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Receptor-mediated gonadotropin action in the ovary

Action of cytoskeletal element-disrupting agents on gonadotropin-induced steroidogensis in rat luteal cells

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The role of the cellular cytoskeletal system of microtubules and microfilaments on gonadotropin-stimulated progesterone production by isolated rat luteal cells has been investigated. Exposure of luteal cells to human choriogonadotropin resulted in a stimulation of cyclic AMP (4–7-fold) and progesterone (3–4-fold) responses. Incubation of cells with the microfilament modifier cytochalasin B inhibited the gonadotropin-induced steroidogenesis in a dose- and time-dependent manner. The effect of cytochalasin B on basal production of steroid was less pronounced. Cytochalasin B also inhibited the accumulation of progesterone in response to lutropin, cholera enterotoxin, dibutyryl cyclic AMP and 8-bromo cyclic AMP. The inhibition of steroidogenesis by cytochalasin B was not due to (*a*) inhibition of ^{125}I-labelled human choriogonadotropin binding to luteal cells, (*b*) inhibition of gonadotropin-stimulated cyclic AMP formation or (*c*) a general cytotoxic effect and/or inhibition of protein biosynthesis. Cytochalasin D, like cytochalasin B, inhibited gonadotropin- and 8-bromo cyclic AMP-stimulated steroidogenesis. Although cytochalasin B also blocked the transport of 3-*O*-methylglucose into luteal cells, cytochalasin D was without such an effect. Increasing glucose concentration in the medium, or using pyruvate as an alternative energy source, failed to reverse the inhibitory effect of cytochalasin B. The anti-microtubular agent colchicine failed to modulate synthesis and release of progesterone by luteal cells in response to human choriogonadotropin. These studies suggest that the cellular microfilaments may be involved in the regulation of gonadotropin-induced steroidogenesis. In contrast, microtubules appear to be not directly involved in this process.

The available evidence suggests that the effects of gonadotropins (lutropin/human choriogonadotropin) on steroidogenesis in the ovary are mediated by gonadotropin receptors and involve a cyclic AMP-dependent mechanism (Menon & Gunaga, 1974). Although the characteristics of gonadotropin receptors have been well defined (Gospodarowicz, 1973; Lee & Ryan, 1973; Haour & Saxena, 1974; Rao, 1974; Menon & Kiburz, 1974; Thambyrajah *et al.*, 1976; Azhar & Menon, 1976; Azhar *et al.*, 1976), the factors regulating their function within plasma membranes or events subsequent to hormone–receptor interaction are still largely unidentified. In recent years, many studies have demonstrated the involvement of a cytoskeletal system of microtubules and microfilaments in various cell-surface-mediated events, including receptor mobility and redistribution, as well as topographical distribution of membrane components (Mitzel & Wilson, 1972; Berlin, 1975; Yahara & Edelman, 1975; De Petris, 1975; Edelman, 1976; Nicolson, 1976; Trifaro *et al.*, 1972; Van Obberghen *et al.*, 1976). It has also been suggested that the microtubular system could be involved in the secretory processes (Wolff & Williams, 1973; Poisner & Bernstein, 1971; Gautvik *et al.*, 1973; Sheterline *et al.*, 1975; Chambaut-Guerin *et al.*, 1978; Banerjee *et al.*, 1976). The participation of microfilaments and microtubules in cellular processes is best demonstrated by the use of pharmacological agents such as colchicine and vinca alkaloids, which cause depolymerization of tubulin (Olmsted & Borisy, 1973; Wilson *et al.*, 1974) and cytochalasins, which disrupt microfilament structures (Wessells *et al.*, 1971; Carter, 1972).

Despite increasing awareness of the importance of the cytoskeletal system in the regulation of cellular processes, the role of microtubules and microfilaments in receptor-mediated gonadotropin action in the ovary has not been explored. The present studies

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therefore have been undertaken to determine the role of microtubules and microfilaments in receptor-mediated gonadotropin stimulation of progesterone synthesis in ovarian tissue. Collagenase-dispersed cells from highly luteinized ovaries have been used as a model system for the present studies. These cells bind ^{125}I-labelled human choriogonadotropin with a high affinity and response to lutropin/human choriogonadotropin with an increase in cyclic AMP and progesterone production. The results demonstrate that cytochalasins inhibit gonadotropin-induced steroidogenesis. In addition, results are presented that suggest that the action of cytochalasin B on steroidogenesis is at a point distal to the gonadotropin–receptor interaction and cyclic AMP formation. Although the involvement of microfilaments in lutropin action in the ovary is documented in the present studies, microtubules may not play any direct role in the process.

