

A Role for Calmodulin in the Regulation of Steroidogenesis

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ABSTRACT Two approaches were used to study the possible role of calmodulin in the regulation of steroid synthesis by mouse adrenal tumor cells: trifluoperazine was used as an inhibitor of calmodulin and liposomes were used to deliver calmodulin into the cells. Trifluoperazine inhibits three steroidogenic responses to both ACTH and dibutyryl cyclic AMP: (a) increase in steroid production, (b) increased transport of cholesterol to mitochondria, and (c) increased side-chain cleavage by mitochondria isolated from cells incubated with ACTH or dibutyryl cyclic AMP. When calmodulin is introduced into the cells via liposomes, steroid synthesis is slightly stimulated. When calmodulin is extensively dialyzed against EGTA, this stimulation is abolished. Ca^{2+} introduced via liposomes was also without effect. However, when both calmodulin and Ca^{2+} are introduced via liposomes (either in separate liposomes or in the same liposomes), steroid synthesis is stimulated. This stimulation does not occur when either anticalmodulin antibodies or EGTA is also present in the liposomes or when trifluoperazine is present in the incubation medium. Calmodulin and Ca^{2+} presented together in liposomes to the cells stimulate transport of cholesterol to mitochondria, and side-chain cleavage activity is greater in mitochondria isolated from cells previously fused with liposomes containing calmodulin and Ca^{2+} than in mitochondria from cells fused with liposomes containing buffer only. These observations suggest that calmodulin may be involved in regulating the transport of cholesterol to mitochondria, a process which is stimulated by ACTH and dibutyryl cyclic AMP and which may account, at least in part, for the increase in steroid synthesis produced by these agents.

The steroidogenic pathway begins with the transport of cytoplasmic cholesterol to the inner mitochondrial membrane, where the side-chain cleavage enzyme converts cholesterol to pregnenolone from which the secreted steroids are formed (1). Studies from a number of laboratories (2, 3), including our own (4-8), have indicated that the transport of cholesterol to the mitochondrial enzyme system is accelerated by ACTH and cyclic AMP and that this response may account, at least partly, for the increase in steroid synthesis produced by these agents.

Several lines of evidence indicate that Ca^{2+} is required for the steroidogenic responses to both ACTH and cyclic AMP (9, 10) and, because cyclic AMP is regarded as a second messenger for ACTH, it seems likely that Ca^{2+} is required for some intracellular event(s) apart from (or beyond) its involvement in the membrane changes that result in enhanced production of the cyclic nucleotide (9, 10). Recent evidence for the widespread role of calmodulin in the regulation of intracellular Ca^{2+} (reviewed in reference 11) led us to determine whether calmodulin is involved in the responses to ACTH and cyclic AMP. For this purpose, an inhibitor of calmodulin (trifluoper-

azine) (12) was used, and liposomes containing calmodulin were prepared as a means of introducing the protein to the interior of the Y-1 cells. Three steroidogenic responses to ACTH and dibutyryl (db) cyclic AMP were studied: production of a representative secreted steroid, 20 α -dihydroprogesterone (5); accumulation of cholesterol in the inner mitochondrial membrane during incubation with aminoglutethimide (to inhibit side-chain cleavage (6, 7); and synthesis of pregnenolone (side-chain cleavage) by mitochondria from cells previously incubated with aminoglutethimide together with ACTH or db cyclic AMP (6, 7). The studies to be reported here demonstrate a role for calmodulin in the regulation of steroidogenesis.

MATERIALS AND METHODS

Cell Culture

Methods for the culture of Y-1 mouse adrenal tumor cells (American Type Culture Collection, Rockville, Md.) and for incubation of cells to measure steroidogenesis have been given previously (4, 5). Cells were cultured in a medium containing serum, but incubation for measurement of steroid synthesis was performed in Eagle's minimal essential medium (EMEM) (5, 13).

Three responses of the cells to ACTH and db cyclic AMP were performed as described elsewhere: production of 20 α -dihydroprogesterone in the medium (4, 5); transport of cholesterol to mitochondria when steroidogenesis is blocked by aminoglutethimide PO_4 (6, 7); and production of pregnenolone by mitochondria isolated from cells previously incubated with aminoglutethimide PO_4 (6-8). Inner mitochondrial membrane was prepared by an established method (14). For measurement of production of 20 α -dihydroprogesterone, zero-time control values (<0.02 nmol/ 10^6 cells) were determined and subtracted from values obtained after incubation for 30 or 60 min.

