Biochimica et Biophysica Acta, 413 (1975) 283–290

BBA 77132

CHOLERAGEN STIMULATES STEROIDOGENESIS AND ADENYLATE CYCLASE IN CELLS LACKING FUNCTIONAL HORMONE RECEPTORS

J. WOLFF and G. HOPE COOK

National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014 (U.S.A.)

(Received June 3rd, 1975)

SUMMARY

Choleragen stimulates steroid secretion and adenylate cyclase in three cell lines, adrenal tumor line (Y-1), a corticotropin-resistant mutant derived from Y-1 called OS-3, and a receptor-deficient Leydig tumor line (I-10). Sensitivity for half-maximal stimulation varies from 3 to 36 pM choleragen, the I-10 line being the most sensitive. Latency before the onset of steroidogenesis is longer in OS-3 and I-10 cells than in the Y-1 line. In both OS-3 and I-10 cells choleragen stimulates adenylate cyclase whether ITP or 5′-guanylylimidodiphosphate is the regulatory cofactor used. In addition to the responses of the receptor-deficient lines, choleragen does not, during its latency, block the response to corticotropin in Y-1 cells; corticotropin does not block binding of ^{125}I-labeled choleragen to Y-1 cells; gangliosides do not interfere with the corticotropin-induced stimulation of Y-1 cells.

We conclude that the corticotropin and choleragen receptors are different.

INTRODUCTION

It has recently been shown that steroid production by adrenal cells in culture (line Y-1) is extremely sensitive to stimulation by choleragen (cholera toxin) [1, 2]. This response was apparently produced through stimulation of adenylate cyclase, and the steroid products were identical to those formed after corticotropin (ACTH) stimulation (20α-dihydroprogesterone and 11β-hydroxy-20α-dihydroprogesterone) [1]. Unlike the response to ACTH, but like the response of other tissues to choleragen, the effect on adrenal cells had a latent period of approx. 60 min and was reversed very slowly upon removal of the toxin and washing, or upon subsequent addition of antibody to the system [1].

It was important to learn whether or not ACTH and choleragen occupied the same receptor. To this end we have employed a spontaneous, near diploid mutant (OS-3) derived from the Y-1 mouse adrenal tumor cell line by Schimmer [3]. This line cannot respond to ACTH, but steroid production is readily stimulated by cyclic AMP, suggesting that the steroidogenic pathway has remained intact. Schimmer [4]

Abbreviation: ACTH, corticotropin.

has shown that these cells exhibit good cyclase activity with F^-, and Londos et al. [5] have demonstrated good response to 5'-guanylylimidodiphosphate, a GTP analogue currently believed to stimulate the catalytic activity at a site other than the F^- site and a probable intermediate in the action of hormones. In the present study we use this cell line, as well as additional data derived from the Y-1 parent to show that the ACTH and choleragen receptors are distinct entities.

METHODS

The ACTH-responsive Y-1 mouse adrenal tumor cell line [6] was obtained from the American Type Culture Association. A mutant of this line that is ACTH resistant, but responds to cyclic AMP with steroidogenesis, was cloned by Schimmer [3] and was generously supplied by him. The lutropin-unresponsive Leydig tumor cell line, I-10, was obtained from the American Type Culture Association [7]. All lines were maintained in 100 mm Falcon petri dishes in Ham's F-10 medium containing 12.5% horse serum, 2.5% fetal calf serum, penicillin (60 μg/ml) and streptomycin (1.35 μg/ml). The atmosphere was water-saturated O_2/CO_2 (95 : 5, v/v) at 37 °C. Medium was changed three times per week.

Choleragen was generously supplied by Dr R. S. Northrup, and prepared by Dr R. A. Finkelstein, University of Texas, under contract to the National Institute of Allergy and Infectious Diseases. The ACTH used was either ACTH (ACTHAR-Armour) or Synacthen ($^{1-24}$ACTH), the 1-24 N-terminal polypeptide of ACTH. The lutropin used was either the National Institutes of Health bovine standard (NIH-LH-B9) generously provided by the NIH study section, or a pure preparation made, and generously provided, by Dr John Pierce. Mixed gangliosides were the gift of Dr E. G. Trams. ^{125}I-labeled choleragen was kindly supplied by Dr A. Levy. It had a specific activity of 25 Ci/g. Binding was assayed on harvested Y-1 cells exposed to choleragen in Dulbecco's phosphate-buffered saline for 15 min at 37 °C. The cells were then separated from the medium by centrifugation in microfuge tubes, medium was thoroughly removed, and the pellets were counted. Non-specific absorption was measured in the presence of excess choleragen (12 μg/ml).

