Proc. Nat. Acad. Sci. USA
Vol. 70, No. 10, pp. 2741–2744, October 1973

Stimulation of Steroid Secretion in Adrenal Tumor Cells by Choleragen

(cholera enterotoxin/adenylate cyclase/adrenocorticotropic hormone)

J. WOLFF, R. TEMPLE, AND G. HOPE COOK

National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Robert W. Berliner, June 14, 1973

ABSTRACT **Choleragen, the pure protein from cholera toxin, stimulates steroid secretion by Y-1 adrenal tumor cells in culture. The secreted steroids are the same as seen after addition of adrenocorticotropic hormone. Half-maximal stimulation occurs at 15 pM; stimulation is essentially irreversible by washing and partially reversible (for about 1 hr) by antibody, and there is a latent period of about 60 min before stimulation is seen. Stimulation of adenylate cyclase occurs at about 30-fold higher choleragen concentrations. Gangliosides inhibit choleragen stimulation when added before but not after the toxin. Lipopolysaccharides from *Escherichia coli*, *Salmonella typhosa*, and *Serratia marcescens* also stimulate steroid secretion, but are less potent than choleragen.**

Evidence has accumulated that indicates that cholera enterotoxin (choleragen) produces some, and perhaps all, of its effects by stimulation of intestinal adenylate cyclase (1–6). Activation appears to require intact cells and is not obtained with homogenates. Moreover, choleragen-induced cyclase stimulation exhibits a considerable latent period, which apparently occurs after the binding step, but which is not understood. Choleragen, which has been purified to homogeneity and has a molecular weight of 84,000 (7), also appears to stimulate adenylate cyclase of other tissues such as fat cells (8), platelets and liver (9, 10) leukocytes (11–14), and thyroid tissue (15). As part of our effort to study the spatial and/or functional relationships between different membrane receptors in Y-1 adrenal cells, we attempted to characterize the response to choleragen by these cells. Choleragen is a more potent stimulator of adrenal-cell adenylate cyclase and steroid production than is adrenocorticotropic hormone (ACTH).

MATERIALS AND METHODS

The Y-1 mouse adrenal-tumor line, which is responsive to ACTH and was introduced into culture by Buonassisi *et al.* (16), was obtained from the American Type Culture Assoc. Cells were maintained in 100-mm (diameter) 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 (135 μg/ml). The atmosphere was water-saturated 5% CO_2–95% air at 37°. Medium was changed every other day.

Choleragen and anticholeragen serum were generously supplied by Dr. R. S. Northrup. Choleragen was prepared by Dr. R. A. Finkelstein, University of Texas, under contract to the National Institute of Allergy and Infectious Diseases. Horse antiserum was also prepared by Dr. R. A. Finkelstein (17). It contained 4000 units/ml, where one unit neutralizes 40 ng of choleragen. Endotoxins were obtained from Difco. Pneumoccus type I polysaccharide, prepared by Ely Lilly Co. under contract to the NIAID, was generously supplied by Dr. R. Horton. Plant gums, ACTH, and dextran sulfate (molecular weight 500,000) were obtained from Sigma; purified type II mixed gangliosides were generously supplied by Dr. E. G. Trams; polyvinyl sulfate was from K & K Laboratories; and poly(L-aspartic acid) (molecular weight 7000–8000) was from Schwartz BioResearch.

Steroid secretion into culture medium was measured either by spectrophotometry (long-term experiments) or by a fluorometric method (18). Steroid products were identified by thin-layer chromatography as 20α-dihydroprogesterone (Δ^4-pregnen-20α-3-one) and 11β-hydroxy-20α-dihydroprogesterone (Δ^4-pregnen-11β,20α-diol-3-one) in four solvents (19). The products resulting from choleragen stimulation were the same as those seen after ACTH stimulation.

Adenylate cyclase was measured in the presence of 50 μM ITP, as described for thyroid membranes (20, 21). Cells were harvested and washed in phosphate-buffered saline (pH 7.4) containing the same choleragen concentrations as the incubation medium. They were then broken with a Dounce homogenizer in 0.25 M sucrose–20 mM Tris·HCl (pH 7.5) and centrifuged at 11,000 × *g* for 10 min. The washed pellet, suspended in buffered sucrose, served as the enzyme and was added to start the reaction.

