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Perspectives in Receptor-Mediated Mineralocorticoid Hormone Action

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I. Introduction

The maintenance of life processes depends on the success with which chemical reactions in the cell can continue in a world that is fundamentally hostile to them. Nearly a century back, the term hormone was coined by Bayliss and Starling from the Greek *hormaein*, meaning to arouse or to stimulate, to explain secretin-stimulated exocrine activity of the pancreas after food intake. This established the principle of coordination between specialized organs by chemical messengers collectively termed hormones (Anfinsen, 1959; Barrington, 1962, 1964).

Life on earth is believed to have evolved approximately 4 billion years ago from 30 or so biologically useful precursors generated from simple inorganic material under the influence of the cosmic energy reaching earth through our solar system (Anfinsen, 1959; Barrington, 1964). Steroids are of universal occurrence, present in all life forms, variously as membrane constituents and/or vitamins, hormones, cytotoxins, and chemical messengers (Clark, 1961; Fieser and Fieser, 1959). Indeed, the remarkable diversity in the biological properties of various steroids is believed to be of the greatest evolutionary importance (Barrington, 1964).

As far back as 1849, Berthold demonstrated that, in birds, the adverse effects of castration on sexual characteristics could be alleviated by testes transplantation. In 1895, Oliver and Schaffer found altered muscle development and blood pressure changes in response to the

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administration of adrenal extracts. These and other observations led Laguesse to coin the term "endocrine" to describe specialized cells that would produce substances transported by blood to the site of action (Barrington, 1962, 1964, 1968).

The possession of endocrine glands distinguishes vertebrates from all other forms of life. To the specialist in animal physiology, endocrinology is inherently concerned with the regulation of vertebrate functions. Steroid distribution, synthesis, metabolism, and cellular action, however, evolved from pathways shared to various degrees by microbes, plants, lower animals, and mammals. The cross-reactivity between various classes of steroids and the production of a steroid hormone at several sites further suggest gradual evolution from processes whose foundations were laid down at the very beginning of earthly life. The extension of the sphere of activity, without further change in the steroid itself, is consistent with the concept of specialized organs developed for the production of, and for responding to, existing hormones (see discussion by Anfinsen, 1959; Barrington, 1962, 1964; Clark, 1961; Fieser and Fieser, 1959).

The present article was conceived as an overview of major trends within the particular context of receptor-mediated mineralocorticoid hormone action. The first few sections provide a brief background to developing a coherent discussion of events expressed through the specific receptor type(s) for the synthesis of ideas in this area of increasing complexity and importance. For clarity and brevity, many areas of contemporary importance had to be left out. To limit the bibliography, extensive use was made of existing specialized books and reviews. This is meant to lead to an appropriate source for further inquiry, and no effort has been made either to be exhaustive or to establish priorities. Therefore, indulgence is asked of everyone concerned.

II. Steroid Synthesis

The diversity of steroids and sterols in lower organisms far exceeds that in higher forms (Agarwal, 1993a; Mahato et al., 1989); indeed, conjugated plant sterols, glycosides, and saponins have no counterpart in animals (Heftman, 1963, 1975). The synthesis of cholesterol from acetate proceeds via different pathways in microbes (Mahato et al., 1989), plants (Benveniste, 1986), and animals (Miller, 1988), but insects depend exclusively on ingested sterols for transformations into hormonally active steroids (Gilmour, 1960). Cholesterol (C27) was finally selected for molecular evolution from a wide range of possibilities because C28 and C29 sterols of lower forms can be neither metabolized nor absorbed from the alimentary tract by the present-day vertebrates. Full oxidation of the side chain of cholesterol to C24 bile acids is also a specialized feature of mammals, whereas lower vertebrates (amphibians and fishes) are endowed with C27 and C28 bile acids (Barrington, 1962, 1964; Ross, 1975).

Cholesterol is the starting material for the genesis of all of the hormonally active steroids (Klyne, 1957; Samuels, 1960). A human adult of 70 kg contains about 140 g cholesterol, primarily in the nervous system, cell membranes, and bile acids (Grant, 1962; Haslewood, 1962). Steroid hormone synthesis occurs in the placenta, gonads, and adrenals, all of which have a common origin in the coelomic epithelium of the embryonic mesoderm which later differentiates into the mesonephric kidney tubules. In fact, the adrenocortical tissue was at first scattered along the dorsal wall and the kidney in elasmobranchs and only later was condensed into the cortical layer of the mammalian adrenal (Barrington, 1964).

Most of the cholesterol for steroidogenesis is derived from plasma low- or high-density lipoproteins, depending on the species. De novo synthesis of cholesterol from acetate is, however, possible in endocrine glands, particularly the adrenal. Steroid hormone synthesis from cholesterol is a complex process, requiring the participation of a cascade of cytochrome P450 enzymes in the endoplasmic reticulum and mitochondria (for reviews, see Bransome, 1968; Hechtor and Pincus, 1954; Miller, 1988; Swann, 1940, and references therein). Some of the salient features are described below for better appreciation of later sections.

Major reactions in steroid hormone biosynthesis are presented schematically in figure 1. Normally, the outermost cortical layer, zona glomerulosa, is the only site of aldosterone synthesis, requiring a synthase found in the rat and possibly the human adrenal (Muller, 1991). Corticosterone is produced by all three layers of the adrenal cortex, but cortisol production is restricted to the two inner layers (Swann, 1940). In lower vertebrates, the adrenal cortex is not differentiated into zones, suggesting that zonation is a recent evolutionary innovation (Shire and Spickett, 1967). Fetal adrenal can synthesize aldosterone (Bird et al., 1965), but this steroid is absent in patients with Addison's disease (Hernando et al., 1967) and after adrenalectomy (Hershberger and Thompson, 1968).

Final steps in aldosterone biosynthesis have not yet been fully elucidated. Although corticosterone appears to be the major precursor, aldosterone synthesis can also proceed from 11-hydroxy and 18-hydroxy derivatives of progesterone via mixed function oxidases (Ross, 1959, 1975; Miller, 1988).

Genetic lesion in these mixed function oxidases leads to the corresponding deficiency syndrome that can be alleviated by the administration of an appropriate steroid (Miller, 1988; New et al., 1982). Extraadrenal distribution of P450c21 can lead to the formation of deoxycorticosterone (Casey and MacDonald, 1982), but the last step in P450c11-mediated catalysis of aldosterone from 18-hydroxycorticosterone appears to be limited strictly to the adrenal (Miller, 1988; Ross, 1975). Redundance of synthetic pathways is actually evident from the fact that

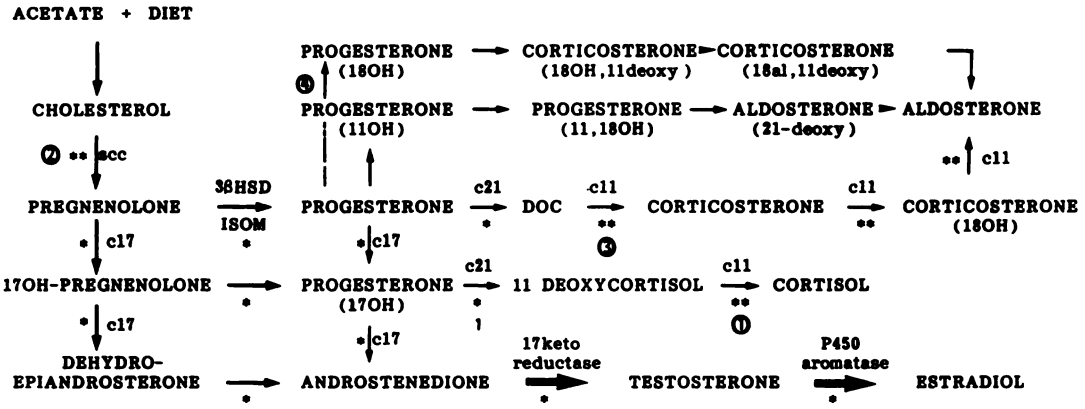

FIG. 1. Biosynthetic pathways for steroid hormones. Cholesterol, synthesized from acetate or absorbed from the digestive tract, is converted to pregnenolone by P450 scc in adrenal mitochondria. Further reactions are catalyzed by P450c17, P450c21, or P450c11, in either the microsomal (*) or the mitochondrial (**) compartment. A non-P450 enzyme(s), bound to the endoplasmic reticulum, possesses dehydrogenase and isomerase (ISOM) activity for three of the steps shown. Aldosterone production is also possible via the conversion of progesterone to its 11- or 18-hydroxyl forms, albeit to a minor extent. The 21-hydroxylase reaction, required for the production of cortisol, is an advanced evolutionary development in higher mammals. Testosterone and estradiol are synthesized principally in the gonads. For further details, see Bransome, 1968; Fieser and Fieser, 1959; Grant, 1962; Hechtor et al., 1953; James, 1992; Klyne, 1957; Miller, 1988; Nebert and Gonzalez, 1987; Ross, 1975; Samuels, 1960. Amphenone B (Peterson et al., 1957; Rosenfeld and Bascom, 1956), aminoglutethimide (Dexter et al., 1967; Fishman et al., 1967), metyrapone (Jenkins et al., 1958; Liddle et al., 1958; Sonino, 1982; Sonino and Merola, 1984), SU-9055 (Bledsoe et al., 1964; Kahnt and Neher, 1962) are some of the chemicals that inhibit steroid synthesis (Parvez et al., 1979), primarily at steps 1 to 4, respectively, but multistep blockade is possible. The toxicity of these inhibitors provokes side effects that limit their clinical utility.

approximately 70 different steroids have been isolated from adrenal extracts, many of which are also synthesized in other specialized glands (Barrington, 1964; James, 1992). Inhibitors for some of these steps are known (fig. 1), but their toxicity limits clinical application (Ross, 1975; Sonino, 1982; Sonino and Merola, 1984).

The superfamily of P450 genes (fig. 1) coalesced about 1.5 billion years ago from at least 10 separate families (one fungal, one bacterial, eight mammalian), exhibiting <40% identity between them (Nebert and Gonzalez, 1987). Yet, they coordinate steroidogenesis from microsomal (P450c17 and P450c21) and mitochondrial (P450scc and P450c11) subsets (Miller, 1988). Considerable species- and tissue-specific differences in these enzyme functions are more than likely.

A 17-amino acid consensus steroid-binding site has been identified in microsomal P450c17 and P450c21 enzyme complexes (Picardo and Miller, 1988). Cloned DNA for these enzymes contains consensus sequences closely resembling those found in genes regulated by cAMP (Montminy et al., 1986), glucocorticoids (Lewis et al., 1987), and the glucocorticoid regulatory element (Jantzen et al., 1987).