Y-1 cells secrete primarily 20α-dihydroprogesterone (pregn-4-en-20α-hydroxy-3-one) and 11β-hydroxy-20α-dihydroprogesterone (pregn-4-en-11β, 20α-diol-3-one) [8]. Since the latter compound was unavailable, the fluorimetrically measured steroid output is expressed in equivalents of 20α-dihydroprogesterone. All results are duplicate determinations on each of at least two dishes and all experiments were done with at least three separate batches of cells. The standard error of the method was nearly always $< \pm 5\%$ of the mean of four determinations done on each point. Protein was determined by the method of Lowry et al. [9] on cells harvested by scraping after washing in phosphate-buffered saline, sedimentation, and subsequent sonication in water.

Adenylate cyclase was determined by the method of Salomon et al. [10]. Cells were exposed to choleragen for 11 h, harvested in 0.25 M sucrose, 3 mM Tris · HCl, pH 7.4, 1 mM dithiothreitol, sedimented, and homogenized in a tight-fitting Dounce homogenizer. The $11000 \times g$ pellet was washed twice in the above medium and then suspended in it. Assays on the fresh material, or that stored in liquid N_2, were carried out under incubation conditions previously described [10].

RESULTS AND DISCUSSION

The OS-3 cells responded well to cyclic AMP and its dibutyryl derivative, which was about twice as potent as cyclic AMP under the present incubation conditions (Fig. 1A). On the other hand, steroid secretion by the OS-3 cells employed here was not increased by addition to the culture medium of very large concentrations of ACTH or synacthen (Fig. 1B). The behavior of this cell line is thus still quite similar to that originally reported by Schimmer [3, 4]. Low concentrations of choleragen stimulated steroid output by OS-3 cells to approximately the same maximum as is obtainable with cyclic AMP (Fig. 1B). The values are not corrected for the latent period that occurs before onset of the steroidogenesis stimulus (see below). This would increase the calculated values by 15–20% with the present incubation times. The concentration required to obtain half-maximal steroid output was 36 pM (3 ng/ml) which is in the range previously reported for the Y-1 cells (15 pM) [1]. Choleragen produced rounding of these cells, as it does in Y-1 cells, and the number of cells rounded corresponded in a rough way to the onset and intensity of steroid secretion. During the course of this work similar results on ACTH-resistant mutants of Y-1 adrenal cells were reported [12, 13].

Unlike the choleragen response of Y-1 cells, OS-3 cells show a longer latent period after exposure to the toxin (Fig. 2). This amounted to approx. 3 h rather than the 1 h delay exhibited by Y-1 cells [1]. Although the response in steroidogenesis to cyclic AMP showed a short lag of approx. 30 min, this was not long enough to account for the difference between these cells and the parent Y-1 strain. Variations

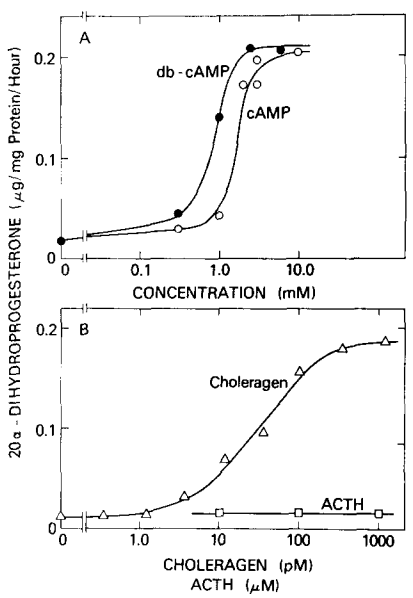

Fig. 1. The steroidogenic response of OS-3 cells to ACTH, cyclic AMP (cAMP) dibutyryl cyclic AMP (db-cAMP) and choleragen. Incubation times are 15–17 h and all points are the means of duplicate determinations on three dishes. Steroid secretion is listed as fluorescence equivalents of 20α-dihydroprogesterone. Note that concentrations are mM for the cyclic nucleotides in A, whereas they are μM ACTH and pM for choleragen (based on a molecular weight of 84000).

