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LEUKOTRIENES AS COMMON INTERMEDIATES IN THE CYCLIC AMP DEPENDENT AND INDEPENDENT PATHWAYS IN ADRENAL STEROIDOGENESIS

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Summary—Aldosterone secretion from adrenal glomerulosa cells can be stimulated by angiotensin II (AII), extracellular potassium and adrenocorticotropin (ACTH). Since the mitochondria can recognize factors generated by AII (cyclic-AMP-independent) and ACTH (cyclic AMP dependent), it is reasonable to postulate the existence of a common intermediate in spite of a different signal transduction mechanism. We have evaluated this hypothesis by stimulation of mitochondria from glomerulosa gland with fractions isolated from glomerulosa gland stimulated with AII or from fasciculata gland stimulated with ACTH; the same fractions were tested using mitochondria from fasciculata cells. Post-mitochondrial fractions (PMTS) obtained after incubation of adrenal zona glomerulosa with or without AII (10^{-7} M) or ACTH (10^{-10} M), were able to increase net progesterone synthesis 5-fold in mitochondria isolated from non-stimulated rat zona glomerulosa. In addition, AII in zona glomerulosa produced *in vitro* steroidogenic fractions that were able to stimulate mitochondria from zona fasciculata cells.

Inhibitors of arachidonic acid release and metabolism blocked corticosterone production in fasciculata cells stimulated with ACTH. This concept is supported by the experiment in which bromophenacyl-bromide and nordihydroguaiaretic acid also blocked the formation of an activated PMTS. In fact, non-activated PMTS, in the presence of exogenous arachidonic acid AA, behaved as an activated PMTS from ACTH stimulated cells.

We suggest that the mechanisms of action of ACTH and AII involve an increase in the release of AA and an activation of the enzyme system which converts AA in leukotriene products.

INTRODUCTION

It is well recognized that steroidogenesis is under complex hormonal control. In the current model of hormone action in steroid-producing tissue, it is proposed that the hormone binds specifically to receptors on the outer surface of the plasma membrane of the respective target cells. How the hormone is able to transmit a specific signal via its interaction with surface receptor is a question which has generated a great deal of experimental data in the last two decades.

Although there are different membrane receptors in each tissue, which are activated to produce a defined steroid, and the information to generate this signal resides in the receptor [1], this signal does not seem to be specific for the cell which contains this receptor [2, 3]. In fact, this concept was demonstrated in a cell-free system assay in which subcellular fractions from steroid-secreting tissues such as rat adrenal zona fasciculata, rat Leydig cells or cultured mouse adrenal carcinoma cell line, could be interchanged to stimulate steroid synthesis [2]. Moreover, electrofused Leydig–adrenal cell hybrids can generate testosterone and corticosterone by activation of either the lutropin (LH) or the ACTH receptors [3]. The same evidence was presented in granulosa cells [4] and by transfer of functional ovarian LH receptors to adrenal fasciculata cells [5]. These data would imply that the mechanism to activate the rate-limiting process in steroidogenesis, the cholesterol side-chain cleavage system, is universal or independent of both the hormone and the tissue. This report demonstrates that, in fact, this may be possible and we suggest that the mechanism of action of a hormone to induce steroidogenesis involves an increase in the release of arachidonic acid (AA) and an activation of the enzyme system which converts AA into leukotriene products in spite of different membrane receptors and different signal transduction mechanisms.

MATERIALS AND METHODS

Preparation and incubation of adrenocortical gland or cells zona fasciculata and zona glomerulosa

Male rats were maintained and supplied with dexamethasone in the drinking water in order to obtain adrenal zona fasciculata as previously described [2, 6–8]. In the case of adrenal zona glomerulosa, dexamethasone blocks the *in vitro* stimulation of aldosterone production with AII but not with ACTH. For this reason adrenal zona glomerulosa was obtained from rats not treated with dexamethasone.

