Vol. 104, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1982 Pages 299-306

RELATIONSHIP BETWEEN INHIBITION OF INTERSTITIAL CELL TESTOSTERONE SYNTHESIS BY CYTOCHALASIN B AND GLUCOSE

E.P. Murono *†, T. Lin*, J. Osterman* and H.R. Nankin*

*Medical Service, Wm. Jennings Bryan Dorn Veterans' Hospital; *Department of Medicine and †Department of Physiology, University of South Carolina, School of Medicine, Columbia, S.C. 29201

Received November 16, 1981

Summary: We examined the possibility that cytochalasin B reduced LH-stimulated testosterone synthesis of Leydig cells in part by inhibiting glucose uptake. In the presence of 5.6mM glucose cytochalasin B (0.1-50μM) inhibited LH-stimulated testosterone synthesis in a dose-dependent manner, while effects with cytochalasin D or cytochalasin E were not evident until inhibitor concentrations reached 1 or 10μM. Increasing levels of glucose (0-5mM) progressively increased LH-stimulated testosterone synthesis by interstitial cells, while cytochalasin B at 1 or 10μM inhibited testosterone synthesis in a dose-dependent manner at each glucose concentration examined. Higher concentrations of glucose (10- 50mM) partially reversed this inhibition. In the absence of glucose LH-stimulated testosterone synthesis was inhibited only when the cytochalasin B concentration reached 10μM. Cytochalasin B (0.1-5μM) inhibited [^{14}C]-2-deoxyglucose uptake by purified Leydig cells in a dose-dependent manner, while 1 or 10μM cytochalasin D or cytochalasin E had only limited effects. These results suggest that cytochalasin B inhibits Leydig cell LH-stimulated testosterone synthesis by at least two mechanisms: by blocking glucose uptake at lower concentrations (0.1-10μM), while at higher concentrations (10-50μM) it probably blocks microfilament function.

INTRODUCTION

Cytochalasin B, a fungal metabolite, has been used extensively to study the role of microfilaments in a variety of cellular functions, including: transport, secretory and motile processes [1,2]. The precise mechanism of CB action on microfilaments is not known; however, recent studies have demonstrated that CB binds to actin, the principal protein of microfilaments, to block microfilament polymerization [3,4,5]. CB also blocks glucose uptake by mammalian cells [6,7], by competitively binding to a high affinity plasma membrane protein involved in the facilitated diffusion of glucose into cells

Abbreviations: LH, luteinizing hormone; Me_2SO, dimethyl sulfoxide; CB, cytochalasin B; CD, cytochalasin D; CE, cytochalasin E; I.C , interstitial cells; buffer I, Medium 199 containing 1 mg/ml of bovine serum albumin at pH 7.4; buffer II, 2.5 mM Hepes containing Krebs Ringer's salts at pH 7.4.

†To whom correspondence should be addressed at: Dept. of Medicine, Bldg. T-28, Veterans' Administration Hospital, Columbia, S.C. 29201.

0006-291X/82/010299-08$01.00/0

[8,9]. Thus, the net effect of CB on cellular function may depend on at least two actions: the limitation of an energy source and the blocking of microfilament function. The overall effect of CB on either process appears to be related to its concentration [3].

We recently reported that CB inhibited LH-stimulated testosterone synthesis by isolated I.C. [10]. Similar inhibitions by CB have been reported on ACTH-stimulated steroid synthesis by the mouse adrenal cell [11] and on LH-stimulated progesterone synthesis by the ovine corpus luteum [12]. Although these inhibitions were attributed to disruption of microfilament function, it was possible that CB was acting also by blocking glucose uptake. The present studies were performed to examine this possibility.

MATERIALS AND METHODS

Materials and animals: CB, CD, CE, bovine serum albumin, Me_2SO, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (Hepes), collagenase (Type 1) and 2-deoxyglucose were purchased from Sigma; [^{14}C]-2-deoxyglucose and [1,2,6,7-^{3}H]-testosterone were from New England Nuclear; Medium 199 containing 1.5 g/L $NaHCO_3$ was from Grand Island Biological Co.; Metrizamide was from Accurate Chemical Co. Human pituitary LH (LH A-3, LER-1549) was provided by the National Pituitary Agency. Sprague-Dawley rats (65-90 days old) were from Zivic-Miller Lab. Testes were quickly removed from decapitated rats, decapsulated and placed on ice.

