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REGULATION OF STEROIDOGENESIS AND PROSTAGLANDIN FORMATION IN ISOLATED ADRENOCORTICAL CELLS: THE EFFECTS OF PREGNENOLONE AND CYCLOHEXIMIDE

SUZANNE G. LAYCHOCK and R. P. RUBIN
Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 23298 U.S.A.

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SUMMARY

The steroidogenic response to ACTH in isolated feline cortical cells was completely blocked by calcium deprivation or cycloheximide, but neither treatment markedly impaired pregnenolone-induced steroid release. While ACTH-evoked steroid release was accompanied by an increase in PGE_2 and $PGF_{2\alpha}$ release, a potently steroidogenic concentration of pregnenolone (3 μm) was unable to augment prostaglandin (PG) release. Cycloheximide slightly obtunded basal PGE_2 and $PGF_{2\alpha}$ release but did not prevent the ACTH-induced increase in PG release. The findings indicate that the critical role of calcium in the steroidogenic process is associated with early events in the interaction of ACTH with the cell membrane; one of the early events includes activation of PG synthesis through a mechanism involving enzyme activation rather than *de novo* synthesis of new enzyme protein.

INTRODUCTION

Although a host of evidence has accrued over the past few decades regarding the mechanism of action of ACTH, it is still not possible at our present state of knowledge to present a unitary hypothesis which is consistent with all of the existing data. However, certain generally accepted concepts have emerged from these studies. The fundamental postulate that ACTH stimulates the conversion of cholesterol to corticosteroid, but not the conversion of pregnenolone to corticosteroid appears well established [1]. In addition, calcium and cyclic AMP appear to participate in the steroidogenic response to ACTH [2]. Calcium may exert its principal action at one or more of the following loci: (a) the interaction of ACTH with its receptor [3]; (b) on the adenylate cyclase–cyclic AMP system [4]; (c) at the level of protein synthesis [5]; (d) directly on mitochondrial steroid biosynthesis [6]; (e) on corticosteroid release [7].

Recent studies from our own laboratory have also implicated prostaglandins (PGs) as mediators of the steroidogenic action of ACTH. Not only does PG augment steroidogenesis [8], but ACTH enhances the turnover, synthesis, and release of PGE_2 and $PGF_{2\alpha}$ by isolated feline adrenocortical cells [9, 10]. The PG response is both time [11] and dose dependent [10] and involves a mechanism which requires calcium [11].

In order to gain additional insight into the mechanism of action of ACTH, especially in regard to the potential roles played by calcium and PGs, the present studies probe the steroidogenic effects of pregnenolone in isolated cortical cells. Pregnenolone, a corticoid precursor, stimulates steroidogenesis by providing a non-limiting substrate source for the enzymatic steps distal to the rate limiting conversion of cholesterol to pregnenolone in the normal steroidogenic sequence [12]. The use of this precursor to stimulate steroid production by circumventing the membrane events triggered by ACTH may help to elucidate the juxtaposition of calcium and PGs in the train of events which culminate in steroid production and release.

Furthermore, in order to gain additional information regarding the mechanism of ACTH-induced PG synthesis in cortical cells, the present experiments also explore the effects of cycloheximide on cortical PG formation. Cycloheximide inhibits steroidogenesis by blocking the formation of a labile protein which is crucial for the rate limiting conversion of cholesterol to pregnenolone [13]. The use of this inhibitor of protein synthesis might help to reveal the nature of the enzymatic mechanisms whereby ACTH enhances PG synthesis, by elucidating whether ACTH elicited PG synthesis is associated with activation of preformed enzyme or with *de novo* synthesis. In addition, it may provide additional knowledge regarding the interrelation between PG synthesis and steroid release, as for example, in regard to whether the formation and release of PGs precedes or is merely a consequence of steroid production.

