Biochimica et Biophysica Acta, 444 (1976) 23—32

BBA 27981

REGULATION OF OVARIAN STEROIDOGENESIS

THE DISPARITY BETWEEN ^{125}I-LABELLED CHORIOGONADOTROPIN BINDING, CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE FORMATION AND PROGESTERONE SYNTHESIS IN THE RAT OVARY

M.R. CLARK and K.M.J. MENON *

Departments of Obstetrics and Gynecology and Biological Chemistry, The University of Michigan Medical Center, Ann Arbor, Mich. 48104 (U.S.A.)

(Received February 16th, 1976)

Summary

The binding of ^{125}I-labelled human choriogonadotropin, formation of cyclic adenosine 3′,5′-monophosphate (cyclic AMP), and synthesis of progesterone were examined in ovarian cells from immature rats. Collagenase dispersed ovarian cells were found to respond specifically to lutropin-like activity. The equilibrium dissociation constant (K_d) for the binding of ^{125}I-labelled choriogonadotropin was $1.7 \cdot 10^{-10}$ M. Progesterone synthesis was increased at least 40-fold and cyclic AMP formation 10-fold in response to maximum hormonal stimulation. The concentration of choriogonadotropin which stimulated progesterone synthesis maximally in Eagle's minimum essential medium — 0.1% gelatin (2 ng/ml), resulted in minimal (less than 30% of maximum) increases in cyclic AMP accumulation and hormone binding. Similarly, binding of choriogonadotropin was not saturated at a hormone concentration (50 ng/ml) that stimulated maximal cyclic AMP formation. These results are consistent with the existence of receptor reserve in the ovarian cell. A marked shift in the dose vs. response relationship for progesterone synthesis occurred when fetal calf serum was used to supplement Eagle's minimum essential medium, however. Under these experimental conditions, progesterone synthesis reached a maximum at a hormone concentration of the same order of magnitude as did cyclic AMP formation. It is concluded that the degree of spare receptor effect observed may depend not only on an absolute amount of excess receptor, but also on the readiness of the system to respond in a given fashion.

* To whom correspondence should be addressed.

Introduction

The widespread disparity between the concentration of effector which can produce maximum specific binding to a cell and the lower concentration which maximally stimulates a cellular response, has had significant impact on theories of effector-target cell interaction (for a review, see ref. 1). The term "spare receptor" has been used to describe the entities responsible for the binding which occurs after the response parameter has been saturated [2]. Spare receptors have been described for drugs [2,3] and polypeptide hormones including corticotropin [4,5], thyrotropin [6], follitropin [7] and lutropin or choriogonadotropin [8—11]. In the testicular system, a disparity between the concentration of ^{125}I-labelled choriogonadotropin required to saturate all the available binding sites and that required for maximally stimulating cyclic AMP and testosterone accumulation has been reported [10].

Since the ovarian steroidogenic response to gonadotropin is also mediated by cyclic AMP [12,13], this study was undertaken to investigate the concentration dependence of choriogonadotropin binding, cyclic AMP production and progesterone formation.

Materials

Minimum essential medium with Earle's salts and 10% fetal calf serum was supplied by Grand Island Biological Co. Purified pigskin gelatin was purchased from Eastman. Cyclic [^{3}H]AMP (16 Ci/mmol) was obtained from Schwarz-Mann and [8-^{14}C]adenine (50—60 Ci/mol) from New England Nuclear. Deoxyribonucelase I (DNAase), Dowex 50-WX4 (200—400 mesh, H^+ form), cyclic AMP and bovine serum albumin fraction V were the products of Sigma Chemical Co. Somatotropin, prolactin, thyrotropin and lutropin (NIH-LH-S18) were made available by the Hormone Distribution Program, National Institutes of Health. Purified human choriogonadotropin (11500 I.U./mg), as well as ^{125}I-labelled choriogonadotropin (50 Ci/μg) and reagents for progesterone radioimmunoassay were provided by Dr. A.R. Medgley, Jr. of this university. Lutropin (LER 1705) was a gift from Dr. Leo. E. Reichert, Jr. of Emory Univeristy, Atlanta, Ga. Aldrich Chemical Co. supplied 1-methyl-3-isobutylxanthine. Sprague-Dawley rats were purchased from Spartan Farms, Hazlett, Mich. Collagenase (Type CLS) was the product of Worthington Biochemical Corp.

