 1975).

The observed increase of I_{sc} at nearly constant tissue resistance cannot serve as strong evidence for hormonal effects on nonconductive pathways. In this case, however, the independence of the two phases could be established by demonstrating that both butyrate and triiodothyronine inhibit the late response without affecting the early one (Truscello et al., 1983; Geering, Gaeggeler & Rossier, 1984). In addition, maximal stimulation of I_{sc} during the late period required higher aldosterone concentrations and the occupation of the low affinity receptors, too (Geering et al., 1985). Thus it is conceivable that the late natriferic response does indeed represent an

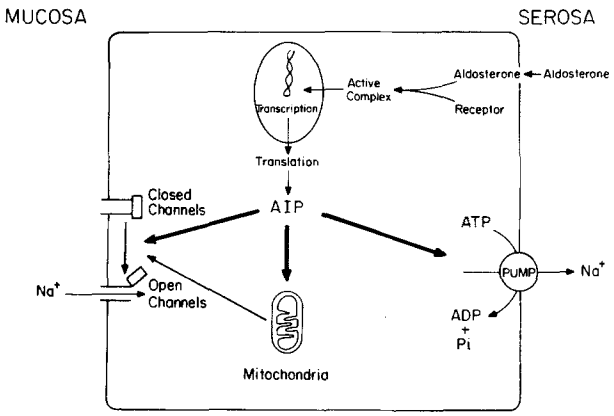

Fig. 3. Sites and mechanisms of aldosterone action. The hormone binds to its receptor in the cytoplasm to form an active complex which diffuses into the nucleus and interacts with chromatin. Altered gene expression leads to the enhanced synthesis of aldosterone-induced proteins (AIP) responsible for all the physiological effects. The AIP function to increase the Na^+ permeability, induce new pumps, and enhance mitochondrial metabolism. The increase in apical Na^+ permeability results from the activation of channels normally resident in the membrane in nonconductive form. This process also depends on the availability of metabolic energy

enhanced rate of basolateral Na^+ pumping, independent of the earlier hormonal effects on apical channels. Two, still missing, pieces of evidence that will establish this model are: (i) demonstration of an aldosterone-induced increase in the specific (Na^+/K^+)ATPase activity (rather than labeling of immunoprecipitate) which correlates with current changes during the late response; (ii) direct determination of the apical permeability (rather than the total tissue resistance) and proving that the increase in I_{sc} is not fully accounted for by apical permeability changes.

VI. Concluding Remarks

Our current understanding of the processes involved in the natriferic action of aldosterone can be described as a partly constructed puzzle. Enough pieces have already been put together to give an impression of what the complete picture is going to look like; however, a few key regions are still empty and more work is required in order to produce and place the pieces that construct them. Thus it is clear that the initial mineralocorticoid-target cell interaction involves an alteration of gene expression and the synthesis of specific proteins which are responsible for the physiological effects. These proteins act separately and presumably independently to facilitate both the passive Na^+ entry and its active extrusion. In addition, the AIP stimulate oxidative phosphorylation in order to fuel the Na^+ pump and enable an energy-dependent activation of channels (Fig. 3).

At short times ($t < 4$ hr) the natriferic action of aldosterone is fully accounted for by an increase in the density of Na^+-conducting channels. After a longer exposure to the hormone, enhanced capacity for Na^+ pumping contributes to the transport changes as well. It appears that the second effect is due to *de novo* synthesis of (Na^+/K^+)ATPase units. On the other hand, hardly any information is available on the nature of the primary activation of channels and the role of AIP in this process. Findings that might help to bridge this major gap in our knowledge are: (i) The aldosterone-induced permeability changes involve proteins which are present in the apical surface prior to its application. (ii) The activation of channels depends on the availability of metabolic substrates. The metabolic stimulation of Na^+ permeability in hormone-treated bladders is fast and reversible. (iii) Aldosterone modifies the cell membrane lipid composition and induces the dephosphorylation of specific protein, presumably cAMP-dependent kinase. (iv) Na^+ channels may be regulated by a methyl transfer reaction and by the cell Ca^{2+}.

The first two findings argue against the hypothesis that aldosterone stimulates transport simply by inducing the synthesis of new Na^+ channels. A more likely possibility would be an aldosterone-induced reaction at the level of the apical membrane, which activates nonconductive, pre-existing, channels or increases the life-time of the channel open state. Such a reaction will either involve covalent modification of proteins and lipids (e.g. methylation, dephosphorylation), or noncovalent interaction of channels with cytoplasmic effectors (Ca^{2+}, H^+, Na^+, or nucleotides). The AIP may control this hypothetical process in many different ways. One possibility is that some of them are enzymes directly involved in modifying the channel protein or its surrounding lipids. Another possibility is that they act indirectly, e.g. by lowering the cytoplasmic Ca^{2+} activity and triggering other events. Clearly many questions remain open, and the mechanism by which mineralocorticoids control Na^+ channels in tight epithelia is far from being understood. The recently available procedures for studying Na^+ channels in isolated apical vesicles (Chase & Al-Awqati, 1983; Garty & Asher, 1985), lipid bilayers (Sariban-Sohraby et al., 1984*b*) and membrane paths (Hamilton & Eaton, 1985), may help to resolve this issue.

What appears to be the characteristic feature in the natriferic action of aldosterone is the coordi-

nated modulation of two or three different sites. In this way the transepithelial transport can be augmented several-fold and not be limited either by the passive entry or by the active exit of Na^+. The now established coordinated action of the hormone pays tribute to the founders of this field by supporting all the early, seemingly conflicting hypotheses.

I wish to thank Prof. M.M. Civan (Department of Physiology and Medicine, University of Pennsylvania), Prof. B. Lindemann (2nd Institute of Physiology, University of Saarland, FDR) and Prof. B.C. Rossier (Institut de Pharmacologie, Universite de Lousanne, Switzerland) for useful discussions, Dr. C. Asher and Mrs. N.B. Garty for editorial assistance, and Miss C. Gross for excellent secretarial assistance.

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