Isolation of interstitial cells or Leydig cells: I.C. were isolated by the collagenase dispersion method of Dufau et al. [13] with slight modifications. The isolated cells were resuspended in either buffer I or buffer II containing varying amounts of glucose to give a final concentration of ~$1x10^6$ cells/ml.

To obtain purified Leydig cells, I.C. were separated using a 20 ml 0-36% Metrizamide gradient according to methods described previously by Conn et al. [14] and Valladares & Payne [15], with slight modifications [16]. The third band of cells consisting primarily of Leydig cells was removed by aspiration, washed twice in 25 ml of buffer II containing no glucose, resuspended in the same buffer II to give a final concentration of ~$4x10^5$ cells/ml.

Incubation of interstitial cells: I.C. were resuspended in either buffer I or buffer II to give a final concentration of ~$1x10^6$ cells/ml. Appropriate amounts of CB, CD or CE were dissolved in 10µl Me_2SO (20 to 100%, to give final concentrations of 0.2 to 1% Me_2SO) to give the final desired concentrations of inhibitor. Control samples received 10µl Me_2SO only. Reactions were initiated by the addition of LH (5 mIU). Each group represents the mean ± standard error (SE) of at least 4 samples each analyzed in duplicate. Samples were incubated for 4h at 34°C in an atmosphere of O_2/CO_2 (95:5, v:v), then centrifuged at 1000xg to settle the cells. The cell-free medium was saved at -20°C for testosterone radioimmunoassay, as described previously [10].

[^{14}C]-2-Deoxyglucose uptake studies using purified Leydig cells: Approximately $4x10^5$ cells/ml in buffer II containing no glucose were preincubated for 15 min at 34°C in a Dubnoff shaker bath in an atmosphere of O_2/CO_2 (95:5, v:v). Next, varying concentrations of CB (0.1-5.0µM), or CD or CE (1-10µM) dissolved in 10µl Me_2SO were added to give final desired concentrations, and the samples were incubated for 10 min. Control tubes received 10µl Me_2SO only. Reactions were initiated by the addition of [^{14}C]-2-deoxyglucose (0.1 mM, 0.2µC) and were continued for 0, 10 and 20 min. Samples were immediately centrifuged at

600xg for 10 min. The cells were washed once in 2 ml of buffer (the total elapsed time was less than 1 min), digested in 1 ml 0.5 N NaOH and then counted in 10 ml scintillation liquid (PCS, Amersham). Each point represents the average of duplicate incubations.
Statistical analysis: Treatment groups for each experiment were compared using Student's t-test.

RESULTS

Effect of varying concentrations of CB, CD or CE on LH-stimulated testosterone synthesis by interstitial cells: Basal testosterone synthesis was 1.45 ± .02 ng/10^6 cells. Treatment with LH increased testosterone synthesis to 9.03 ± .29 ng/10^6 cells, which is designated here as the 100% control. Varying concentrations of CB (0.1, 1.0, 10 or 50μM) significantly inhibited LH-stimulated testosterone synthesis in a dose-dependent manner to 90 ± 3, 72 ± 4, 56 ± 4 and 36 ± 5% of control, respectively (Fig. 1, Panel A). Comparable concentrations of CD or CE (0.1-50μM) inhibited LH-stimulated testosterone synthesis in a similar dose-dependent manner (Fig. 1, Panels B and C, respectively), however, statistically significant reductions were not observed until CD or CE concentrations reached 10 ($P<.02$) and 1.0μM ($P<.01$), respectively. At the highest concentration of CD or CE (50μM), LH-stimulated testosterone synthesis was 72 ± 3 and 71 ± 2% of control, respectively.

Effect of increasing (0-5 mM) glucose concentrations on testosterone synthesis by interstitial cells and effect of cytochalasin B: In the absence or presence of LH, increasing concentrations of glucose progressively increased testosterone synthesis by I.C which reached a plateau between 2.5 and 5 mM glucose (Fig. 2, Panel A, lower and upper curves, respectively). In the presence of LH, at peak testosterone synthesis (5mM glucose), there was a 1.8-fold increase over the zero glucose concentration value. The addition of 1 or 10μM CB to LH-treated cells exposed to increasing concentrations of glucose (0-5mM), inhibited testosterone synthesis dose-dependently at each concentration of glucose (Fig. 2, Panel B).

