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IMPLICATION OF MICROTUBULES AND MICROFILAMENTS IN THE RESPONSE OF THE OVARIAN ADENYLATE CYCLASE-CYCLIC AMP SYSTEM TO GONADOTROPINS AND PROSTAGLANDIN E_2

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SUMMARY

Addition of anti-actin serum or cytochalasin B (3 μg/ml) to the medium abolished the stimulatory effect of LH and of choleragen, and inhibited the action of FSH, but not of PGE_2, on cyclic AMP production in cultured rat Graafian follicles. Colchicine and anti-sera to BSA, tubulin or smooth-muscle myosin, as well as anti-actin serum absorbed with actin, had no effect on the follicular response to LH, but anti-tubulin serum and colchicine inhibited the response to FSH and PGE_2. The inhibitory effect of cytochalasin B on LH-action was fully reversed 24 h after transfer of the follicles to drug-free medium. Neither anti-actin serum nor cytochalasin B had any effect on the binding of ^{125}I-hCG by the follicular cell membrane. The results suggest that microfilaments, but not microtubules, are intimately involved in the process of LH- and choleragen-stimulated ovarian adenylate cyclase activity. By contrast, the action of PGE_2 is dependent on microtubule assembly, while the action of FSH seems to depend on both these components of the cytoskeleton.

INTRODUCTION

The role of cytoskeletal system in lateral mobility of cell surface receptors within a fluid plasma membrane has been extensively studied during recent years (1-11). Microtubules as well as microfilaments are closely associated with the plasma membrane of many cells (8,12-21), and are probably involved in the control of receptor mobility and redistribution (3,4,8-11). Such movement of receptor molecules appears to play an important part in the interaction of the hormone-receptor complex with the catalytic

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Abbreviations used: LH, luteinizing hormone; FSH, follicle stimulating hormone; PGE_2, prostaglandin E_2; hCG, human chorionic gonadotropin; BSA, bovine serum albumin

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moiety of adenylate cyclase (6,7,22,23).

Current evidence for the involvement of the cytoskeleton in locomotion of cell substances and organelles is based mainly on the pharmacological action of cytochalasins, which are believed to impair microfilament function (8,24,25), and of colchicine, which causes depolymerisation of microtubules (26). Cytochalasin B inhibits the stimulatory effect of TSH and ACTH on thyroidal and adrenal secretion (27-29). Colchicine was reported to inhibit the clustering of binding sites for concanavalin A on epithelial cells of toad bladder in response to vasopressin (30) and to inhibit insulin action on lipid and glycogen synthesis (31).

The follicular response to gonadotropins depends on functional coupling of receptors located on the outer cell surface to the membrane-bound enzyme adenylate cyclase (32). Microtubules and microfilaments are widely distributed in the Graafian follicle (33-36). In the present study, we examined whether the cytoskeletal system participates in gonadotropin- and prostaglandin-stimulated ovarian cyclic AMP production. To clarify this question, we used the drugs colchicine and cytochalasin B, as well as specific antibodies to actin and tubulin.

MATERIALS AND METHODS

Materials. LH (NIH-LH-S19) was kindly made available by the NIAMD, N.I.H., Bethesda, Maryland, FSH (G4-150C; 50 x NIH-FSH-S1) by Prof. H. Papkoff and PGE_2 by Dr. J. Pike of the Upjohn Co., Kalamazoo, Michigan. Purified cholera toxin was prepared under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by Dr. R.A. Finkelstein, The University of Texas, Southwestern Medical School, Dallas, Texas.

3-Isobutyl-1-methylxanthine (IBMX), cytochalasin A, B and D were purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. The cytochalasins were dissolved in dimethylsulfoxide (DMSO: Fluka AG, Buchs SG, Switzerland), as stock solutions of 5 mg/ml.

Highly purified human chorionic gonadotropin (hCG; 14,000 i.u./mg, Serono, Rome, Italy) was used for iodination and hCG (5000 i.u./mg, Organon, Holland) was used as unlabelled hormone in competition studies. Colchicine was purchased from Merck, Germany.