III. Hormone Availability and Metabolism

Only 50 to 70% of the circulating aldosterone is bound to serum proteins, such as the transcortin, orosmucoid, and albumin, whereas its precursors bind these globulins avidly (Daughaday et al., 1961; Davidson et al., 1962; Meyer et al., 1961; Sandberg et al., 1960). As much as 90% of free aldosterone is removed during just one passage through the liver, and its half-life of only 35 min contrasts with 120 min for that of cortisol (Peterson, 1959; Wolff and Torbica, 1963). A mean plasma level of only 1 nM aldosterone must compete with nearly 1000-, 100-, and 30-fold greater levels of cortisol, corticosterone, and progesterone, respectively, for binding to the receptor (Coghlan and Scoggins, 1967; Fraser et al., et al., 1966; James, 1992; Johnson et al., 1957); considerable diurnal variation has also been well documented (Muller, 1991; Pincus, 1943). Mean daily secretion rates of aldosterone, corticosterone, and cortisol in an adult man have been estimated as 0.05 to 0.15, 2 to 5, and 15 to 30 mg, respectively (Muller and O'Conner, 1958; Yates and Urquhart, 1962), but adrenal pathology can modify these substantially (Moon, 1961; Soffer et al., 1961).

Steroids are reduced to dihydro and tetrahydro derivatives in the liver, conjugated with glucuronic acid in both hepatic and renal parenchyma, and finally excreted by the kidney. The filtered, free steroid (90 to 95%; aldosterone or cortisol) is reabsorbed (Flood et al., 1967; Siegenthaler et al., 1964). As expected, hepatic and/or renal disease alters these parameters considerably, such that the assessment of urinary steroid excretion is a much valued diagnostic tool (Fraser et al., 1966; Moon, 1961; Peterson, 1960; Soffer et al., 1961).

IV. Interspecies Distribution of Steroids

This subject has been thoroughly reviewed (Barrington, 1964, 1968. Hechtor and Pincus, 1954; Heftman, 1975; Mahato et al., 1989; Sandor and Mehdi, 1979; Schopf, 1977, and references therein), but major traits are recapitulated here for developing a unified perspective.

In microbes, plants, and lower organisms, rich diver-

sity of steroid synthesis and transformation is not restricted to specialized cells (Heftman, 1975; Mahato et al., 1989). Acquisition of a closed circulatory system by protochordates 1 billion years ago probably necessitated the development of an adrenal cortex for the regulation of homeostasis. The beginning of a terrestrial life and the development of homoiothermy (warm bloodedness) necessitated further adjustments in steroid secretion and action.

Members of cyclostomata (slime eels and hagfishes) stay isoosmotic with their environment, and little is known regarding corticosteroids produced by these organisms, although cortisol and corticosterone (fig. 2) have been demonstrated in some cases (Sandor, 1969). Higher up, elasmobranchs (dogfish, sharks, rays) additionally produce 1_{α}-hydroxycorticosterone which is presumed to be a mineralocorticoid; aldosterone (fig. 2) is first detected in teleosts (bony fish) that also produce cortisol and corticosterone (Sandor et al., 1976). The complexity increases in birds, reptiles, amphibians, and mammals in which 11-deoxy and 18-hydroxy-11-deoxy corticosterone are physiologically active. Furthermore, cortisol is absent from rodents and most of the premammalian phyla (Barrington, 1964, 1968; Sandor et al., 1976; Sandor and Mehdi, 1979).

It becomes immediately clear from the foregoing that the present-day distinction between glucocorticoids and mineralocorticoids is largely irrelevant in the evolutionary history of steroid action. Mineralocorticoid function is assured by cortisol in teleosts (Henderson et al., 1975) and by corticosterone in salt-secreting marine birds (Thomas and Philips, 1975). Rather, the relevant target cells have specialized during approximately the past 2 billion years to respond to ubiquitous steroids synthesized by fusion of pathways (Miller, 1988; Nebert et al., 1987).

A curious dichotomy occurred at the burst of metazoan evolution in the precambrian period 500 million years ago. Arthropods are contemporary to crustaceans, as evident from moulting induced in these two phyla by ecdysteroids (Karlson, 1974; Sandor and Mehdi, 1979). However, in insects, steroid biosynthesis ceased altogether, and ingested cholesterol was subsequently used for further transformations (Gilmour, 1960; Karlson, 1967).

The position of progesterone as the major precursor of hormonal steroids (fig. 1) appears somewhat paradoxical. In addition to its peculiar role in female placental mammals, it also possesses antiglucocorticoid activity, particularly in vitro (Agarwal et al., 1987; Boucheix and Agarwal, 1979; Naylor and Rosen, 1982; Sakiz, 1987). Progesterone also exhibits antimineralocorticoid activity in vivo (Landau, 1979; Landau and Lugibihl, 1958; Wambach and Higgins, 1979) and, along with its numerous synthetic derivatives, has become an invaluable tool to delineate various receptor-mediated events (Agarwal and Lazar 1991). The nature of cross-reactivity among these three groups of steroids will be elucidated further in later sections.

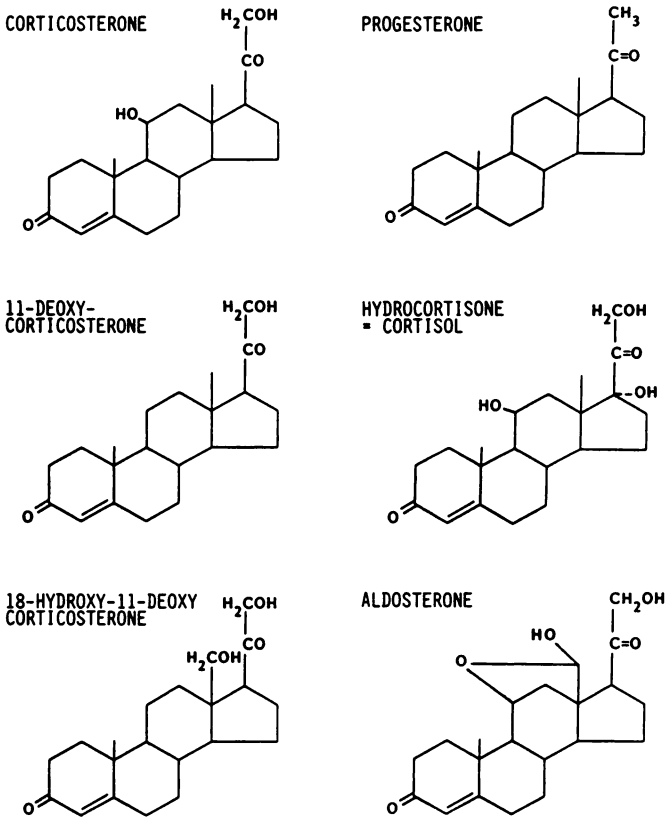

FIG. 2. Chemical structure of major adrenal steroids used to study gluco- and/or mineralocorticoid action. Progesterone is the precursor for the synthesis of the majority of steroid hormones (fig. 1). It exhibits antiglucocorticoid activity, particularly in vitro (Agarwal et al., 1987; Naylor and Rosen, 1982), and its antimineralocorticoid action in vivo has been well documented (Landau, 1979; Landau and Lugibihl, 1958). Corticosterone and cortisol are the major glucocorticoids in mammals but ensure mineralocorticoid function in lower phyla and some mammalian tissues. The 11-deoxy and the 18-hydroxy derivatives of corticosterone exhibit weak mineralotropic activity in mammals but are bifunctional in premammalian organisms. Aldosterone makes its first appearance with the beginning of terrestrial warm bloodedness. Cortisol is absent from rodents but is synthesized in several classes of fishes (Barrington, 1964, 1968; Sandor, 1969; Sandor et al., 1976, 1983; Sandor and Mehdi, 1979). The mineralocorticoid action of cortisol in teleosts (Henderson et al., 1975) and of corticosterone in birds (Thomas and Philips, 1975) has been well documented. Furthermore, approximately 30 different steroids alter growth, sugar uptake, and permeability to ions in several yeasts and bacteria (Conway and Hingerty, 1953; Hechtor and Lester, 1960; Lester et al., 1958). More recently, algae, too, were found to respond to some of these steroids (Agarwal, 1993a; Mirshahi et al., 1992a,b). Such considerations are consistent with the idea of evolution from a common ancestral precursor whose specialization into mineralocorticoids is still incomplete. The affinity of these molecules for the MCR, in relation to their biological potency, is summarized in table 1.

V. Mineralocorticoid Antagonists

Although progesterone exhibits natriuretic effects in vivo (table 1), its gestational action obviously excludes any possible therapeutic application but should be borne

in mind when treating pregnant women with high levels of circulating progesterone (Landau, 1979; Landau and Lugibihl, 1958; Wambach and Helber, 1982; Wambach and Higgins, 1979). Interestingly, the "ideal" synthetic progestin R 5020 (fig. 3) and other derivatives of this series do not exhibit antagonist activity (table 1).

The bifunctional steroid RU 38486 (fig. 3) is endowed with both antiglucocorticoid and antigestational activity (Agarwal, 1993b,c; Agarwal et al., 1987). It has been used for the treatment of hypertensive disorders mediated by glucocorticoids (Grunfeld et al., 1985), but it does not antagonize mineralocorticoid-mediated sodium absorption (table 1).

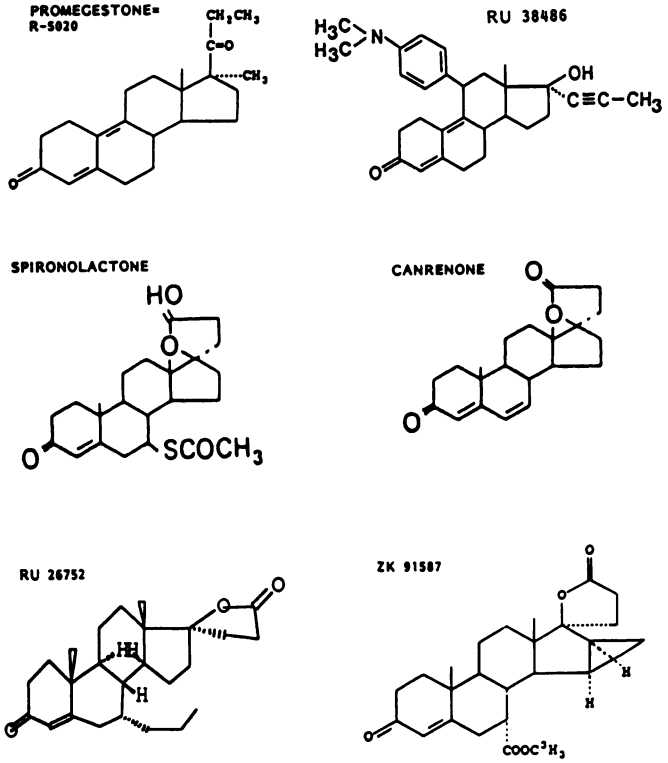

FIG. 3. Antagonists for adrenocortical hormone action. RU 38486 is a bifunctional derivative endowed with both antiglucocorticoid and antigestational activity (Agarwal, 1993b; Agarwal et al., 1987; Nédélec et al., 1986; Philibert, 1984). It is the only steroid in actual clinical use in this context (Sakiz, 1987; Nieman and Loriaux, 1987; Philibert et al., 1991) and has become an indispensable tool to distinguish between glucocorticoid and mineralocorticoid types of hypertension (Grunfeld et al., 1985; Clore et al., 1992). Promegestone (R-5020) is a synthetic progestin that is devoid of antimineralocorticoid activity (Wambach, 1987; Wambach and Higgins, 1979) but that has been successfully used to photolabel various classes of receptors (Mirshahi et al., 1992a–d). Spironolactone and its metabolite canrenone are in clinical use to antagonize the mineralocorticoid type of hypertension, despite numerous side effects and negligible affinity for the MCR (Ramsay, 1979, 1982; Ramsay and McGinnes, 1984). The introduction of a propyl (RU 26752) or a methoxycarbonyl (ZK 91587) residue in the 7_α-configuration of spironolactone increased the affinity for the MCR (Losert et al., 1986). ZK 91587 has now been marketed as the ligand specific for the MCR, but its activity in vitro is more potent than its action in vivo (Agarwal, 1993b; Agarwal and Lazar, 1991). These two spirolactones have, nevertheless, permitted the elucidation of receptor-mediated processes because natural steroids exhibit cross-reactivity with several classes of carriers (Agarwal and Kalimi, 1988a–c; Nédélec et al., 1986; Wambach and Stenzel, 1984). The structure-activity relationship of these derivatives is summarized in table 1.