MATERIALS AND METHODS

Minimal Eagle's medium (MEM) was purchased from the Grand Island Biological Company; Grand

Island, NY. Cycloheximide and pregnenolone were obtained from Sigma Chemical Company. [^{3}H]-$PGF_{2\alpha}$ (175 Ci/mmol) and [^{3}H]-PGE_2 (117 Ci/mmol) were purchased from Amersham-Searle and New England Nuclear, respectively. Unlabeled prostaglandins were obtained through the courtesy of Dr. J. E. Pike of the Upjohn Company; and β-1-24 ACTH was a generous gift from Ciba Pharmaceuticals.

Cortical cells were isolated from cat adrenal glands using a trypsinizing procedure described by Rubin and Warner[14] and modified to contain 0.2% bovine serum albumin (fatty acid free) and 0.02% trypsin inhibitor in the final incubation medium. Cell aliquots, approximating 2.5×10^5 cells/ml were incubated for 90 min at 37° in the presence or absence of appropriate stimulatory or inhibitory agents. Subsequent to incubation, the cell suspensions were centrifuged at 6000 *g* for 10 min at 4°, and the resulting supernatants analyzed for corticosteroid by competitive protein binding[15], and for prostaglandin by direct radioimmunoassay using $PGF_{2\alpha}$ antibody[16] or PGE_2 antibody[11]. Pregnenolone and cycloheximide in the concentrations employed did not interfere with ligand binding in either the steroid or PG assay.

In the experiments concerned with calcium deprivation the basic medium was Kreb's bicarbonate solution fortified with vitamins plus amino acids. The concentrations of all constituents were identical to those in MEM, except that when appropriate, calcium was excluded from the medium; EGTA, (0.4 mM) which was added to both normal and calcium-free media, was used to chelate residual calcium.

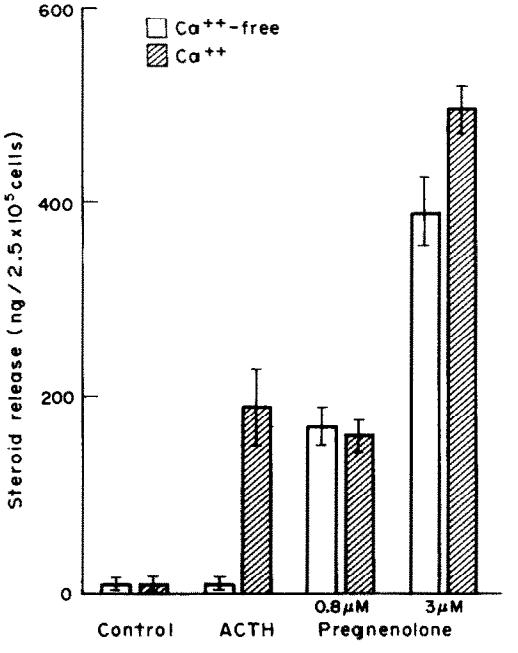

Fig. 1. The effect of calcium deprivation on steroid release induced by ACTH or pregnenolone. Equal numbers of cells (2.5×10^5) were incubated for 60 min in normal or calcium-free medium in the presence or absence of ACTH (250 μU) or pregnenolone. The cells were centrifuged, the supernatant extracted with 5 ml methylene chloride and assayed for corticosteroid. Each vertical bar represents mean steroid release (±S.E.M.) derived from triplicate beakers from the same preparation. The data shown are representative of two such experiments.

RESULTS

Comparison of the effects of pregnenolone and ACTH on steroid release

Isolated cortical cells incubated with pregnenolone or ACTH responded with a brisk rise in steroid release (Fig. 1). The response to pregnenolone was concentration dependent and consisted of approximately a 40-fold increase at the higher concentration employed. In the same experiments ACTH augmented steroid release by about 20-fold (Fig. 1). Although only one ACTH concentration was used in this particular study, previous work has shown that this concentration (250 μU) evokes near maximal steroid production [8].