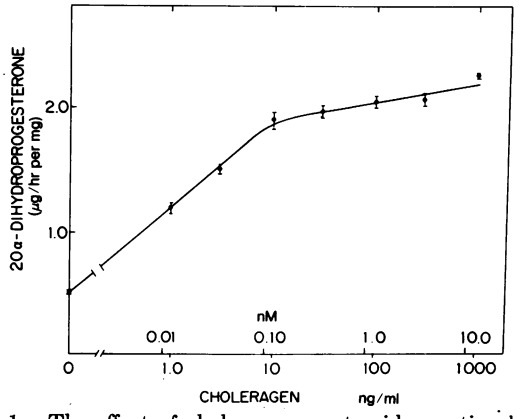

FIG. 1. The effect of choleragen on steroid secretion by Y-1 adrenal tumor cells. Cells were incubated for 16 hr in fresh F-10 medium containing 12.5% horse serum, 2.5% fetal-calf serum, and the indicated amounts of choleragen. The assay is by the spectrophotometric method. All points are quadruplicate determinations ±SE of the mean.

Abbreviation: ACTH, adrenocorticotropic hormone.

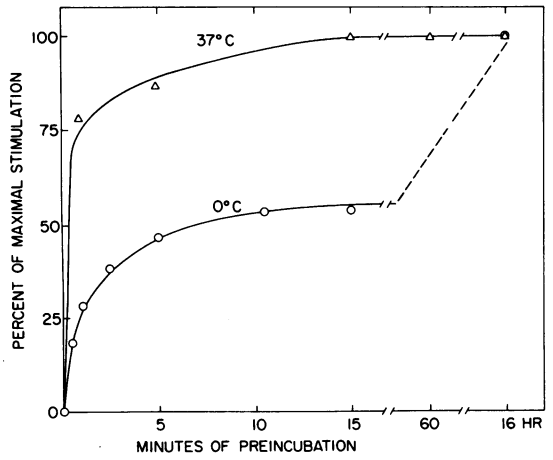

FIG. 2. Reversibility of choleragen stimulation of steroid secretion by washing. Y-1 cells were bathed in fresh F-10 medium at either 37° or 0° and exposed to 50 ng/ml of choleragen for different time periods. At the end of the period medium was removed; dishes were washed with two 5.0-ml portions of medium and then with two 5.0-ml portions of phosphate-buffered saline at the temperatures indicated. They were then placed in F-10 containing 12.5% horse serum, and 2.5% fetal-calf serum at 37° for 16 hr. Media were assayed spectrophotometrically. For the 16-hr incubation (and preincubation combined), choleragen was present throughout. All values are means of quadruplicate determinations.

RESULTS AND DISCUSSION

Choleragen stimulation of steroid production in Y-1 adrenal tumor cells is concentration dependent (Fig. 1). The concentration required for half-maximal stimulation from three separate concentration curves averaged 1.3 ng/ml. On the basis of a molecular weight of 84,000 this amounts to a concentration 15 pM. Half-maximal stimulation with ACTH occurs at 0.5–1 mU/ml (16) and was 1.5 mU/ml in our sublines. This amounts to a concentration of 1.0–2.0 nM. There is thus a markedly greater sensitivity to choleragen. Although greater stability of choleragen may contribute to this difference, a similar difference in potency was observed in short-term (2- or 3-hr) secretion periods. No additive effect on steroid secretion was seen when maximally effective concentrations of ACTH and choleragen were added to each other (Table 1). The order of potency of choleragen on Y-1 cells appears to be greater than that seen with intestinal mucosa (3–6) but is comparable to that observed with leukocytes (12, 13).

TABLE 1. *Lack of additive effects of ACTH and choleragen stimulations of steroid secretion*

Treatment	Concentration	Steroid secretion (μg/mg per hr)
Control	—	0.66
ACTH	10 mU/ml	2.47
Choleragen	30 ng/ml	2.92
ACTH + choleragen	10 mU/ml + 30 ng/ml	3.14

Y-1 cells were incubated in F-10 medium containing 12.5% horse serum and 2.5% fetal-calf serum for 16 hr. Spectrophotometric assay.