Rabbit antiserum to rabbit muscle actin was the gift of Drs. A. Schreiber and J. Hoebeke, Free University, Brussels and rabbit antiserum to calf-brain tubulin was the gift of Dr. U. Littauer and Mrs. I. Gozes, Department of Neurobiology, The Weizmann Institute of Science. Goat antiserum to rat uterine myosin was the gift of Drs. D. Wallach and I. Pastan, N.I.H. Rabbit anti-BSA was prepared by Dr. Y. Weinstein.

Follicle culture. Three-month-old Wistar derived rats of the departmental colony were sacrificed between 8.00-12.00 h on the day of proestrus. Large intact Graafian follicles were isolated and cultured in Eagle's medium as previously described (37).

Binding of ^{125}I-hCG to follicles was assayed as previously described (32,38).

Assay of cyclic AMP. After culture with test substance(s), the follicles were washed with cold saline and incubated with the hormone specified for 30' in Krebs Ringer bicarbonate buffer containing 1 mg/ml glucose and IMBX 0.1 mg/ml. The cyclic AMP content of the tissue was then assayed by a modification (39) of the Gilman method (40).

RESULTS

Anti-actin serum added to the culture medium abolished the response of isolated rat Graafian follicles to LH or FSH as judged by cyclic AMP production (Fig. 1), but did not affect the follicular response to PGE_2. The inhibition of LH action by anti-actin serum was counteracted by prior incubation of anti-actin serum with exogenous actin (Table 1). Sera directed against smooth-muscle myosin, anti-tubulin or BSA had no effect on the response to LH (Table 1), but the anti-tubulin serum blocked FSH- and PGE_2-stimulated ovarian cyclic AMP production (Table 1).

Addition of cytochalasin B (3 µg/ml) to the medium likewise abolished the subsequent response of cultured follicles to LH with respect to cyclic AMP formation. At the dose of 1 µg/ml, cytochalasin B reduced the response to LH after 24 h culture by 70% (Fig. 2). The effect of cytochalasin B was gradual: at 3 µg/ml inhibition was 50% after 6 h and 93% after 24 h of culture (Fig. 3). The response to FSH decreased by 60% after 24 h culture with cytochalasin B (3 µg/ml), but the follicles remained fully responsive to PGE_2 throughout this culture period (Fig. 2). Cytochalasin D (3 µg/ml) inhibited the stimulatory effect of LH by 50%, and cytochalasin A was only slightly inhibitory (30%).

When follicles were incubated with cytochalasin B (3 µg/ml) for 24 h, subsequent incubation for 8 h in drug-free medium resulted in only 15% recovery of the response to LH, but after 24 h full recovery of the responsiveness to LH was observed. In another experiment, follicles were incubated for 24 h with anti-actin serum, and thereafter without anti-actin serum and with added actin in an attempt to strip antibody bound to endogenous actin. No recovery