Methods

Cell suspension. Cells were prepared from the ovaries of 25—26-day-old rats by a modification of the previously described procedure [14]. All manipulations were carried out in plastic or siliconized glass vessels. After excision, ovaries were placed in minimal essential medium supplemented with 10% fetal calf serum, or for the experiments shown in Figs. 2 and 3, with 0.1% pigskin gelatin. They were then trimmed of excess fat, weighed and quartered into minimal essential medium with approx. 500 units/ml collagenase and 0.4 mg/100 ml DNAase at 50 mg tissue per ml. The minced tissue was digested at 37° C under O_2/CO_2 (95 : 5, v/v) with shaking for 2 h. Mechanical dispersal was provided by pipetting the suspension up and down in a needleless 1 ml plastic

syringe 30 times at 30, 60 and 120 min of incubation. The cells were then centrifuged at room temperature at 600 × *g* for 3 min and resuspended in fresh medium. Two additional washes were performed. The cells were finally resuspended in medium at 50 mg original wet weight of tissue per ml, which gave approx. $3 \cdot 10^7$ cells per ml. Cells were counted in a hemocytometer and viability determined by dye exclusion following staining in 0.02% nigrosin. DNA was estimated by the method of Burton [15] for corrections for differences in cell yields between experiments. However, such corrections did not materially alter the results, since the yield routinely varied by less than 5%. The number of replicate experiments, each performed in duplicate, is given in parentheses in the figure legends.

Cyclic AMP measurements. Approx. $3 \cdot 10^6$ cells in 0.1 ml of cell suspension was incubated at 37°C for 30 min (unless stated otherwise) under O_2/CO_2 (95 : 5, v/v) in a final volume of 300 µl with or without test substances. The 1-methyl-3-isobutylxanthine was present unless stated otherwise, at 0.5 mM to inhibit cyclic AMP degradation (1-methyl-3-isobutylxanthine did not alter the position of the dose vs. response curve for cyclic AMP; data not shown). For studying the incorporation of [^{14}C]adenine into cyclic [^{14}C]AMP, the prelabelling technique was employed [16,17,19]. After a preincubation period of 45 min in the presence of 3.3 µCi/ml of [^{14}C]adenine, the cells were centrifuged as above and resuspended in fresh medium for the test incubations. At the end of the test incubations, 0.2 ml of cold 10% trichloroacetic acid containing cyclic [^{3}H]AMP (10000 cpm) for determination of recovery and carrier cyclic AMP (0.1 mmol) was added. The precipitated protein was removed by centrifugation at 5000 × *g* for 10 min. The supernatant was freed of trichloroacetic acid by three extractions with 1 ml of ethyl ether each time, and placed on a Dowex 50W column (0.6 × 4 cm) [19]. This column was eluted with 2.5 ml of water and the eluate containing labelled ATP and ADP discarded. An additional 3 ml water wash was performed and to this eluate was added 0.3 ml each of 0.125 M $ZnSO_4$ and 0.125 M $Ba(OH)_2$ on ice. After 10 min the $BaSO_4$ precipitate was removed by centrifugation at 2000 × *g* for 10 min and a 2 ml aliquot of the supernatant counted. The scintillation fluid contained 50 mg POPOP (*p*-bis-[2-(5-phenyloxazolyl)]-benzene), 4 g PPO (2,5-diphenyloxazole), 500 ml of Triton X-100, and 500 ml of toluene. Single determinations were made on duplicate samples for each experiment. Alternatively, the mass of cyclic AMP formed was determined by trichloroacetic acid extraction and protein binding assay as described by Gilman [20] and modified by Brooker [21]. Two determinations were performed on replicate 20-µl aliquots of duplicate samples for each experiment.

