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THE ROLE OF MICROFILAMENTS IN THE RESPONSE OF LEYDIG CELLS TO LUTEINIZING HORMONE

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SUMMARY

Highly purified anti-actin was prepared from sera of patients suffering from hepatitis. The antibody was entrapped within negatively charged vesicles made from phosphatidylcholine and phosphatidylserine (9:1 molar ratio). Leydig cells prepared from testes of adult rats were incubated for one hour with liposomes containing buffer (control) or buffer with anti-actin. Cells were washed and incubated with saline (control), LH (50 μg/ml) or db cyclic AMP (2 mM) for 30 min. Entrapped anti-actin inhibited three steroidogenic responses of the cells to LH: (i) increased production of testosterone by Leydig cells, (ii) increased side-chain cleavage (cholesterol → pregnenolone) by isolated mitochondria prepared from cells previously incubated with either of these stimulating agents, and (iii) increase in the cholesterol content of inner mitochondrial membranes isolated from cells incubated with aminoglutethimide (an inhibitor of side-chain cleavage) and LH. Free anti-actin (no liposomes) produced no more than slight inhibition, while boiled anti-actin, human IgG, anti-actin with excess actin and bovine serum albumin captured within liposomes were all without effect on the response to LH. In addition, anti-actin was without effect on side-chain cleavage when added to isolated mitochondria and on the conversion of [^{3}H]-pregnenolone to [^{3}H]-testosterone. These findings, together with previous evidence, strongly suggest that LH and cyclic AMP stimulate side-chain cleavage of cholesterol (and hence steroidogenesis) by a mechanism involving actin—presumably in microfilaments; the responding microfilaments promote transport of cholesterol to mitochondria.

INTRODUCTION

Current views concerning the mechanism by which trophic hormones accelerate synthesis of steroids in adrenal and gonadal cells center around the idea that these hormones influence a rate-determining step in the steroidogenic pathway. Early studies demonstrated that this slow step involves the conversion of cholesterol to pregnenolone (side-chain cleavage) [1–3]. However, the acute or early response to trophic stimulation appears to result not from acceleration of enzymatic activity, but rather from transport of substrate (cholesterol) to the side-chain cleavage system in mitochondria [4–6]. Considerable interest is therefore associated with the intracellular and intramitochondrial transport of cholesterol. Studies from this laboratory showed that cytochalasin [6, 7], but no anti-tubular agents [8], inhibit the steroidogenic response of adrenal cells to ACTH. The evidence suggested that intracellular transport of cholesterol involves the contractile activity of microfilaments and that this process is accelerated by ACTH [7]. The isolation of highly purified anti-actin from human serum [9] and recent advances in the use of liposomes to introduce various substances (including proteins) into cells [10], have provided the opportunity to examine the role of actin in the response of steroidogenic cells to trophic stimulation by a more direct approach than that based upon the use of metabolic inhibitors. The studies reported here concern the steroidogenic response of Leydig cells to LH.

Abbreviations used: Aminoglutethimide: α-(*p*-aminophenyl)-α-ethylglutarimide db cyclic AMP: N^6, $O^{2'}$-dibutyryladenosine 3′,5′-monophosphate 20α-dihydroprogesterone: 20α-hydroxy-4-pregnen-3-one; pregnenolone: 3β-hydroxy-5-pregnen-20-one; testosterone: 17β-hydroxy-4-androsten-3-one.

MATERIALS AND METHODS

Preparation and incubation of Leydig cells. Leydig cells were prepared from testes of adult rats and incubated by the methods described by Dufan and Catt[11]. Testosterone was measured by radioimmunoassay [11]. Unless otherwise stated, 10^7 cells were incubated with phosphate buffered saline in a final volume of 2 ml. Cells were washed by resuspending pellets following centrifugation at 900 *g* for 10 min.

Preparation and incubation of mitochondria. Mitochondria were prepared as described elsewhere [7] and pregnenolone was measured by radioimmunoassay [7]. Aminoglutethimide was used to inhibit side-chain cleavage [12] in two types of experiments: firstly, the inhibitor was used to study the response of Leydig cells to LH by measuring production of pregnenolone by isolated mitochondria, and secondly, aminoglutethimide was used to measure accumulation of cholesterol in the inner mitochondrial membrane. In the first type of experiment, cells were incubated with aminoglutethimide (0.76 mM) with or without LH and after thirty minutes the cells were

cooled (4°C) and mitochondria were prepared in medium containing the inhibitor. The inhibitor was removed by washing mitochondria in phosphate buffered saline (without inhibitor) and mitochondria were then incubated at 30°C for various periods of time. The relevant methods have been described [6] and optimal conditions were found in preliminary studies (e.g. duration of incubation, concentration of inhibitor, etc.). In the second type of study, mitochondria were prepared as before from cells incubated with aminoglutethimide (0.76 mM) with or without LH. In this case, inner mitochondrial membrane was prepared [13] and cholesterol content was determined by gas chromatography [14].