TABLE 1
*Structure-activity correlation of mineralotropic steroids**

Derivative	Affinity in vitro	Potency in vivo
Aldosterone	100	100
Deoxycorticosterone	100	100
Corticosterone†	10–20	?
Cortisol†	10–20	?
Progesterone	20	>90
Promegestone	5–10	0
RU 38486	0	0‡
Spironolactone	<10	>90
Canrenone	1	45
RU 26752	57	50
ZK 91587	400	180

* The affinity in vitro was determined by Scatchard analysis in presence of a steroid competing for binding to the aldosterone-renal receptor complex (= 100). The biological potency in vivo was assessed by the ability of the steroid to alter urinary Na^+/K^+ ratio in adrenalectomized rats (Kagawa, 1960). This table was compiled from Beaumont and Fanestil, 1983; Fraser and Lantos, 1978; Nédélec et al, 1986; Agarwal et al, 1987, Sakiz, 1987; Wambach, 1987; Arriza et al, 1988; Agarwal and Lazar, 1991, and has been developed in detail in the text.

† It should be borne in mind that brain MCR binds corticosterone and cortisol just as well as aldosterone, contrary to the situation in the cytosol of peripheral organs where clear aldosterone preference is evident; recombinant MCR binds these steroids equally well.

‡ RU 38486 can influence this parameter via the GCR.

Synthetic spirolactones have been the agents of choice for mineralocorticoid antagonism in vivo (table 1). Spironolactone and its metabolite canrenone (fig. 3) have been in clinical use for more than three decades for the management of several forms of hypertensive disease (Ramsay, 1979, 1982; Ramsay and McInnes, 1984). Adverse side effects associated with these drugs, such as gynecomastia, hyperkalemia, menstrual abnormalities, and impotence in men, led to the synthesis of RU 26752 and ZK 91587 (fig. 3). The biological potency of these latter derivatives in vivo was not in direct correlation with their affinity for the MCR† in vitro (table 1), but ZK 91587 has actually emerged as the MCR-specific steroid (Agarwal and Lazar, 1991; Sutanto and deKloet, 1991; Wambach and Stenzel, 1984).

VI. Physiological Action of Mineralocorticoids

Since the isolation of the principal substance responsible for the "mineral activity" in beef adrenal extract (Grundy et al., 1952), the mechanism of action of aldosterone has been the subject of much experimental effort. Although the term "mineralocorticoid" has now generally come to denote aldosterone action, it should be clear from the preceding sections that this appellation is not accurate. In fact, the influence of several cortical hor-

† Abbreviations: MCR, mineralocorticoid receptor; GCR, glucocorticoid receptor; HSP, heat shock protein; DOC, deoxycorticosterone; ACTH, adrenocorticotropic hormone; DBD, DNA-binding domain; HBD, hormone-binding domain; HRE, hormone response element; NTD, N-terminal domain.

mones on cell permeability, the exchange of ions, and metabolite uptake was noted in a number of systems approximately four decades ago (fig. 4).

A. Microbes

In yeasts and bacteria, 30 different steroids were found to antagonize growth and uptake of sugars, ions, and amino acids, leading to altered cell permeability. DOC was the most effective steroid in inhibiting sodium transport, but cortisone also exhibited considerable activity. The analogy with mammalian systems is therefore evident (Conway and Hingerty, 1953; Hechtor and Lester, 1960; Lester et al., 1958).

Four decades later, it was shown that spironolactone analogues inhibited the growth of the photosynthetic alga *Chlamydomonas*, and this was antagonized by aldosterone (Agarwal, 1993a; Mirshahi et al., 1992a,b). Unpublished observations from this laboratory have extended these observations to vascular plants (*Nicotiana tabacum* and *Solanum tuberosum*), paving the way for an entirely new interpretation of the phylogeny of steroid hormone action. Because mammalian steroids are frequently found in higher plants, further studies are clearly required to fully appreciate the significance of these observations (Agarwal, 1993a).

B. Amphibians

Because aldosterone, identified as the mammalian mineralocorticoid back in 1952, made its first appearance at the dawn of the terrestrial life (see preceding sections), it was logical to delineate its activity in amphibian systems. The epithelial layer of the frog skin (Bishop et al., 1961; McAfee and Locke, 1961; Sawyer, 1956) and the mucosal cells lining the urinary (apical) surface of the toad bladder (Crabbé, 1963; Sharp and Leaf, 1966a,b) present permeability barriers for sodium ions.

In the toad bladder, sodium enters passively from the mucosal (urinary) side and is extruded via a "pump" at the serosal (peritoneal) side. This electrogenic transport across a chemical and electrical gradient, where sodium influx is not coupled to the movement of other ions, generates an electrical potential that can be quantitated as a short-circuit current (Crabbé, 1963; Sharp and Leaf, 1966a,b). Steroids neither induced the formation of more pumps nor provided more energy to power them. Rather, hormones enhanced the synthesis of a protein that facilitates sodium entry at the mucosal lining, analogously to the bacterial permeases (Cohen and Monod, 1957). Cortisol, corticosterone, DOC, 9_{α}-fluorocortisol, and 18-hydroxy-DOC also stimulated active sodium transport, albeit less effectively than aldosterone. Furthermore, sodium channels affected by steroid hormones were distinct from those that are stimulated by vasopressin, leading to a summation of the two signals. Pyruvate and acetoacetate are consumed in the process of transporting 2.7 sodium ions per high-energy phosphate bond (Crabbé, 1963; Sharp and Leaf, 1966a,b).

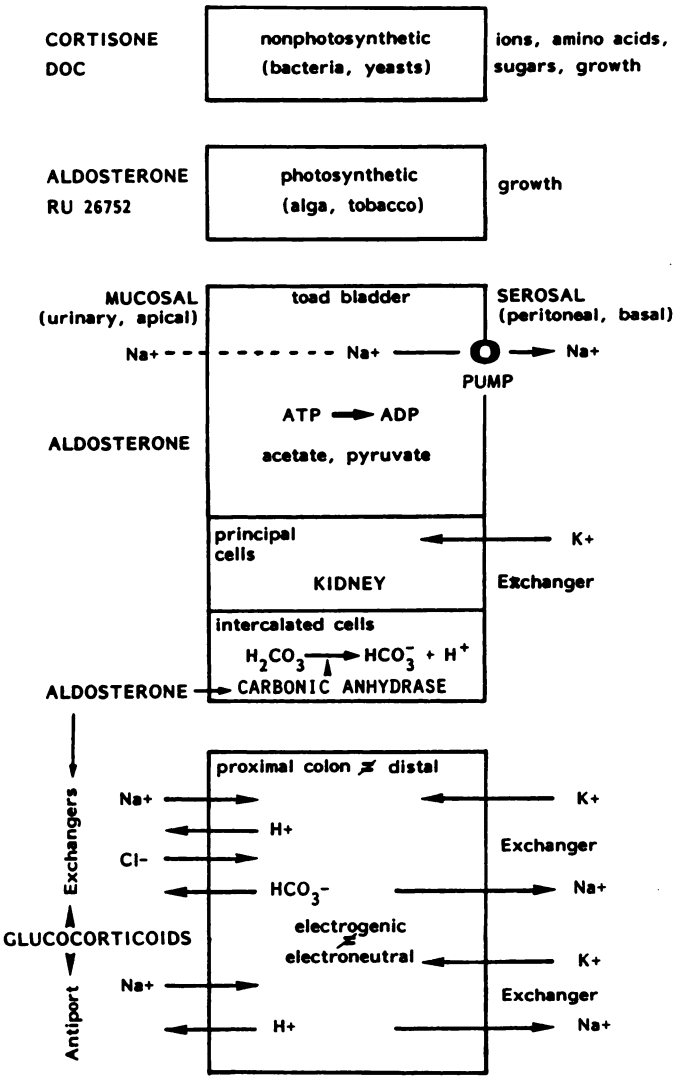

FIG. 4. Physiological expression of mineralotropic action in major cell types. In yeasts and bacteria, 30 different steroids altered growth and metabolite uptake. DOC was the most potent steroid, and even cortisone was effective (Conway and Hingerty, 1953; Lester et al., 1958). Aldosterone and spirolactones (RU 26752) inhibited growth in photosynthetic cells (Agarwal, 1993a; Mirshahi et al., 1992a,b). In the toad bladder, sodium enters passively (dashed line) from the mucosal (urinary, apical) side and is extruded via a pump at the serosal (peritoneal, basal) side. This electrogenic transport, influenced by aldosterone as well as several glucocorticoids, consumes acetate and pyruvate to transport 2.7 Na^+ ions per ATP consumed (Sharp and Leaf, 1966a,b). In the principal cells of the kidney and intestinal epithelium, the electrogenic sodium absorption is linked to electrogenic K^+ secretion, possibly via a Na^+/K^+ exchanger; parallel uptake of Cl^- leads to electrical neutrality but an expanded extracellular fluid volume (Lipton and Edelman, 1971; Marver and Kokko, 1983). Aldosterone increases H^+ secretion by a direct action on carbonic anhydrase in the intercalated cells of the kidney and possibly the Na^+/K^+ antiport in the proximal colon where it also stimulates electroneutral sodium transport by activating $Na^+/H^+/Cl^-/HCO_3^-$ exchangers, which are depressed by this hormone in the distal segment (Ganong and Murlow, 1958; Hulter et al., 1979). Glucocorticoids induce Na^+/K^+/ATPase units in the proximal tubule, modulate electroneutral NaCl absorption in the renal collecting duct, and regulate Na^+/H^+ and Cl^-/HCO_3^- antiporters (Bastl and Hayslett, 1992; Clore et al., 1992).