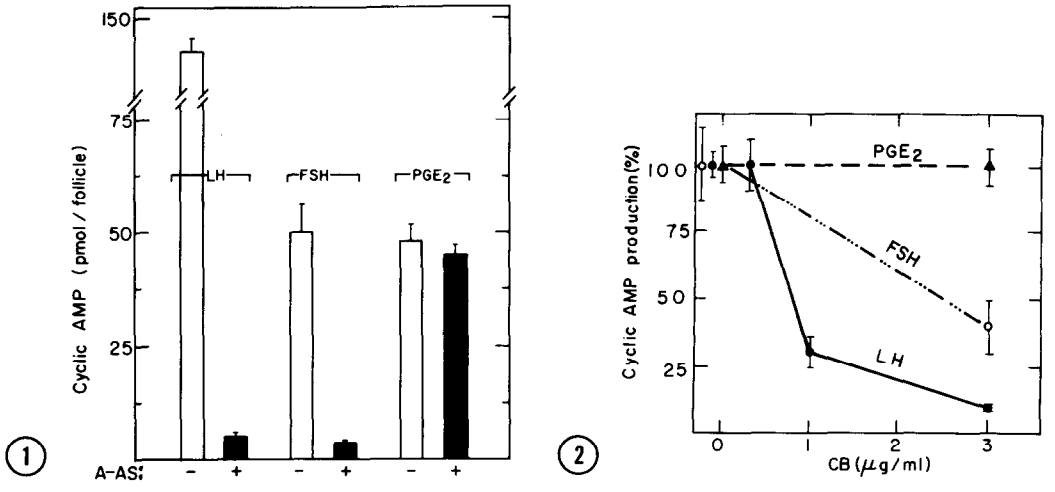


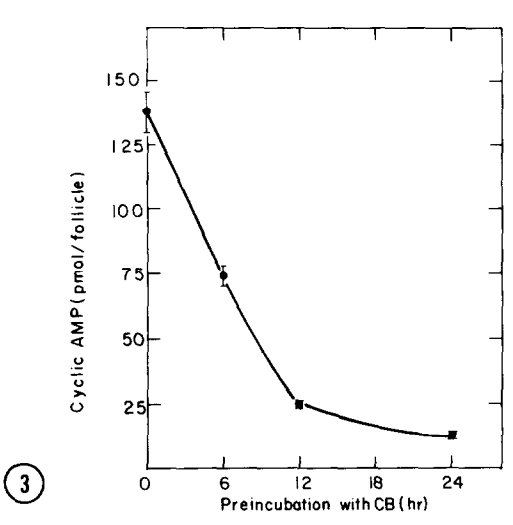
Fig. 1. Selective inhibition by anti-actin serum (A-AS) of gonadotropin-stimulated cyclic AMP production by cultured Graafian follicles. Incubation for 24 h. LH, 10 μg/ml; FSH, 0.5 μg/ml; PGE_2, 10 μg/ml. A-AS, anti-actin serum (20 μl/ml). Vertical brackets, ± S.E.M. (n = 4).

Fig. 2. Selective inhibition by cytochalasin B (CB) of gonadotropin-stimulated cyclic AMP production by cultured Graafian follicles. Incubation for 24 h. Maximal stimulation in absence of cytochalasin B taken as 100%: LH (10 μg/ml), 131 ± 7; FSH (0.5 μg/ml), 56 ± 8; PGE_2 (10 μg/ml), 44 ± 4 pmol/follicle. Vertical brackets, ± S.E.M. (n = 12).

Table 1. Suppression of hormone-stimulated follicular cyclic AMP production in vitro by agents affecting the cytoskeleton. Vertical brackets ± S.E.M. (n = 8).

Additions to medium	pmol cyclic AMP/follicle (mean ± S.E.M.)			
Challenge (30 min) / Initial culture (24 h)	LH (10 μg/ml)	FSH (0.5 μg/ml)	PGE_2 (10 μg/ml)	Control
Control	110 ± 4	50 ± 6	36 ± 2	
Anti-myosin serum	117 ±10	N.d	N.d	
Anti-tubulin serum	129 ± 7	20 ± 2	10 ± 2	
Anti-tubulin serum + tubulin	N.d	53 ± 4	N.d	2 ± 0.4 (n = 56)
Anti-actin serum	9 ± 1	4 ± 0.2	34 ± 3	
Anti-actin serum + actin	95 ± 2	N.d	N.d	
Anti-BSA serum	114 ±15	N.d	N.d	

N.d - not determined



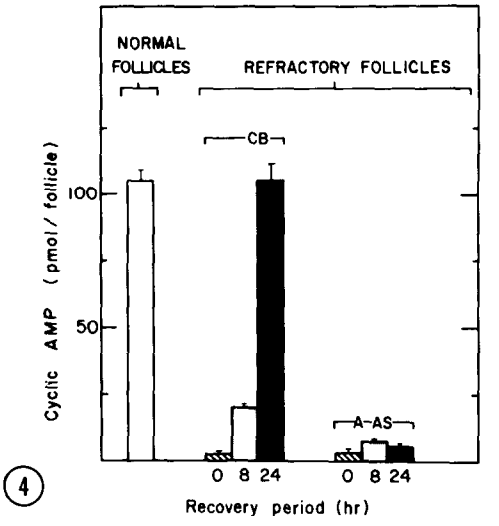


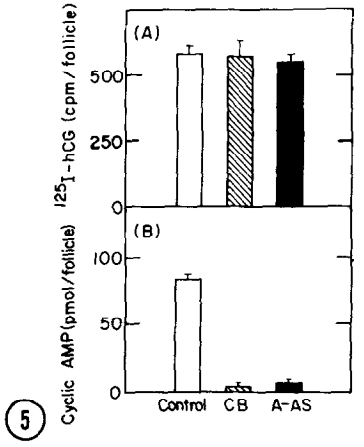
Fig. 3. Time-course of cytochalasin B (CB) inhibition of LH-stimulated cyclic AMP formation by cultured Graafian follicles. LH, 10 µg/ml; cytochalasin B, 3 µg/ml. Vertical brackets, ± S.E.M. (n = 8).