Liposomes

PREPARATION: Lipid vesicles (liposomes) were prepared according to the method of Papahadjopoulos et al. (15) using their system number 1 (phosphatidylglycerol, phosphatidylcholine, and cholesterol [1:4:5 molar ratio]) and following the procedure exactly as described. Substances to be incorporated were added to the buffer in which liposomes were prepared (potassium phosphate-buffered saline [PBS]). Liposomes containing calmodulin were prepared with Ca^{2+} -free PBS containing calmodulin (1 mg/ml). Liposomes containing Ca^{2+} were prepared with PBS containing calcium chloride 5 mM. Liposomes were separated from unincorporated material by chromatography on Sephadex G-50. The eluting buffer contained EGTA 1 mM when Ca^{2+} -free liposomes were prepared and EGTA was subsequently removed by dialysis of the vesicles. After this treatment, the concentration of total calcium in the dialyzed calmodulin was <0.1 nmol/mg of protein as measured by atomic absorbance. Moreover, when liposomes were prepared in the buffer used for dialysis (before dialysis), no effect was observed on steroid synthesis when these liposomes were incubated with Y-1 cells. Unless otherwise stated, the concentration of $CaCl_2$ incorporated into liposomes was 5 mM. The liposomes used in these studies showed a capture volume of 12.2-13.6 (range of six determinations) μ l/mg of lipid amounting to 59-64% of the aqueous phase. These values were determined by published methods (16). That fusion occurs between liposomes and Y-1 cells has been demonstrated by the use of [3H]cyclic AMP (6). Uptake of [3H]cyclic AMP after fusion was examined in this study and was found to increase with time for 1 h. It is not possible at present to determine the extent of cellular uptake of the contents of liposomes because many liposomes adhere to the cells but may not necessarily fuse with them in the sense of making their contents available to the cytoplasm. When the cells are washed four times after fusion (with PBS), the combined washes were found to contain 15-20% of the phospholipid initially added to the cells.

FUSION OF LIPOSOMES WITH CELLS: Liposomes were incubated with Y-1 cells in monolayer culture in plastic dishes for 1 h as a routine. Zero-time controls (cells washed immediately after addition of liposomes and then incubated for determination of steroid production) were performed in all experiments. Steroid production by these controls was the same as that of cells not incubated with liposomes. Fusion was performed by washing cells with PBS and adding the liposomes in EMEM. Cells were kept at 37°C for 1 h. The medium was then removed and the cells were washed with PBS and incubated for various times in EMEM to measure steroid production or cholesterol transport.

Materials

Calmodulin was prepared from rat testes exactly as described by Dedman et al. (17). The purified material showed a single band on sodium dodecyl sulfate (SDS) gels and was shown to form a single band on double diffusion with anticalmodulin purified by affinity chromatography, using purified calmodulin as ligand (see below). Calmodulin was shown to stimulate brain phosphodiesterase with a specific activity of 22,000 U/mg protein, which is comparable to that reported by Dedman et al. (17). Trifluoperazine (Stellazine) and trifluoperazine sulfoxide were generously provided by Smith, Kline, and French Laboratories (Philadelphia, Pa.). Other chemicals were obtained from sources published elsewhere (4, 5, 6, 8).

Anticalmodulin antibody raised in sheep to calmodulin from rat testes and purified by affinity chromatography was generously provided by Dr. A. R. Means, Baylor College of Medicine. This material has been fully characterized (18).

Miscellaneous

The method of measuring incorporation of [$2-^{14}C$]acetate into [^{14}C]cholesterol has been given elsewhere (19).

RESULTS

Influence of Trifluoperazine on Steroidogenic Responses to ACTH and db Cyclic AMP

PRODUCTION OF 20 α -DIHYDROPROGESTERONE: It can be seen from Fig. 1 that trifluoperazine (50 μ M) strongly inhibits the stimulation of steroid synthesis produced by ACTH (A) and db cyclic AMP (B) in Y-1 adrenal tumor cells. The figure also shows that a chemically modified form of trifluoperazine (trifluoperazine sulfoxide) is without effect on the response to ACTH (C). Fig. 2 shows the effects of various concentrations of trifluoperazine on these responses. From this and two additional experiments (not shown), it was calculated that the concentration of trifluoperazine required to inhibit these responses by 50% is ~ 15 μ M for both ACTH and db cyclic AMP. Previous studies have shown that production of 20 α -dihydroprogesterone is representative of total steroid output by Y-1 cells and that the doses of the two stimulating agents (ACTH and db cyclic AMP) used here are supramaximal (4, 5).