C. Mammals

The influence of aldosterone on sodium absorption has been studied in the epithelial cells of the kidney (Edelman and Fimognari, 1968; Marver and Kokko, 1983; Snart and Taylor, 1978; Clore et al., 1992), intestine (Levitan and Ingelfinger, 1965; Shields and Elmslie, 1962), sweat glands (Conn, 1963; Shuster, 1962), and salivary glands (Denton et al., 1959).

In the distal nephron of mammalian kidneys, sodium enters passively from the mucosal side lining the lumen and is extruded into the interstitial space through a pump in the serosal lining. The sodium pump behaves as a Na^+/K^+/ATPase using cellular ATP generated by pyruvate or acetoacetate (Edelman, 1979; Skou, 1965), much as occurs in amphibian epithelia (see preceding section).

The effect of aldosterone in the kidney was initially believed to be limited to all segments of the cortical collecting duct (Marver and Kokko, 1983; Stokes et al., 1981). Recently, medullary cells from rat kidney, too, permitted electrogenic Na^+ transport (Husted et al., 1990), and mRNA for MCR was present all along the rabbit nephron (Turla et al., 1993). Steroids influenced cortical sodium absorption as follows: 9_α-fluorocortisol > DOC = cortisol = corticosterone > prednisolone, whereas cortisone was ineffective; progesterone and spironolactone antagonized the effect of aldosterone in the kidney (Marver et al., 1974; Sakauye and Feldman, 1976) and the toad bladder (Sharp and Leaf, 1966a,b) but did not influence the basal rate of salt absorption. This biological response has become a standard tool to test the antialdosterone activity of spirolactones (Kagawa, 1960; Losert et al., 1986; Nédélec et al., 1986; Nishino et al., 1988).

The sodium channel protein has now been characterized by a number of biochemical techniques. It is a heteromeric assembly of five to seven subunits (700 to 750 kDa total mass) one of which is phosphorylated in the presence of arginine-vasopressin, whereas another subunit may be methylated as a consequence of aldosterone action. Aldosterone apparently increases the synthesis of some of the subunits and citrate synthase (Bastl and Hayslett, 1992; Verrey, 1990), but these may be indirect effects. Calcium decreases sodium permeability but does not interact directly with the sodium channel (Benos et al., 1987; Schafer and Hawk, 1992).

Differences between amphibian and mammalian systems actually outnumber the apparently similar mechanism of salt conservation by mineralocorticoids. A linear correlation was observed between net Na^+ absorption and electrogenic K^+ secretion in the kidney and colon, and the two processes may actually be linked by the coordinated action of Na^+/K^+ exchangers in renal principal cells (Bastl and Hayslett, 1992; Foster et al., 1983; Marver and Kokko, 1983; Stokes, 1981). These are compatible with hyperkalemia during adrenal insufficiency and negative K^+ balance in patients with syndromes of mineralocorticoid excess (Conn, 1961; James, 1992). On the other hand, sodium absorption in amphibia is not coupled to the loss of potassium from the cell (Sharp and Leaf, 1966a,b).

Na^+ uptake is electrically compensated for by a parallel uptake of Cl^-, leading to an expansion of the extracellular fluid volume in all cases (Lipton and Edelman, 1971; Sharp and Leaf, 1966a,b). Chronic mineralocorticoid treatment also stimulated chloride uptake from the lumen of the kidney tubule (Hanley and Kokko, 1978), but no comparable studies have been reported in amphibia.

Metabolic acidosis is commonly associated with adrenal insufficiency (James, 1992; Kuwahara et al., 1992). Aldosterone increases H^+ secretion by a direct action on ATPase-dependent carbonic anhydrase in the intercalated cells of the collecting tubules, by altering the Na^+/K^+ antiporter in the proximal colon and possibly by increasing proton pumps in the apical membrane, all of which are independent of hyperkalemia or salt balance regulated via the principal cells (Bastl and Hayslett, 1992; Ganong and Murlow, 1958; Hulter et al., 1979; Koeppen, 1987). In amphibia, salt absorption was said to be independent of H^+ secretion (Sharp and Leaf, 1966a,b), but these effects may actually be linked (Al-Awqati et al., 1976; Ludens and Fanestil, 1974). Increased H^+ secretion permits the production and release of ammonia (Snart and Taylor, 1978).

Adrenal insufficiency in patients with Addison's disease leads to diminished urine concentration, and this can be alleviated by mineralocorticoid repletion (Uffeman and Schrier, 1972). Mineralocorticoids may play a permissive role for the cAMP-mediated hydroosmotic effect of the antidiuretic hormone. Studies of the toad bladder cells have suggested an interaction between adrenocorticoids and antidiuretic hormones, but the underlying mechanisms remain to be elucidated (Lipton and Edelman, 1971).

Aldosterone also stimulated electroneutral sodium transport in the proximal colon, most likely by activating Na^+/H^+ and Cl^-/HCO_3^- exchangers which were depressed in the distal colon; only the electrogenic component is stimulated by aldosterone in the latter segment of the intestine (Bastl and Hayslett, 1992). Such mechanisms in the amphibian bladder have not been explored.

VII. Mineralotropic Action of Glucocorticoids

Steroids that predominantly exert a glucocorticoid type of action are known to influence Na^+/K^+/ATPase activity by pathways distinct from those activated by mineralocorticoids (Bartter and Fourman, 1962; Clore et al., 1992). In the large intestine, glucocorticoids modulate NaCl absorption via the Na^+/H^+ antiport and probably the $Cl^-/HCO_3^-/OH^-$ antiport (Garg et al., 1985). In the proximal tubule, which is not sensitive to mineralocorticoids, glucocorticoids increase the number of functional Na^+/K^+/ATPase units. Indirect evidence suggests that glucocorticoids, unlike aldosterone, modulate the electro-

neutral NaCl absorption in all segments of rat collecting duct (Bidet et al., 1987; Kinsella, 1990; Turnamian and Binder, 1990).

Cortisol and corticosterone, therefore, appear to act in a manner complimentary to the action of aldosterone. Glucocorticoids regulate electroneutral sodium absorption in the large intestine when the plasma mineralocorticoid level is low. Release of aldosterone during volume depletion stimulates electroneutral NaCl absorption in the proximal colon but inhibits that in the distal colon. Aldosterone-induced electrogenic sodium absorption and active potassium secretion may be necessary during urgent situations, whereas glucocorticoids may regulate the normal, daily patterns (Bastl and Hayslett, 1992).

Distinction between glucocorticoid- and mineralocorticoid-induced hypertensive states is rather clear-cut (Clore et al., 1992). Thus, spirolactones (Kalimi et al., 1990; Opoku et al., 1991) and RU 38486 (Grunfeld et al., 1985) antagonize mineralocorticoid- and glucocorticoid-specific hypertension, respectively.

VIII. Control of Steroid Secretion

Decreases in plasma Na^+ and hyperkalemia stimulate aldosterone secretion by a direct action on the adrenal in a number of species (Ganong et al., 1958, 1966, and references therein). This is consistent with the inhibitory effect of hypervolemia due to ACTH secretion during Cushing's disease (Veldhuis and Melby, 1981). An increase in arterial pressure and/or cardiac output can also stimulate the pineal, leading to enhanced aldosterone secretion (fig. 5). Stress-induced ACTH release can also lead to hyperaldosteronism (Melby, 1989; Schambelan and Biglieri, 1972).

The aforementioned stimuli also provoke the liberation of renin from the juxtaglomerular cells in the kidney. Renin-induced formation of angiotensin stimulates aldosterone secretion by a direct action on the adrenal and by interaction with mechanisms sensitive to ACTH, although glucocorticoid secretion is unaltered (Blair-West et al., 1963; Tiemmermans et al., 1993).

Interestingly, ACTH coordinates the secretion of both groups of steroid hormones. The glucocorticoid secretion is sensitive to smaller amounts of ACTH than those required for aldosterone release from the adrenal. The sensitivity of the adrenal to ACTH is altered by Na^+ and water such that feedback inhibition of aldosterone output is possible even in the presence of high levels of plasma glucocorticoids (Fraser and Lantos, 1978; Kater et al., 1985; Sanchez et al., 1990; Stockigt and Scoggins, 1987). Mediators from the immune system alter glucocorticoid release (fig. 5), but their influence on aldosterone output remains unexplored (Bateman et al., 1989). Atrial peptides, dopamine and somatostatin, also alter aldosterone secretion (Tiemmermans et al., 1993).

IX. Mechanisms of Hormone Discrimination

Because the distinction between glucocorticoids and mineralocorticoids appears to be a rather recent, and as yet incomplete, development, some mechanism(s) must ensure class specificity in vivo. Altered availability of the biologically active hormone in vivo could be one possible mechanism ensuring appropriate physiological action.

The conversion of glucocorticoids to inactive 11-keto metabolites by the enzyme 11_β-hydroxysteroid dehydrogenase may causally permit the mineralocorticoid to occupy its specific receptor which may otherwise be masked by far greater levels of circulating glucocorticoids (Funder et al., 1988; Monder 1991). Furthermore, a decrease in the activity of this enzyme could lead to glucocorticoid overload with physiological symptoms of apparent mineralocorticoid excess (Clore et al., 1992). However, a direct correlation between change in 11_β-hydroxysteroid dehydrogenase activity and receptor occupancy by mineralocorticoids remains to be proven, and a large number of considerations argue against the simplicity of this sort of reasoning.

First, by immunostaining techniques, the enzyme does not colocalize with the aldosterone receptor in situ, perhaps due to tissue-specific isoforms (Whorwood et al., 1993), is more abundant in nontarget tissues (liver, renal proximal tubule), and is absent altogether from some of the targets (Funder, 1993). Second, glucocorticoid response is possible in liver despite high 11_β-hydroxysteroid dehydrogenase activity (Monder and Shackleton, 1984). Finally, it is difficult to explain glucocorticoid action in tissues that also respond to mineralocorticoids (Bastl and Hayslett, 1992; Clore et al., 1992).

Enhanced activity of 6_β-hydroxylase produces derivatives with potential glucocorticoid agonist activity leading to hypertensive syndromes (Clore et al., 1992). The physiological action of 6_β derivatives of cortisol or corticosterone is believed to proceed via the occupancy of a group of sites unrelated to the classical receptors for either glucocorticoids or mineralocorticoids (Duncan et al., 1988). This mechanism may be important during glucocorticoid overproduction after stress but has little, if any, relevance during normal homeostasis.