Materials and methods

Materials

Purified human choriogonadotropin (11 500 units/mg) was kindly provided by Dr. R. Canfield (Columbia University College of Physicians and Surgeons, New York, NY, U.S.A.) through the Center for Population Research, National Institutes of Child Health and Human Development, Bethesda, MD, U.S.A. Ovine lutropin (NIH-LH S18) and bovine lutropin (NIH-LH B9) were gifts of the Hormone Distribution Program, National Institutes of Arthritis, Metabolism, and Digestive Diseases, Bethesda, MD, U.S.A. Cholera enterotoxin was purchased from Schwarz/Mann, Orangeburg, NY, U.S.A. Cytochalasin B, cytochalasin D and colchicine were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Dibutyryl cyclic AMP, cyclic AMP and 8-bromo cyclic AMP were supplied by the Sigma Chemical Co., St. Louis, MO, U.S.A. 3-*O*-[^{3}H]Methylglucose (sp. radioactivity 1.2 Ci/mmol) and [2,3-^{3}H]proline (sp. radioactivity 37.1 Ci/mmol) were the products of International Chemical and Nuclear Corp., and New England Nuclear Corp. respectively. Medium 199, medium 109, foetal bovine serum and horse serum were purchased from Grand Island Biological Company, Grand Island, NY, U.S.A. ^{125}I-labelled human choriogonadotropin was prepared as described by Dufau *et al.* (1973).

Methods

Pretreatment of rats. Rats (24–26 days old) obtained from the Spartan Research Animals Inc., Haslett, MI, U.S.A., were used in the present studies. Highly luteinized ovaries from these rats were obtained by using the regimen described by Parlow (1958). This involved subcutaneous injection of rats with 50 i.u. of pregnant-mare's-serum gonadotropin (Gestyl, Organon) followed 56 h later by 25 i.u. of human choriogonadotropin (Sigma) on day 0.

Preparation of luteal cells. At 6–7 days after the injection of human choriogonadotropin, the animals were killed by cervical dislocation, the ovaries removed, freed of connective tissues, weighed and placed in medium 199 containing 2% (w/v) bovine serum albumin. Collagenase dispersed luteal cells were prepared by a slight modification of the procedure described previously from this laboratory (Kawano *et al.*, 1975; Clark & Menon, 1976). Briefly, luteinized ovaries were cut into small pieces (approx. 1 mm^3) with scissors and transferred to a 50 ml plastic beaker. The tissue was suspended at a concentration of 50 mg per ml of medium 199/0.1% bovine serum albumin, containing 500 units of collagenase/ml and 4 mg of deoxyribonuclease/ml. The samples were incubated for 60 min at 37°C in an atmosphere of O_2/CO_2 (19:1). At 30 and 60 min of incubation, cells were dissociated by flushing the tissue and medium 30 times through a 1 ml plastic syringe. After 60 min of incubation, the cell suspension was centrifuged at 600 ***g*** for 3 min at room temperature. The sedimented cells were washed twice with medium 199 containing 0.1% bovine serum albumin and then resuspended to a concentration of approx. 10^7 cells/ml. The cells were counted by using a haemocytometer and viability as checked by the Trypan Blue-exclusion procedure was in the range 80–95%. DNA was determined by the colorimetric procedure of Burton (1956).

Incubation condition for progesterone production by luteal cells. Unless otherwise stated, portions (0.1 ml) of cells were incubated in a final volume of 0.4 ml of medium 199 containing 0.1% bovine serum albumin in the presence or absence of added hormone and/or cytochalasins or colchicine or other substances. Incubations were carried out at 37°C in an atmosphere of O_2/CO_2 (19:1), usually for 3 h. Other applicable details are given in individual Tables and Figures.

Radioimmunoassay for progesterone. After incubation as described above, the sample tubes were placed in a boiling-water bath for 3 min, followed by the addition of 0.6 ml of water and 10 μl of [1,2-^{3}H]progesterone (approx. 10000 c.p.m.) to monitor recovery, and the samples left in the cold overnight. After 12 h, the samples were extracted with light petroleum (b.p. 30–60°C) and assayed for progesterone by radioimmunoassay as described previously (Kawano *et al.*, 1975; Clark & Menon, 1976).

Incubation conditions for gonadotropin and cholera enterotoxin stimulation of cyclic AMP. Portions (0.1 ml) of cells were incubated in a final volume of 0.4 ml of medium 199 containing 0.1% bovine serum albumin in the presence of 0.5 mM-3-isobutyl-1-methylxanthine, and, where required,

human choriogonadotropin, lutropin, cholera enterotoxin and/or cytochalasin B were also added. After incubation for 2h in the presence of O_2/CO_2 (19:1), the cells and media were processed and assayed for cyclic AMP by the method of Gilman (1970) as described previously (Azhar & Menon, 1979*a*).