INTRACELLULAR TRANSPORT OF CHOLESTEROL: When side-chain cleavage is blocked by aminoglutethimide, mitochondrial cholesterol cannot enter the steroidogenic path-

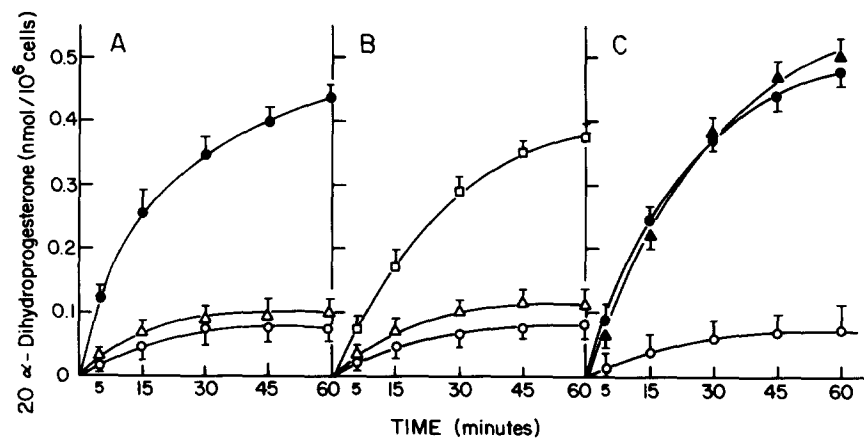


FIGURE 1 Effect of trifluoperazine on the steroidogenic response of Y-1 cells to ACTH (A) and db cyclic AMP (B). Cells were incubated in EMEM with and without ACTH (40 mU/ml (A) or db cyclic AMP (1 mM) (B) and trifluoperazine (50 μ M) for the times shown. After incubation, the concentration of 20 α -dihydroprogesterone in the medium was determined by radioimmunoassay. Values shown are means and ranges for duplicate determinations. In C, trifluoperazine sulfoxide (50 μ M) was used in place of trifluoperazine. \circ , control; \bullet , ACTH; \square , db cyclic AMP; \triangle , ACTH (or db cyclic AMP) plus trifluoperazine; and \blacktriangle , ACTH plus trifluoperazine sulfoxide.

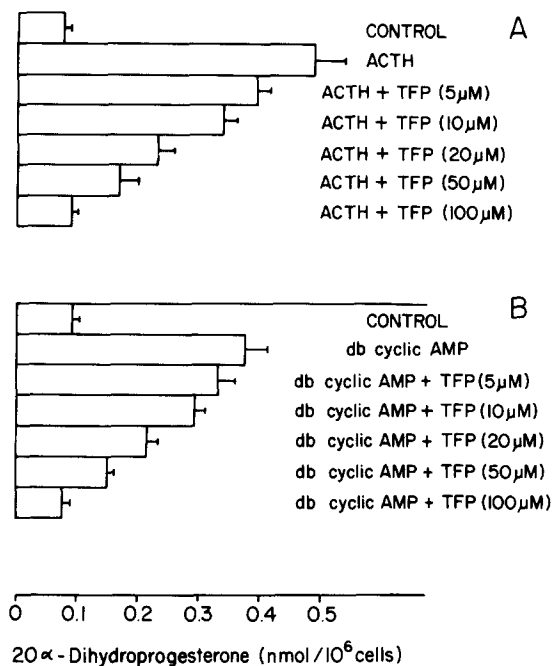


FIGURE 2 Inhibition of the steroidogenic responses to ACTH (A) and db cyclic AMP (B) by various concentrations of trifluoperazine. Experiments were performed as described under Fig. 1. The duration of incubation was 30 min. Values are means and ranges of duplicate determinations. TFP, trifluoperazine.

way so that intracellular transport of cholesterol can be measured by determining the amount of mitochondrial cholesterol per milligram of membrane protein as a function of time (6-8). We have made these measurements on inner mitochondrial membrane where the side-chain cleavage system is situated (14). Fig. 3 shows that no demonstrable accumulation of cholesterol occurs in the inner mitochondrial membrane of unstimulated cells during a period of 15 min. By contrast, considerable accumulation of cholesterol is seen when the cells incubated with aminoglutethimide are stimulated by ACTH (A) or db cyclic AMP (B). In both cases, this stimulation is almost completely inhibited by trifluoperazine (50 mM).

SYNTHESIS OF PREGNENOLONE BY ISOLATED MITOCHONDRIA: ACTH and db cyclic AMP added to Y-1 cells do not accelerate side-chain cleavage of cholesterol in isolated mitochondria prepared from such cells and incubated in vitro (8). However, if side-chain cleavage is blocked by aminoglutethimide while the cells are under stimulation by either of these agents, isolated mitochondria from the cells show increased side-chain cleavage (production of pregnenolone) when incubated at 30°C after washing to remove aminoglutethimide (6-8). Fig. 4 shows that this response is inhibited by trifluoperazine (A and B). However, when trifluoperazine is added to the isolated mitochondria from cells previously stimulated by ACTH in the presence of aminoglutethimide (but without trifluoperazine), it is without effect on the response to ACTH (Fig. 4 C).