Anti-actin. Anti-actin was prepared from sera obtained from patients suffering from chronic aggressive hepatitis. Sera were purified by affinity chromatography using actin from rabbit skeletal muscle as ligand [9]. Highly purified actin was prepared from rabbit skeletal muscle by an established method [15]. Methods for characterizing anti-actin have been published [9, 16]. In one study, unpurified serum was used.

Preparation of liposomes. Liposomes were prepared by minor modification of a method reported from one of our laboratories [17]. Phosphatidylcholine and phosphatidylserine (9:1 molar ratio) (10 mg) were mixed and dried under nitrogen. Sodium phosphate buffer (4 ml) (5 mM; pH 7.4) containing anti-actin was added and the mixture gently shaken for two minutes. After standing for one hour at room temperature, the mixture was sonicated under nitrogen for 10 min in bursts of 2 min each using a 1.8 cm titanium probe at the full setting of an MSE 60 W sonicator. The sample was then purified by chromatography on Sephadex G50 as described previously [17]. The protein-containing vesicles appeared in the void volume (well separated from anti-actin) and were subjected to centrifugation at 100,000 *g* for 60 min. The pellet was resuspended in 2 ml of phosphate buffered saline pH 7.4 and kept at 4°C before use in the present studies. More than 90% of the protein and lipid appeared in the pellet. In some studies, liposomes containing buffer only were used as control for liposomes with anti-actin.

Miscellaneous. Conversion of [7α-^{3}H]-pregnenolone to [^{3}H]-testosterone by Leydig cells was measured by the method described previously for conversion of [^{3}H]-pregnenolone to [^{3}H]-20α-dihydroprogesterone by adrenal cells [6].

Chemicals. The sources of chemicals used have been published previously [6, 7].

RESULTS

Characterization of anti-actin antibody

The anti-actin used in these studies has been characterized by the following criteria: (a) binding to sepharose-actin during purification [9]; (b) single band of precipitation with actin on double diffusion in agar [9]; (c) single band of precipitation on immunoelectrophoresis [10]; (d) classical line of identity between anti-actin and both crude and purified actin [9]; (e) on immunofluorescence, the antibody binds specifically to I bands of striated muscle and not to other muscle proteins [9].

Characterization of liposomes

The liposomes used in these studies are negatively charged unilammellar vesicles of approximately 300 Å diameter [17]. The liposomes are well separated from free anti-actin by chromatography on Sephadex G50 before use in these studies. It will be shown elsewhere* that anti-actin is trapped within the vesicles and not loosely associated with the external surface of the liposomes and that furthermore, cyclic AMP entrapped in such vesicles is admitted to the interior of adrenal tumor cells where it produces its well known physiological effects.

Efficiency of incorporation of anti-actin into liposomes was 5–10%; this corresponds to incorporation of 80 to 160 mg protein in one preparation.

Influence of anti-actin on the response to LH

(i) *Synthesis of testosterone.* It can be seen from Fig. 1 that anti-actin, presented to Leydig cells in liposomes, inhibits the steroidogenic response to LH (A) and that to db cyclic AMP (B) as judged by production of testosterone. Figure 1C also shows that liposomes containing only buffer cause no more than minimal inhibition of the response to LH. In Fig. 1A, the effect of anti-actin on the production of testosterone without LH is not shown because the curve overlaps the control or unstimulated values and has therefore been omitted for the sake of clarity.

(ii) *Production of pregnenolone by isolated mitochondria.* When adrenal cells are incubated with aminoglutethimide (an inhibitor of side-chain cleavage [12] and hence steroidogenesis), with and without ACTH production of pregnenolone by isolated mitochondria (washed to remove aminoglutethimide) is greater in the case of mitochondria from cells incubated with ACTH than those from cells incubated without ACTH [18]. A similar response is seen with cells incubated with db cyclic AMP [18]. No response to ACTH is seen in mitochondria unless side-chain cleavage is inhibited while the cells are incubated with the trophic hormone [18]. It appears that ACTH and cyclic AMP stimulate transport of cholesterol to mitochondria. This cholesterol accumulates while side-chain cleavage is inhibited; when mitochondria are washed to remove aminoglutethimide, the organelles show increased side-chain cleavage—this increase is presumably due to the presence of accumulated cholesterol resulting from the action of the stimulating agents on the whole cells [18]. Figure 2 shows that the same response is seen with Leydig cells incubated with aminoglutethimide with and without

* P. F. Hall, C. Charponnier, M. Nakamura and G. Gabbiani. Manuscript in preparation.