Binding of human choriogonadotropin to luteal cells. The method used for the binding assay of ^{125}I-labelled human choriogonadotropin to luteal cells was identical with that described by Clark & Menon (1976) and Azhar *et al.* (1978).

Measurement of 3-O-[^{3}H]methylglucose uptake by luteal cells. The uptake of 3-*O*-[^{3}H]methylglucose by luteal cells was measured by a slight modification of the procedure described by Czech *et al.* (1973). The luteal cells were washed twice with Krebs–Ringer bicarbonate buffer (without glucose) containing 2% bovine serum albumin (Umbreit *et al.*, 1951) and then suspended in the same buffer. Portions of cells (0.1ml; approx. 10^6 cells) in triplicate were incubated with or without 50μM-cytochalasin B or cytochalasin D for 10min at room temperature. The reaction was started by the addition of 20μl of 3-*O*-[^{3}H]methylglucose (1μCi) to give a final concentration of 50μM. After incubation at room temperature for various times, incorporation was stopped by the addition of 3ml of ice-cold Krebs–Ringer bicarbonate buffer containing 0.1% bovine serum albumin, and the mixture was filtered through Whatman GF/C filter pre-soaked with buffer. The filters were washed with 2 × 15ml of Krebs–Ringer bicarbonate buffer containing 0.1% bovine serum albumin and dried and then counted for radioactivity after the addition of 10ml of scintillation fluid (4g of Omnifluor/litre of toluene) in a Beckman LS 230 scintillation spectrophotometer. Non-specific binding of radioactivity to filters was checked by filtering the cells in 3ml of Krebs–Ringer bicarbonate buffer containing 0.1% bovine serum albumin immediately after the addition of 3-*O*-[^{3}H]methylglucose (time 0min). The results are presented as sugar uptake from the difference of total uptake minus the radioactivity associated with the filters at zero time.

Protein-synthesis measurements. Luteal-cell protein synthesis was measured by following the incorporation of [^{3}H]proline into cellular proteins. For these studies, luteal cells were washed twice and suspended in proline-free medium 109/0.1% bovine serum albumin. Portions of cells (0.1ml; approx. 10^6 cells) were incubated in a final volume of 0.4ml of medium 109 containing 0.1% bovine serum albumin, 50μM-[^{3}H]proline (5μCi) and, where required, cytochalasin B. After incubation for 2–3h, the cells were centrifuged at 600*g* for 3min at 4°C and washed with 2 × 2ml of ice-cold medium 109/0.1% bovine serum albumin, containing unlabelled 0.1mM-proline. After the last washing, the cells were suspended in 2ml of 10% (w/v) trichloroacetic acid and 0.2ml of 1% bovine serum albumin was added as a carrier protein. After allowing the suspension to remain in the cold for 2h, the tubes were centrifuged and sedimented proteins were washed twice with 2ml of 10% trichloroacetic acid, heated at 90°C for 20min, cooled and filtered through Whatman GF/C filters. The filters were washed successively with 2 × 10ml of 10% trichloroacetic acid and 10ml of a mixture of ethanol/diethyl ether (1:1, v/v). The dried filters were counted for radioactivity after the addition of 10ml of scintillation fluid, as described in the previous section.

Results

Effect of incubation condition and cytochalasin B on steroidogenesis

Results presented in Table 1 show the effect of cytochalasin B (50μM) on human choriogonadotropin-stimulated progesterone production by rat luteal cells. Addition of cytochalasin B resulted in inhibition of gonadotropin-induced steroidogenesis, with minimal effect on basal progesterone production. Preincubation with the inhibitor was not necessary to document inhibition of human choriogonadotropin-induced progesterone synthesis.

Effect of incubation time and cytochalasin B concentration

Increasing cytochalasin B concentration in the incubation medium resulted in a concentration-dependent inhibition of human choriogonadotropin-stimulated steroid production (Fig. 1). Concentration of cytochalasin B as low as 10μM significantly decreased progesterone production and this effect was increased with increasing concentration of cytochalasin B. The concentration of cytochalasin B required to produce 50% inhibition in gonadotropin-induced steroidogenesis was 35μM. In contrast, 50μM or higher concentrations of cytochalasin B led to 20–50% inhibition of basal steroid synthesis. Dimethyl sulphoxide (in which cytochalasin B was dissolved) itself had no effect either on basal or human choriogonadotropin-stimulated progesterone accumulation.