Determination of progesterone accumulation. Cells were incubated as described above for cyclic AMP measurements, with the exception that 1-methyl-3-isobutylxanthine was not present. Following incubation (for 2 h, unless otherwise noted) the reaction was stopped by placing the sample tubes in a boiling water bath for 3 min, after which the precipitated protein was removed by centrifugation at 4°C for 10 min at 5000 × *g*. The supernatants were frozen at −20°C for later assay in duplicate of 10- and 25-µl aliquots by the radioimmunoassay described by Bajpai et al. [14] using an antiserum characterized by Niswender [22]. In brief, appropriate standards or samples were incubated

for 16 h in buffer (0.14 M NaCl, 0.01 M sodium phosphate, and 1 : 10000 merthiolate at pH 7.0) with approx. 30000 cpm of tracer and progesterone antiserum (at a final dilution of 1 : 16000). The tracer was the carbon-11 tyrosine methyl ester derivative of progesterone which had been iodinated with ^{125}I. The antiserum was similarly raised against a protein conjugate with progesterone attached to protein via the carbon-11 position. The first incubation was followed by a second 16 h incubation with anti-rabbit γ-globulin (1 : 90 final dilution) and the final precipitate collected by centrifugation at 1500 $\times$ *g* for 30 min. The precipitates were counted directly with a Searle Gamma Counter Model 1195. Analysis of the radioimmunoassay data was made utilizing the computer program described by Duddleson et al. [30] or using a Wang Programable Calculator, Model 600-6.

Measurement of ^{125}I-labelled choriogonadotropin binding. Binding of gonadotropin to ovarian cells was determined by a modification of the assay of Cuatrecasas [31] for insulin binding to fat cells. Ovarian cells were incubated as above with ^{125}I-labelled choriogonadotropin in test tubes which had been precoated with 5% bovine serum albumin. Binding was terminated at the desired times by dilution (3 : 1) with cold medium and immediate centrifugation at 4°C for 3 min at 500 $\times$ *g*. The medium was removed and the pelleted cells washed twice with 1 ml of fresh media and counted directly on a gamma counter (Searle Analytic, Model 1195). Specific binding was calculated by correcting for binding in the presence of greater than a 1000-fold excess of unlabelled hormone (non-specific binding), with both specific and non-specific binding assays performed in duplicate or quadruplicate. Non-specific binding was generally less than 10% of the total binding.

Calculation of the specific activity of ^{125}I-labelled choriogonadotropin. The specific activity of the ^{125}I-labelled choriogonadotropin was calculated from the quantity of radioactivity recovered with protein following iodination (A.R. Midgley, Jr., personal communication) and corrected for the fact that only approx. 60% of this radioactivity would bind to plasma membranes from bovine corpora lutea (determined in our laboratory) or from rat ovaries (J. Duncan, personal communication). Alternatively, the specific activity was measured by comparison of the displacement of a trace amount of ^{125}I-labelled choriogonadotropin from rat ovarian cells by ^{125}I-labelled choriogonadotropin (self-displacement), with the displacement by unlabelled hormone (Midgley, Jr., A.R., personal communication).

Determination of equilibrium dissociation constant. The binding of ^{125}I-labelled choriogonadotropin was carried out as described above. The free hormone concentration was calculated by correcting the total quantity added for that bound both specifically and non-specifically, and for the fact that only 60% of the radioactivity could be bound. The bound hormone was calculated from the radioactivity bound, with no correction necessary for inactive hormone. Analysis of the bound to free ratio as a function of the bound hormone was made by a Scatchard plot [23], as described by Birnbaumer et al. [24].