As previously reported [8], cells incubated in a calcium-deprived medium failed to respond to ACTH with a rise in steroid release (Fig. 1). In fact, in the absence of calcium, the amount of steroid synthesized and released by ACTH was no greater than that released without ACTH (Fig. 1). By contrast, calcium deprivation did not discernibly alter the steroidogenic response to the lower concentration of pregnenolone (0.8 μM) (Fig. 1). Thus, in the absence and presence of calcium, pregnenolone-facilitated steroid release was 161 and 171 ng, respectively (Fig. 1). At the higher pregnenolone concentration (3 μM), calcium deprivation reduced steroid release from 499 to 393 ng—a reduction of only 21%.

The effects of pregnenolone and ACTH on PG release

ACTH evoked an increase in PGE_2 and $PGF_{2\alpha}$ release (Table 1). When the ACTH-stimulated PG value of each experiment was expressed as a percent of its unstimulated control, ACTH elicited a 22% increase in PGE_2 release and a 128% increase in $PGF_{2\alpha}$ release (Table 1). By contrast, the potently steroidogenic concentration of pregnenolone failed to augment $PGF_{2\alpha}$ or PGE_2 release when the data were expressed in terms of concentrations of PG released from pregnenolone-treated cells as a percent of PG released from unstimulated cells (Table 1).

The effects of cycloheximide

Incubation of cortical cells with cycloheximide completely blocked the steroidogenic effects of ACTH (Fig. 2). On the other hand, the steroidogenic response to pregnenolone was unaffected by the same concentration of cycloheximide (Fig. 2), indicating that the inhibitor was not impairing the general viability of these cells.

Unstimulated cells incubated with cycloheximide tended to release less PGE_2 and $PGF_{2\alpha}$ than in its absence (Table 2), although only the $PGF_{2\alpha}$ values

Table 1. Comparative effects of ACTH and pregnenolone on prostaglandin formation

	Expt.	PGE_2 pg	PGE_2 % of basal	$PGF_{2\alpha}$ pg	$PGF_{2\alpha}$ % of basal
	1. Basal	205	—	280	—
	ACTH	225	110	401	143
	Pregnenolone	122	60	152	54
	2. Basal	218	—	681	—
	ACTH	266	122	819	120
	Pregnenolone	222	102	603	89
	3. Basal	92	—	113	—
	ACTH	117	127	232	205
	Pregnenolone	97	105	114	101
	4. Basal	269	—	431	—
	ACTH	344	128	1014	235
	Pregnenolone	259	96	232	54
	5. Basal	—	—	91	—
	ACTH	—	—	396	435
	Pregnenolone	—	—	96	106
Mean	Basal	196 ± 37	—	319 ± 109	—
±S.E.	ACTH	238 ± 47	122 ± 4	572 ± 147	228 ± 56
	Pregnenolone	175 ± 39	91 ± 10	239 ± 94	81 ± 11

After cortical cells were incubated for 90 min in MEM, in the presence or absence of ACTH (250 μU) or pregnenolone (3 μM), PG analysis was carried out by direct assay of the incubation media using either PGE_2 or $PGF_{2\alpha}$ antiserum. Basal PGE_2 and $PGF_{2\alpha}$ values are significantly different from corresponding ACTH-stimulated values as determined by paired t test ($P < 0.05$), but are not significantly different from corresponding values obtained with pregnenolone ($P > 0.5$).

were significantly different when analyzed by paired observation t test ($P < 0.05$). When PG values for cycloheximide-treated samples in each experiment were expressed as a percent of those obtained in the absence of inhibitor, the average amount of PGE_2 and $PGF_{2\alpha}$ released in the presence of cycloheximide was 84 ± 9% and 82 ± 6% of control, respectively.