Results presented in Fig. 2 show the influence of incubation time on the cytochalasin B effect. In the absence of inhibitors, human choriogonadotropin-induced progesterone production reached maximum values at 2h, whereas a 1h incubation time was required to attain maximum basal production of steroid. Addition of cytochalasin B (50μM) led to a significant inhibition of human choriogonadotropin-stimulated progesterone accumulation at 30min of incubation and this effect persisted up to 3h. Cytochalasin B also decreased basal steroid

Table 1. *Effect of incubation conditions on cytochalasin B inhibition of progesterone production by luteal cells in response to human choriogonadotropin*

Portions of rat luteal cells (approx. 10^6 cells) were pre-incubated in 0.36ml of medium 199/0.1% bovine serum albumin in the presence or absence of cytochalasin B (50μM). After pre-incubation at indicated times, 40μl of human choriogonadotropin (100ng/ml) or 40μl of medium (basal) was added and incubations continued for additional period of 3h. At the end of the second incubation, samples were processed for progesterone production as described in the Materials and methods section. Results are means ± S.E.M. Abbreviation used: N.S., not significant.

Condition	Progesterone (ng/μg of DNA) None	Progesterone (ng/μg of DNA) Cytochalasin B (50μM)
Pre-incubation with cytochalasin B, 0min		
Basal	1.77 ± 0.06	1.55 ± 0.09 (N.S.)
Human choriogonadotropin (10ng/ml)	6.88 ± 0.32	3.09 ± 0.21 ($P < 0.001$)
Pre-incubation with cytochalasin B, 30min		
Basal	1.45 ± 0.07	1.22 ± 0.09 (N.S.)
Human choriogonadotropin (10ng/ml)	6.8 ± 0.21	2.56 ± 0.14 ($P < 0.001$)
Pre-incubation with cytochalasin B, 60min		
Basal	1.83 ± 0.1	1.39 ± 0.02 (N.S.)
Human choriogonadotropin (10ng/ml)	6.94 ± 0.35	2.46 ± 0.11 ($P < 0.001$)

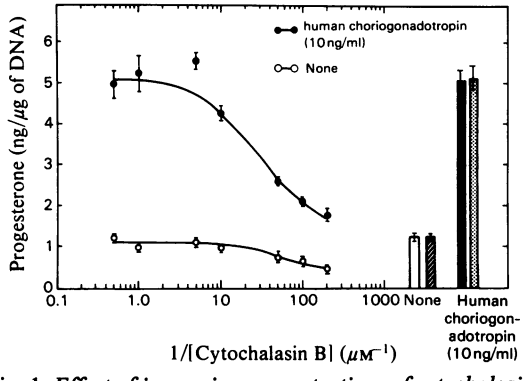

Fig. 1. *Effect of increasing concentrations of cytochalasin B on gonadotropin-induced steroidogenesis*

Portions of cells (2×10^6 cells) were incubated with or without human choriogonadotropin and indicated concentrations of cytochalasin B (in 4μl of dimethyl sulphoxide). After incubation for 3h, the samples were assayed for progesterone by radioimmunoassay. □, ■, Without dimethyl sulphoxide; ■, ▦, with dimethyl sulphoxide (1%, v/v). Points represent means and the error bars represent S.E.M.

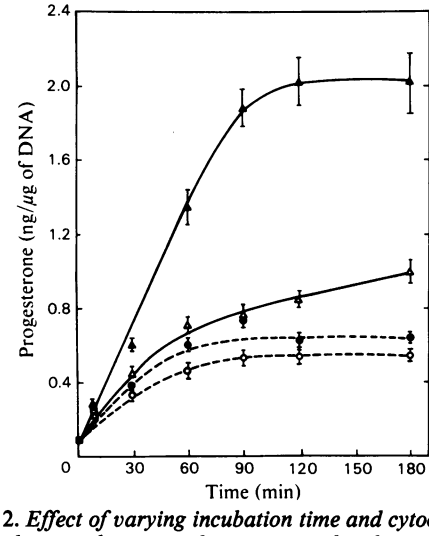

Fig. 2. *Effect of varying incubation time and cytochalasin B on human choriogonadotropin-stimulated progesterone production*

Incubation conditions were the same as given in the legend to Fig. 1, except that the incubations were terminated at indicated time periods, processed and assayed for progesterone by radioimmunoassay. Points represent means with the bars indicating ± S.E.M. Symbols: ●, none; ▲, human choriogonadotropin (10ng/ml); ○, cytochalasin B (50μM); △, cytochalasin B + human choriogonadotropin.

production at all time points except at 10min of incubation. However, the effect on basal production was less pronounced compared with human choriogonadotropin-stimulated progesterone synthesis.