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Assay for extramitochondrial steroidogenic factors

A sensitive cell-free assay was performed as previously described [7] (Fig. 1). The homogenates of adrenal zona fasciculata or glomerulosa were centrifuged at 800 **g** for 10 min. The pellets containing cell debris and membranes (pre-mitochondrial fractions from unstimulated or ACTH-stimulated adrenal) were washed once with 0.5 ml of 270 mM mannitol, 1 mM Tris–HCl, pH 7.4, medium A. From the 800 **g** supernatant, the mitochondrial fractions were obtained by centrifugation of 9000 **g** for 10 min, washing off the pellets in medium A and resuspended them in 200 μl of medium A per adrenal. The 9000 **g** supernatant was further centrifuged at 105,000 **g** for 60 min to obtain the 105,000 **g** pellet and cytosol. The following fractions were used throughout the experiments: PF = 800 **g** + 105,000 **g** pellets, PMTS = 9000 **g** supernatant, cytosol = 105,000 **g** supernatant and mitochondria = 9000 **g** pellet. Mitochondrial steroidogenesis was allowed to proceed in the presence of 0.27 mM Metopyrone [9] and 1.6 mM 1-(β-guanidinoethyl)-3-(4-pyridyl)indol sulfate (Ba 40.028) [10] as inhibitors of 11β-, 18-, and 19-hydroxylation, and 21-hydroxylation, respectively, without addition of an electron donor.

RESULTS AND DISCUSSION

Adrenal zona glomerulosa was incubated with or without AII (10^{-7} M) in a low K^+ medium as described [11] or in the presence or in the absence of 10^{-10} M ACTH at 37°C for 90 min. Aldosterone was assayed in the incubation medium and the values were 3.9 ± 0.1 vs 1.25 ± 0.05 ng/incubation vial in the presence or absence of AII, and 5.9 ± 0.3 ng/incubation vial in the presence of ACTH (mean ± SD, $n = 3$). At the end of the incubation, the tissue was homogenized as described in Materials and Methods and the post-mitochondrial supernatant (PMTS) and the mitochondrial fraction were isolated. The PMTS derived from AII- or ACTH-treated preparations were incubated with mitochondria from non-stimulated adrenal zona glomerulosa. PMTS-AII was able to increase net progesterone synthesis 5-fold in comparison with post-mitochondrial fraction from non-treated adrenal zona glomerulosa (PMTS). PMTS derived from ACTH-treated adrenal zona glomerulosa was also able to increase net progesterone formation 4.5-fold (control = 1.4 ± 0.26; AII = 7.5 ± 0.10; ACTH = 6.2 ± 0.15 ng of progesterone/incubation).

The heterologous recombination using ACTH-dependent PMTS from zona fasciculata incubated with mitochondria from zona glomerulosa also stimulates steroid synthesis (20.58 ± 0.81 vs 2.57 ± 0.40 ng/adrenal with or without 10^{-10} M ACTH, respectively). The heterologous stimulation can be understood and interpreted on the basis of a cyclic AMP signal being a common intermediate in the ACTH stimulation of zona glomerulosa and fasciculata. However, AII activated PMTS (PMTS-AII) from zona glomerulosa is also able to recognize heterologous mitochondria isolated from adrenal zona fasciculata. A 4-fold increase in the net progesterone production can be observed (control = 1.10 ± 0.05; AII = 4.49 ± 0.14 ng of progesterone/incubation). These mitochondria, now from zona fasciculata, recognize cyclic-AMP-dependent steroidogenic factors and also, AII cyclic-AMP-independent factors from zona glomerulosa.