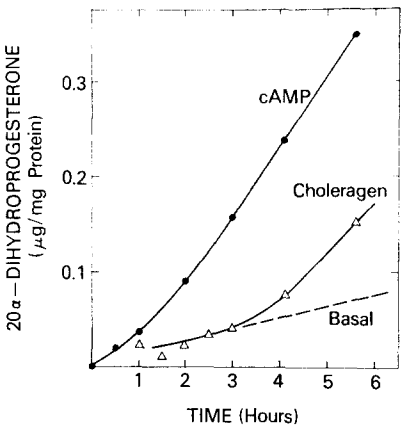

Fig. 2. Time curve for the cyclic AMP and choleragen responses of OS-3 cells. Dishes were washed twice with 37 °C medium and exposed to 3 mM cyclic AMP or 30 μg/ml of choleragen. Steroid output is listed as fluorescence equivalents of 20α-dihydroprogesterone. 1-ml samples were then taken at the intervals indicated and assayed fluorimetrically. All results are the means of three dishes.

in latent period for the choleragen response have been reported in other tissues [14–18] but the reason for it remains to be explained.

Evidence has been presented that the choleragen receptor on cell surfaces requires the presence of GM_1 ganglioside and that this ganglioside, added exogenously, can prevent the biological responses to choleragen [19, 20]. Added ganglioside might thus be expected to differentiate between these two stimulators. As is the case with the Y-1 cell line [1], mixed gangliosides, containing ganglioside GM_1, readily prevented the choleragen effect on steroidogenesis or cell shape, if added before the toxin. The same concentration of gangliosides had no effect on the steroidogenic response of Y-1 cells to ACTH (Table I). On the other hand, the steroidogenic response of OS-3 cells was inhibited to the same extent as that of Y-1 cells when choleragen was used.

While evidence has previously been presented that choleragen-induced steroidogenesis in Y-1 cells proceeds by way of the adenylate cyclase system [1], this

TABLE I

THE EFFECT OF GANGLIOSIDES ON THE STIMULATION OF STEROIDOGENESIS IN Y-1 AND OS-3 CELLS

Mixed gangliosides added 10 min before stimulators. Incubation time 13 h. Steroid measured fluorimetrically and expressed as equivalents of 20α-dihydroprogesterone (mean ±S.E.). The results are expressed as μg steroid/mg per h.

Stimulator	Y-1 Cells Ganglioside		OS-3 Cells Ganglioside	
	0	12 μg/ml	0	12 μg/ml
Control	0.14±0.002	0.15±0.005	0.010±0.003	0.010±0.002
ACTH (25 munits/ml)	0.75±0.009	0.76±0.015	—	—
Choleragen (0.36 nM)	0.57±0.001	0.17±0.016	0.138±0.006	0.011±0.001

TABLE II

THE EFFECT OF CHOLERAGEN TREATMENT ON ADENYLATE CYCLASE OF STEROID SECRETING CELLS

Cells were exposed to choleragen (300 μg/ml) for 11 h. They were then harvested in buffered 0.25 M sucrose, 1 mM dithiothreitol containing the same concentration of choleragen (where indicated), and were homogenized in a loose fitting Dounce homogenizer. The 8000 × *g* pellet was washed twice in the same medium and was then used to start the reaction. Gpp(NH)p stands for 5′-guanylylimidodiphosphate, ITP for 5′-inosine triphosphate.

Cell line	Nucleoside triphosphate (50 μM)	Adenylate cyclase (nmol/mg per 10 min)	
		Control	Choleragen (300 ng/ml)
Y-1	ITP	0.11	1.56
	Gpp(NH)p	1.06	1.77
OS-3	ITP	0.04	1.20
	Gpp(NH)p	0.36	1.05
I-10	ITP	0.07	0.49
	Gpp(NH)p	0.30	0.46

had to be established for the OS-3 cell line since steroid production can be stimulated apparently without involvement of changed cyclic AMP levels [8]. As shown in Table II, excess choleragen caused activation of adenylate cyclase in membranes from OS-3 cells that persisted through the preparative procedure. It resembled the stimulation seen in Y-1 cells. The relative degree of stimulation was greater when ITP was the nucleotide rather than 5′-guanylylimidodiphosphate (Gpp(NH)p); this is primarily the result of a large stimulatory effect of Gpp(NH)p on basal activity as noted previously [5]. In the presence of choleragen there was much less difference between the nucleotides. Choleragen-stimulated cells were, however, less responsive to F^- than control cells and showed lower yields of cyclic AMP with F^- than without (data not shown).