Effect of cytochalasin B on lutropin-, cholera enterotoxin-, dibutyryl cyclic AMP- and 8-bromo cyclic AMP-stimulated steroidogenesis

Cytochalasin B was also tested for its ability to inhibit steroidogenesis in response to lutropin, cholera enterotoxin and cyclic AMP analogues. The results presented in Table 2 show that inclusion of cytochalasin B in the incubation medium significantly decreased the stimulation of steroidogenesis by the above agents. Of particular interest was the inhibition produced in response to dibutyryl cyclic

Table 2. *Effect of cytochalasin B on human choriogonadotropin-, lutropin-, cholera enterotoxin-, 8-bromo cyclic AMP- and dibutyrylcyclic AMP-stimulated progesterone production in rat luteal cells*

Rat luteal cells (1.6×10^6 cells) were incubated in a final volume of 0.4ml of medium 199 containing 0.1% bovine serum albumin and indicated concentrations of human choriogonadotropin, lutropin, cholera enterotoxin, 8-bromo cyclic AMP and dibutyryl cyclic AMP in the presence or absence of cytochalasin B. After incubation for 3h, the samples were assayed for progesterone by radioimmunoassay, as described in the Materials and methods section. Results are means ± S.E.M. Numbers in parentheses are P values calculated using student's t test. Abbreviation used: N.S., not significant.

Addition	Progesterone (ng/μg of DNA)	
	None	Cytochalasin B (50μM)
Experiment 1		
None	0.62 ± 0.07	0.5 ± 0.04 (N.S.)
Human choriogonadotropin (10ng/ml)	2.35 ± 0.18	1.05 ± 0.12 ($P < 0.001$)
Lutropin (100ng/ml)	2.21 ± 0.23	0.86 ± 0.07 ($P < 0.001$)
Cholera enterotoxin (1μg/ml)	1.87 ± 0.09	0.88 ± 0.08 ($P < 0.001$)
Experiment 2		
None	0.56 ± 0.05	0.46 ± 0.02 (N.S.)
Human choriogonadotropin (10ng/ml)	2.23 ± 0.09	1.25 ± 0.07 ($P < 0.001$)
8-Bromo cyclic AMP (1.5mM)	2.40 ± 0.14	1.33 ± 0.01 ($P < 0.001$)
Dibutyryl cyclic AMP (1.5mM)	2.48 ± 0.08	1.24 ± 0.03 ($P < 0.001$)

AMP and 8-bromo cyclic AMP as these results suggested its action at a point after cyclic AMP formation. This was further confirmed by following the effect of cytochalasin B on ^{125}I-labelled human choriogonadotropin binding to cells and gonadotropin-stimulated cyclic AMP accumulation.

Effect of cytochalasin B on ^{125}I-labelled human choriogonadotropin binding and human choriogonadotropin-stimulated cyclic AMP accumulation

Since the initial event in gonadotropin action is binding of hormone to cell-surface receptor (Menon & Gunaga, 1974), we first checked the possibility of cytochalasin B having any effect on ^{125}I-labelled human choriogonadotropin binding activity. The results showed that it has no effect on binding at the membrane level. Additional treatment of cells with cytochalasin B also failed to modify the equilibrium dissociation constant (K_d) or hormone-binding sites on luteal cells (results not shown). As shown in Fig. 3, cytochalasin B treatment did not affect subsequent production of cyclic AMP in response to lutropin, human choriogonadotropin or cholera enterotoxin. Similarly, basal production of cyclic AMP remained unaltered both in the presence and absence of cytochalasin B.

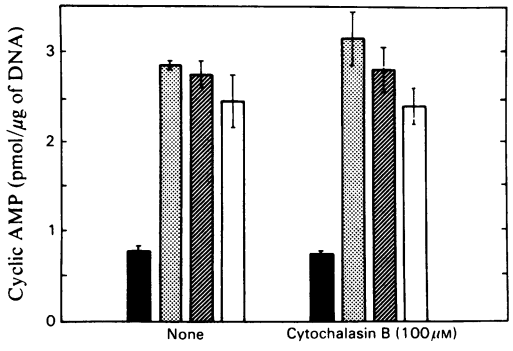

Fig. 3. *Stimulation of cyclic AMP production by rat luteal cells in response to lutropin, human choriogonadotropin and cholera enterotoxin*

Incubation conditions were the same as those described in the Materials and methods section. ■, basal; ⁞⁞⁞, human choriogonadotropin (100ng/ml); ▨, lutropin (1μg/ml); □, cholera toxin (1μg/ml). Results are means with S.E.M. indicated by error bars.

Effect of cytochalasin B on protein synthesis

Results presented in Table 3 show that up to 50μM concentrations of cytochalasin B had no significant effect on luteal-cell protein synthesis as measured by [^{3}H]proline incorporation. At a concentration of 100μM, however, 20% inhibition of protein synthesis was observed. As expected, cytochalasin B (50μM) effectively blocked the human choriogonadotropin - stimulated steroidogenesis (Table 3). In contrast, protein-synthesis inhibitors blocked both [^{3}H]proline incorporation into proteins and human choriogonadotropin-induced progesterone production.