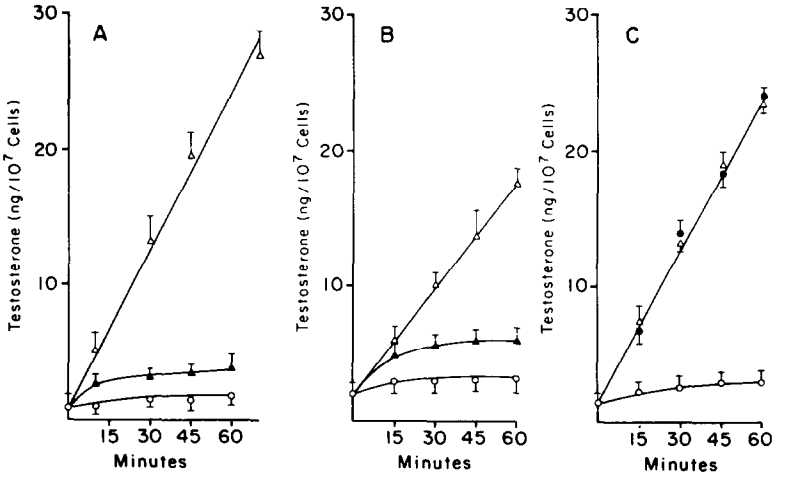

Fig. 1. Effect of anti-actin in liposomes on the steroidogenic response of Leydig cells to LH (A) or db cyclic AMP (B). Leydig cells were incubated for 1 h with liposomes containing buffer or buffer plus anti-actin (10 μg of antibody protein/ml of incubation medium). Medium was removed, cells washed, and fresh medium containing saline (control) or LH (50 μg/ml) or cyclic AMP 2 mM, but no liposomes were added. Incubation was continued for 30 min when the production of testosterone was measured by radioimmunoassay [11]. (C) shows the effect of adding liposomes containing buffer only on the response to LH; in this case, the control and one series of flasks with LH (●——●) were incubated without liposomes during the first incubation (1 h); during the second incubation (30 min), saline or LH was added as shown. Values are means and ranges of duplicate determinations. O——O Liposomes with buffer; no LH or db cyclic AMP. △——△ Liposomes with buffer; LH or db cyclic AMP. ▲——▲ Liposomes with anti-actin; LH or db cyclic AMP. ●——● No liposomes; LH. In these symbols, additions to the first incubation are shown to the left of the semicolon, and those to the second incubation to the right. In *c* values for no liposomes and no LH are omitted for the sake of clarity.

LH (A) or db cyclic AMP (B). These responses were inhibited by anti-actin (Fig. 2). Liposomes not containing anti-actin were without effect on these responses, and liposomes containing anti-actin do not inhibit side-chain cleavage by mitochondria from untreated Leydig cells when added directly to isolate mitochondria (data not shown).

(iii) *Transport of cholesterol to inner mitochondria membrane.* Figure 3 shows that when Leydig cells are incubated with aminoglutethimide, with and without LH, cholesterol content of the inner mitochondrial membrane is higher in membranes derived from cells incubated with LH than those from cells incubated without this hormone. In the present studies, the cholesterol content of the inner mitochondrial membrane was measured since the side-chain cleavage enzyme is situated in that part of the mitochondrion [13]. It can also be seen from Fig. 3. that liposomes containing anti-actin inhibited this response. These studies measure transport of cholesterol to mitochondria because the subsequent conversion of cholesterol to pregnenolone is inhibited by aminoglutethimide.

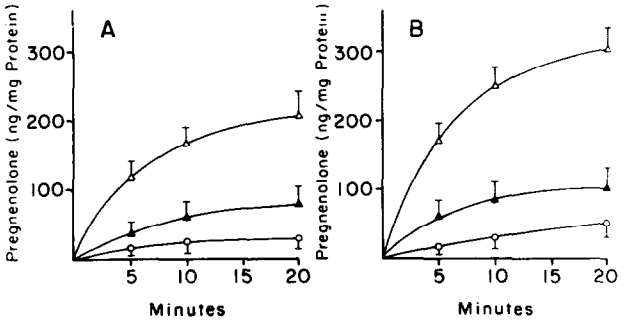

Fig. 2. The effect of anti-actin on side-chain cleavage by mitochondria. Leydig cells were incubated for 1 h with liposomes as described under Fig. 1. A second incubation was performed for 30 min with aminoglutethimide (0.76 mM) and saline (control) or LH (50 μg/ml) or db cyclic AMP (2 mM) (no liposomes). Mitochondria were then prepared and incubated for the times shown; these procedures are described in greater detail in reference 18. O——O Liposomes with buffer; no LH or db cyclic AMP. △——△ Liposomes with buffer; LH or db cyclic AMP. ▲——▲ Liposomes with anti-actin; LH or db cyclic AMP. The additions in the first incubation are to the left of the semicolon; those for the second incubation are to the right.