Results

Characterization of cell suspension

Preparation of cells by the technique described here produced a similar via-

bility, as determined by nigrosin exclusion, as that previously reported from this laboratory [18]. The yield and responsiveness to hormone were more consistent with the mild collagenase treatment used in these studies. In fact, the minimum concentration of choriogonadotropin (50 pg/ml) which produced a detectable increase in progesterone synthesis was comparable to that which elicited a similar response in the sensitive mouse Leydig cell preparation [25] The amount of DNA per cell, 7.6 ± 0.5 pg, agreed with values reported by Liu and Gorski [26] for a cell suspension of rabbit ovaries.

Ovarian cells responded specifically to lutropin-like activity. Those samples with lutropin-like activity (lutropin and choriogonadotropin) produced maximal increases over control values. Somatotropin and prolactin did not stimulate progesterone synthesis nor did insulin (data not shown).

Time course of ^{125}I-labelled choriogonadotropin binding, cyclic [^{14}C]AMP formation and progesterone synthesis

As shown in Fig. 1 the formation of cyclic [^{14}C]AMP in the presence of 0.5 mM 1-methyl-3-isobutylxanthine was immediate and marked, rising to a value approx. 10-fold over controls in 15 min. Control values increased only slightly even though phosphodiesterase inhibitor was included in the incubation. Determination of the mass of cyclic AMP generated showed a similar 10-fold increase in the presence of 0.5 mM 1-methyl-3-isobutylxanthine. In the absence of 1-methyl-3-isobutylxanthine, a smaller increase in cyclic AMP was observed. Progesterone synthesis in the absence of 1-methyl-3-isobutylxanthine, on the other hand, increased less rapidly, but rose steadily and at an approximately linear rate for 60 min (Fig. 1).

The time course of ^{125}I-labelled choriogonadotropin binding of ovarian cells revealed that hormone binding approached equilibrium after 1 h of incubation, similar to choriogonadotropin binding to testis receptor [9]. Unlabelled choriogonadotropin displaced 50% of the ^{125}I-labelled choriogonadotropin at approx.

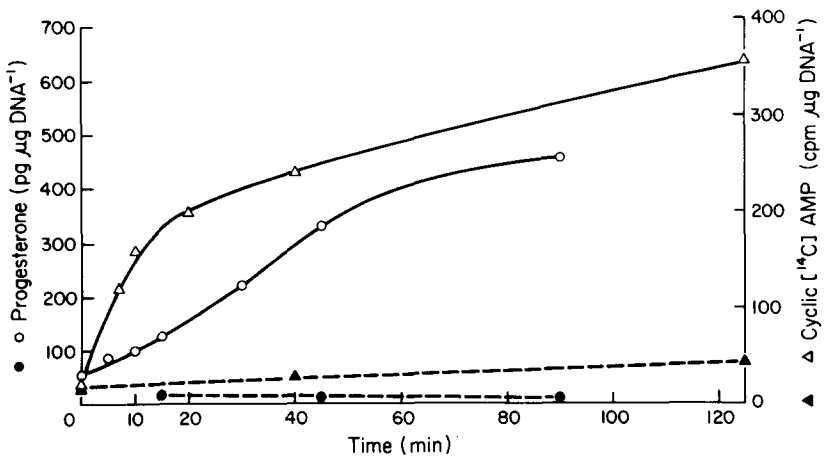

Fig. 1. Time course of cyclic [^{14}C]AMP formation and progesterone synthesis. Ovarian cells were incubated as described in Methods without (●,▲) and with (○,△) 67 ng/ml choriogonadotropin. Cyclic [^{14}C]-AMP (△,▲) [4] and progesterone (○,●) [2] were assayed at the indicated times.

40 ng/ml. The equilibrium dissociation constant, calculated from the graphical treatment of the above data according to Scatchard [23], was $1.7 \cdot 10^{-10}$ M (Fig. 2).