Comparison of effects of cytochalasin B and cytochalasin D on 3-O-[^{3}H]methylglucose uptake and steroidogenesis

Since cytochalasin B is known to affect the transport of sugars into mammalian cells (Estensen

Table 3. *The effect of cytochalasin B, emetine and cycloheximide on protein synthesis and human choriogonadotropin-stimulated steroidogenesis in luteal cells*

[^{3}H]Proline incorporation into luteal cellular proteins was carried out as described in the Materials and methods section. Other details were same as given in the legend to Table 2. Results are means ± S.E.M.

Additions	Progesterone (ng/μg of DNA) Basal	Progesterone (ng/μg of DNA) Human choriogonadotropin (10ng/ml)	[^{3}H]Proline incorporation into proteins (pmol/μg of DNA)
None	1.04 ± 0.04	3.71 ± 0.28	1.93 ± 0.08
Dimethyl sulphoxide (1% v/v)	0.97 ± 0.13	3.85 ± 0.18	1.92 ± 0.05
Cytochalasin B (10 μM)	—	—	1.73 ± 0.04
Cytochalasin B (50 μM)	0.74 ± 0.02	1.82 ± 0.08	1.78 ± 0.11
Cytochalasin B (100 μM)	—	—	1.55 ± 0.04
Emetine (1 μM)	0.63 ± 0.03	0.9 ± 0.02	0.18 ± 0.03
Cycloheximide (10 μM)	0.70 ± 0.07	1.51 ± 0.09	0.12 ± 0.01

& Plagemann, 1972; Czech *et al.*, 1973; Lin *et al.*, 1978), whereas cytochalasin D usually has no effect on the transport process but is more effective as a microfilament modifier (Tannenbaum *et al.*, 1977), it was of interest to determine the effect of these agents on 3-*O*-[^{3}H]methylglucose transport and gonadotropin-stimulated progesterone synthesis in our system. Results presented in Fig. 4 show that, as expected, cytochalasin B inhibited the uptake of 3-*O*-[^{3}H]methylglucose transport by rat luteal cells. Under identical experimental conditions, cytochalasin D did not affect sugar transport in luteal cells. When tested for their effect on human choriogonadotropin- and 8-bromo cyclic AMP-stimulated steroidogenesis, as shown in Fig. 4, both cytochalasin B and cytochalasin D led to a decrease in progesterone production, although the latter was less effective. These observations clearly demonstrate that the inhibitory effects of cytochalasin B on sugar transport and steroidogenesis are separable and represent independent events.

Attempted reversal of the inhibitory effect of cytochalasin B by various agents

To rule out the possibility that the inhibitory effect of cytochalasin B on steroidogenesis was due to inhibition of glucose transport, we attempted to reverse this effect by increasing glucose concentration or by the addition of an alternative energy source such as pyruvate. Addition of increasing concentrations of glucose up to 0.1 M or 20 mM-pyruvate did not reverse cytochalasin B-mediated inhibition of steroid production. Similarly, phlorizin, which affects cellular metabolism of glucose (Czech *et al.*, 1973) up to 10 mM concentration had no demonstrable effect on either basal or human choriogonadotropin - stimulated steroidogenesis (results not shown). Results shown in Table 4 compare the effects of the composition of the incubation media and cytochalasin B on progesterone synthesis. Incubation of cells in media containing no cholesterol (medium 109) or with cholesterol (medium 199) did not alter basal or human choriogonadotropin-stimulated steroid pro-

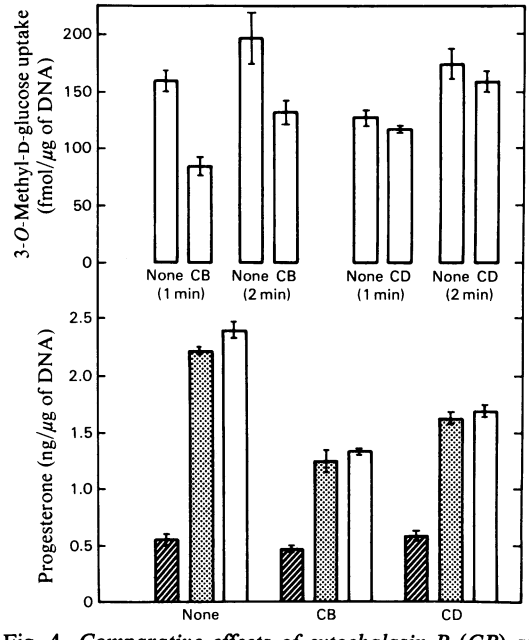

Fig. 4. *Comparative effects of cytochalasin B (CB) and cytochalasin D (CD) on 3-O-[^{3}H]methylglucose transport and gonadotropin- or 8-bromo cyclic AMP-stimulated steroidogenesis*

Measurements of 3-*O*-[^{3}H]methylglucose transport into luteal cells were carried out as described in the Materials and methods section. Other details were the same as those given in the legend to Table 2. ▨, basal; ▥, human choriogonadotropin (10ng/ml); □, 8-bromo cyclic AMP (1.5 mM)-stimulated progesterone production.

