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THE BIOSYNTHESIS OF ALDOSTERONE

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Summary—The cellular mechanisms for aldosterone biosynthesis are incompletely understood. Although the enzymes involved are now well characterized, the dynamics of aldosterone secretion in a variety of rat adrenal preparations are not consistent with the concept that freshly synthesized corticosterone is an important intermediate. In whole glomerulosa tissue preparations, aldosterone is more readily formed from endogenous precursors than from an added radioactive precursor, such as [^{3}H]pregnenolone, and in the *in situ* perfused gland preparation, aldosterone responses to stimulation, for example by ACTH, are significantly more rapid than those of corticosterone, suggesting a tissue source of steroid substrate for aldosterone production other than corticosterone. The only steroid which is stored in rat adrenal glomerulosa tissue to any extent is 18-hydroxydeoxycorticosterone (18-OH-DOC), and this pool has been located in plasma membrane fractions. It is lost on preparation of collagenase dispersed glomerulosa cells. Since dispersed glomerulosa cell preparations produce significantly less aldosterone, relative to corticosterone, than incubated intact whole glomerulosa, it is plausible that this tissue pool (which is not found in the inner zones) is the immediate precursor for aldosterone formation.

Further evidence shows that trypsin, which stimulates aldosterone (and 18-hydroxycorticosterone) production in rat intact glomerulosa tissue, but not in dispersed cells, stimulates translocation of protein kinase C to the plasma membrane. It is plausible that one function of protein kinase C in the rat adrenal zona glomerulosa is to mobilize membrane sequestered 18-OH-DOC for conversion to aldosterone.

INTRODUCTION

The biosynthetic pathway by which secreted aldosterone is formed in the zona glomerulosa of the adrenal cortex is not resolved, although there are several possibilities. It was established soon after the original characterization of aldosterone that corticosterone may be a substrate for its formation [1], and subsequently there have been many studies on the regulation of aldosterone production which utilize this precursor [2–4]. In this way, two sites for control of aldosterone biosynthesis were recognized, one at the site of cholesterol side chain cleavage (the early pathway), and the second, the late pathway site, between corticosterone and aldosterone. Later, the finding that cytochrome *P*-450 is involved, and thus the reaction depends on hydroxylation [5], and that aldosterone and 18-hydroxycorticosterone (18-OH-B) are synthesized concomitantly under many conditions suggested that 18-OH-B is an intermediate in the synthesis of aldosterone and that the pathway involved the hydroxylation of corticosterone at C-18 followed by dehydrogenation [4, 6] (possible pathways are illustrated in Fig. 1). This view was refined to suggest that 18-OH-B is converted by a second hydroxylation, and that 18-dihydroxycorticosterone is then converted to aldosterone through spontaneous loss of water [7, 8]. With the resolution of cytochrome *P*-450 structures and functions, it has now become clear that a single species, $P\text{-}450_{11\beta/18}$, catalyses both 11$\beta$- and 18-hydroxylations and thus a reconstituted 11β/18 hydroxylase system from bovine and porcine tissue contains all of the enzyme activities required to convert deoxycorticosterone (DOC) to aldosterone [9, 10]. In the rat the existence of two $P\text{-}450_{11\beta/18}$ enzymes has been reported, and while one, which occurs throughout the cortex, results in corticosterone and 18-hydroxydeoxycorticosterone (18-OH-DOC) formation, only the glomerulosa specific form yields aldosterone,