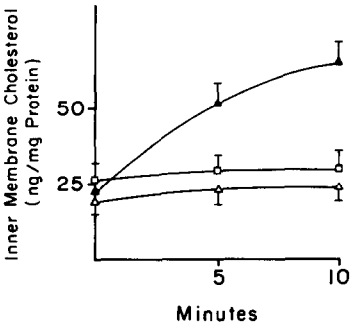

Fig. 3. Effect of anti-actin on the response of inner mitochondrial membrane cholesterol to LH. The first incubation of Leydig cells was performed as described under Fig. 1. After washing, cells were incubated with fresh medium containing aminoglutethimide (0.76 mM) and saline (control) or LH (50 μg/ml). After the times shown, inner mitochondrial membrane was prepared [13] and the cholesterol content of the membrane was determined by gas/liquid chromatography [14]. △——△ Liposomes with buffer; no LH. ▲——▲ Liposomes with buffer; LH. □——□ Liposomes with anti-actin; LH. First incubation to the left of the semicolon and second to the right.

(iv) *Effect of various proteins on the response of Leydig cells to LH.* The table shows that the response of Leydig cells to LH is no more than slightly inhibited by "free" anti-actin and by liposomes plus anti-actin (as opposed to liposomes *containing* anti-actin). The statistical significance of these small differences has not been examined, but they are at best trivial in contrast to the effect of liposomes containing entrapped anti-actin. Crude anti-actin (serum in liposomes) inhibited the response to LH, but entrapped human (normal) IgG was without effect (Table 1). The table also shows that the inhibitory action of anti-actin was overcome by actin in the same liposomes as the antibody.

(v) *Anti-actin and conversion of* [^{3}H]-*pregnenolone to* [^{3}H]-*testosterone.* When Leydig cells were incubated with [7α-^{3}H]-pregnenolone (1.0×10^6 cpm/10^7 cells), [^{3}H]-testosterone was isolated in confirmation of earlier studies from this laboratory [3]. Addition of anti-actin in liposomes was without effect on this conversion, e.g. 217,000 ± 2500 and 219,000 ± 2000 cpm—means and ranges for triplicate determinations when the first incubation medium contained liposomes with buffer and liposomes with anti-actin, respectively. In confirmation of earlier studies [3], addition of LH to Leydig cells was without effect on the conversion of [^{3}H]-pregnenolone to [^{3}H]-testosterone.

DISCUSSION

The anti-actin used in these studies has been characterized by a number of criteria, including binding to highly purified actin [9, 16]. The criteria of purity for the actin itself have also been published [15]. Previous studies have demonstrated specific binding of this anti-actin to microfilaments of fibroblasts [15]. In studies to be reported,† it will be shown that the anti-actin is entrapped within liposomes and not bound to the exterior of the vesicles. The same report reveals that the liposomes deliver cyclic AMP to adrenal Y-1 cells where the nucleotide exerts its physiological actions. Negatively charged vesicles were chosen for these experiments because they are believed to be

† Ibid.

Table 1. Influence of various substances on the steroidogenic response of Leydig cells to LH and db cyclic AMP

1st Incubation	2nd Incubation	Testosterone* ng/10^6 cells/min
Liposomes–buffer	—	0.5 ± 0.2
Liposomes–buffer	LH (50 μg/ml)	6.2 ± 0.6
Liposomes–buffer	db cyclic AMP (2 mM)	5.8 ± 0.5
Liposomes–anti-actin	LH (50 μg/ml)	0.7 ± 0.2
Liposomes; anti-actin	LH (50 μg/ml)	5.9 ± 0.4
Liposomes–buffer	Anti-actin	6.0 ± 0.3
Liposomes–hepatitis serum	LH (50 μg/ml)	1.2 ± 0.1
Liposomes–human IgG	LH (50 μg/ml)	6.4 ± 0.6
Liposomes–boiled anti-actin	LH (50 μg/ml)	5.9 ± 0.4
Liposomes–serum albumin	LH (50 μg/ml)	6.5 ± 0.7
Liposomes–anti-actin + actin	LH (50 μg/ml)	6.1 ± 0.3

* Values represent means and SEM for duplicate determinations.