Effect of hormone concentration on choriogonadotropin binding, cyclic AMP formation and progesterone synthesis

The concentration dependence of ^{125}I-labelled choriogonadotropin binding, cyclic AMP production and progesterone synthesis is shown in Fig. 3. This experiment was performed in minimal essential medium using 0.1% gelatin as a protein substitute for fetal calf serum. Gelatin was used in order to achieve a chemically defined medium. The time course for ^{125}I-labelled choriogonadotropin binding, cyclic [^{14}C]AMP formation, and progesterone synthesis were unchanged in this medium compared to that supplemented with fetal calf serum (data not shown). The disparity between the binding, cyclic AMP and progesterone curves was striking. Measurement of cyclic AMP by the protein binding method did not alter these results (data not shown). There was a 20-fold difference between the concentrations of choriogonadotropin required to cause half maximal stimulation of cyclic AMP and progesterone accumulation. A lesser separation existed between the dose respone curves for the ^{125}I-labelled choriogonadotropin binding and cyclic AMP accumulation.

When the concentration dependence of the above parameters was determined in minimum essential medium-fetal calf serum, however, somewhat different results were obtained (Fig. 4). The curves for hormone binding and cyclic AMP formation were not shifted. However, the concentration of choriogonadotropin which produced 50% of the maximum response on the progesterone curve had shifted from 0.2 to 0.7 ng/ml. This effect was reproducible and corresponded to an approx. 3-fold increase in hormonally stimulated proges-

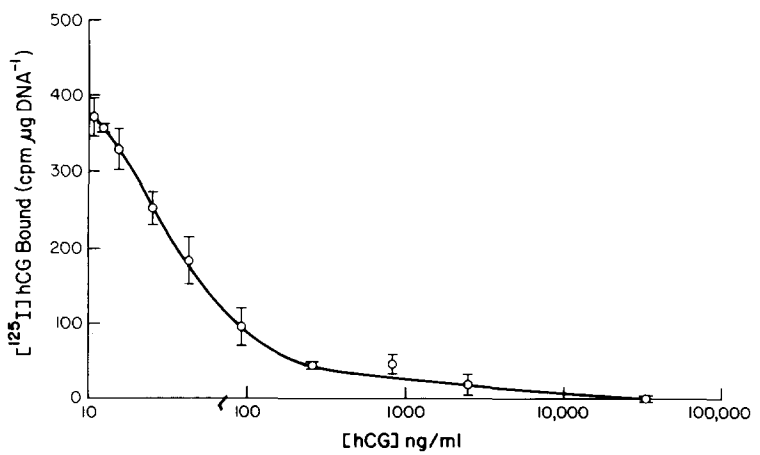

Fig. 2. Displacement of ^{125}I-labelled choriogonadotropin by unlabelled choriogonadotropin. Cells were incubated with 200000 cpm (3.3 ng) of ^{125}I-labelled choriogonadotropin in the presence of the indicated concentration of unlabelled hormone. Incubations were carried out for 1 h in quadruplicate. Specific binding was calculated by correcting total binding for counts bound in a 1000-fold excess of unlabelled choriogonadotropin [1]. Results are the mean ± S.E.