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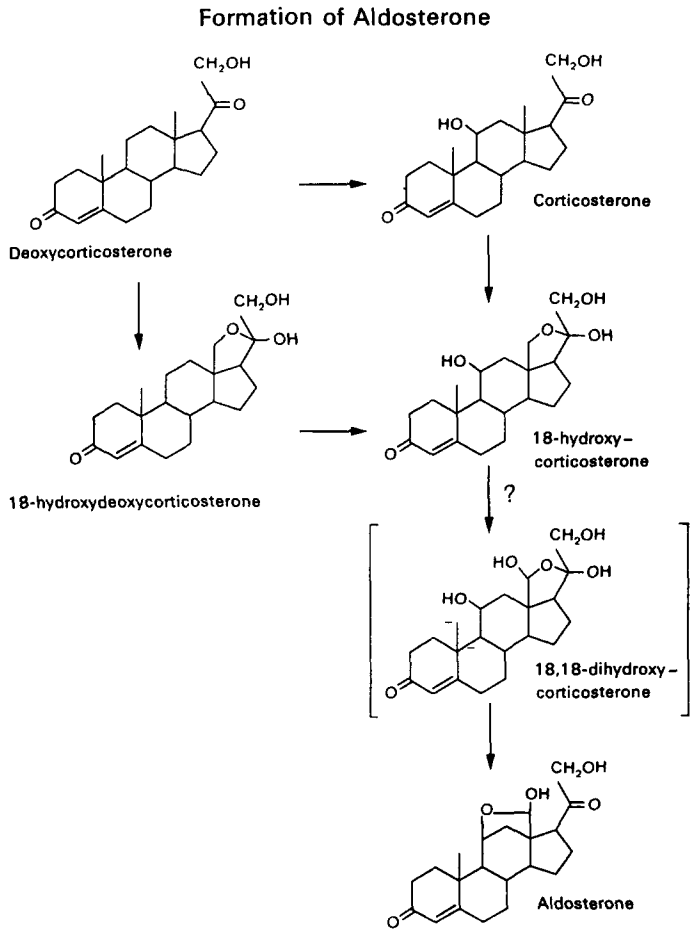

Fig. 1. Suggested pathways for aldosterone biosynthesis. It is possible that a single species of cytochrome $P450_{11\beta/18}$ may catalyse all of the steps between DOC and aldosterone (see the text).

and this species of *P*-450 has thus been termed "aldosterone synthase" [11–13].

Although exogenously added corticosterone may be transformed into aldosterone, with this recent knowledge it is not at all clear that it is an obligatory intermediate, or indeed whether it is necessary that it is formed at all. Certainly, many studies show that, at least in the rat, not all of the aldosterone formed can be accounted for by the corticosterone route, and evidence exists to suggest alternatives. One obvious point is that 18-OH-DOC is an equally valid intermediate, and various studies have shown that it too can be converted to aldosterone and 18-OH-B [14–16] (Fig. 1), as may 18-deoxyaldosterone [17].

THE *IN VITRO* PREPARATION

These studies have been carried out using a variety of *in vitro* preparations. For all of these reasons, there is clearly considerable difficulty in reaching any firm conclusion about what may actually occur in the intact cell *in vivo*. Work from our laboratory has frequently drawn attention to the variations which are attributable to the choice of experimental model. For one example, the yield of aldosterone which can be obtained under either basal or stimulated conditions varies massively according to which method is applied. Information derived from a survey of 70 papers published prior to 1985 [18], showed that relative yields of aldosterone and corticosterone obtained from rat adrenal tissue by different methods gave quite distinctly different results. *In vitro*, the stimulated output of aldosterone from whole tissue preparations, or from intact capsular preparations (which contain all of the zona glomerulosa as well as the connective tissue capsule, and some fasciculata contamination) is approximately equivalent to rates of aldosterone secretion into the adrenal vein under conditions of surgical stress. On the other hand, when dispersed capsular cell

preparations are used (and this has been the most commonly applied methodology since the techniques were introduced some 20 years ago), outputs of aldosterone are significantly lower. In contrast, although no *in vitro* preparation gives yields of corticosterone as high as those found *in vivo* (and the reasons for this have been discussed elsewhere, Ref. [19]), there is no significant difference between intact tissue and dispersed cell preparations in levels of corticosterone output. Similar data for aldosterone and corticosterone production by whole adrenal glands under maximally stimulated conditions which were obtained in our own laboratories are shown in Fig. 2. As a consequence, when capsular tissues alone were incubated, ratios of yields of corticosterone to aldosterone are considerably higher in dispersed glomerulosa preparations than when the intact tissue is incubated *in vitro* (Fig. 3).