These results can be interpreted on the basis of a

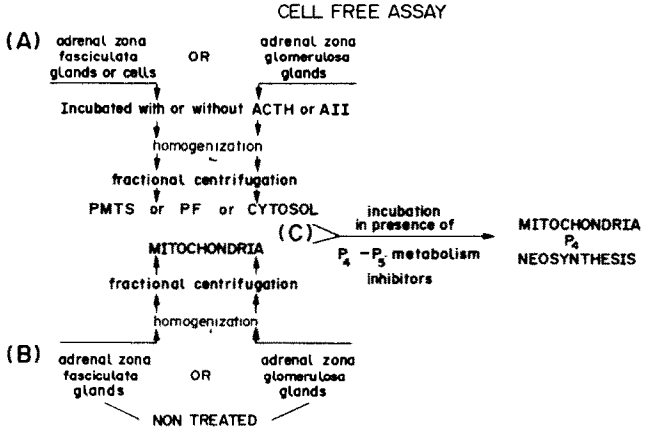

Fig. 1. Assay for extramitochondrial steroidogenic factors. (A) Adrenal zona fasciculata glands or cells incubated with or without ACTH, or adrenal zona glomerulosa glands incubated with or without ACTH or AII, were washed and subjected to fractional centrifugation to isolate the different fractions: PMTS – 9000 **g** supernatant; PF = 800 **g** + 105,000 **g** pellets; cytosol = 105,000 **g** supernatant. (B) Non-treated adrenal zona glomerulosa or zona fasciculata glands were homogeneized and fractionally centrifuged to obtain the 9000 **g** pellet (free of the 800 **g** pellet), rich in mitochondria. (C) Subcellular fractions obtained in A were incubated with mitochondria from B, in the presence of pregnenolone–progesterone metabolism inhibitors. Progesterone synthesis was measured by radioimmunoassay. For details see Materials and Methods.

common intermediate in AII and ACTH action in spite of different membrane receptors and different signal transduction mechanisms.

Lipoxygenase, but not cycloxygenase products of arachidonic acid (AA) metabolism may mediate the action of AII [12]. In order to study whether arachidonic acid may be the common intermediate in the actions of AII and ACTH, the effects of bromophenacyl bromide (BPB) and nordihydroguaiaretic acid (NDGA), inhibitors of AA release and metabolism respectively [13–17], were studied.

Adrenal zona fasciculata was incubated in the presence or in the absence of BPB or NDGA for 15 min at 37°C prior to the addition of ACTH. Corticosterone was measured in the incubation medium. These compounds inhibit ACTH-stimulated corticosterone production. NDGA (5×10^{-9} M) is able to totally abolish corticosterone production stimulated with ACTH in adrenal zona fasciculata cells (Fig. 2). BPB (5×10^{-11} M) also totally abolished ACTH-stimulated corticosterone production (control = 117.0 ± 3; BPB = 78 ± 5; ACTH = 330 ± 30; BPB + ACTH = 96 ± 18 ng of corticosterone/incubation).

In an attempt to further elucidate the intracellular effect of BPB and NDGA in the hormonal control of steroid synthesis, a sensitive cell-free assay was used [7], as described in Materials and Methods. In this assay, adrenal zona fasciculata cells were incubated in the presence or in the absence of 10^{-10} M ACTH. After the incubation, the post-mitochondrial fractions (PMTS) were isolated as described in Materials and Methods and incubated with mitochondria from non-stimulated adrenal zona fasciculata in the presence of 10^{-10} M BPB or 5×10^{-9} M NDGA. Neither BPB nor NDGA were able to inhibit the mitochondria progesterone synthesis produced by incubation with ACTH-dependent steroidogenic factors contained in PMTS from ACTH-stimulated cells (control = 3.7 ± 0.3; ACTH = 91.4 ± 2.9; BPB + ACTH = 94.6 ± 5; NDGA + ACTH = 90.8 ± 3.7 ng of progesterone/incubation). However, when BPB and NDGA are incubated prior to ACTH stimulation of intact isolated adrenal zona fasciculata cells, they produce the inhibition of corticosterone production, and they also inhibit the production of a PMTS capable of activating mitochondria steroidogenesis in a cell-free assay (Table 1). Moreover, BPB and NDGA completely block the formation of steroidogenic factors *in vitro*. In this assay, cytosol from ACTH-stimulated adrenal zona fasciculata was recombined with the 800 ***g*** and 105,000 ***g*** pellets obtained from non-stimulated adrenal zona fasciculata, as described in Materials and Methods. Both BPB and NDGA totally blocked the activation of the pellet fraction by an activated cytosol and consequently the activation of adrenal mitochondria, incubations in the absence of particulate fraction, are shown as controls of other results (Table 1).