Leydig tumor cells

Stimulation of steroid production could also be elicited in the I-10 Leydig tumor cell line developed by Shin [7]. Steroidogenesis (mostly 20α-hydroxy-4-pregnen-3-one and some progesterone) was reported to be stimulated by cyclic AMP but not by lutropin, thyrotropin or human choriogonadotropin [7]. However, as shown in Fig. 3, the I-10 cells available to us were not entirely unresponsive to lutropin, although they required very large concentrations of the hormone to produce even a small steroidogenic response. We have also observed stimulation with impure human choriogonadotropin, again at huge concentrations. Whether this implies reversion from the original clones or increased sensitivity of the present assay remains to be clarified. Like the previous report [7], our clone of I-10 cells was very sensitive to cyclic AMP and exhibited half maximal steroid output at 2 μM cyclic AMP (Fig. 3).

Choleragen produced a maximal response like that of cyclic AMP. The sensitivity of the I-10 cells of the toxin was even greater than that of the Y-1 adrenal cells and yielded half maximal stimulation at 3–10 pM choleragen in various experiments (Fig. 3). The latent period before onset of steroidogenesis was of the same order as

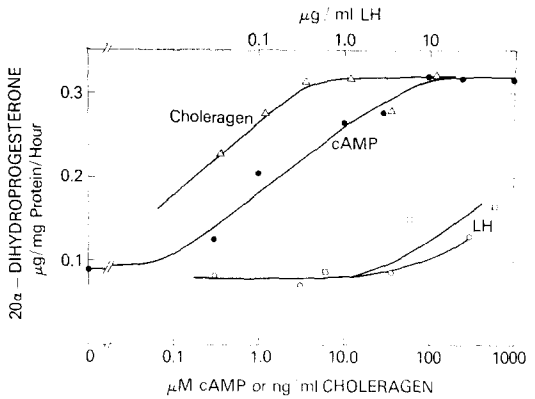

Fig. 3. The steroidogenic response of I-10 cells in response to choleragen, cyclic AMP or lutropin (LH). Washed cells were exposed for 16 h and the medium was then assayed for steroid content on the basis of fluorescence equivalent to standard solutions of 20α-dihydroprogesterone extracted and treated in an identical manner. The open circles are NIH standard. The open squares are results obtained with purified lutropin obtained from Dr. John Pierce. All results are means of triplicates.

that for the OS-3 cells, i.e. about 3–4 h. Mixed gangliosides blocked the choleragen response (data not shown). That choleragen also stimulated the adenylate cyclase system in these cells is shown in Table II. As in the other two cell lines, ITP permits the demonstration of a greater relative choleragen effect whereas the maximum obtained was not a function of the nucleotide. Together with the high sensitivity toward cyclic AMP shown by these cells, these data suggest that choleragen probably stimulates steroidogenesis in the I-10 cell line through the adenylate cyclase system.

The above data on OS-3 and I-10 cells are consistent with the interpretation that the receptor domains for ACTH or lutropin and choleragen are probably different. Additional means were sought to confirm such a hypothesis. An approach to the question of any possible sharing of these receptor sites is afforded by the fact that, despite rapid binding [1, 20], choleragen exhibits a latent period of approx. 60 min before steroidogenesis is stimulated [1]. Since ACTH acts immediately, choleragen might then block ACTH stimulation in Y-1 cells if it shared the receptor domain with the hormone. That this did not occur is shown in Table III, in which

TABLE III

THE EFFECT OF CHOLERAGEN ON THE RESPONSE TO ACTH IN Y-1 CELLS

Incubation time 45 min. Calculated to hourly rate ±S.E. Choleragen, where present, was added 10 min before the ACTH.

Treatment	Steroid secretion (μg/mg per h)
No additions	0.21±0.016
Choleragen (0.25 μg/ml)	0.22±0.014
ACTH (25 (munits/ml))	2.49±0.086
Choleragen+ACTH	2.53±0.130
$^{1-24}$ACTH (2.4 μM)	2.07±0.067
Choleragen+Synacthen	1.99±0.094

the 45-min steroid production, following Synacthen or ACTH addition, is compared in the absence and presence of excess choleragen. The presumptive conclusion is thus that the sites for ACTH or choleragen are different.