Effect of higher (5-50mM) glucose concentrations on cytochalasin B inhibited LH-stimulated testosterone synthesis: Higher concentrations of glucose gradually decreased LH-stimulated testosterone synthesis (Fig. 3, Panel A). At 50mM glucose testosterone synthesis was 74 ± 1% of control (cells incubated in 5mM glucose). The inhibition of testosterone synthesis by 1μM CB (79 ± 1% of control) was partially reversed by 10-30mM glucose (Fig. 3, Panel B). However, with higher concentrations of glucose, testosterone synthesis was significantly less than levels observed at either 5 or 20mM glucose. Similarly, the

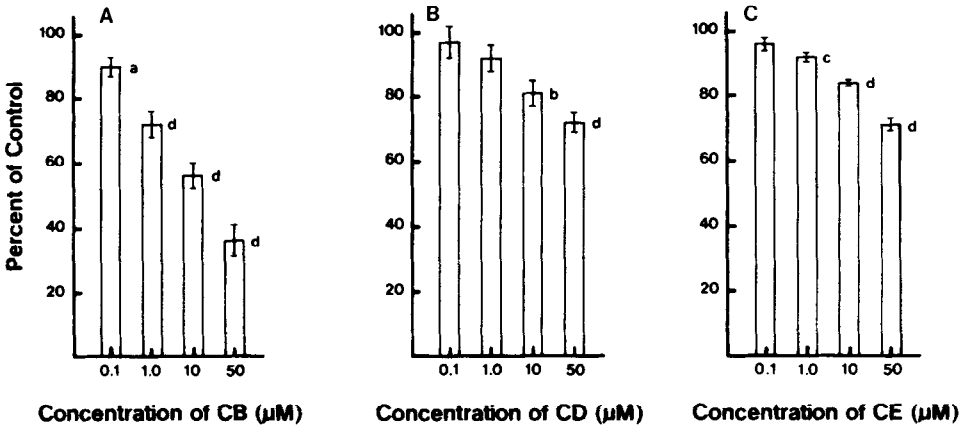

Fig. 1. Effect of cytochalasins on LH-stimulated testosterone synthesis by interstitial cells. Each group represents the mean of at least eight determinations from two experiments. Interstitial cells (~1x10^6/ml) were incubated in buffer I for 4h at 34°C under O_2/CO_2 (95/5, v/v). A. Effect of cytochalasin B; B. Effect of cytochalasin D; C. Effect of cytochalasin E; a=P<.05, b=P<.02, c=P<.01, d=P<.001

inhibition of testosterone synthesis by 10μM CB (48 ± 1% of control) was partially reversed by higher concentrations of glucose (Fig. 3, Panel C), peaking at 30mM glucose. However, androgen synthesis was reduced at 40 and

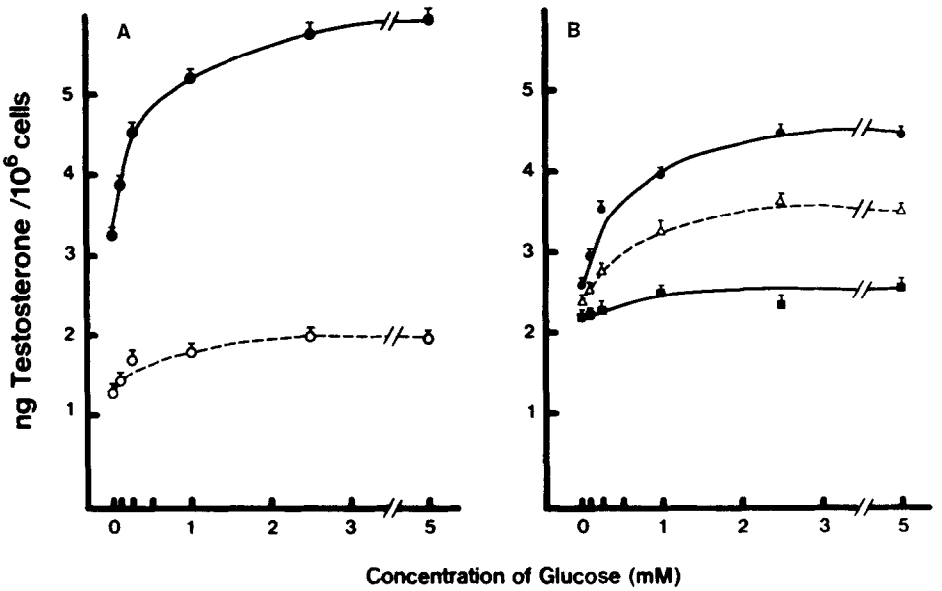

Fig. 2. Effect of increasing glucose concentrations (0-5mM) on testosterone synthesis by interstitial cells. Interstitial cells (~1x10^6/ml) were incubated in buffer II containing varying concentrations of glucose. A. Incubations were performed in the absence of LH (—o—) or in the presence of 5mIU LH (—●—). B. Incubations were performed in the presence of LH alone (—●—), LH plus 1μM cytochalasin B (—▲—), or LH plus 10μM cytochalasin B (—■—). Each point represents the mean of 4 incubations ± SE.