More recently, it has been shown that steroid hormones bind to cell membranes and activate the sodium/proton exchanger within 1 to 2 min. The inositol triphosphate/calcium pathway is also activated during the same period. The membrane binding is 10,000-fold more selective for aldosterone than for cortisol, does not involve the synthesis of RNA or protein, and is not inhibited by spirolactones (McEwen, 1991; Wehling et al., 1992). These parameters are just the opposite of the genomic effects described earlier which were inhibited by spirolactones (Edelman and Fimognari, 1968). The contribution of the membrane-bound sites in aldosterone action remains uncertain and controversial. By far the largest body of evidence favours the idea that the steroid-

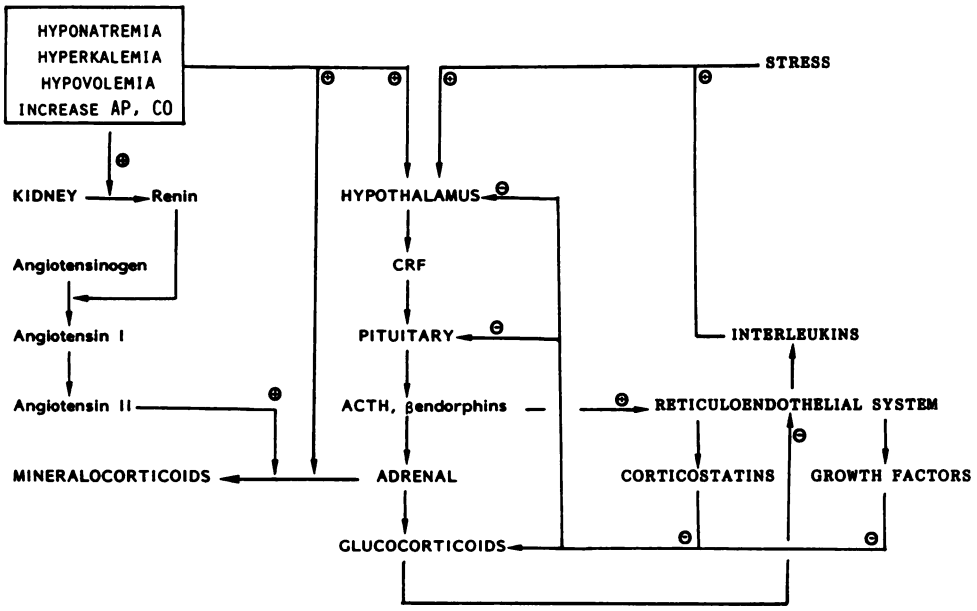

FIG. 5. Secretion of adrenocortical hormones in vivo. Decrease in plasma sodium, hyperkalemia, increase in arterial pressure (AP) and/or cardiac output (CO) are all potent stimuli for the secretion of aldosterone. They may influence the adrenal directly, via the diencephalon (pineal?), or through the release of renin from juxtaglomerular cells in the kidney (Blair-West et al., 1963; Ganong et al., 1966; Melby, 1989; Schambelan and Biglieri, 1971). Stress-induced ACTH release is the common denominator regulating the secretion of both gluco- and mineralocorticoids via the classical endocrine system. Adrenal sensitivity to ACTH can be altered by electrolyte concentration in the extracellular fluid. Even minute amounts of ACTH can stimulate the adrenal to secrete aldosterone, whereas larger amounts are required for glucocorticoid secretion, thereby permitting separate feedback controls (Fraser and Lantos, 1978; Kater et al., 1985; Sanchez et al., 1990; Stockigt and Scoggins, 1987). The immune system exerts horizontal and vertical control of ACTH production at several levels. The cytokines (interleukins, lymphokines, tumour necrosis factor) stimulate ACTH levels not only via the liberation of corticotropin-releasing factor (CRF) from the hypothalamus but also from diffuse neuroendocrine system in ovaries, placenta, testes, etc. ACTH and endorphins modulate the activity of various cell types of the reticuloendothelial system (killer cells, lymphocytes, macrophages). The stimulatory effect of glucocorticoids on many of these cells is antagonized by corticostatins and growth factors produced by them (Bateman et al., 1989), but the influence of such stimuli on aldosterone production has not been assessed.

receptor complex acts as a transcription factor. The remainder of this overview will be devoted to understanding the cellular and molecular aspects of the MCR in relation to other carriers in this superfamily of proteins that activate transcription of tissue-specific genes by binding to DNA via zinc fingers (Agarwal, 1992, 1993b,c; Arriza et al., 1987; Evans, 1988).

X. Cellular Receptor

Several decades ago, many hypotheses were advanced to explain steroid hormone action. These included altered DNA template activity by steroid intercalation between base pairs, modification of kinetics by direct association with enzymes, etc. In retrospect, it is amusing to learn of early reluctance to accept the idea that steroid hormone action is mediated via a soluble, non-membrane-bound, receptor. The synthesis of tritiated steroids by Jensen et al., the development of scintillation counters by Packard, and the foresight of Pincus were collectively responsible for the acceptance of the receptor concept. In this model, the binding of a hormone to the cytoplasmic receptor will lead to the transfer of the complex to the nuclear compartment, finally culminating in the synthesis of tissue-specific proteins (Jensen et al., 1966; Jensen, 1992).

Whereas almost all steroid hormones exert a multiplicity of actions on a number of tissues, mineralocorticoids are unique in being rather specific to the regulation of salt balance. This effect fulfilled the criteria for a receptor-mediated event, viz, the binding to tissue extracts was of high affinity but of low capacity; a good correlation was established between maximal physiological effect and plasma level of aldosterone; the hormonal response was evident only after a variable lag phase during which the synthesis of RNA and protein were required. These led to the generally accepted model in which the binding of aldosterone to its receptor will induce the formation of tissue-specific proteins (Edelman and Fimognari, 1968; Sharp and Leaf, 1966a,b), much as is seen with ecdysone action (Karlson and Sekeris, 1966).

A. Kidney

The physiological action of mineralocorticoid agonists and antagonists in the kidney has already been summarized. The renal MCR from different species has been studied by several groups, largely with the aid of saturation binding and Scatchard analysis. The natural hormone, aldosterone, binds with high affinity (K_d 3 to 4

nM, 37°C) to a relatively small number of sites (10 to 20 fmol) in the cortical portion of the collecting duct (Edelman and Fimognari, 1968; Herman et al., 1968). Adrenalectomy increases renal MCR, in keeping with the negative feedback control of receptors by steroid hormones (Grekin and Sider, 1980). Interestingly, the medullary portion of rabbit kidney, too, tested positive for the MCR both in affinity and capacity (Marver, 1980) and possessed mRNA for MCR (Turla et al., 1993); rat renal medulla permitted electrogenic Na^+ transport (Husted et al., 1990).

A number of glucocorticoid and mineralocorticoid agonists can occupy the MCR as well as the GCR. Altered salt balance can sometimes be corrected by specific antagonists, thereby identifying the underlying receptor type (Koshida et al., 1990; Lan et al., 1982; Ulick et al., 1983). At the present time, RU 38486 is the only steroid with viable anti-GCR potency that does not exhibit any affinity for the MCR and does not antagonize MCR-mediated actions (Agarwal et al., 1987; Grunfeld et al., 1985; Nédélec et al., 1986). The biological potency of spirolactones in vivo (table 1) is species dependent and does not parallel the affinity for kidney MCR in vitro; therefore, these spironolactone derivatives have no clinical future (Agarwal and Lazar, 1991; Nédélec et al., 1986; Sutanto and DeKloet, 1991).

Some authors observed that the spironolactone-MCR complex did not migrate into the nucleus, contrary to what was seen with the aldosterone-MCR complex (Marver et al., 1974; Sakauye and Feldman, 1976), in keeping with the classical model. Paradoxically, by autoradiography, no evidence was found for thermodependent nuclear translocation of the aldosterone-MCR complex (Farman et al., 1984), whereas the spironolactone-MCR complex migrated into the nucleus (Bonvalet et al., 1991).

More recently, ZK 91587 was found to destabilize the MCR at elevated temperatures and to antagonize the transformation of the MCR into a DNA-binding form (termed activation) in rat kidney cytosol. RU 26752 did not influence the activation process but destabilized the MCR (Agarwal and Kalimi, 1988a,b). These derivatives may help elucidate autoradiographic localization.

A polyclonal antiserum, characterized by Western blots, photoaffinity, precipitation, and macroaggregation, revealed rich staining of principal cells in the cortical collecting duct of rat kidney, whereas the intercalated cells were MCR negative (Mirshahi et al., 1992c); these findings confirm the physiological studies cited above. Interestingly, the glomerular region in bovine kidney also stained positively for this receptor (Mirshahi et al., 1992c). MCR immunoreactivity in the rat kidney cortical duct was also noted with an antiserum directed against synthetic peptides (Rundle et al., 1989) and by the antiidiotype (Lombes et al., 1990), but neither serum was analyzed rigorously. The staining pattern was identical in normotensive and genetically hypertensive rats (unpublished observations). The nature of aldosterone-induced proteins (Yost et al., 1982), however, remains to be elucidated.

B. Cardiovascular System

Adrenocortical steroids influence a number of physiological functions in the cardiovascular system such as inotropism, peripheral resistance, venous return, ventricular ejection, myocardial contractility, etc. (Akatsuka et al., 1974; Kornel et al., 1987; Maempel, 1974; Stumpf, 1990; Vargish et al., 1974). An excess of mineralocorticoids leads to myocardial fibrosis, followed by ventricular remodeling (Brilla et al., 1990; Weber et al., 1992). Aldosterone modifies cardiac and vascular Na^+/K^+/ATPase gene expression (Garwitz and Jones, 1982; Ikeda et al., 1991; Lin et al., 1990; Llaurado et al., 1983; Worcel and Moura, 1987), increases β-adrenergic activity in cultured arterial cells (Jazayeri and Meyer, 1989), and regulates the function of the atrial natriuretic peptide (Ganguly, 1992; Geiger et al., 1990; Mulay et al., 1993; Ota et al., 1992). The Na^+/Ca^{2+} exchanger, widely distributed over the surface of rat and guinea pig hearts, could be a target for steroids (Kieval et al., 1992).

Approximately 15 years ago the presence of MCR in rat myocardium was demonstrated by biochemical techniques (Agarwal and Philippe, 1979). A decade later, both the atrial and ventricular compartments of rat heart were estimated to contain high-affinity (K_d approximately 1 nM), low-capacity (10 to 12 fmol/mg protein) binding sites for aldosterone (Barnett and Pritchett, 1988; Pearce and Funder, 1987). The binding of aldosterone in rabbit aorta (Kornel et al., 1982) and human arterial cells (Scott et al., 1987) did not show characteristics typical of the MCR. Furthermore, the aortic cells from both salt-resistant and salt-susceptible rats exhibited comparable aldosterone-binding components (Nichols et al., 1985).