Influence of Calmodulin and Ca²⁺ on the Steroidogenic Responses to ACTH and db Cyclic AMP

PRODUCTION OF 20 α-DIHYDROPROGESTERONE: It will be seen from Fig. 5 that calmodulin and Ca²⁺ (separately)

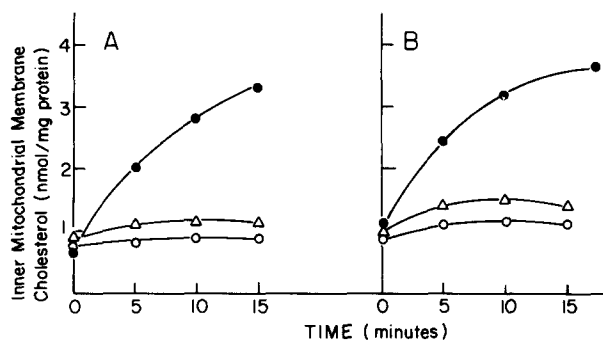


FIGURE 3 Influence of trifluoperazine on the increase in intracellular transport of cholesterol produced by ACTH (A) and by db cyclic AMP (B). Side-chain cleavage was inhibited by incubation of Y-1 cells with aminoglutethimide PO₄ (0.76 mM) for 15 min before addition of ACTH (40 μM) or db cyclic AMP (1 mM) with and without trifluoperazine (50 μM). After incubation inner mitochondrial membrane was prepared (14). The cholesterol content of the membrane was measured by gas/liquid chromatography (8). ○, control; ●, ACTH or db cyclic AMP; and Δ, ACTH or db cyclic AMP plus trifluoperazine.

appear to cause slight increases in the production of 20 α-dihydroprogesterone when introduced into Y-1 cells via liposomes. The possible significance of these small differences was not tested statistically. When calmodulin was extensively dialyzed against EGTA (1 mM) and used after dialysis to remove EGTA, no stimulation of steroid production was observed, suggesting that the small stimulation seen with untreated calmodulin may have resulted from bound Ca²⁺. When calmodulin and Ca²⁺ were added together in liposomes,¹ stimulation of steroid production was observed (Fig. 5). Such stimulation by calmodulin-Ca²⁺ was seen in two additional experiments (P < 0.01). This response was inhibited by trifluoperazine. When ACTH or db cyclic AMP was added together with liposomes containing calmodulin and Ca²⁺, no additive effect was observed. However, when a submaximal concentration of ACTH or db cyclic AMP was used, stimulation was additive (Fig. 6).

Further studies of this response are shown in Table I. The two agents calmodulin and Ca²⁺ were less effective when administered in separate liposomes, and "free" calmodulin and "free" Ca²⁺ (not in liposomes) were without effect. Anticalmodulin antibody (fivefold molar excess) inhibited the action of calmodulin-Ca²⁺. EGTA also prevented stimulation by calmodulin-Ca²⁺ (Table I). In addition, calmodulin-Ca²⁺ in liposomes partly overcomes inhibition by a low concentration of trifluoperazine.

INTRACELLULAR TRANSPORT OF CHOLESTEROL: Fig. 7 shows that calmodulin-Ca²⁺ presented to Y-1 cells in liposomes accelerates transport of cholesterol to inner mitochondrial membrane (A). This response is inhibited by trifluoperazine and does not occur when aminoglutethimide is absent from the medium "▲."

SYNTHESIS OF PREGNENOLONE BY ISOLATED MITOCHONDRIA: Mitochondria isolated from cells incubated with calmodulin-Ca²⁺ in liposomes and aminoglutethimide in the incubation medium showed increased production of pregnenolone when incubated at 30°C after removal of aminoglutethimide (Fig. 7 B). Calmodulin-Ca²⁺ was without effect in the

¹ Calmodulin-Ca²⁺: this expression refers to calmodulin associated with calcium in contrast to dialyzed calmodulin.