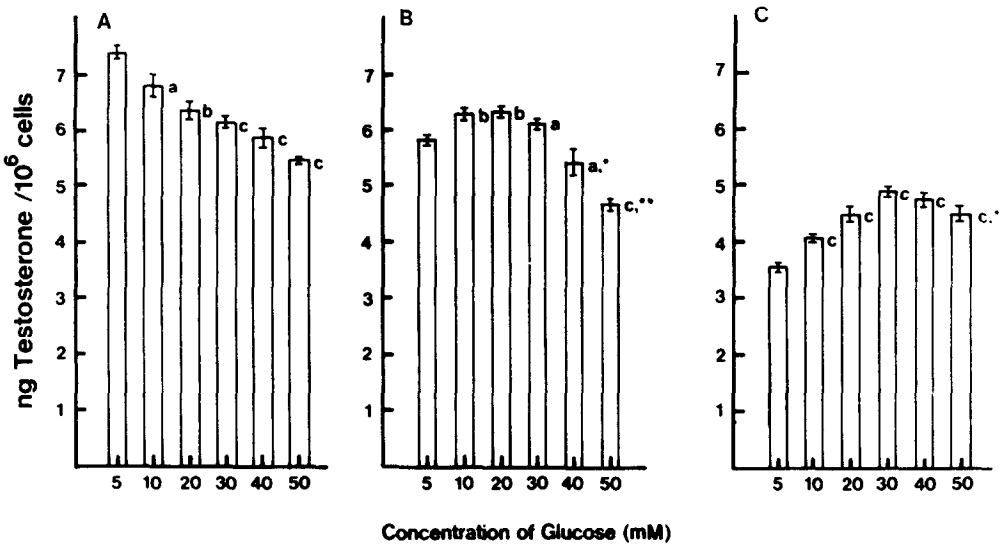

Fig. 3. Effect of increasing concentrations of glucose on LH-stimulated testosterone synthesis by interstitial cells in the absence or presence of cytochalasin B. A. Incubations were performed in the absence of cytochalasin B; a=P<.05, b=P<.01, c=P<.001 when each was compared with group incubated with 5mM glucose. B. Incubations were performed in the presence of 1μM cytochalasin B; a=P<.05, b=P<.01, c=P<.001 when compared with group incubated with 5mM glucose; *P<.05, **P<.001 when compared with group incubated with 20mM glucose. C. Incubations were performed in the presence of 10μM cytochalasin B; c=P<.001 when compared with group incubated with 5mM glucose; *=P<.05 when compared with group incubated with 30 mM glucose.

50mM, which probably reflect the inhibitive effects of high glucose concentrations as demonstrated in Panel A.

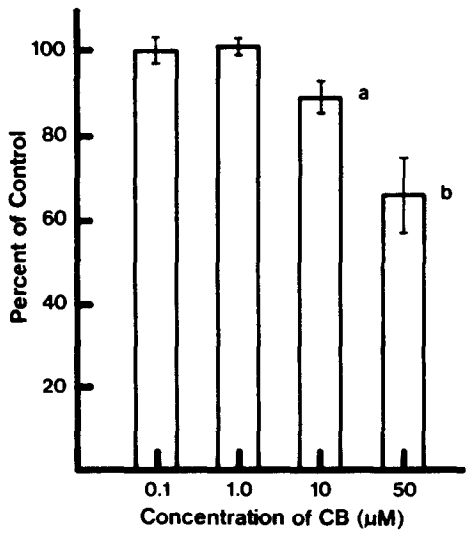

Fig. 4. Effect of cytochalasin B on LH-stimulated testosterone synthesis by interstitial cells incubated in the absence of glucose.