Fig. 4. Recovery of responsiveness to LH of Graafian follicles made refractory by culture with cytochalasin B (CB; 3 µg/ml) or anti-actin serum (A-AS; 20 µl/ml). After 24 h, fresh medium devoid of drug or antibody was substituted and the follicles were challenged with LH (10 µg/ml) after the time-lapse indicated and the tissue level of cyclic AMP was determined. Normal follicles were cultured for 48 h in control medium. Vertical brackets, ± S.E.M. (n = 8).

of the LH-response was observed in these follicles within 24 h (Fig. 4).

Culture of follicles with either anti-actic serum or with cytochalasin B (3 µg/ml) for 24 h did not affect the subsequent binding of ^{125}I-hCG to follicle cells, although LH-stimulated cyclic AMP formation was sharply reduced (90% inhibition; Fig. 5).

Colchicine (3 µg/ml) did not impair the response to LH during 2-24 h incubation, but prevented the stimulatory effect of FSH and of PGE_2 on follicular cyclic AMP accumulation (Fig. 6). Inhibition of FSH action was evident even after exposure of the follicles to 0.1 µg/ml colchicine for 6 h, and at higher concentrations (3 µg/ml) partial inhibition was seen after 1 h and complete blockade after 2 h (Fig. 6). Choleragen-stimulated cyclic AMP pro-



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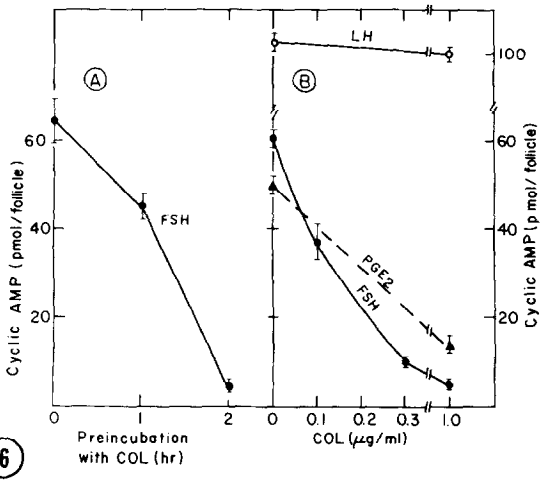

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Fig. 5. Binding of ^{125}I-hCG (A) and cyclic AMP accumulation (B) in follicles rendered refractory to LH action by cytochalasin B (CB; 3 μg/ml) or anti-actin serum (A-AS; 20 μl/ml) during 24 h of culture. The follicles were challenged with LH (10 μg/ml) 2½ h after addition of a trace amount of ^{125}I-hCG. Vertical bars, ± S.E.M. (n = 8).

Fig. 6. Selective inhibition by colchicine (Col) of FSH- and PGE_2-stimulated cyclic AMP production by Graafian follicles: (A) colchicine, 3 μg/ml; (B) Incubation for 6 h. FSH, 0.5 μg/ml; PGE_2, 10 μg/ml; LH, 10 μg/ml. Vertical brackets, ± S.E.M. (n = 4).

duction was inhibited by cytochalasin B and by anti-actin serum but not by colchicine or anti-tubulin, i.e. this agent behaved in all respects like LH.

DISCUSSION

It is well established that ovarian LH binding sites are distributed on the outer cell membrane (38,41) and that a coupling mechanism must be operative (22,23,32) to transmit the signal carried by the LH-receptor complex to the adenylate cyclase moiety which is believed to be localized on the inner part of the plasma membrane (42-44). Since gonadotropins stimulate adenylate cyclase in purified plasma membranes (45), the coupling agent should be an integral part of the plasma membrane. The present results seem to implicate the microtubule-microfilament system in this coupling process.