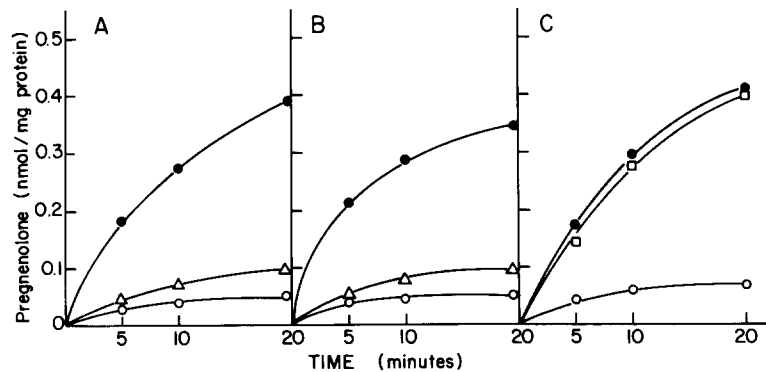


FIGURE 4 The influence of trifluoperazine on the production of pregnenolone by mitochondria isolated from Y-1 cells previously incubated with aminoglutethimide. Y-1 cells were incubated for 60 min with aminoglutethimide (0.76 mM) with and without ACTH (40 mU) or db cyclic AMP (1 mM) with and without trifluoperazine (50 μ M). After incubation, mitochondria were prepared from the cells in the presence of aminoglutethimide (0.76 mM) at 0°C for the times shown. The mitochondria were then washed twice to remove aminoglutethimide and incubated at 30°C for the times shown. Pregnenolone was measured in samples of medium by radioimmunoassay (8). In C, cells were incubated with ACTH and aminoglutethimide; trifluoperazine (50 μ M) was added to the isolated mitochondria. O, control; ●, ACTH or db cyclic AMP; Δ, ACTH or db cyclic AMP plus trifluoperazine; and □, ACTH; trifluoperazine added to mitochondria.

TABLE I
The Influence of Calmodulin and Various Agents on Steroid Production by Y-1 Adrenal Cells

Addition to medium	Liposomes		20 α -Dihydroprogesterone nmol/10 ⁶ cells
	I	II	
—	Buffer	—	0.08 \pm 0.02
—	Calmodulin	—	0.44 \pm 0.02
—	Ca ²⁺	—	0.23 \pm 0.04
—	Calmodulin-Ca ²⁺	—	0.38 \pm 0.2
—	Calmodulin	Ca ²⁺	0.18 \pm 0.3
—	Calmodulin-anti-calmodulin-Ca ²⁺	—	0.12 \pm 0.05
—	Calmodulin-Ca ²⁺ -EGTA	—	0.11 \pm 0.04
Ca ²⁺	—	—	0.08 \pm 0.01
Calmodulin	—	—	0.07 \pm 0.02
Trifluoperazine (20 μ M)	—	—	0.04 \pm 0.02
Trifluoperazine (20 μ M)	Calmodulin-Ca ²⁺	—	0.18 \pm 0.1
Trifluoperazine (50 μ M)	—	—	0.02 \pm 0.01
Trifluoperazine (50 μ M)	Calmodulin-Ca ²⁺	—	0.06 \pm 0.02

This experiment was performed as described under Fig. 1, except that in some dishes two kinds of liposomes (I and II) were added. The concentrations of substances in the liposomes or in the medium were as follows: calmodulin 1 mg/ml; anticalmodulin 40 μ g/ μ g calmodulin; Ca²⁺ 5 mM; EGTA 1 mM. Each addition of liposomes was 200 μ l (10 μ mol of phospholipid) in a total volume of 2 ml incubation medium. The calmodulin used was exhaustively dialyzed against EGTA (1 mM). Values shown are means and ranges of duplicate determinations.

absence of aminoglutethimide (\blacktriangle) and when added to mitochondria from unstimulated cells (control, \square). Stimulation of pregnenolone production by calmodulin-Ca²⁺ was prevented by trifluoperazine added to the medium in which cells were incubated (Fig. 7 B, Δ) but not when added to isolated mitochondria.

Miscellaneous

When Y-1 cells were incubated with [7 α -³H] pregnenolone

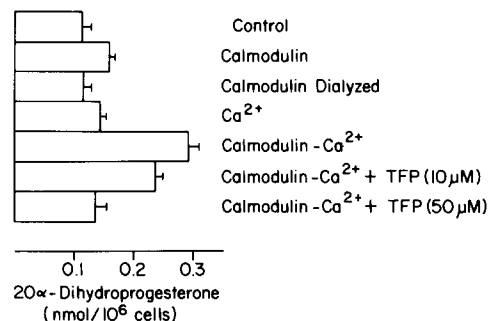


FIGURE 5 The influence of calmodulin and Ca²⁺ on steroidogenesis by Y-1 cells. Cells were incubated for 60 min with liposomes containing buffer or buffer plus the additions shown. At the end of this time, medium was removed, cells were washed twice with PBS at 0°C, and fresh medium was added with liposomes (200 μ l; 10 nmol of phospholipid/dish) containing buffer (control) or the additions shown. Liposomes were prepared as described under Materials and Methods. Incubation was continued for 30 min and 20 α -dihydroprogesterone was measured in samples of medium. Dialyzed calmodulin refers to calmodulin extensively dialyzed against EGTA (1 mM). Trifluoperazine was added to the medium "free", i.e., not in liposomes. Other details are given under Materials and Methods. The values shown represent means and ranges for duplicate determinations.