Leydig cells were incubated with liposomes containing buffer or buffer plus the various additions shown (liposomes-) or liposomes containing buffer and "free" anti-actin (Liposomes; anti-actin) for one hour. Medium was changed, cells washed and fresh medium containing saline (control) or LH (50 μg/ml) or db cyclic AMP (2 mM) or free anti-actin was added and cells were incubated for 30 min. Production of testosterone was measured by radioimmunoassay [11]. Liposomes were only present in the first incubation. Proteins other than LH were present in a final concentration of 10 μg/ml of incubation medium. Hepatitis serum was added in amounts of 15 μl per ml. Actin was present in a weight ratio of 2:1 relative to anti-actin. Values are means and ranges of duplicate determinations. Symbols: "—" refers to proteins in liposomes, and ";" means liposomes containing buffer, i.e., the following protein was *not* in the liposomes.

accepted by cells as the result of membrane fusion rather than phagocytosis [19].

The evidence presented here indicates that the observed inhibition of the action of LH on Leydig cells results from the effect of anti-actin itself. Apart from the purity of the anti-actin, the following control experiments support this view: boiled anti-actin normal human IgG and anti-actin with excess actin are all without effect on the action of LH (Table 1). It is also interesting to notice that unpurified hepatitis serum (from which the anti-actin was prepared) inhibits the action of LH and that "free" anti-actin produces no more than a trivial effect; presumably free anti-actin penetrates the plasma membrane very poorly if at all. One report from another laboratory suggests that free anti-actin inhibits the action of LH on ovarium cells [20]. In this case, detailed characterization of the antibody was not presented.

Anti-actin inhibits three well-defined steroidogenic responses to LH: (i) increased production of testosterone by Leydig cells (Fig. 1), (ii) increased production of pregnenolone by isolated mitochondria from Leydig cells incubated with LH (Fig. 2), and (iii) accumulation of cholesterol in inner mitochondrial membranes when side-chain cleavage is inhibited (Fig. 3). The last two of these effects indicate that anti-actin prevents some response to LH which in the uninhibited cell leads to transfer of cholesterol to mitochondria. In this respect, it is important to notice that anti-actin is without effect on side-chain cleavage when added directly to mitochondria and on the remainder of the steroidogenic pathway (pregnenolone → testosterone) when added to cells (Results). It would appear that the effect of anti-actin is confined to steps which precede side-chain cleavage. Earlier studies from this laboratory [6, 7], which have been recently confirmed [21], indicate that cytochalasin B inhibits the action of ACTH on adrenal cells again at some step before side-chain cleavage. Reasons were given for believing that cytochalasin B acts on microfilaments to prevent the accelerated transport of cholesterol to mitochondria produced by ACTH [6, 7]. These findings with LH and Leydig cells are entirely consistent with studies of ACTH and adrenal cells (4, 6–8, 22).

Cytochalasin B is known to exert non-specific effects on cell membranes [23]. The possibility that liposomes containing anti-actin may do the same must be considered. If the action of either of these agents resulted from non-specific effects on the plasma membrane, one would expect the effect of db cyclic AMP to be uninfluenced by these agents, since the nucleotide is believed to be formed in the plasma membrane and to act within the cell to stimulate steroidogenesis. It is clear, however, that the action of db cyclic AMP is also inhibited by cytochalasin B [6, 7] and by anti-actin in liposomes (Figs. 1B and 2B).

The most reasonable interpretation of the present data would seem to involve uptake of liposomes by Leydig cells and intracellular release of anti-actin which would, in turn, bind to actin, presumably in microfilaments. These changes would take place during the first incubation. Subsequent addition of LH or db cyclic AMP fails to stimulate steroid synthesis because the response to these agents require activity of microfilaments. The normal response in uninhibited cells results in enhanced transport of cholesterol to the side-chain cleavage enzyme system in the inner mitochondrial membrane. The binding of anti-actin prevents the microfilaments from responding and hence limits transport of cholesterol to the enzyme.

The experiments reported here do not provide the basis for speculation concerning the mechanism by which microfilaments promote transport of cholesterol within the cell. Certainly the well known rounding of adrenal tumor cells in response to ACTH [24] is likely to involve contractile elements within these cells [25]. Moreover, the response to ACTH does not involve microtubules [8]. To the extent that these findings may apply to other steroidogenic cells, they limit the nature of the changes which must occur within the cytoplasm of steroidogenic cells to permit the usual response to trophic hormones to take place and emphasize the importance of intracellular transport of cholesterol in the process of steroidogenesis.

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