ACTH-enhanced PGE_2 release was unimpaired by cycloheximide; in fact, in the presence of this inhibitor, ACTH produced a slightly greater facilitation of PGE_2 release over its corresponding control value (134%) than the facilitation observed in the absence of cycloheximide (74%) (Table 2). When the responses to ACTH in the presence and absence of cycloheximide were compared in each experiment, they also were found to be comparable (111 ± 19%). In the presence of cycloheximide ACTH augmented $PGF_{2\alpha}$ release by an average of 49% (Table 2). This modest increase was manifest only if the ACTH-induced $PGF_{2\alpha}$ release in the presence of inhibitor was expressed as a percent of basal cycloheximide-treated samples, since the average amount of $PGF_{2\alpha}$ released by ACTH in the presence of inhibitor (441 pg) approximated basal $PGF_{2\alpha}$ levels in the absence of inhibitor (459 pg) (Table 2).

DISCUSSION

Although calcium obviously plays a fundamental role in the mode of action of ACTH, its principal site of action in the intact cell has not been clearly defined. Moreover, the interaction of this cation with other putative mediators, such as cyclic AMP and PGs, has so far defied elucidation (cf. 2). The present experiments have shown that the role of calcium in the steroidogenic process must be associated with one or more of the early events taking place at the cell

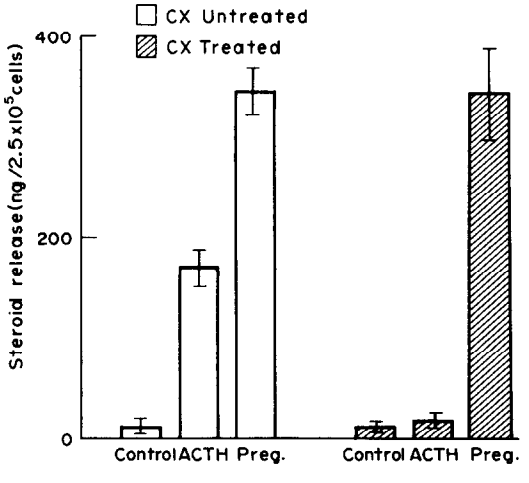

Fig. 2. The effect of cycloheximide on steroid release from isolated adrenocortical cells. Cells were incubated for 90 min in Minimal Eagle's medium in the presence or absence of ACTH (125 μU) or pregnenolone (3 μM) and/or cycloheximide (CX) (10 μM). Each vertical bar represents mean steroid release (±S.E.M.) derived from 5 different preparations.

Table 2. Comparison of the effects of cycloheximide on PGE_2 and $PGF_{2\alpha}$ formation

Incubation Time Additions	PGE_2(pg)			$PGF_{2\alpha}$(pg)		
	Basal	ACTH	% of basal	Basal	ACTH	% of basal
None	232 ± 46	285 ± 59	174 ± 53	459 ± 124	670 ± 152	163 ± 24
Cyclohex.	155 ± 26	329 ± 113	234 ± 95	348 ± 94	441 ± 92	149 ± 32

All PG values were derived from experiments described in Fig. 2 by direct assay of incubation medium using appropriate antisera. The values represented as percent of basal in the presence of ACTH—with or without cycloheximide—were obtained from their corresponding control values with or without cycloheximide. Mean values (±S.E.M.) were calculated from 5 different preparations.

membrane as a consequence of the interaction of ACTH with its receptor. This conclusion is supported by the finding that when hormone–receptor interactions are bypassed using pregnenolone as the steroidogenic agent, the calcium requirement for steroid production and release is essentially obviated. The inability of pregnenolone to enhance PG synthesis and its refractoriness to cycloheximide (see also 12), support the notion that this corticoid precursor is, unlike ACTH, acting intracellularly to augment steroidogenesis by providing a substrate source.