Table 4. *Lack of effect of sera on cytochalasin B inhibition of steroidogenesis*

Rat ovarian cells, after initial isolation in medium 199, were washed with medium 109 containing 0.1% bovine serum albumin and finally suspended in medium 199, medium 109 alone, or containing indicated concentrations of foetal bovine serum or foetal horse serum. After the addition of cytochalasin B and/or human choriogonadotropin, the samples were incubated for 3h. After incubation, the samples were extracted with light petroleum and assayed for progesterone by radioimmunoassay. Results are means ± S.E.M.

	Progesterone (ng/μg of DNA)			
	Basal	Cytochalasin B (50μM)	Human choriogonadotropin (10ng/ml)	Cytochalasin B (50μM) + human choriogonadotropin (10ng/ml)
Medium 109 containing bovine serum albumin (0.1%, w/v)	1.12 ± 0.06	0.85 ± 0.01	3.79 ± 0.1	1.66 ± 0.06
Medium 199 containing bovine serum albumin (0.1%)	1.15 ± 0.15	1.02 ± 0.17	4.46 ± 0.31	1.6 ± 0.07
Medium 109 containing foetal bovine serum (15%, v/v)	1.93 ± 0.15	1.56 ± 0.22	6.10 ± 0.20	2.49 ± 0.17
Medium 109 containing horse serum (15%, v/v)	8.98 ± 0.38	4.20 ± 0.11	10.0 ± 0.48	5.5 ± 0.56

duction. Cytochalasin B inhibited the steroidogenic response of cells to human choriogonadotropin in both media. Similarly, addition of horse serum (a source of cholesterol) enhanced steroidogenesis 2–3-fold (S. Azhar & K. M. J. Menon, unpublished work), but failed to prevent the inhibitory effect of cytochalasin B. These results suggest that cytochalasin B may not affect the transport of the steroid precursor cholesterol from the incubation medium to the luteal cells.

The effect of microtubule modifier on progesterone production

The effect of colchicine, an agent causing depolymerization of microtubules, on gonadotropin-induced steroidogenesis in luteal cells was also investigated. Addition of different concentrations of colchicine from 10μM to 1mM showed no effect on total steroid production under basal conditions or in the presence of human choriogonadotropin (10mg/ml). Similarly, preincubation of luteal cells with different concentrations of colchicine also was without any effect on subsequent production of progesterone in response to human choriogonadotropin (results not shown).

Discussion

The present studies were aimed to investigate the role of the cellular cytoskeletal system in gonadotropin-induced synthesis of progesterone by rat luteal cells. Exposure of luteal cells to cytochalasin B produced a decrease in steroidogenesis evoked by gonadotropins or other substances. This inhibitory action of cytochalasin B was both concentration- and time-dependent. However, unlike other systems (Van Obberghen *et al.*, 1976; Zor *et al.*, 1978), the effect of microfilament modifier did not require preincubation conditions to inhibit luteal steroidogenesis. Zor *et al.* (1978) have reported that the microfilaments, not microtubules, are intimately involved in lutropin- and choleragen-stimulated adenylate cyclase activity in cultured Graafian follicles. In this respect our results differ from those of Zor *et al.* (1978). The reason for the apparent discrepancy between our findings and those of Zor *et al.* (1978) might be partly due to the fact that we have utilized an acutely dispersed cell preparation from luteinized rat ovary, whereas Zor *et al.* (1978) have employed cultured Graafian follicles.

The cytochalasin B effect on eukaryotic cells involves either transport or motile processes depending on the concentration of the drug used. At relatively lower doses (0.1–5μM) this drug inhibits hexose transport by both a competitive (Estensen & Plagemann, 1972; Kletzien *et al.*, 1972; Taylor & Gagneja, 1975) and a non-competitive manner (Zigmond & Hirsch, 1972; Bloch, 1973; Czech *et al.*, 1973). At higher concentrations (1–100μM), cytochalasin B has been shown to produce changes in cell morphology and inhibition of microfilament function and related motile processes (Wessells *et al.*, 1971; Carter, 1972; Sanger & Holtzer, 1972). In the present studies, the effective range of cytochalasin B (10–50μM), as well as various other experimental evidence, provides support for the assumption that the effect of this drug was due to interference with microfilament system and not due to inhibition of glucose transport or a non-specific toxic effect.