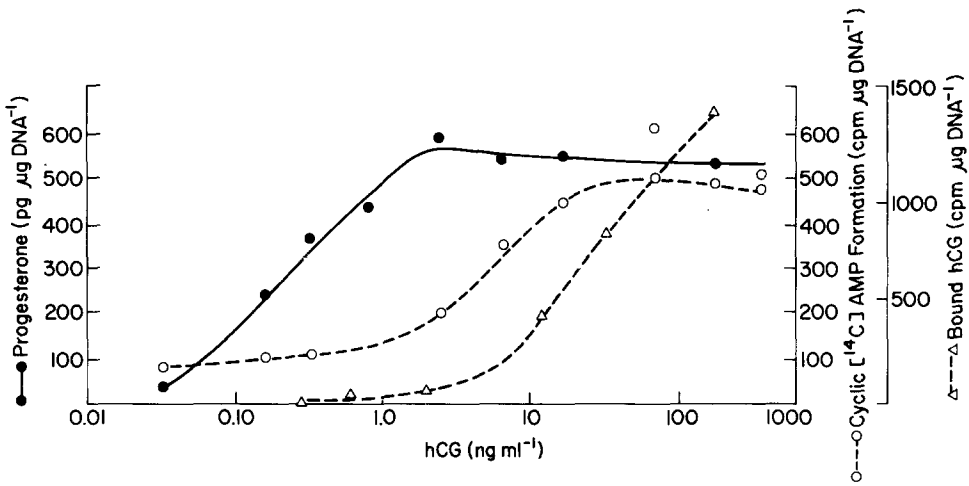

Fig. 3. ^{125}I-labelled choriogonadotropin binding, cyclic [^{14}C]AMP formation and progesterone synthesis in minimum essential medium-gelatin as a function of choriogonadotropin concentration. Ovarian cells were incubated in minimum essential medium-0.1% gelatin in the presence of 50 pg/ml to 500 ng/ml of unlabelled choriogonadotropin and cylic [^{14}C]AMP formation (○) [2] and progesterone synthesis (●) [2] were measured as described in Methods. Binding of ^{125}I-labelled choriogonadotropin was also measured at the indicated concentrations of labelled hormone (△) [2].

terone levels without an effect on basal levels. Another change in the progesterone curve was that it approached a maximum at a hormone concentration of the same order of magnitude as that for maximum binding and cyclic AMP formation. The presence of fetal calf serum appears to increase the upper limit on progesterone production, allowing close but non-linear correlation of ste-

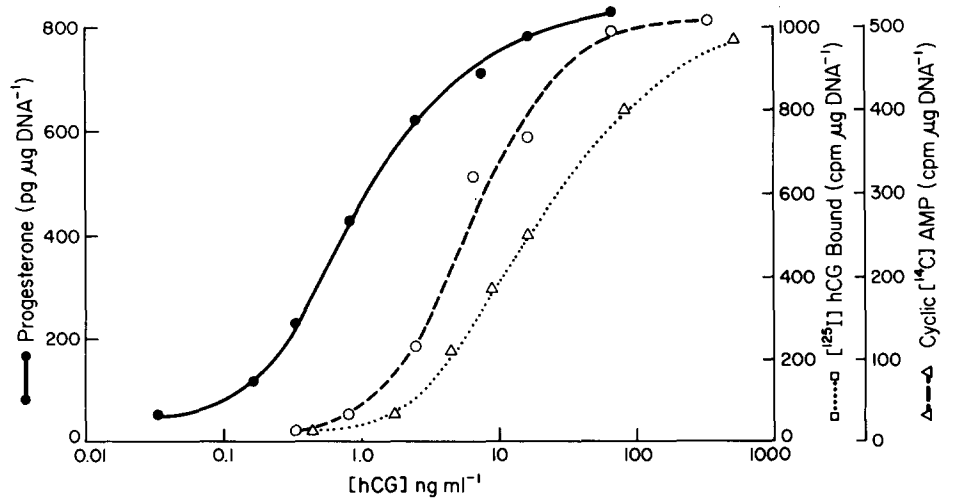

Fig. 4. ^{125}I-labelled choriogonadotropin binding, cyclic [^{14}C]AMP formation, and progesterone synthesis in response to choriogonadotropin in minimum essential medium-fetal calf serum. Ovarian cells were exposed to different concentrations of unlabelled choriogonadotropin in medium containing 10% fetal calf serum. Cyclic [^{14}C]AMP formation (△) [3] and progesterone synthesis (○) [4] were measured as described in Methods. Binding of ^{125}I at the concentrations of labelled hormone shown was also measured (□) [3].

TABLE I

THE EFFECT OF 1-METHYL-3-ISOBUTYLXANTHINE ON PROGESTERONE SYNTHESIS

Cells were incubated as described in Methods with 0.5 M 1-methyl-3-isobutylxanthine or 6.7 ng/ml choriogonadotropin. The untreated control progesterone level was 90 (83—97) pg/μg DNA (mean and range of duplicates) at zero time.