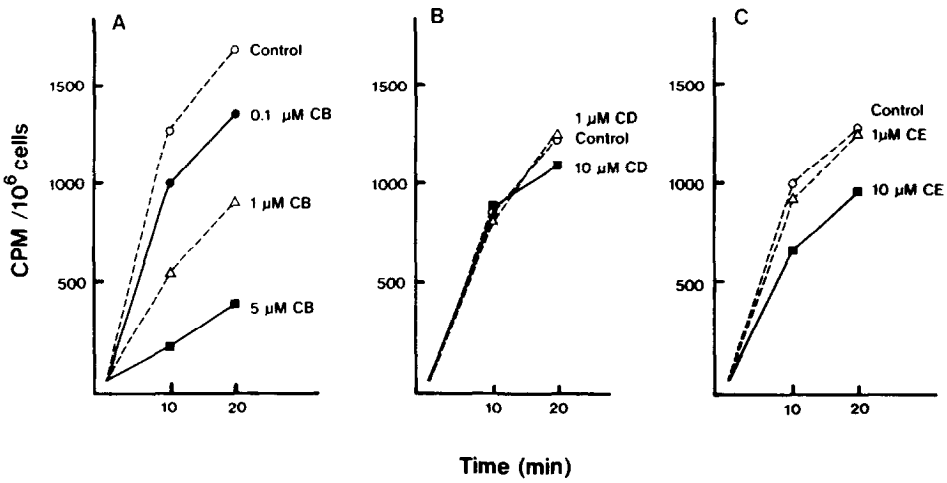

Fig. 5. Effect of cytochalasin B, cytochalasin D or cytochalasin E on [^{14}C]-2-deoxyglucose uptake by purified Leydig cells.

Effect of cytochalasin B on LH-stimulated testosterone synthesis by interstitial cells incubated in medium lacking glucose: In the absence of glucose 0.1 or 1.0μM CB had no effect on LH-stimulated testosterone synthesis (Fig. 4). However, at higher concentrations of CB (10 and 50μM), testosterone synthesis was inhibited significantly in a dose-dependent manner.

Effect of cytochalasins on [^{14}C]-2-deoxyglucose uptake by purified Leydig cells: The effect of varying concentrations of CB (0.1-5.0μM) on [^{14}C]-2-deoxyglucose uptake by Leydig cells is presented in Fig. 5, Panel A. A dose-dependent inhibition of [^{14}C]-2-deoxyglucose uptake was observed. CD or CE at 1 or 10μM had no or only a minimal effect on [^{14}C]-2-deoxyglucose uptake by Leydig cells (Fig. 5, Panels B and C, respectively).

DISCUSSION

The present results suggest that lower concentrations of CB inhibit Leydig cell LH-stimulated testosterone synthesis by inhibiting glucose uptake. This is demonstrated by several lines of evidence: 1) in the absence of glucose LH-stimulated testosterone synthesis is approximately one half the level observed in the presence of 5.0 mM glucose, 2) increasing low concentrations of glucose (0-5mM) progressively increased LH-stimulated testosterone synthesis and which was inhibited dose-dependently by 1 or 10μM CB at each concentration of glucose added, 3) in the absence of glucose, only higher concentrations of CB (10 or 50μM) inhibited LH-stimulated testosterone synthesis, while in the presence of physiological glucose concentration the inhibition was dose-dependent at all concentrations of CB (0.1-50μM) added, 4) the

inhibition of LH-stimulated testosterone synthesis by 1μM or 10μM CB in 5mM glucose is partially overcome by some higher glucose concentrations, and 5) CB inhibits [^{14}C]-2-deoxyglucose uptake by purified Leydig cells.

We previously reported that CB inhibited LH-stimulated testosterone synthesis by I.C. by blocking microfilament function [10]. The present results suggest that CB inhibits testosterone synthesis by at least two mechanisms: 1) primarily blocking glucose uptake at lower concentrations (0.1-10μM), and 2) inhibiting microfilament function at higher concentrations (10-50μM).

In this regard, low concentrations of CB have been demonstrated to inhibit glucose uptake in a number of cellular systems [9,17,18], while higher concentrations have been associated with inhibition of cellular motility, by presumably blocking microfilament function [2,3]. In the former case the effect of CB appears to be related to its competitive binding to a plasma membrane protein involved in the facilitated diffusion of glucose [9,19,20]; in the latter, it appears to be related to its binding to actin, the major protein of microfilaments [4,21].

The present results contrast with studies reported in mouse adrenal tumor cells where CB similarly inhibited steroid biosynthesis in the absence or presence of glucose [11], and where CB had no effect on total cellular ATP levels [22]. We have shown that in the absence of glucose inhibition is dependent on the concentration of CB added, with only higher concentrations of CB (10-50μM) inhibiting testosterone synthesis. In the former paper [11], the concentration of CB added was not reported, and it is possible that they utilized higher concentrations. With respect to the latter paper [22], total cellular ATP levels may not reflect changes in specific ATP or cofactor (NAD^+, NADPH) pools involved in steroid biosynthesis.