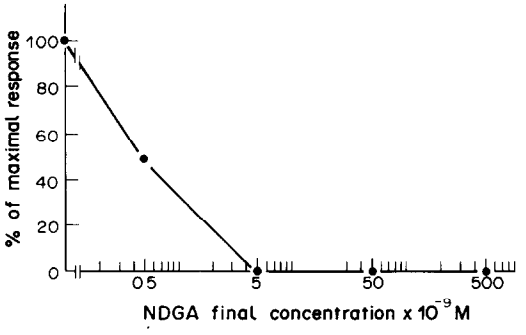

Fig. 2. Inhibition of corticosterone production by NDGA. Rat adrenal fasciculata cells were incubated for 15 min at 37°C with increasing concentration of NDGA, followed by 180 min incubation in the presence or in the absence of 10^{-10} M ACTH. Corticosterone production was measured by radioimmunoassay in the medium as previously described [6, 7].

It seems likely, from the experiments described above, that an increase in arachidonic acid concentration into the PMTS fraction may contribute to the action of ACTH on steroidogenesis; if this is true, exogenous arachidonic acid in combination with non-activated PMTS should increase mitochondrial progesterone synthesis.

Exogenous arachidonic acid, in a dose-dependent response (10^{-6}–10^{-3} M) was able to stimulate progesterone synthesis in adrenal mitochondria, in the presence of a non-stimulated PMTS fraction. In addition, the presence of NDGA abolished the effect of exogenous arachidonic acid (Fig. 3) but BPB had no effect on the action of AA.

In all cases studied the effect of the inhibitors BPB

Table 1. Production of steroidogenic factors by the recombination, in a cell-free system assay, of particulate fraction and mitochondria from non-stimulated adrenal with cytosol from ACTH-treated adrenal fasciculata cells

Subcellular fractions		Progesterone (ng/incubation)
Incubations in presence of PF		
Cytosol from:		
–		3.1 ± 0.3
+		82.5 ± 4.5
+	/BPB	1.7 ± 0.4
+	/NDGA	2.1 ± 0.6
Incubations in absence of PF		
Cytosol from:		
–		2.7 ± 0.3
+		42.4 ± 1.9
+	/BPB	40.6 ± 1.4
+	/NDGA	41.3 ± 1.6

*Adrenal zona fasciculata cells were treated with (+) or without (–) 10^{-10} M ACTH. After the incubation, the cells were homogenized and their cytosol fractions were obtained as indicated in Materials and Methods. The fractions were recombined *in vitro* with or without PF (particulate fraction) and mitochondria from control cells; progesterone production was determined by radioimmunoassay. Values are expressed as mean ± SD, $n = 3$.

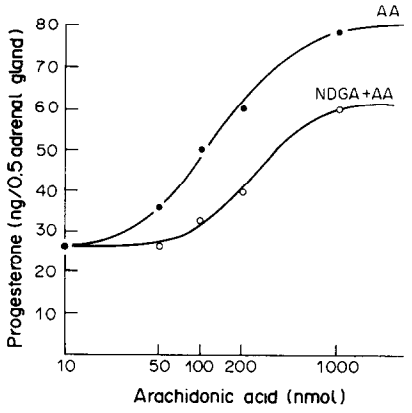

Fig. 3. Arachidonic acid (AA) stimulation of progesterone mitochondria neosynthesis. Mitochondria were isolated, as described in Fig. 1(B), from adrenal zona fasciculata. Incubation with AA was done in the presence of PMTS according to the assay for extramitochondrial steroidogenic factors, as described in Materials and Methods. NDGA was added 40 min prior to the incubation with AA. Progesterone synthesis was measured by radioimmunoassay.

and NDGA are only apparent when they are used at least 15 min prior to the activation step.

All these studies can be understood and interpreted on the basis of a cyclic AMP signal being a common intermediate. However, this does not seem to be the case in the stimulation of steroid by AII and ACTH in the adrenal zona glomerulosa. Here, AII and ACTH have two different signals transduction mechanism, cyclic-AMP-independent and dependent, respectively. Therefore, it seems likely that in this case the common intermediate is located after the signal transduction event.