There are other points of difference between the whole tissue and the dispersed cell preparation. One observation in particular may throw light on the nature of the biosynthetic pathway for aldosterone production. Examination of the profile of steroids formed *in vitro* by the intact capsule when stimulated (for example by ACTH) shows that 18-OH-DOC secretion uniquely is not significantly increased, whereas corticosterone, aldosterone and 18-OH-B outputs are stimulated. In contrast, in collagenase-dispersed capsular cells, 18-OH-DOC secretion is stimulated by ACTH while aldosterone, though stimulated, is only secreted at the low rates which are characteristic of this preparation [20, 21]. These two observations may

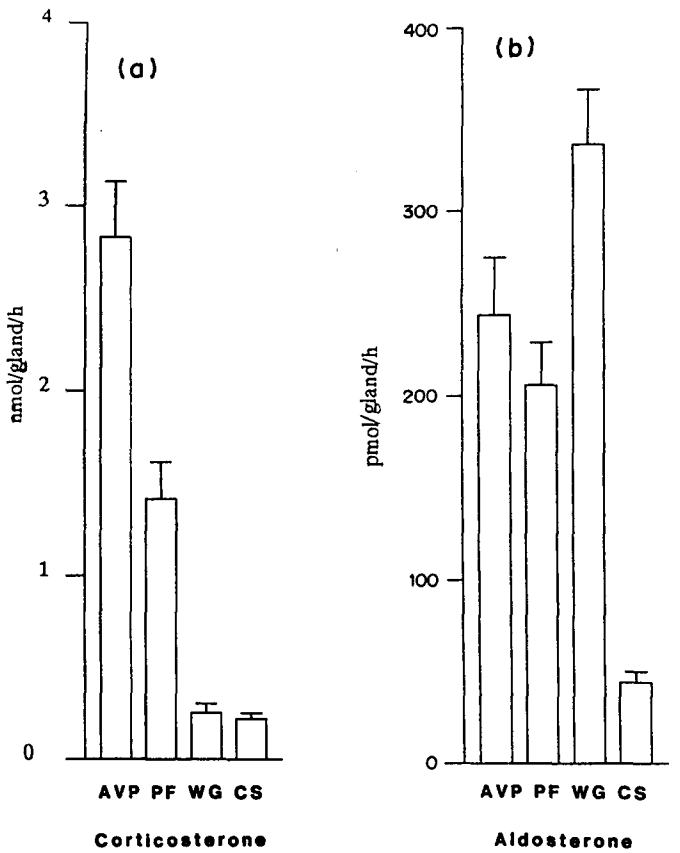

Fig. 2. Examples of maximally stimulated rates of (a) corticosterone and (b) aldosterone output by rat adrenal glands in different experimental conditions. These were: adrenal vein plasma samples (AVP), the *in situ* perfused adrenal system (PF), and *in vitro* incubation, either as whole adrenal gland tissue not enzymically dispersed (WG), or in whole adrenal collagenase dispersed cell suspensions (CS), using standard methodologies (see Refs [2, 21, 23]), previously unpublished data. It is clear that, compared with the *in vivo* situation, *in vitro* output of corticosterone is impaired, and reasons for this have been discussed elsewhere[19]. However, aldosterone output is similar in adrenal vein plasma, the perfused adrenal and non-dispersed tissue incubation, but relatively impaired in dispersed cells. When capsular outputs are compared, the corticosterone/aldosterone ratio is thus much higher for dispersed than for non-dispersed tissue (see Fig. 3). Similar data have been summarized from a series of about 70 papers published prior to 1985 [18]. Values are means ± SE, $n = 20$.