Antimineralocorticoids reversed a number of physiological effects mentioned above. For example, spironolactone prevented the reactive fibrosis and scarring seen in primary hyperaldosteronism (Weber et al., 1992). These studies should be interpreted with caution because spironolactone exhibits negligible affinity for the MCR (table 1) and influences a number of other receptor types and cell processes (Agarwal and Lazar, 1991; Nédélec et al., 1986; Sutanto and DeKloet, 1991). An analogue of RU 26752 (fig. 3) blocked Na^+ influx as well as K^+ permeability in arterial smooth muscle (Worcel and Moura, 1987). RU 26752 and ZK 91587 influenced rat myocardial MCR in much the same manner as the renal receptor (Agarwal and Kalimi, 1988c; Lazar et al., 1990a).

A polyclonal antiserum permitted precipitation, aggregation, and Western blot analysis of rat cardiac MCR that was also revealed by photochemistry; immunofluorescence suggested perinuclear localization of MCR in

myocytes (Mirshahi et al., 1992d). Here, bovine atria appeared more rich in MCR-specific blots than did the ventricular compartment, confirming earlier investigations in which atria concentrated more dexamethasone and estradiol than did the ventricles (Stumpf, 1990; Lin et al., 1990). The demonstration of MCR in myocytes and large vessels, with the aid of an antiidiotype (Lombes et al., 1992), should be interpreted with caution because these authors did not show control sections stained with nonimmune serum. Also, aldosterone was bound to the MCR along with the HSP which is generally believed to dissociate in the presence of the steroid, especially during lengthy centrifugation (Couette et al., 1992). Finally, arterial cells do not apparently contain the MCR (Kornel et al., 1982; Scott et al., 1987).

C. Gastrointestinal Tract

The influence of steroid hormones on the gastrointestinal tract has a rich, long, and varied history (Bastl and Hayslett, 1992). In a recent study, continuous infusion of aldosterone stimulated electroneutral Na^+ absorption and K^+ secretion in rat proximal colon, which was prevented by spironolactone; glucocorticoids, too, increased Na^+ and Cl^- absorption in the intestine, but the receptor type(s) involved in this process were not identified (Turnamian and Binder, 1990). In the past, specific antagonists have permitted identification of the receptor involved in the physiological responses (Kalimi et al., 1990; Opoku et al., 1991; Worcel and Moura, 1987) and may be useful with the intestine.

Aldosterone binds with high affinity (K_d 0.6 nM) to sites in the proximal and distal sections of rat colon in the estimated range of 50 to 60 fmol/mg protein (Binder et al., 1986). Duodenum, jejunum, and ileum also exhibit aldosterone-specific binding, but their affinity and capacity were not determined (Pressley and Funder, 1975). MCR mRNA was found to be expressed throughout the gastrointestinal tract, with highest levels in the distal segment (Fuller and Verity, 1990). To date, there has been no histochemical analysis of the MCR in this organ, despite the availability of various antisera.

The similarity between the hydrodynamic properties of the chicken intestinal receptor and rat renal MCR (Oblin et al., 1989; Schulman et al., 1986) may not be valid; MCR-specific spirolactones should have been used because natural steroids exhibit notoriously elevated cross-reactivity for avian GCR (Sandor et al., 1983).

D. Lung

Adrenocortical steroids are known to influence lung maturation and development (Ballard, 1989). Lung possesses receptors for glucocorticoids (Agarwal and Philippe, 1977; Giannopoulos et al., 1974) and gonadal hormones (Morishige and Uetake, 1978). More than a decade ago, rat lung was found to contain about 2 fmol MCR/mg protein and an affinity of <1 nM for aldosterone (Krozowski and Funder, 1981). More recently, the presence of MCR in pulmonary cytosol was demonstrated with the aid of an anti-MCR antibody (Agarwal and Mirshahi, 1992), although this tissue was negative for MCR mRNA (Loffreda et al., 1992). The cell type(s) containing the MCR and the role of this receptor in the lung remain to be explored. In any event, the MCR is distributed more extensively than had been believed (fig. 6).

E. Glandular Tissues

Excretory epithelium in the parotid, pancreas, salivary, mammary, and sweat glands contributes to electro-

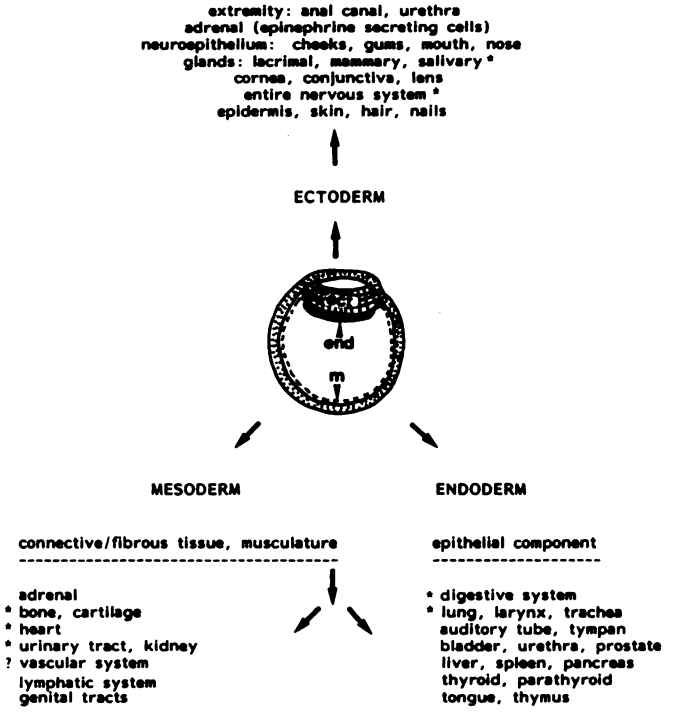

FIG. 6. Tissue distribution of the MCR. In the center, the early blastocyst is shown with its three germ layers, ectoderm (ect), endoderm (end), and mesoderm (m). The two former layers give rise to the epithelial lining of a number of organs, but the structural component (connective/fibrous tissue, musculature, blood vessels, bones) in all cases is of mesodermal origin (Warwick and Williams, 1973). In addition to some convincing evidence for pulmonary (Agarwal and Mirshahi, 1992) and brain (Agarwal et al., 1993) MCR, endodermal (Schulman et al., 1986; Oblin et al., 1989) and ectodermal (Shuster, 1962) epithelia have been mostly studied with the aid of nonspecific radioligands or by a poorly characterized antibody (Sasano et al., 1992). In fact, the presence of MCR has been most thoroughly demonstrated by specific ligands, immunohistochemistry, and photoaffinity in organs of mesodermal origin such as the heart (Agarwal and Philippe, 1979; Agarwal and Kalimi, 1988c; Mirshahi et al., 1992d), the kidney (Agarwal and Kalimi, 1988b; Mirshahi et al., 1992c) and the bone (our unpublished observations). MCR prefers corticosterone or cortisol in ectodermal neurons (Beaumont and Fanestil, 1983), whereas aldosterone and spirolactones bind to this receptor in organs of endodermal or mesodermal ancestry (Agarwal and Lazar, 1991; Sutanto and DeKloet, 1991); the sequences of cloned MCR from human kidney (Arriza et al., 1987) and rat brain (Patel et al., 1989) are, however, identical. The assumption that MCR (*) is limited to epithelial linings is, therefore, just as fallacious as the "type I and II" designation (Funder and Sheppard, 1987). Modern tools of molecular biology have been used only rarely in this field and may help resolve some of these controversies.

lyte homeostasis (Denton et al., 1959; Shuster, 1962). In these organs, the affinity and the capacity of MCR have not been the subject of precise analysis, particularly with the aid of potent and specific steroids (fig. 3). Recently, MCR-specific immunoreactivity was observed in the cells of the excretory ductal system in some of these organs (Sasano et al., 1992). Because the antibody was raised against a synthetic peptide, its specificity for the MCR remains equivocal, and the results should, therefore, be interpreted with caution, more so because even a Western blot analysis was not performed to assess the receptor size. Northern blot analysis and in situ hybridization would be particularly welcome in this context.

F. Brain

Corticosteroids influence the growth, development, maturation, and differentiation of mammalian brain in an important manner (Bohn, 1980; Doupe and Patterson, 1982; Meyer, 1985). Whereas glucocorticoids are generally believed to mediate stress and tonic effects, mineralocorticoids probably regulate salt intake and blood pressure (DeKloet and Reul, 1987; Magarinos et al., 1986). Interestingly, brain MCR in vivo is occupied by endogenous glucocorticoids (corticosterone in rodents, cortisol in humans), whereas MCR in peripheral organs exhibits selectivity for aldosterone (Funder and Sheppard, 1987; McEwen et al., 1986). Brain is a rich source of MCR in the mammal (about 51 fmol/mg protein), and aldosterone binding exhibits high affinity (K_d 0.9 nM) which, in the dog, can be displaced by corticosterone > aldosterone = cortisol > ZK 91587 > RU 26752 > spironolactone (Beaumont and Fanestil, 1983; Reul et al., 1990). Therefore, these antagonists do not appear suitable to study the brain MCR of ectodermal origin because they may bind to sites other than this receptor (Sutanto and De Kloet, 1991), contrary to the situation in the peripheral organs of endodermal or mesodermal origin (fig. 6).

In autoradiographic studies, aldosterone was concentrated primarily in the limbic regions (McEwen et al., 1986), but the mRNA for MCR was widely distributed in the adult rat (Herman et al., 1989; VanEekelen et al., 1991) and human (Seckl et al., 1991) brain. This observation was confirmed in the developing rat brain with the aid of an antiserum against synthetic peptides whose specificity for the MCR remains to be ascertained (Ahima et al., 1991).

Recently, an anti-MCR-specific serum revealed richest staining in the cerebellum, followed by hippocampus, and several hypothalamic foci in adult rat brain (Agarwal et al., 1993). This antibody readily recognized radioactive aldosterone bound specifically to the MCR in the presence of RU 38486, which was used to saturate the GCR. However, radioactive RU 26752 apparently binds to sites other than the MCR (Beaumont and Fanestil, 1983; Reul et al., 1990) and went unrecognized by the antibody, confirming the specificity of the antiserum (Agarwal et al., 1993).

Interestingly, cloned MCR binds both gluco- and mineralocorticoids with comparable affinity in vitro (Arriza et al., 1988). In addition, the primary structure of MCR from rat brain (Patel et al., 1989) is identical with that from human kidney (Arriza et al., 1987). Thus, the confusing nomenclature of type I (corticosterone-preferring MCR) and type II (GCR) sites (Funder and Sheppard, 1987) is no longer justified, and its extension to peripheral organs that prefer natural mineralocorticoids is actually wrong. Rather, posttranslational modifications of a primary gene product appear to be responsible for the organ-specific differences. In other words, peripheral organs have progressed farther in recognizing aldosterone as a mineralocorticoid than has the brain in which glucocorticoids may perform both functions, as occurs in organisms at lower levels of evolution (Barrington, 1968; Sandor et al., 1976). The B7 cell line from brain microvessels was used to delineate some of these interactions, but the data should be interpreted with caution because lung was negative for MCR mRNA (Loffreda et al., 1992), despite the demonstration of pulmonary MCR by immunochemistry (Agarwal and Mirshahi, 1992) and ligand binding (Krozowski and Funder, 1981).