A possible intermediate could be calcium since its role is known in both mechanisms [6, 11, 18–20]. However, an activated post-mitochondrial fraction can still produce the *de novo* synthesis of progesterone in the presence of 1 mM EGTA [7]. These results suggest that the common activator of the mitochondria is not calcium *per se* but some product after the intracellular calcium movement. These observations are similar to the one in which a low concentration of an inhibitor of phospholipase A_2 action, "quinacrine", inhibits reversibly the AII-induced increase in aldosterone secretion without altering the AII-induced mobilization of calcium [12].

ACTH causes a similar increase in the rate of calcium efflux as AII when extracellular calcium is present, but not if it is absent. Therefore it was concluded that ACTH does not induce the mobilization of calcium from an intracellular pool [19, 21].

The molecular nature of this intermediate is still unclear; however, the fact that the inhibitors of arachidonic acid release and metabolism can block aldosterone synthesis in glomerulosa cells stimulated with angiotensin [12] or ACTH, and corticosterone synthesis in fasciculata cells stimulated with ACTH, suggest that arachidonic acid may be the common intermediate in the angiotensin II and ACTH action upon steroid synthesis. This concept is supported by the experiment in which BPB and NDGA also blocked the formation of an activated post-mitochondrial fraction (Table 1), but did not block, in the cell-free system assay, the mitochondrial activation by an ACTH-dependent PMTS. Moreover, non-activated PMTS, in the presence of exogenous AA, behaves as an activated PMTS from ACTH-stimulated cells increasing progesterone synthesis (Fig. 3).

The intracellular compartment in which all the enzyme activities are located is still unsolved, but experiments to determine it are ongoing. However, the results presented are still relevant since we can produce steroids in a test tube and study each individual step to produce the side-chain cleavage in intact mitochondria.

The inhibitory effect of BPB and NDGA on corticosterone production has been previously reported [22]. In addition, evidence has been presented showing that exogenous AA significantly increases aldosterone secretion in isolated glomerulosa cells [12], and also that caffeic acid, an inhibitor of lipoxygenase activity, also inhibited AII mediated aldosterone production [12]. In the same paper [12], it has been shown that AII causes a release of free arachidonic acid from glomerulosa cells. These results are in agreement with previous data showing that AII causes a rapid breakdown of phosphatidyl inositol-4,5 biphosphate and generates the diacylglycerol, rich in arachidonic acid [23, 24]. The diacylglycerol and/or phosphatydic acid may serve as an additional source of arachidonic acid.

It has also been demonstrated that ACTH does not activate the specific phospholipase C, responsible for the hydrolysis of phosphatidyl inositols. Hence, ACTH does not generate diacylglycerol and activate protein kinase C. In contrast, there are some reports describing an activation of phosphatidyl inositol turnover in adrenal zona fasciculata by ACTH [25, 26]. Therefore, it is still controversial how ACTH via a cyclic-AMP-dependent mechanism can produce arachidonic acid release. It is known that cyclic-AMP can generate arachidonic acid by means of the increase in phosphatidylcholine synthesis via plasma membrane methylation, and that phosphatidyl-choline can be a precursor of arachidonic acid [27].

Nevertheless, endogenous phospholipids isolated from particulate adrenal fractions of ACTH-treated rats or exogenous phospholipids also stimulate steroidogenesis *in vitro* [28]. Moreover, recent findings [25] demonstrated that ACTH via cyclic AMP rapidly increases phosphatidic acid synthesis by a cycloheximide-sensitive process, and changes in steroidogenesis correlate very well with changes in phospholipid turnover. In addition, it has been shown that reconstitution of enzymes into membranes differing in the fatty acyl side-chains of phosphatidylcholine resulted in large differences in