(0.1 μ g: 1.1 \times 10⁶ cpm/dish) the production of 20 α -dihydro-³H] pregnenolone was not altered by either calmodulin-Ca²⁺ or trifluoperazine (control 217,000 \pm 8,000; calmodulin-Ca²⁺ 218,000 \pm 6,000; trifluoperazine 219,000 \pm 8,000 cpm/10⁶ cells; means and ranges for duplicate determinations). Neither agent influenced the incorporation of [¹⁴C]acetate into cholesterol during incubation with the labeled substrate for one hour (data not shown). Vesicles containing calmodulin-Ca²⁺ were prepared from a variety of phospholipids including phosphatidylethanolamine and phosphatidylcholine in various mixtures and from various sources (synthetic and animal) (16). Such vesicles produce the same responses in Y-1 cells qualitatively but were less potent, presumably because of lower capture volume (not shown). The absence of cholesterol from the liposomes did not affect their ability to influence the Y-1 cells. Moreover, addition of cholesterol, in a variety of solvents, to Y-1 cells does not affect steroid synthesis (data not shown).

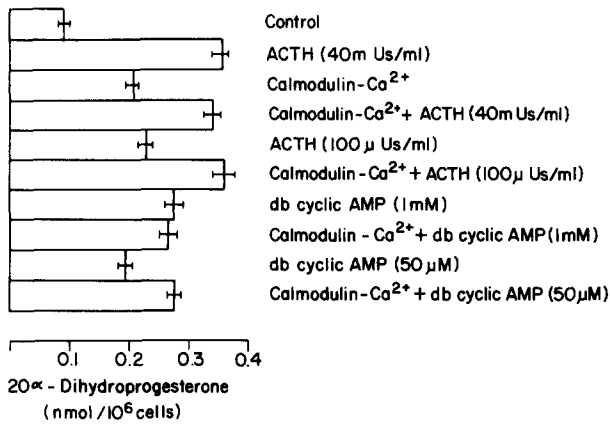


FIGURE 6 The effects of ACTH, db cyclic AMP, and calmodulin-Ca²⁺ in various combinations on the production of 20 α -dihydroprogesterone by Y-1 adrenal tumor cells. Experiments were performed as described under Fig. 5. Values are means and ranges of duplicate determinations.

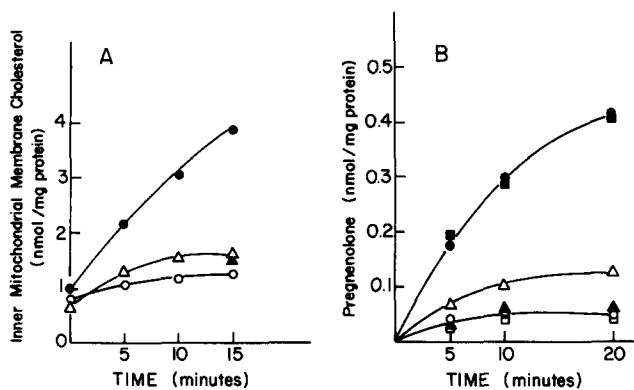


FIGURE 7 The influence of calmodulin with calcium on the accumulation of cholesterol in the inner mitochondrial membrane and on production of pregnenolone by isolated mitochondria. Y-1 cells were incubated with liposomes containing buffer (control) or buffer with calmodulin and Ca²⁺ (200 μ l/dish) as described under Fig. 5. The incubation medium contained trifluoperazine (50 μ M) where indicated and aminoglutethimide (0.76 mM). After incubation for the times shown in A, inner mitochondrial membrane was prepared and the cholesterol content was measured. In B, pregnenolone production was determined after removal of aminoglutethimide as described under Fig. 4. O, control; ●, calmodulin-Ca²⁺; Δ, calmodulin-Ca²⁺ plus trifluoperazine; □, control plus calmodulin-Ca²⁺ added to mitochondria; and ▲, calmodulin-Ca²⁺ and no aminoglutethimide. ■, calmodulin = Ca²⁺; trifluoperazine (50 μ M) added to isolated mitochondria.

DISCUSSION

Two lines of evidence presented here suggest that calmodulin plays some role in the regulation of steroid synthesis by Y-1 adrenal tumor cells. First, trifluoperazine inhibits the stimulating effects of both ACTH and db cyclic AMP on steroid production in these cells. Inhibition by trifluoperazine is concentration-dependent and occurs at low concentrations within the range seen when inhibition of isolated enzymes requiring calmodulin is studied (12, 20). Moreover, chemical modification of the inhibitor (sulfoxide derivative) results in greatly diminished binding to phosphodiesterase (21) and loss of inhibition of the steroidogenic responses to ACTH and db cyclic AMP. These observations suggest that calmodulin is necessary for the steroidogenic responses to ACTH and db cyclic AMP.