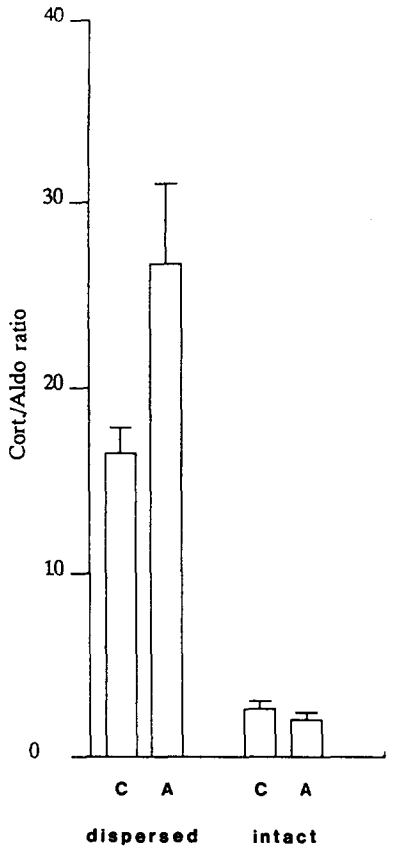

Fig. 3. Corticosterone/aldosterone ratios in control (C) and maximally ACTH stimulated [A, ACTH (1–24), 10^{-9} mol per l] intact capsule and dispersed capsular cell incubations. The ratios are invariably considerably higher in the dispersed cell preparation. Data not previously published, J. P. Hinson and G. P. Vinson.

well be related, and suggest that 18-OH-DOC can serve as an efficient aldosterone precursor in intact tissue, but that its utilization is disrupted in dispersed cells.

In an examination of the causes of such differences between preparations, the effects of a number of proteases, and also of variations in the usual preparative procedures on capsular steroidogenesis, were examined. It was found that in collagenase (or hyaluronidase) treated cells, disruption of aldosterone output follows the process of mechanical dispersal, filtration and centrifugation, and is not attributable to the actions of the proteases alone. The situation is complex, and attempts to restore cell–cell contact by culturing previously dispersed cells on contracting collagen gels were not successful in restoring aldosterone outputs [20]. However, the studies revealed another, rather spectacular, property of rat glomerulosa tissue: trypsin is itself a potent stimulator of steroidogenesis in the intact tissue preparation, but not in dispersed cells [20, 22]. This led at the time to the concept that aldosterone itself, as well as other steroids, such as 18-OH-B and 18-OH-DOC, might be stored within the tissue in steroid–protein complexes.

THE *IN SITU* PERFUSED GLAND

Because of the problems of interpreting *in vitro* data in physiological terms, the technique of *in situ* perfusion of the intact gland was developed [23]. The objective here was to maintain the integrity of the structure of the gland, while making it possible to control the delivery of stimulants, and to measure the hormonal output, with great precision.

The detailed characteristics of the method and the data which may be obtained have been described in a number of publications [24, 25]. In the present context, there are two important features which throw light on the regulation of aldosterone production in particular. In the first place, aldosterone secretion responds to glomerulosa specific stimulants, such as angiotensin II, and low concentrations of α-melanocyte stimulating hormone (α-MSH), but corticosterone does not [24]. From this it can be concluded that corticosterone is not normally secreted by the intact zona glomerulosa, a situation quite unlike *in vitro* incubation of glomerulosa tissue either as intact tissue or dispersed cells, in which corticosterone is always a major product, responding to the usual stimulants [26]. Clearly this reinforces the view that even the most modest tissue dispersal such as that associated with intact capsular incubations brings disruption of the pathway to some extent. The other feature of the perfused gland responses is that this system is particularly sensitive to angiotensin II stimulation, giving significant enhancement of aldosterone secretion with concentrations of angiotensin II at least two orders of magnitude lower than required in *in vitro* preparations [25]. The reasons for this appear to be complex, and probably only partly related to the greater efficiency of aldosterone biosynthesis in the intact gland system [27]. However, the time course of aldosterone response to stimulation for example by ACTH is usually much more rapid than that of corticosterone (Fig. 4) in this system, which certainly suggests that corticosterone is not an obligatory intermediate in aldosterone biosynthesis.