Additional evidence derives from preliminary studies in Y-1 cells on the binding of ^{125}I-labeled choleragen [1, 20]. ACTH had no effect on the binding process up to concentrations of 37.5 μM (the highest level tested). This again suggests that there are no interactions between these two receptors in the Y-1 cell. However, since the binding of choleragen appears to be reversible with difficulty upon dilution, etc. [19, 20], these results cannot be interpreted unequivocally.

Several conclusions seem permissible from the experiments presented above. (A) Although the latent periods for response show some differences, all three steroid-secreting cell lines show a similar sensitivity to choleragen with respect to the concentration required to enhance steroid secretion to half-maximal values (3–36 pM). (B) The receptor for choleragen is different from the receptor for ACTH (and lutropin) and there appears to be very little interaction between these two receptors. The evidence for this may be summarized as follows. (1) The loss of functional hormone receptors in no way influences the ability of OS-3 or I-10 cells to respond to the toxin. (2) The toxin, which is bound rapidly [1, 20], does not interfere with the response to ACTH in Y-1 cells when this is tested during the latency of the choleragen effect (Table II). (3) ACTH did not influence the binding of labeled choleragen to Y-1 cells. (4) Mixed gangliosides did not affect the response to ACTH in Y-1 cells, but did block the response to choleragen. (5) Finally, very recently, Haksar et al. [21] have shown that treatment of adrenal cells with neuraminidase enhanced the cyclic AMP and steroid responses to choleragen while it diminished these responses to ACTH.

Thus, taken together, the five pieces of evidence strongly support the view that the receptors for ACTH and choleragen are separate and different from each other.

ACKNOWLEDGEMENT

We are indebted to Mr William Hardy for his expert assistance.

REFERENCES

1 Wolff, J., Temple, R. and Cook, G. H. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2741–2744
2 Donta, S. T., King, M. and Sloper, K. (1973) Nat. New Biol. 243, 246–247
3 Schimmer, B. P. (1969) J. Cell. Physiol. 74, 115–122
4 Schimmer, B. P. (1972) J. Biol. Chem. 247, 3134–3138
5 Londos, C., Salomon, Y., Lin, M. C., Harwood, J. P., Schramm, M., Wolff, J. and Rodbell, M. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3087–3090
6 Buonassisi, U., Sato, G. and Cohen, A. I. (1962) Proc. Natl. Acad. Sci. U.S. 48, 1184–1190
7 Shin, S. I. (1967) Endocrinology 81, 440–448
8 Temple, R. and Wolff, J. (1973) J. Biol. Chem. 248, 2691–2698
9 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
10 Salomon, Y., Londos, C., Rodbell, M. (1974) Anal. Biochem. 58, 541–548
11 Wolff, J. and Jones, A. B. (1971) J. Biol. Chem. 246, 3939–3947
12 Wishnow, R. M. and Feist, P. (1974) J. Infect. Dis. 129, 690–695

13 Donta, S. T. (1974) J. Infect. Dis. 129, 728–731
14 Schafer, D. E., Lust, W. D., Sircar, B. and Goldberg, N. D. (1970) Proc. Natl. Acad. Sci. U.S. 67, 851–856
15 Sharp, G. W. G. and Hynie, S. (1971) Nature 229, 266–269
16 Kimberg, D. V., Field, M., Johnson, J., Henderson, A. and Gershon, E. (1971) J. Clin. Invest. 50, 1218–1231
17 Lichtenstein, L. M., Henney, C. S., Bourne, H. R. and Greenough, W. B. (1973) J. Clin. Invest. 52, 691–697
18 Mashiter, K., Mashiter, G. D., Hauger, R. L. and Field, J. B. (1973) Endocrinology 92, 541–549
19 Van Heyningen, W. E., Carpenter, C. J., Pierce, N. F. and Greenough, W. B. (1971) J. Infect. Dis. 124, 415–418
20 Cuatrecasas, P. (1973) Biochemistry 12, 3547–3581
21 Haksar, A., Maudsley, D. V. and Peron, F. C. (1972) Biochim. Biophys. Acta 381, 308–325