Calmodulin-Ca²⁺ stimulates steroid production by the Y-1 cells, and each of these agents is less effective or ineffective alone. It is clear that the limited stimulation by calmodulin alone results from the presence of bound Ca²⁺ which can be removed by EGTA. Stimulation was less effective when the two agents were added in separate liposomes and was abolished by a fivefold molar excess of anticalmodulin (Table I). Moreover, the effect of calmodulin was prevented by trifluoperazine and the inhibitory effect of trifluoperazine was partly overcome by calmodulin-Ca²⁺ (Table I).

The site in the steroidogenic pathway at which trifluoperazine causes inhibition is the same as that at which calmodulin-Ca²⁺ stimulates, namely, the transport of cholesterol to mitochondria. When Y-1 cells are incubated with aminoglutethimide, ACTH increases the concentration of cholesterol in the inner mitochondrial membrane. When such mitochondria are incubated after removal of aminoglutethimide, production of pregnenolone is greater by mitochondria prepared from cells incubated with ACTH or db cyclic AMP than those from unstimulated cells. Both responses are prevented by trifluoperazine and similar responses are seen when ACTH is replaced by calmodulin-Ca²⁺. Cells incubated with ACTH or db cyclic AMP (8) or calmodulin-Ca²⁺ but without aminoglutethimide show neither increased levels of cholesterol in inner mitochondrial membrane nor increased side-chain cleavage by isolated mitochondria (Fig. 7). Again, neither trifluoperazine nor calmodulin-Ca²⁺ alters the rate of incorporation of acetate into cholesterol by Y-1 cells. In this connection, it is important to notice that neither trifluoperazine nor calmodulin-Ca²⁺ (nor ACTH and db cyclic AMP) influences side-chain cleavage when added to isolated mitochondria, nor do these agents affect the remainder of the steroidogenic pathway (pregnenolone \rightarrow 20 α -dihydroprogesterone). Clearly, both agents influence the transport of cholesterol to the inner mitochondrial membrane where the side-chain cleavage enzyme is to be found. ACTH, db cyclic AMP, and calmodulin-Ca²⁺ stimulate this transport and trifluoperazine inhibits the stimulation produced by all three agents. Once the cholesterol has reached the inner mitochondrial membrane, it is beyond the influence of all four agents (the three stimulating agents and the inhibitor).

We previously reported that intracellular transport of cholesterol to mitochondria in Y-1 cells involves the action of actin and hence, presumably, microfilaments (6, 7, 22). It would be consistent with what is known concerning the roles of troponin in skeletal muscle and calmodulin in other tissues that calmodulin-Ca²⁺ may influence the function of nonmuscle actin (reviewed in reference 23).

Although liposomes have been used in these studies with success, it is important to consider some limitations in this approach. Calmodulin without Ca²⁺ may be capable of limited stimulation and the two agents are less effective when added in separate liposomes (Table I). Again, calmodulin-Ca²⁺ is only partly successful in overcoming inhibition by trifluoperazine. It is likely that these limitations arise from intracellular barriers to the movement of calmodulin and Ca²⁺ within the cell. For example, the distribution of calmodulin entering the cell from one liposome may not be the same as that of Ca²⁺ entering from another liposome. However, mixing calmodulin with Ca²⁺ in the same liposomes allows the protein to carry saturating levels of Ca²⁺ with it through the cell. Presumably, the distribution of trifluoperazine in the cell is less restricted than that of calmodulin and Ca²⁺. These ideas are consistent with the belief that calmodulin serves to regulate local levels of Ca²⁺

within the cell (11). Because nothing is known concerning the distribution of calmodulin introduced into the cell by liposomes, this approach does not reveal whereabouts in the cell calmodulin acts or whether ACTH influences the distribution of endogenous calmodulin.

These considerations raise the question of how calmodulin- Ca^{2+} causes responses by the cell similar to those seen with ACTH and cyclic AMP. Calmodulin shows no additive effect above the levels of steroid production resulting from maximal stimulation by these two agents but does increase the response to submaximal levels of ACTH or db cyclic AMP (Fig. 6). These observations are consistent with a role for calmodulin- Ca^{2+} in the steroidogenic responses to the hormone and its second messenger. However, the experiments reported here do not indicate that an action of the stimulating agents via calmodulin- Ca^{2+} is the only action of the hormone and its messenger.

It appears that at least one important action of ACTH and cyclic AMP is to increase the transport of cholesterol to the mitochondrial membrane where side-chain cleavage occurs. We are now examining the molecular basis by which calmodulin- Ca^{2+} and actin serve to express the stimulating effect of ACTH and db cyclic AMP.