In order to unravel the mechanism by which calcium regulates steroidogenesis, it is essential that the effects of this cation be understood in relation to the intact cell. The present findings that cycloheximide inhibited calcium-dependent ACTH-induced steroidogenesis, but not calcium-independent pregnenolone-induced steroidogenesis, do not rule out some primary action of calcium on protein synthesis [5], which appears to be activated by ACTH and required for steroidogenesis [13]. Neither do these findings discredit the hypothesis advanced by *in vitro* studies that calcium exerts a direct role in regulating the mitochondrial biosynthesis of steroids [6]. But if calcium were exerting its primary action in the intact cell either by regulating protein synthesis or by directly affecting steroid biosynthesis, then calcium deprivation should similarly blunt the steroidogenic response to other stimulating agents—exclusive of pregnenolone—as well as ACTH; and a number of studies indicates that this is not the case [8, 11, 17]. For example, steroidogenesis elicited by exogenous cyclic AMP appears minimally dependent upon extracellular calcium [8, 17]. Thus, the overwhelming evidence relating calcium to events associated with membrane-bound adenylate cyclase [4, 17, 18], and additionally supported by such findings as those presented in the present study, point to the initial and possibly primary locus of calcium action in the intact cell at the level of the cortical cell membrane. A model which attempts to account for the effects of calcium in the mechanism of action of ACTH has been previously presented [4].

Another implication of the present study relates to the role of PGs in the mode of action of ACTH and its interaction with calcium. The inability of pregnenolone to augment PG synthesis despite marked steroidogenic activity, not only reveals that the induction of PG synthesis by ACTH is a consequence of some action of the hormone at the cell membrane prior to conversion of cholesterol to corticosteroid, but also implies that PG formation is not an obligatory event during all phases of steroid biosynthesis; it also corroborates previous studies demonstrating that steroid production and release induced either by ACTH [10, 11] or by butyryl cyclic AMP [11] are not singularly dependent upon enhanced PG formation. On the other hand, using indomethacin to alter PG formation, a functional relationship between steroid and PG release was found [10], which suggests that although PGs may not be obligatory intermediates, they may act somehow to modulate steroid production and/or release. These findings together with those revealing that ACTH-facilitated PG synthesis is also a calcium-dependent process [11] has special relevance here. For although the central position of calcium in steroidogenesis cannot, in all probability, relate to its functional role in regulating PG synthesis—since PG synthesis is not a *sine qua non* for ACTH-induced steroid production—the fact that ACTH appears to trigger PG synthesis by a calcium-dependent action on the cortical cell membrane [11] further underscores the critical role of calcium in the diverse signals generated by hormone–receptor interaction.

The other important aspect of this investigation pertains to the effects of cycloheximide on PG synthesis. It is clear from the present studies that although basal PG levels are reduced by cycloheximide, ACTH-induced PG formation is not markedly affected by a cycloheximide concentration which completely blunts steroid production and release. Not only do these results imply that PG formation and steroid production are not inextricably linked but also that the fundamental action of ACTH on the basic synthesizing enzyme systems which control the formation of PGE_2 and $PGF_{2\alpha}$, and probably other PGs as well, involves enzyme activation rather than *de novo* synthesis of new enzyme protein. However, since cycloheximide is known as a general inhibitor of microsomal, but not mitochondrial protein synthesis [19], the observed effects of cycloheximide on PG levels may be a consequence of several sites of action. For example, cycloheximide may also be exerting its actions through enzyme systems concerned with PG metabolism as well as synthesis.

Accordingly, PGE_2 is readily converted to other PG species, including its 15-keto derivative by PG dehydrogenase [20] and to $PGF_{2\alpha}$ by α-keto reductase [21]. Since recent evidence revealed that renal PG dehydrogenase is a labile enzyme with a half-life of 1 h [22] some of the net effects of cycloheximide on PGE_2 levels, for example, could also be explained on the basis of impaired metabolism as well as altered synthesis.

Finally, it has not yet been established whether ACTH enhances PG synthesis by a direct action on PG synthetase and/or by an action on plasma membrane phospholipase which may augment PG formation by making precursor fatty acids available. Therefore, further studies are now in progress to identify and localize the enzyme systems which are principally responsible for enabling ACTH to express its effects on the mechanisms controlling PG synthesis. Knowledge of these enzyme systems and the possible functional role of calcium in regulating their activity may provide additional salient information concerning the early signals generated by the action of ACTH.

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