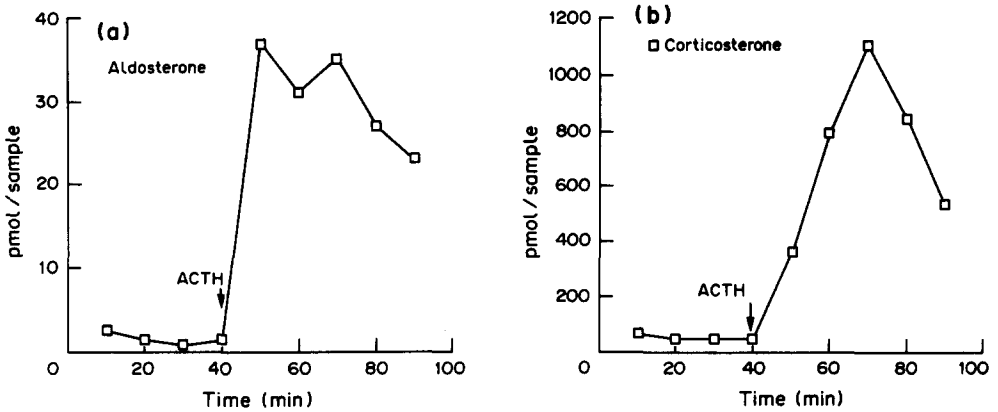

Fig. 4. Time course for (a) aldosterone and (b) corticosterone responses to ACTH stimulation [ACTH(1–24), 300 fmol bolus] in the *in situ* perfused rat adrenal gland system [22]. The aldosterone response reaches maximal output before corticosterone. A hypothesis to explain this is that corticosterone is synthesized from cholesterol *de novo*, whereas aldosterone is more rapidly formed from tissue sequestered 18-OH-DOC; see text.

SEQUESTERED STEROID POOLS IN THE ZONA GLOMERULOSA

The concept that there may exist different pools of steroid within the glomerulosa which may have different metabolic fates arose from the observation that, in studies on the conversion of radioactive precursors, such as [^{3}H]pregnenolone, by intact capsule preparations, yields of aldosterone were invariably low compared with those of other products, such as corticosterone, whereas it was produced in fairly large amounts from endogenous precursors [28]. In other words, in experiments in which the isotopic incorporation into these products was measured simultaneously with their mass, specific activities of aldosterone were much lower than those of corticosterone or DOC. They were, however, similar to those of 18-OH-DOC. Some typical results are illustrated in Table 1. These data too suggest that 18-OH-DOC rather than corticosterone is the aldosterone precursor. It was reasoned that such a situation could only arise when 18-OH-DOC and corticosterone were formed and maintained in separate compartments within the tissue. One possibility is that one or other of these pools of steroid is associated with a macromolecule or macromolecules. To study this, the technique of incubation with dialysis was developed, and this suggested that 18-OH-DOC in particular, and possibly other steroids including DOC and aldosterone, were associated with such a complex, whereas corticosterone was not [28]. The subsequent finding that aldosterone and 18-OH-B (but not 18-OH-DOC) are released from intact glomerulosa tissue by trypsin, drew attention to the possibility that these two steroids rather than 18-OH-DOC may be bound to protein, as discussed above. However, exhaustive studies do not support the concept of large reserves of aldosterone and 18-OH-B within the tissue, whereas much data supports the existence of a substantial pool of sequestered 18-OH-DOC [29, 30].

The nature of this sequestration is still unresolved. One point is clear, neither binding studies, nor the size of the pool are consistent with the concept of a high affinity/low capacity 18-OH-DOC binding protein, with characteristics like those of a receptor or plasma transport protein. Exhaustive studies designed to reveal such binding by incubating high specific activity tritiated 18-OH-DOC with a variety of different

Table 1. Yields and specific activities of steroid products formed from [7α-^{3}H]progesterone [Amersham Int., U.K., 1 μCi (Sp. act. 6.9 Ci/mmol) per flask] after incubation with rat intact adrenal capsules

	Mass	Sp. act. (cpm/ng)
Corticosterone	193 ± 50	96 ± 24
18-OH-DOC	168 ± 35	20 ± 4
Aldosterone	125 ± 15	12 ± 3