Stimulation of steroidogenesis by lutropin/human choriogonadotropin involves first binding to ovarian cell-surface receptors and subsequently cyclic AMP plays a major role in this process (Menon & Gunaga, 1974). In an effort to localize the site of action of cytochalasin B, we examined its effect on ^{125}I-labelled human choriogonadotropin–luteal-cell-surface interaction and gonadotropin-stimulated cyclic AMP response. Our results show that under experimental conditions in which progesterone production was inhibited, cytochalasin B did not interfere with ^{125}I-labelled human choriogonadotropin binding to gonadotropin receptors. Moreover, steroidogenesis in response to cholera enterotoxin, an agent that mimics the action of several hormones in target cells by stimulating adenylate cyclase (Van Heyningen, 1977; Vaughan & Moss, 1978), was also inhibited by cytochalasin B. Since cholera toxin binds to specific cell-surface receptors (Van Heyningen, 1977; Vaughan & Moss, 1978) that are different from the lutropin/human choriogonadotropin receptors in the ovary (Azhar *et al.*, 1978; Azhar & Menon, 1979*b*), the inhibition of cholera enterotoxin-stimulated steroidogenesis by cytochalasin B further supports our conclusion that its effect is not due to an interaction with gonadotropin receptors. Furthermore, basal and 8-bromo cyclic AMP-induced steroidogenesis (which bypasses the receptor step) was also inhibited by cytochalasin B. The results reported in the present paper also show that accumulation of cyclic AMP in response to lutropin/human choriogonadotropin or cholera enterotoxin is not modulated by cytochalasin B, thus suggesting that the inhibition of steroidogenesis is not due to inhibition of adenylate cyclase.

Although cytochalasins, notably cytochalasin B, have been demonstrated to inhibit hexose transport (Estensen & Plagemann, 1972; Kletzien *et al.*, 1972; Zigmond & Hirsch, 1972; Bloch, 1973; Czech *et al.*, 1973; Taylor & Gagneja, 1975) besides their well established effect on microfilaments (Wessels *et al.*, 1971), in our system the inhibitory action of cytochalasin on steroidogenesis was not due to impairment of energy metabolism of cells as demonstrated by its lack of effect on 3-*O*-methylglucose transport. Similarly, increasing glucose concentration in the incubation medium or using pyruvate as an alternative energy source did not reverse the inhibitory action of cytochalasin B, further supporting our statement that inhibition of steroidogenesis was not due to inhibition of the cellular energy-generating process. These results are consistent with those reported in other systems, in which cytochalasin D has been shown to disrupt microfilaments (Wessels *et al.*, 1971; Mousa & Trevithick, 1977) without detectable effect on glucose transport (Tannenbaum *et al.*, 1977). Extensive studies reported by Lin *et al.* (1978) suggest that the inhibitory effects of cytochalasin B on hexose transport and on cell morphology are unrelated events and probably mediated by interaction of drugs to specific and independent receptors on the cell surface.

The inability of the anti-microtubular drug colchicine to modify steroidogenesis suggests that microtubules may not be directly involved in the synthesis and/or release of progesterone in rat luteal tissue. In this respect, our data are consistent with those of Zor *et al.* (1978), who demonstrated that colchicine had no effect on the response of rat Graafian follicles to lutropin, but inhibited the response to follitropin and prostaglandin E_2. In contrast, injection of ewes *in vivo* with colchicine has been reported to decrease blood progesterone concentration with a parallel increase in luteal tissue progesterone content (Gemmell & Stacy, 1977). Similarly employing ovine luteal slices, Sawyer *et al.* (1979) observed a slight decrease in lutropin-stimulated progesterone secretion in the presence of colchicine.

Since lutropin has been suggested to increase mitochondrial supply of unesterified cholesterol (Robinson *et al.*, 1975) and possibly stimulate movement of cholesterol to the site of side-chain cleavage (Toaff *et al.*, 1979), it is conceivable that the cytoplasmic microfilaments might be involved in these processes. In this respect, in the mouse adrenal tumour cells (Y-1), cytochalasin B has been known to inhibit corticotropin-induced steroidogenesis and involvement of microfilaments has been suggested at the level of cholesterol transport into mitochondria (Mrotek & Hall, 1977). By contrast, employing the same Y-1 cells, Cortese & Woolf (1978) not only demonstrated stimulation of steroidogenesis by cytochalasins, but also proposed that this drug acts by enhancing the availability of cholesterol bound to high-density lipoprotein for steroidogenesis. In the present studies, addition of horse sera or foetal bovine serum (a source of cholesterol in the form of lipoproteins) along with human choriogonadotropin stimulated steroidogenesis by 1.5–2-fold over the value that was exhibited by human choriogonadotropin alone. However, cytochalasin B did not enhance the steroidogenesis observed in the presence of serum, but in fact inhibited the serum-stimulated steroid accumulation. From these studies, we conclude that cytoplasmic microfilament structures could be involved in gonadotropin-induced steroidogenesis, whereas microtubular structures probably are not directly participating in this process.

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