G. Isolated Cells

Mononuclear leukocytes contain binding sites whose affinity for aldosterone (K_d 1.4 nM) is comparable to the kidney MCR (Armanini et al., 1985). Furthermore, MCR downregulation is seen in patients with mineralocorticoid excess (Armanini et al., 1987, 1988). These are to be contrasted with the non-MCR membrane sites which have a K_d of 0.1 nM or less (Wehling et al., 1992).

B- and T-lymphocytes from human spleen, too, contain high-affinity (K_d approximately 2 nM) aldosterone-binding sites (approximately 174 per cell). Because whole cells were used, it was not possible to assess whether the binding was associated with the membrane or represented the genomic receptors in the cytoplasmic and/or nuclear compartment (Armanini et al., 1988).

In unpublished observations from this laboratory, the cytosolic MCR has been detected in osteoblasts from rat calvaria. MCR positivity was evident by the binding of specific spirolactones, immunocytochemistry, photoaffinity, and after the amplification of total RNA by polymerase chain reaction. Furthermore, aldosterone inhibited osteoblast cell growth in vitro, an action that was prevented by RU 26752. Thus, MCR is present in cells of mesodermal origin just as well as the layers derived from the embryonic ectoderm or the endoderm (fig. 6). Osteoblasts contain a $Na^+/K^+/Cl^-$ cotransporter that is influenced by a number of calcitropic hormones (Whisenant et al., 1991), and this may be affected by mineralocorticoids because bone contributes to the regulation of acid-base balance in an important manner (Green and

Kleeman, 1991). Such considerations provide important new opportunities for further work with this mesodermal tissue.

H. Nonmammalian Vertebrates

Because aldosterone makes its first appearance with the advent of terrestrial life, steroids now fulfilling glucocorticoid-specific functions in the mammal were also active as mineralocorticoids prior to the teleosts, as described above. Although the toad bladder was the model of choice to study sodium homeostasis in the past, it has not been analyzed in recent years using modern techniques and probes.

The A6 cell line derived from toad kidney has attracted much attention as a model for mineralocorticoid action (Rafferty, 1969). However, aldosterone was used to assess the presence of both the GCR and MCR by autoradiography and radioligand binding; therefore, the nature of receptors in A6 cells remains equivocal (Claire et al., 1989). Because aldosterone-mediated electrogenic Na^+ transport in A6 cells was blocked by RU 38486, the effects appear to be GCR mediated; unfortunately, the influence of the potent antimineralocorticoids was not analyzed (Schmidt et al., 1993).

Lower intestinal osmoregulation/electrolyte homeostasis is very important in euryhaline vertebrates such as teleosts, marine reptiles, and marine birds in which the distinction between glucocorticoids and mineralocorticoids is often blurred (Sandor, 1969). This is also true to some extent in the chicken (Skadhauge, 1981), whereas nasal glands contribute to osmoregulation in the duck (Anfinsen, 1959). These sources yield 2 to 3000 fmol/mg protein that binds aldosterone with an affinity of 1 to 5 nM (DiBattista et al., 1983). Here again, synthetic spirolactones (fig. 3), MCR-specific antibodies, and search for mRNA with known probes may be useful to assess the nature of the binding sites.

XI. Receptor Heterogeneity

Before the modern era of molecular biology, the cellular receptor was studied almost exclusively by radioligand-binding assays consisting of competition experiments in the presence of various agonists and antagonists, Scatchard plots, association/dissociation kinetics, etc. Little attention was generally paid to the influence of steroids and cellular environment on the conformation(s) of the receptor protein.

With the aid of a number of chromatographic techniques, it soon became evident that the MCR was able to assume a number of molecular forms whose genesis was tissue, steroid, and species dependent (Agarwal, 1976, 1978). These observations were confirmed independently in a contemporary study (Strum et al., 1975) and soon extended to all receptor classes (Agarwal, 1977). In addition, these receptor forms did not reflect the "intracellular transcortin," an appelation much in vogue at that time to account for all observations that did not fit into the accepted model of one steroid-one receptor site (Amaral et al., 1974). Similarly, endogenous proteolysis, limited in time and space, could not explain the origin of receptor multiplicity (for detailed references, see Agarwal, 1979).

Thus, two decades ago, it was not possible to answer the question whether receptor heterogeneity reflects different proteins or modified forms of a primary gene product. Receptor purification and/or cloning were required to elucidate this with certainty. It took about 15 years to tackle this problem with the aid of a number of different developments, the most important of which, perhaps, was the availability of MCR-specific ligands.

XII. Receptor Purification

Glucocorticoids and mineralocorticoids nonspecifically occupy the receptor for the other hormone class, and the MCR is unstable in vitro, particularly at room temperature (Agarwal, 1978; Arriza et al., 1987; Lan et al., 1982). In all likelihood, therefore, the product obtained after lengthy passages through an affinity resin and gel permeation in the presence of aldosterone (Lombes et al., 1987), or following chromatography on heparin-Sepharose (Weisz et al., 1986), probably represents either the GCR or the progesterone receptor. These techniques were uniformly negative in our hands when the MCR-specific ligands RU 26752 and ZK 91587 were used in place of aldosterone (Lazar et al., 1990a,b). We consequently developed a simple procedure consisting of cytosol saturation with [^{3}H]RU 26752 at 4°C, passage through phosphocellulose, activation at room temperature, and batch elution from DNA-cellulose, in that order. The final product, obtained within 6 to 8 h, was resolved as a single, homogeneous band on polyacrylamide gels from both rat heart (Lazar et al., 1990a) and kidney (Lazar et al., 1990b).

The purified, activated MCR singularly eluted in 0.017 M sodium phosphate from the DEAE-cellulose-52 resin, irrespective of the occupying steroid (Lazar et al., 1990a,b), whereas the unactivated receptor present in crude cytosol eluted in 0.006, 0.024, and 0.06 M phosphate in the presence of aldosterone, RU 26752, and R 5020, respectively (Agarwal and Kalimi, 1988a–c). Receptor heterogeneity may, therefore, stem from posttranslational modifications, contrary to the suggestion, made with a poorly characterized product (Lombes et al., 1987), that crude and partially purified MCRs are similar.

XIII. Anti-Receptor Antibodies

In the past two decades, various types of antibodies have been used to delineate receptor structure and function (Taub and Greene, 1992). The steroid ligand covalently linked to a carrier has been used as an immunogen to screen for idiotypes that can subsequently be used for the genesis of antiidiotypes (Cayanis et al., 1986). The idiotype binds the steroid in a rather specific manner, contrary to the receptor that accepts a number of ligands

with varying degrees of affinity (Agarwal and Cayanis, 1986). Because of poor characterization of the antiserum and consistent lack of adequate controls caution should be used when interpreting the data for MCR (Lombes et al., 1990, 1992).

Synthetic sequences of cloned proteins have been used to engender antibodies supposedly specific to the MCR, despite the fact that the conserved domain structure shares close structural homology among various members of the receptor superfamily (Laudet et al., 1992). These antisera, too, were not characterized for specificity to MCR but have been used for immunoperoxidase staining (Rundle et al., 1989; Sasano et al., 1992).

Antibodies to the native receptor are expected to be directed against the tridimensional conformation of the whole protein and thus be specific to it. This antiserum was also characterized for specificity to the MCR by Western blots, photoaffinity, aggregation, and precipitation of the radioligand-receptor complex. Immunoperoxidase staining with this antibody in the brain (Agarwal et al., 1993) and peripheral tissues (Mirshahi et al., 1992c,d) revealed a pattern somewhat unlike that observed with other antisera and may form a means of identifying MCR-responsive targets.

XIV. Cloning and Molecular Structure

The MCR is a member of a family of 30 or more proteins whose primary structure suggests a complex evolutionary history involving gene duplication and swapping between domains of vertebrate and insect origins. A functional analysis by site-directed mutagenesis led to the estimated ancestry of 500 million years beginning at the arthropod/vertebrate dichotomy during the early burst of metazoan evolution of the precambrian era (Barrington, 1964; Laudet et al., 1992). Because some photosynthetic cells also respond to hormonal steroids, much in the same way as animal cells (Mirshahi et al., 1992a,b), the principles of zinc finger-mediated transcription regulation were probably laid down at the very origin of life processes (Agarwal, 1993a).

The MCR is the largest member in this superfamily of proteins consisting of 984 residues that are identical from both the human kidney (Arriza et al., 1987) and rat brain (Patel et al., 1989). This amounts to an estimated molecular mass of 107 kDa which could vary depending on the extent of glycosylation (Pagano et al., 1993).

The composition of the NTD is highly variable, exhibiting little homology among various receptor classes, and this has formed the theoretical basis for the genesis of receptor-specific antibodies (Taub and Greene, 1992). The highly acidic NTD may discriminate between specific and nonspecific contacts with DNA, modulate the magnitude of transactivation, and determine nucleotropism via the nuclear localization 1 signal (Picard et al., 1988; Luisi et al., 1991).

The approximately 60 residues (15 kDa) of the DBD exhibit up to 90% homology across the superfamily of 30 proteins (Evans, 1988; Laudet et al., 1992). The two zinc fingers of the DBD interact with the HRE in the DNA in a helix-turn-helix configuration, but only the C-terminal finger ensures dimer interface for complex stability in the major groove (Berg, 1989; Luisi et al., 1991, for reviews).

A hydrophilic hinge region of 40 to 70 amino acids links the DBD to the HBD. The hinge region starts with a sequence facilitating nucleotropism, and this is followed by a nuclear localization stretch (NL2 or Tau 2) toward the C-terminal end, as shown schematically in figure 7.

The HBD of approximately 250 residues exhibits a rather conserved structure within various members of this superfamily. Initially, it was believed that all agonists and antagonists bind to the one and the same active site, but aldosterone-binding sequences may be different from those that bind spirolactone derivatives (Agarwal and Kalimi, 1988a–c; Agarwal and Lazar, 1991; Luzzani and Glasser, 1984). The technique of photoaffinity labeling, in which the steroid is covalently linked to the MCR (Agarwal and Mirshahi, 1992; Agarwal, 1993a–c; Mirshahi et al., 1992a–d), may help identify the amino acid(s) that bind the ligand(s).

Hydrophobic cluster analysis (Ojasoo and Raynaud, 1987; Varloot et al., 1992) has revealed a proline-rich pocket in the HBD of androgen receptor, GCR, MCR, and progesterone receptor that may explain the functional cross-reactivity between these hormone classes (Agarwal, 1992; Philibert, 1984). This has permitted the molecular modeling of steroid analogues with dissociated activity (Philibert et al., 1991).