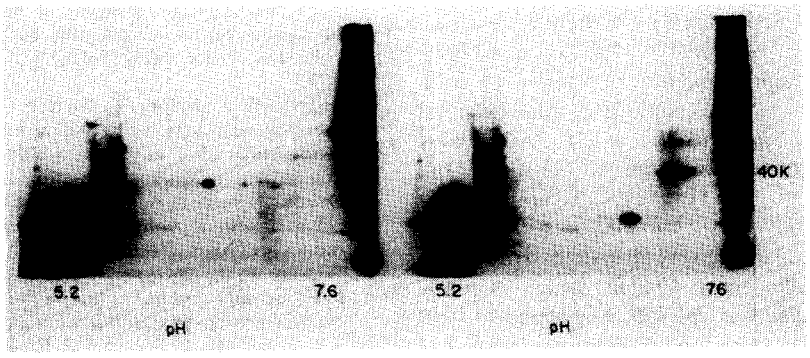

Fig. 4. Two-dimension gels autoradiogram of ^{32}P labelled proteins isolated from adrenal zona fasciculata cells treated with: (A) none, (B) 10^{10} M ACTH. Cells were incubated with 1 mCi of ^{32}P for 30 min followed by 10 min incubation with ACTH. Adrenal proteins were isolated by TCA-acetone precipitation and subjected to two-dimensional gel electrophoresis with a pH range from 5 to 8 and 5–17.5% acrylamide gradient. On the right of each panel are the autoradiograms of an aliquot of the original sample running only in the second dimension.

the activity of side-chain cleavage system, and that the activity was correlated with high spin optical spectrum of the cytochrome [29]. Moreover, an effector lipid binding site(s) on cytochrome P-450$_{scc}$ when occupied by the appropriate phospholipid affects binding of cholesterol to the enzyme; this site appears to accommodate either two phosphatidyl-cholines or one cardiolipin [30].

Those findings together with these and previous [2, 7, 8, 28] results, suggest that stimulation of AA release by ACTH and cyclic AMP may be closely related to the soluble cyclic-AMP-dependent protein kinase system [8], and the formation of a cycloheximide-sensitive steroidogenic factor(s) of phospholipid nature which enables the entire cascade of events including the transport of phospholipids to the mitochondria via a cytosolic hormone-independent phospholipid exchange protein. This protein is ACTH independent and it was shown to be heat sensitive, not dialyzable, trypsin resistant and eluated from a Sephadex G-100 column with an apparent mol. wt of 28,000 ± 3700 Da, $n = 3$ [28]. Exogenous extramitochondrial phospholipids from rat adrenal are able to stimulate mitochondrial steroidogenesis, particularly when endogenous phospholipids are prepared from ACTH-treated rats (control = 41 ± 9; ACTH = 1215 ± 103 pg of progesterone/0.04 adrenal) [2]. In addition, it has been recently proposed that in several systems a protein of relative molecular mass 40 kDa, which is phosphorylated *in vivo* and *in vitro*, was found to be a phospholipase inhibitor, and its activity is regulated by phosphorylation [31, 32]. This process may cause the activation of the mitochondrial cholesterol side-chain-splitting enzyme. Two-dimension gel electrophoresis, but not one-dimension analysis, of ^{32}P incorporation in adrenal cells shows a protein of an apparent mol. wt of 40 kDa, being phosphorylated under the action of ACTH (Fig. 4). An ACTH-dependent dephosphorylation can also be detected at pI 6.58 (Fig. 4, panel A); the spot observed at pI 7.15 with 40 kDa apparent mol. wt in panel B (ACTH treatment), could also be the first site phosphorylation of a multiple site phosphorylated protein with pI 6.58. In the same figure, a protein of apparent mol. wt of 28 kDa is also phosphorylated. This protein can be seen also in one-dimensional gel electrophoresis and is similar to the one previously described (Ref. [33] and Orme-Johnsson, personal communication on the 2nd Conf. in Adrenal Cortex, 1986, Newport, CA). We therefore suggest that the mechanism of action of ACTH, AII and probably LH, involves an increase in the release of AA and an activation of the enzyme system which converts AA in leukotriene products. Both ACTH and AII may have a common intermediate in spite of different membrane receptors and different signal transduction mechanisms.

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