Time (min)	Progesterong (pg/μg DNA)	
	1-Methyl-3-isobutylxanthine	Choriogonadotropin
15	208 ± 15	198 (190—206)
30	247 ± 3	282 (260—304)
60	285 ± 8	443 (384—502)

roidogenesis with cyclic AMP formation over a wider range of hormone concentrations. A close correlation between gonadotropin binding and progesterone production has been reported under similar experimental conditions [32].

Also consistent with the result that small increases in cyclic AMP formation give rise to substantial increases in progesterone production, was the effect of 1-methyl-3-isobutylxanthine on progesterone synthesis. Slight increases in cyclic AMP formation due to the presence of 1-methyl-3-isobutylxanthine alone produced progesterone synthesis nearly equal to that of choriogonadotropin alone (Table I) up to 30 min of incubation.

Discussion

These experiments were designed to utilize a cell suspension system to correlate on a time and concentration basis, the binding of choriogonadotropin with cyclic AMP production and progesterone synthesis. Choriogonadotropin binding and cyclic AMP formation occurred rapidly, followed by progesterone synthesis, consistent with the second messenger role for cyclic AMP. It was found that wide disparities existed between the concentration vs. effect curves for hormone binding, cyclic AMP production and progesterone synthesis in chemically defind medium. Stimulation of progesterone synthesis had reached a maximum at concentrations of choriogonadotropin which elicited minimal increases in choriogonadotropin binding or cyclic AMP formation. This phenomenon of disparity between concentration effect curves for binding and cyclic AMP production and/or endpoint response has been reported for a number of hormonally responsive systems [3—11].

One possible explanation for the results is the theory that excess or spare receptors exist, which may not be coupled to adenylate cyclase and/or steroidogenesis [1—10]. Such spare receptors could account for the small (2-fold), but consistently observed difference, between the choriogonadotropin binding and cyclic AMP formation dose vs. response relationships. Alternatively, formation of cyclic AMP may be controlled by a feedback regulator [27], which could prevent activation of adenylate cyclase by all the occupied receptors.

The results obtained in minimum essential medium supplemented with fetal calf serum, however, support the alternative explanation. The fact that the ob-

served disparity between cyclic AMP formation and progesterone synthesis was lessened when the cells were exposed to a variety of anabolic stimuli found in serum, supports the hypothesis that such disparities are caused by other factors such as available concentrations of substrates, cofactors, activators or inhibitors involved the cascade of reactions leading from binding to physiological response [4,8]. The importance of NADPH, for instance, in the synthesis of progesterone has been demonstrated with rabbit ovarian homogenates [28]. Alternatively, since it is known that progesterone is metabolized rapidly to other steroids, fetal calf serum may exhibit a protecting effect by inhibiting this process [29]. Thus all of the receptors may be coupled to cellular events, but the response elicited by that coupling is dependent upon the cell's ability to act. This readiness, as expressed by intracellular levels of substrates, intermediates and the like, could in turn be controlled by serum factors, thus establishing a new level of control beyond that of gonadotropin or receptor concentration alone.

Acknowledgements

The authors wish to express their thanks to Dr. A. Rees Midgley, Jr. of this university for his helpful suggestions regarding this manuscript. Supported by N.I.H. grant HD06656 and General Research Support grant RR05383. Portions of the study were taken from the thesis of M.R. Clark submitted to the Graduate School of the University of Michigan in partial fulfillment of the requirements of the Ph.D. degree. M.R.C. is a pre-doctoral fellow of the Ford Foundation.