Yields, uncorrected for recovery, were calculated for individual samples per pair of glands after derivatization (acetates of corticosterone and DOC, and γ-lactones of aldosterone and 18-OH-DOC) and paper chromatography, so that recoveries for isotope content and mass of steroid formed from endogenous precursors were identical. Isotope content was assayed by liquid scintillation spectrometry, mass by gas liquid chromatography. Broadly comparable yields of steroid from endogenous precursors contrast with the much higher yields of radioactive corticosterone compared with radioactive aldosterone and 18-OH-DOC. The data, previously published in Ref. [28], show that the 18-oxygenated steroids are much more readily formed from endogenous precursors than from the added radioactive pregnenolone, and their specific activities suggest their biosynthetic relationship.

preparations, including whole cells, and subcellular fractions, have failed, and this is consistent with the failure of added radioactive steroid to penetrate the endogenous precursor pool, in the aldosterone biosynthesis experiments described above ([30], and G. P. Vinson, unpublished observations).

However, good evidence on the location of the tissue sequestered 18-OH-DOC has now been obtained. Fractionation of homogenized zona glomerulosa tissue, and of an enriched plasma membrane preparation, by density gradient centrifugation revealed that tissue 18-OH-DOC banded in high density (1.063–1.21 g/ml) fractions, as did the tissue protein, whereas tissue corticosterone, aldosterone and 18-OH-B behaved like free steroid, and banded in fractions of low density (< 1.006 g/ml). In dramatic confirmation of the inability of exogenously added steroid to penetrate the endogenous steroid pool, tritiated 18-OH-DOC behaved like free steroid, binding to the low density fractions, even when it had previously been incubated with homogenized glomerulosa tissue overnight (Fig. 5). Not only did these results confirm that 18-OH-DOC is the major sequestered steroid in the rat adrenal zona glomerulosa, but they also showed that the sequestration is attributable to the association of 18-OH-DOC with a high density component of the plasma membrane [30].

This is a startling discovery. Not only is the cellular site of steroid sequestration unexpected, it suggests, if the hypothesis we have developed is correct, the involvement of the plasma membrane in the steroidogenic and steroid secretory process, an involvement which previously would not previously have been suspected. However, it is interesting in this context to note that at about the same time, other workers showed that progesterone in the porcine ovary is also plasma membrane associated [31].

The reason why the rat glomerulosa is particularly sensitive to the means of tissue preparation, and why aldosterone biosynthesis can be disrupted in dispersed tissue now becomes apparent. It has previously been conjectured that dispersed cells produce less aldosterone because the sequestered steroid reserve is lost. Certainly dispersed glomerulosa cells contain very little 18-OH-DOC, or any other steroid. Clearly, now it is known that the sequestered store in question lies in the plasma membrane, its sensitivity to mechanical disturbance can be understood.

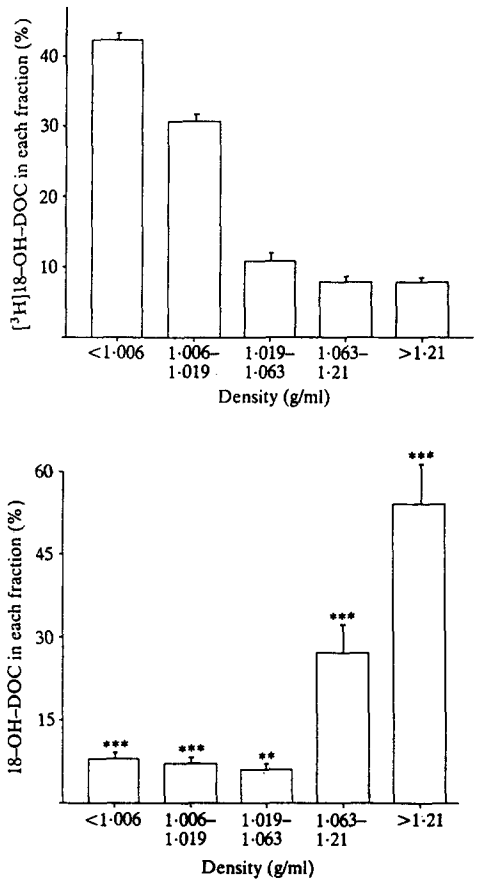

Fig. 5. Salt density gradient fractionation of free [3H]18-OH-DOC (upper figure) and membrane sequestered 18-OH-DOC obtained from rat adrenal capsules. Free steroid migrates in the low density fractions, while membrane sequestered 18-OH-DOC migrates as though associated with a macromolecule. Other tissue steroids, and all steroids released into incubation media, behave like free 18-OH-DOC. Data originally published in Ref. [31].