At least three possibilities exist to explain the ability of cytochalasin B or anti-actin serum to abolish gonadotropin-stimulated cyclic AMP production:

a) inhibition of LH binding to its receptor; b) impairment of the coupling mechanism or of lateral mobility of the receptor within the membrane; c) inhibition of the catalytic moiety of adenylate cyclase. The first of these can be ruled out, since cytochalasin B or anti-actin serum did not interfere with the specific binding of LH to cell surface receptors on follicle cells (Fig. 5). By contrast, cytochalasin B was found to inhibit the binding of insulin and growth hormone (46). Although we did not measure adenylate cyclase activity in membrane preparation from cytochalasin B or anti-actin serum-treated follicles, the inability of either cytochalasin B or anti-actin serum to inhibit stimulation of cyclic AMP formation by PGE argue against the possibility that adenylate cyclase was directly affected. It is more likely, therefore, that the microfilament system, on which both cytochalasin B and anti-actin serum presumably exert their action, plays a role in the coupling between the LH-receptor complex and adenylate cyclase. A similar relationship may apply to FSH action, since this too was susceptible to inhibition by cytochalasin B and anti-actin serum.

It is interesting to compare the refractory state produced by cytochalasin B or anti-actin serum to desensitization which results from prolonged incubation of Graafian follicles with LH (32,47). Since in this case too, neither the number of receptors for LH nor the catalytic activity of adenylate cyclase were reduced during the early refractory period, we proposed that an impairment of the coupling machinery is responsible for the observed desensitization (32).

The time-course (6-12 h) of cytochalasin B action on LH stimulation of ovarian cyclic AMP production is longer than the time (1-3 h) necessary for the inhibition of adrenal and thyroid secretion in response to ACTH or TSH (27-29) or to the time required for cytochalasin B to reduce the number of receptors to insulin or growth hormone on lymphocytes (46). This difference may reflect poor penetration of cytochalasin B to the follicular wall, or suggest that cytochalasin B inhibits cyclic AMP formation by a mechanism other than that of deranging microfilament function. Cytochalasin B at higher con-

centrations than used in our experiments (> 10 μg/ml) is toxic to many cells (48), specifically if incubation proceeds for several hours (48). However, when cultured follicles desensitized by cytochalasin B were transferred to drug-free medium, full recovery of the response to LH in terms of stimulation of cyclic AMP formation was observed. This seems to argue against a non-specific toxic effect of cytochalasin B on the follicle. Moreover, the effect of cytochalasin B was selective, since PGE_2 stimulation was not impaired by cytochalasin B under conditions causing 90% inhibition of LH action. Although we have no direct evidence that the effect of cytochalasin B is a result of microfilament derangement, this possibility is corroborated by the finding that anti-actin serum had a similar inhibitory action and that this action of anti-actin serum is reduced by treatment of the antiserum with actin. These conclusions accord with the suggestion made by Helmreich (11) on theoretical grounds that actin may serve as a coupler between hormone-receptor complex and adenylate cyclase.

It is assumed that the intact cell is largely impermeable to large proteins such as antibodies. It was therefore surprising to find that actin antibodies, which probably neutralized cellular actin, are able to block the action of LH on follicle cells. This may suggest either that actin is localized on the outer surface of the plasma membrane and is related to the LH-receptor, or that during 6-24 h of culture some cell damage occurred which permits penetration of anti-actin serum into the cell. Cytochalasin B does bind to the outer cell surface (49,50), but the precise localization of actin in relation to the plasma membrane is still a matter of controversy (18,19,51).

Colchicine has been widely used in the analysis of microtubule involvement in lectin receptor mobility and the capping phenomenon (9). In the present experiments, colchicine suppressed the stimulatory effect of FSH and PGE_2, but not that of LH, on follicular cyclic AMP accumulation. Likewise, antiserum to tubulin inhibited FSH and PGE_2 action, but not that of LH. Again, the apparent accessibility of tubulin in intact cells to antibody is somewhat

surprising but there is evidence for the presence of tubulin in the plasma membrane (13,14,16,19,21). The latency of the colchicine effect was much shorter than that of the cytochalasin B effect.

The results permit the following tentative conclusions: (i) microfilament seems to play an essential role in the coupling of gonadotropin (LH and FSH)-receptor complexes to the catalytic moiety of adenylate cyclase, but are not involved in the action of PGE_2 on follicular cyclase; (ii) microtubules may be important in mediating the action of FSH and PGE_2, but not of LH, on follicular cyclase. The different characteristics of the coupling mechanisms for various hormones impinging on adenylate cyclase invite further investigation.

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