References

1 Rodbard, D. (1973) Adv. Exp. Med. Biol. 36, 342—364
2 Stephenson, R.P. (1956) Br. J. Pharmacol. 11, 379—393
3 Paton, W.D.M. (1970) CIBA Symposium on Molecular Properties of Drug Receptors (Porter, R. and O'Connor, M., eds.), pp. 3—32, J.A. Churchill, London
4 Sayers, G., Beall, R.J. and Seelig, S. (1972) Science 175, 1131—1133
5 Lang, U., Karlangis, G., Vogel, R. and Schqyzer, R. (1974) Biochemistry 13, 2626—2633
6 Boeynaems, J.M., VanSande, J., Pochet, R. and Dumont, J.E. (1974) Mol. Cell Endocrinol. 1, 139
7 Means, A.R., MacDOugall, E., Soderling, J.R. and Corbin, J.D. (1974) J. Biol. Chem. 249, 1231—1238
8 Hechter, O. and Braun, T. (1972) Structure-Activity Relationships of Protein and Polypeptide Hormones (Margoulies, M. and Greenwood, F.C., eds.), No. 241, pp. 212—227, Excerpta Medica, Amsterdam
9 Lindner, H.R., Tsafriri, O., Lieberman, M.E., Zor, U., Koch, Y., Bauminger, S. and Barnes, A. (1974) Recent Progress in Hormone Research (Greep, R.O., ed.), Vol. 30, pp. 79—138, Academic Press, New York
10 Catt, K.J. and Dufau, M.L. (1973) Nat. New Biol. 244, 219—221
11 Koch, Y., Zor, U., Chobsieng, P., Lamprecht, S.A., Pomerantz, S. and Lindner, H.R. (1974) J. Endocrinol. 61, 179—191
12 Marsh, J.M., Butcher, R.W., Savard, K. and Sutherland, E.W. (1966) J. Biol. Chem. 241, 5436—5440
13 Marsh, J.M. and Savard, K. (1966) Steroids 8, 133—138
14 Bajpai, P.K., Dash, R.J., Midgley, Jr., A.R. and Reichert, Jr., L.E. (1974) J. Clin. Endocrinol. Metab. 38, 721—724
15 Burton, K. (1956) Biochem. J. 62, 315—323
16 Kuo, J.F. and DeRenzo, E.C. (1969) J. Biol. Chem. 244, 2252—2260
17 Shimizu, H., Daly, J.W. and Creveling, C.R. (1969) J. Neurochem. 16, 1609—1619
18 Kawano, A., Gunaga, K.P. and Menon, K.M.J. (1975) Biochim. Biophys. Acta 385, 88—100
19 Krishna, G., Weiss, B. and Brodie, B.B. (1968) J. Pharmacol. Exp. Ther. 163, 379—385
20 Gilman, A.G. (1970) Proc. Natl. Acad. Sci. U.S. 67, 305—312

21 Brooker, G. (1974) Methods of Biochemical Analysis (Glick, D., ed.), Vol. 22, pp. 95—121, John Wiley and SOns, New York
22 Niswender, G.D. (1973) Steroids 22, 413—424
23 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660—672
24 Birnbaumer, L., Pohl, S.L. and Kaumann, A.J. (1974) Advances in Cyclic Nucleotide Research (Greengard, P. and Robison, G.A., eds.), Vol. 4, pp. 240—281, Raven Press, New York
25 Van Damme, M.-P., Robertson, D.M. and Diczfalusy, E. (1974) Acta Endocrinol. 77, 655—671
26 Liu, T.-C. and Gorski, J. (1971) Endocrinology 88, 419—426
27 Ho, R.-J. and Sutherland, E.W. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1773—1777
28 Scoon, V. and Major, P.W. (1972) J. Endocr. 52, 147—159
29 Armstrong, D.T., Kraemer, M.A. and Hixon, J.E. (1975) Biol. Reprod. 12, 599—608
30 Duddleson, W.G., Midgeley, Jr., A.R. and Niswenden, G.D. (1972) Comput. Biomed. Res. 5, 205—217
31 Cautracasas, P. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1264—1268
32 Midgley, Jr., A.R. and Dash, R.J. (1974) J. Steroid Biochem. 4, 348; Abst. 4th Int. Congr. Hormonal Steroids, Mexico City, September 2—7, 1974