In a previous study the inhibition of LH-stimulated testosterone synthesis by anti-actin [23] indicated the involvement of microfilaments in the response of I.C. to tropic hormone stimulation. We propose that higher concentrations of CB inhibit LH-stimulated testosterone synthesis by a similar mechanism, i.e., by blocking microfilament function. This is supported by our studies using CD or CE which previously have been reported to block motility (microfilament) related functions of cells but had only minimal effects on glucose uptake [24,25]. In the present study CD or CE inhibited LH-stimulated testosterone synthesis only at higher concentrations (10 and 1μM, respectively) and had only minimal effects on [^{14}C]-2-deoxyglucose uptake by Leydig

cells. Furthermore, in the absence of glucose higher concentrations of CB (10 and 50μM) inhibited LH-stimulated testosterone synthesis by I.C.

ACKNOWLEDGEMENTS

We thank Jeff Lane and Katherine Gettys for their technical assistance, Dorothea Barwick for the typing of the manuscript and Reid Hearn for the illustrations. This work was supported by the Veterans' Administration, and by National Institute of Aging Grant No. 1 R01 AG0121703.

REFERENCES

1. Carter, S.B (1967) Nature 213:261-264.
2. Wessells, N.K., Spooner, B.S., Ash, J.F., Bradley, M.O., Luduena, M.A., Taylor, E.L., Wrenn, J.T. and Yamada, K.M. (1971) Science 171, 135-143.
3. Lin, S. and Spudich, J.A. (1974) J. Supramole. Struct. 2, 728-736.
4. Flanagan, M.D. and Lin, S. (1980) J. Biol. Chem. 255, 835-838.
5. Brenner, S.L. and Korn, E.D. (1980) J. Biol. Chem. 255, 841-844.
6. Kletzien, R.F., Perdue, J.F. and Springer, A. (1972) J. Biol. Chem. 247, 2964-2966.
7. Zigmond, S.H. and Hirsch, J.G. (1972) Science 176, 1432-1434.
8. Estensen, R.D. and Plagemann, P.G.W. (1972) Proc. Nat. Acad. Sci. U.S.A. 69, 1430-1434.
9. Kletzien, R.F. and Perdue, J.F. (1973) J. Biol. Chem. 248, 711-719.
10. Murono, E.P., Lin, T., Osterman, J. and Nankin, H.R. (1980) Biochim. Biophys. Acta 633, 228-236.
11. Mrotek, J.J. and Hall, P.F. (1975) Biochem. Biophys. Res. Commun. 64, 891-896.
12. Silavin, S.L., Moss, G.E. and Niswender, G.D. (1980) Steroids 36, 229-241.
13. Dufau, M., Mendelson, C.R. and Catt, K.J. (1974) J. Clin. Endocrinol. Metab. 39, 610-613.
14. Conn, P.M., Tsuruhara, T., Dufau, M. and Catt, K.J. (1977) Endocrinology 101, 639-642.
15. Valladares, L.E. and Payne, A.H (1979) Proc. Nat. Acad. Sci. U.S.A. 76, 4460-4463.
16. Lin, T., Murono, E., Osterman, J., Allen, D.O. and Nankin, H.R. (1980) Steroids 35, 653-663.
17. Bloch, R. (1973) Biochemistry 12, 4799-4801.
18. Traverna, R.D. and Langdon, R.G. (1973) Biochim. Biophys. Acta 323, 207-219.
19. Lin, S. and Spudich, J.A. (1974) J. Biol. Chem. 249, 5778-5783.
20. Jung, C.Y. and Rampal, A.L. (1977) J. Biol. Chem. 252, 5456-5463.
21. Lin, S. and Snyder, C.E. (1977) J. Biol. Chem. 252, 5464-5471.
22. Mrotek, J.J. and Hall, P.F. (1977) Biochemistry 16, 3177-3181.
23. Hall, P.F., Charponnier, C., Nakamura, M. and Gabbiani, G. (1979) J. Steroid Biochem. 11, 1361-1366.
24. Atlas, S.J., Magargal, W.W. and Lin, S. (1980) J. Receptor Res. 1, 113-135.
25. Cuppoletti, J., Mayhew, E. and Jung, C.Y. (1981) Biochim. Biophys. Acta 642, 392-404.