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REFERENCES

1. Hall, P. F. 1970. Gonadotrophic regulation of testicular function. *In* Androgens of the Testis. K. B. Eik-Nes, editor. Marcel Dekker, Inc., New York. 73-115.
2. Garren, L. D., G. N. Gill, H. Masui, and G. J. Walton. 1971. On the mechanism of action

- of ACTH. *Recent Prog. Hor. Res.* 27:433-478.
3. Mahaffee, D. R. C. Reitz, and R. L. Ney. 1974. The mechanism of action of adrenocorticotrophic hormone. *J. Biol. Chem.* 249:227-233.
4. Mrotek, J., and P. F. Hall. 1975. The influence of cytochalasin B on the response of adrenal tumor cells to ACTH and cyclic AMP. *Biochem. Biophys. Res. Commun.* 64:891-896.
5. Mrotek, J., and P. F. Hall. 1977. Response of adrenal tumor cells to ACTH: site of inhibition by cytochalasin B. *Biochemistry.* 16:3177-3181.
6. Hall, P. F., C. Charponnier, M. Nakamura, and G. Gabbiani. 1979. The role of microfilaments in the response of adrenal tumor cells to ACTH. *J. Biol. Chem.* 254:9080-9084.
7. Hall, P. F., C. Charponnier, M. Nakamura, and G. Gabbiani. 1979. The role of microfilaments in the response of leydig cells to luteinizing hormone. *J. Steroid Biochem.* 11:1361-1366.
8. Nakamura, M., M. Watanuki, B. E. Tilley, and P. F. Hall. 1980. The effect of ACTH on intracellular cholesterol transport. *J. Endocrinol.* 84:179-188.
9. Kuo, T. H., C. T. Ou, and T. T. Tchen. 1965. The effect of calcium on the stimulation of corticosterone biosynthesis by dibutyryl-C-AMP in cultures of ATCC cell line Y-1. *Biochem. Biophys. Res. Commun.* 65:190-195.
10. Lefkowitz, R. J., J. Roth, and I. Pastan. 1970. Effects of calcium on ACTH stimulation of the adrenal: separation of hormone binding from adenyl cyclase activation. *Nature (Lond.)* 228:864-866.
11. Means, A. R., and J. R. Dedman. 1980. Calmodulin—an intracellular calcium receptor. *Nature (Lond.)* 285:73-84.
12. Fertel, R. M., and B. Weiss. 1976. Properties and drug responsiveness of cyclic nucleotide phosphodiesterases of rat lung. *Mol. Pharmacol.* 12:678-687.
13. Watanuki, M., and P. F. Hall. 1979. Stimulation of uptake of cholesterol ester into adrenal tumor cells by ACTH and other agents. *FEBS (Fed. Eur. Biochem. Soc.) Letters.* 101:239-244.
14. Yago, N., S. Kobayashi, S. Sekijama, H. Kurokawa, Y. Iwai, I. Suzuki, and S. Ichii. 1970. Further studies on the submitochondrial localization of cholesterol side-chain cleaving enzyme system in hog adrenal cortex by sonic treatment. *J. Biochem. (Tokyo)* 68:775-783.
15. Szoka, F., and D. Papahadjopoulos. 1978. Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. U. S. A.* 75:4194-4198.
16. Papahadjopoulos, D. 1978. Methods for preparing liposomes for cell fusion. *Ann. N. Y. Acad. Sci.* 308:1-11.
17. Dedman, J. R., J. D. Porter, R. L. Jackson, J. D. Johnson, and A. R. Means. 1977. Physicochemical properties of rat testis Ca^{2+} -dependent regulator protein of cyclic nucleotide phosphodiesterase. *J. Biol. Chem.* 252:8415-8422.
18. Dedman, J. R., M. J. Welsch, and A. R. Means. 1978. Ca^{2+} -dependent regulator: production and characterization of a monospecific antibody. *J. Biol. Chem.* 253:7515-7521.
19. Hall, P. F. 1963. The effect of ICSH on the biosynthesis of testicular cholesterol from acetate- ^{14}C . *Biochemistry.* 2:1232-1238.
20. Levin, R. M., and Weiss, B. 1976. Mechanism by which psychotropic drugs inhibit cyclic AMP phosphodiesterase of brain. *Mol. Pharmacol.* 12:581-589.
21. Weiss, B., R. Fertel, R. Tighn, and P. Uzunov. 1974. Selective alteration of the activity of the multiple forms of AMP phosphodiesterase of rat cerebrum. *Mol. Pharmacol.* 10:615-625.
22. Hall, P. F., C. Charponnier, and G. Gabbiani. 1979. Role of actin in the response of leydig cells to luteinizing hormone. *In* Testicular Development, Structure, and Function. A. Steinberger and E. Steinberger, editors. Raven Press, New York. 229-235.
23. Kretsinger, R. H. 1980. Structure and function of calcium-modulated proteins. *CRC Crit. Rev. Biochem.* 8:119-174.