THE STEROIDOGENIC ACTION OF TRYPSIN

Yet other differences between whole capsule and dispersed cell preparations exist. Extensive studies have demonstrated that the calcium/phosphatidyl inositol messenger system is active in the zona glomerulosa, and is particularly responsive to angiotensin II stimulation. Furthermore, angiotensin II, like phorbol ester stimulation, causes translocation of protein kinase C to the plasma membrane, suggesting that diacylglycerol also functions as a messenger in the glomerulosa system. Nevertheless, most groups have been unable to show any stimulation of steroidogenesis by phorbol esters in the rat zona glomerulosa [32]. Most studies utilize the dispersed cell preparation however, and in contrast, when the whole capsule preparation is used, the gland responds sensitively to the phorbol ester TPA, and also more sensitively to the

calcium ionophore A23187 than dispersed cells. Significantly, it is the two "late pathway" products, aldosterone and 18-OH-B which respond most strongly, the same two steroids whose production is most compromised by the disruptive process of cell dispersal [33].

The tissue sensitivity of the phorbol ester/protein kinase C and calcium ionophore response, and its resemblance to the tissue sensitivity to trypsin stimulation suggest they may act through the same mechanism. Addressing this problem, it was found that inhibitors of both protein kinase C and phospholipase C also inhibit the stimulation of aldosterone output by trypsin. Direct assay confirmed that trypsin stimulates the translocation of protein kinase C to the cell membrane. This stimulation was not due to the generation of the Ca^{2+}/phospholipid independent protein kinase C fragment, but possibly to the enhancement of phospholipase C activity [34].

This action of trypsin does not negate the possibility of its direct proteolytic action on a steroid–protein complex. However, the coexistence of the sequestered pool of 18-OH-DOC and protein kinase C in the plasma membrane in the stimulated gland strongly present the possibility that 18-OH-DOC is mobilized as an aldosterone precursor by protein kinase C activity.

CONCLUSIONS

While the enzymes concerned with aldosterone biosynthesis have now been well characterized, there is still doubt about the mechanisms which are involved in ensuring that aldosterone secretion from the zona glomerulosa is quantitatively and temporally appropriate to the physiological demand, and furthermore, that the concomitant secretion of other steroids, such as corticosterone, with possibly inappropriate biological activity, does not occur at the same time. A plausible mechanism would be that a tissue sequestered store, either of aldosterone itself or of a close precursor, is mobilized by physiological stimulation. The plasma membrane located store of 18-OH-DOC, which is found only in the zona glomerulosa in the rat, could easily serve in such a role. The plasma membrane is a hitherto unsuspected location for a steroid pool, but its relevance becomes clear when its possible relationship to protein kinase C is considered, since it is now well established that protein kinase C activation, by transfer to the plasma membrane, is a consequence of zona glomerulosa stimulation, particularly by angiotensin II.

The physical nature of the components which retain 18-OH-DOC in the cell membrane of the zona glomerulosa are as yet unknown. Clearly the interactions between the steroid and other components of the system are unlike any which occur for example between steroid and receptor, or plasma binding protein, since there is no exchange between steroid formed within the cell and that added exogenously: added radioactive steroid does not penetrate the endogenous pool.

The interesting possibility arises that there may be other hitherto undescribed non-exchangeable steroid–macromolecule interactions of this type. In this context, we have recently shown that the trypsin induced release of oestrogen from breast tumour homogenates is attributable to a sequestered pool of steroid, in this case possibly in the 8S soluble receptor [35]. It seems plausible that other examples will be found.

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