The HBD also forms the site of association of a number of proteins, such as HSP90 and immunophilins (Smith and Toft, 1993), but sequences involved in this interaction have not been identified. A number of receptor-mediated processes, including HSP90-assisted folding and receptor activation, are believed to require ATP (Beckman et al., 1990). Amino acids 745 to 780 in the HBD of the MCR correspond to ATP-binding sequences (Rossmann fold) found in serine proteinase inhibitors (Varloot et al., 1992). Clusters 90 to 100, 110 to 130, and 490 to 500 are rich in aspartic acid and serine, compounds whose chromophores may form putative calcium-binding sites (J. P. Mornon, personal communication).

We have recently observed the binding of both calcium and ATP to MCR immunopurified from bovine kidney. Analysis by far-ultraviolet circular dichroism spectra showed that MCR contains 33% α-helices and 30% β-sheets, an observation compatible with a relatively flat conformation of the native protein (Pagano et al., 1993). Thus, cross-talk between several protein families may be involved in the coordination of steroid hormone action at the cellular level.

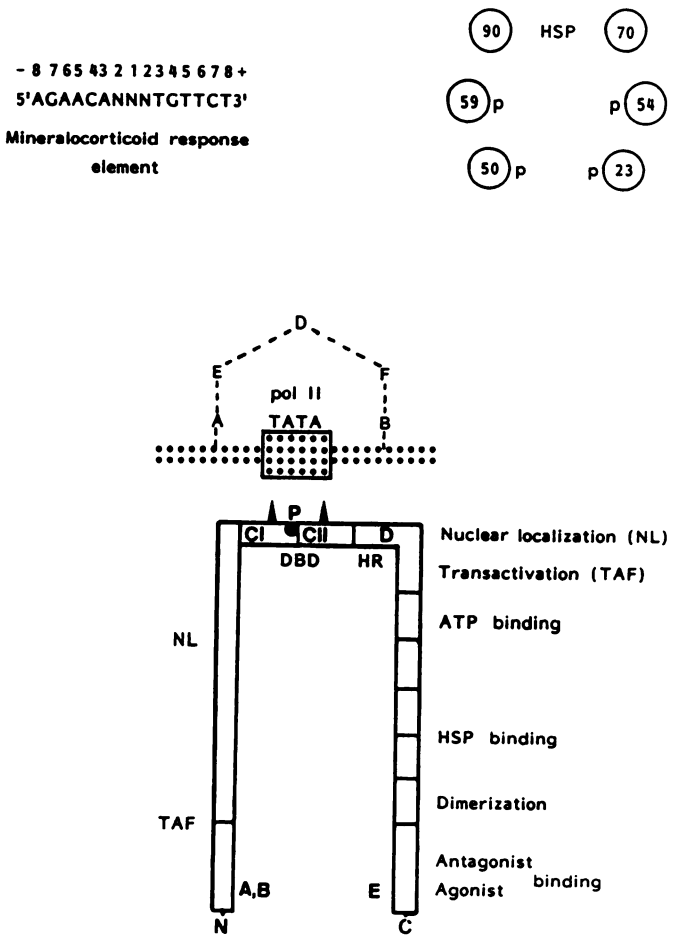

FIG. 7. Transcription activation by steroid hormone receptors. With the aid of site-directed mutagenesis, the receptor has been divided into five functional domains (A to E). For the MCR, the N-terminal (A/B) domain consists of about 600 residues and contains sequences for nuclear localization (NL1) and transcription activation (TAF1). The C-terminal HBD of about 250 residues may possess distinct sequences for agonist and antagonist binding in the E-region. The HBD is also endowed with sequences for dimerization (leucine zippers?), association with proteins of various molecular sizes (59, 54, 50, 23 kDa, etc.), and binding of ATP (Rossmann folds) and calcium. The TAF2 and NL2 sequences of the HBD are immediately adjacent to a hydrophilic stretch of 40 to 70 amino acids, termed the hinge region (HR), that connects the HBD to the DBD. The two zinc fingers (CI and CII) of the DBD recognize the mineralocorticoid response element in the reporter gene via a "P" box at the end of CI; CII contributes to the stability of dimerization. In the resting state, the access to all but the E-domain is occluded by the associated proteins. The binding of a ligand to E initiates activation leading, successively, to the dissociation of HSP and other proteins, receptor dimerization along the response element, and the formation of an initiation complex at the proximal promoter (TATA box) for polymerase (pol II) mediated mRNA synthesis that involves several transcription factors (A to F). Hormone antagonists permit receptor binding to DNA, but subsequent events are blocked. For further details and reviews, see Agarwal, 1992, 1993-a–c; Arriza et al., 1987; Evans, 1988; Rupprecht et al., 1993; Pearce and Yamamoto, 1993.

XV. Intracellular Localization and Transactivation

The interaction of a ligand with the E-region of the HBD (fig. 7) releases the inhibition exerted by associated proteins, such as HSP90, permitting the receptor to form stable dimers (K_d 1 nM) or tetramers (K_d 10 pM) via the two zinc fingers at the HRE in the proximal promoter (TATA box). Although the androgen receptor, GCR, MCR, and progesterone receptor share the same HRE, the activation of the reporter gene is specific to the particular class of hormone (Berg, 1989; Freedman, 1992).

Residues 105 to 440 in the NTD of the MCR may permit discrimination from the GCR type of responses (Pearce and Yamamoto, 1993). Studies with chimeric human GCR/MCR hybrids revealed that the NTD of the latter provides weaker transactivation stimulus than that of the former and may even be inhibitory (Rupprecht et al., 1993).

It was believed at one time that antisteroids would interfere with the conformational change required for MCR binding to the DNA. This view was challenged by the observation that MCR activation was possible in the presence of RU 26752 (Agarwal and Lazar, 1991; Agarwal and Kalimi, 1988a–c). One study suggested destabilization of the MCR by spironolactone-mediated HSP90 release (Couette et al., 1992) in contrast to the positive role played by HSP90 in transactivation (Picard et al., 1990).

The intracellular localization of the innate steroid receptors has become a matter of contemporary controversy. Originally, the arrival of a steroid was believed to activate the cytoplasmic receptor leading to internalization of the complex into the nucleus (Jensen et al., 1966), but estrogen and progesterone receptors are now believed to be inherently nuclear (Rajendran and Parikh, 1987). The original model appears valid for the GCR and possibly for the MCR, because the two share close structural and functional kinship (for reviews, see Agarwal, 1992; Evans, 1988; Picard et al., 1988). In any event, the nonactivated receptor is believed to be loosely bound to the nuclear matrix and diffuses into the cytosolic compartment during homogenization for assays in vitro; the activated receptor binds tightly to DNA and can be eluted only with a high concentration of salt. Such considerations do not alter the fundamentals of transactivation but should be kept in mind for further elucidation.

XVI. Epilogue

Steroids are present in all life forms as membrane constituents and/or vitamins, hormones, cytotoxins, and chemical messengers. Diversity in steroid structure and function is believed to be of the greatest evolutionary significance. Steroidogenesis in plants is not limited to specialized organs. In animals, cells specializing in steroid synthesis have a common origin in the embryonic mesoderm. Ten separate families (one fungal, one bacterial, and eight mammalian) of P450 genes apparently coalesced approximately 2 billion years ago, leading to the present-day pattern of steroid synthesis (fig. 1). This

is consistent with the concept of specialized cells developing for response to existing hormones without further change in the steroid itself.

Aldosterone synthesis is first noted in teleosts and is limited strictly to the adrenal, which, in higher mammals, further differentiates into zona glomerulosa. This steroid is relatively unique because it possesses a rather limited sphere of physiological action which, in contemporary endocrinology, has become identified with mineralocorticoid response. Precursors of aldosterone, collectively termed glucocorticoids by mammalian endocrinologists, ensure mineralocorticoid function in all life forms in which aldosterone is absent (fig. 2). Even in mammals, steroids with a predominantly glucocorticoid type of action regulate the daily pattern of mineral balance, whereas aldosterone is supposedly required in urgent situations (fig. 4). In excess, aldosterone can exert a glucocorticoid type of action. The secretion of both types of steroids is coordinated by ACTH, but each category of hormone also has its own regulatory stimuli for specific feedback control (fig. 5).

Systems of signal transduction in the cell, too, appear to have a common ancestry. The primary structure of the 30 steroid hormone receptors cloned to date suggests a complex evolutionary history that dates back to the early burst of metazoan evolution of the precambrian era 500 million years ago. Gene duplication and domain swapping between proteins of vertebrate and insect origins led to the genesis of the steroid receptor superfamily known to us today. Interestingly, enzymes involved in steroid biosynthesis possess sequences resembling those found in genes regulated by cAMP and glucocorticoid hormone response elements. Such observations may have far reaching significance regarding the physiology of zinc finger-mediated transcription activation (fig. 7).

In peripheral organs (heart, kidney, lung), the MCR exhibits a clear preference for aldosterone. Brain MCR, however, prefers natural glucocorticoids (corticosterone in the rat, cortisol in the human) despite the fact that the primary structure of this protein from both sources is identical. Therefore, peripheral organs have progressed farther in recognizing aldosterone as a mineralocorticoid than has the brain, in which glucocorticoids can perform both functions, as in lower phyla. This heterogeneity clearly reflects posttranslational modifications, but it generated the confusing nomenclature of type I and II receptors, which is invalid even for the brain; its extension to MCR in peripheral organs is mere foolishness. Because cells of diverse origins possess MCR and respond physiologically to aldosterone mineralocorticoid action appears to be more widely spread than had hitherto been believed (fig. 6).

The cross-reactivity evident from the foregoing means that aldosterone is not an appropriate ligand for its receptor. Synthetic spirolactones, with marked affinity and specificity for the MCR, are now available (table 1). These have permitted MCR purification and, together with the receptor-specific antiserum, should help identify MCR-specific targets. On the other hand, the physiological action of spirolactones in photosynthetic cells indicates an active site on the receptor that was present even at a time when cell signaling was still in its primordial state. Photochemical labeling of the MCR with an appropriate ligand (fig. 3) can unravel some of these relationships.

Considerations summarized in this overview assume even greater importance in various syndromes of hormone deficiency or hormone excess. Here again, receptor-specific antagonists have helped us understand underlying mechanisms. Molecular modeling may permit the synthesis of derivatives targeted against any one of the numerous receptor functions (steroid recognition, nuclear translocation, transactivation). Because the same HRE is shared by GCR and MCR, crystal analysis of the agonist/antagonist-receptor complex is required to discriminate signal from noise.

Computer simulation has revealed that the steroid receptor superfamily contains sequences capable of binding a number of mediators involved in cell signaling, such as calcium and ATP. Experimental proof for this has also been obtained and suggests an interaction between several families of proteins. Molecular biology will have come of age if it can reverse the contemporary tide of overspecialization and emphasize the organismal approach in unraveling the mystery of the living whole.

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