TABLE 2. *Reversal of choleragen-stimulated steroid secretion in Y-1 cells by antibody*

Choleragen (20 ng/ml)	Addition of antibody (min) Before choleragen	Addition of antibody (min) After choleragen	Steroid produced (μg/mg per hr)
—	0	0	0.45
—	2		0.40
+	2		0.47*
+		1	0.50
+		5	0.61
+		20	0.78
+		60	1.14
+		15 hr	1.32

Spectrophotometric assay for 15-hr incubation after antibody addition. Antibody was present in 8-fold excess. In the last sample antibody was added just before harvesting. All experiments were at 37°.

* Identical results were obtained if antibody and choleragen were mixed just before addition to cells.

In an attempt to measure reversal of the reaction, cells were exposed to 50 ng/ml of choleragen for various time periods at 37° and 0°. They were then washed with two changes of phosphate-buffered saline and two changes of fresh medium and subsequently incubated in fresh medium for 16 hr to measure steroid production. Binding was rapid at 37°, and the effect on steroid production was virtually irreversible after 1 min of exposure followed by washing (Fig. 2), as for intestinal mucosa (22, 23) and leukocytes (12). The reaction could be slowed at 0° but was still half-maximal between 5 and 10 min at this temperature.

In contrast to the washing procedure, choleragen remained available to reversal by addition of antibody for a longer time. Even at 20 min, more than half of the expected stimulation of steroid production could still be prevented by antibody (Table 2). The duration of the reversibility appears to be longer than that seen in intestinal mucosa (19, 20), leukocytes (14), and thyroid tissue (15).

The choleragen effect is characterized by a latent period of 15–60 min in various tissues (2–4, 8, 9, 13, 15). This latency was elucidated in Y-1 adrenal cells with the fluorometric assay. When confluent cultures of adrenal tumor cells were exposed to 200 ng of choleragen per ml, steroid production was not stimulated for about 60 min even with this very large concentration (Fig. 3). In contrast, ACTH exhibited no latency. Once the latent period had ended, the two agents stimulated steroid secretion at comparable rates. The latent period was not a function of the concentration of choleragen, since exposure to 30 ng of choleragen per ml yielded an identical delay before steroid production was stimulated. The latent period is also not a function of the assay method since ACTH-stimulated steroid secretion can be detected in a few minutes. The data show, furthermore, that the stimulation persists for at least 15–18 hr after removal of choleragen. Whether this is the result of persistent binding or is due to the persistence of stimulation in subsequent steps for the biosynthetic pathway for steroids is currently being studied.

Although the usual mechanism for stimulation of steroid production probably occurs by way of the hormone receptor–

TABLE 3. *Effect of bacterial lipopolysaccharides and plant gums on steroid secretion*

Compound	Concentration (μg/ml)	% Maximal steroid secretion
Choleragen	0.0013	50
Lipopolysaccharides		
E. coli (0111:B_4) (B)	0.005	50
S. marcescens (T)	0.01	50
S. typhosa (0901) (B)	0.03	50
Gums		
Arabic	1000	0
Tragacanth	1000	0
Ghatti	1000	0
Karaya	1000	0
Xanthan	120	50
Pneumococal type I	20	0
polysaccharide	84	10

Spectrophotometric assay; incubation time was 16 hr.

adenylate cyclase–cyclic AMP pathway (24), we have recently shown that equivalent degrees of stimulation of steroid formation and secretion *can* result without detectable intervention of this pathway (19). It was, therefore, important to ascertain whether or not choleragen stimulated the adenylate cyclase system in Y-1 adrenal tumor cells in a manner analogous to that reported for other tissues (3, 4, 6, 10, 12, 15). With sufficient concentrations of choleragen, adenylate cyclase can be stimulated 30- to 40-fold, i.e., up to about $^3/_4$ of the fluoride-stimulated response (Fig. 4). Unlike ACTH, activation of cyclase by choleragen persisted through the washing procedure and preparation of crude membranes, and the presence of choleragen in the washing medium is not necessary. In contrast to ACTH, addition of choleragen to crude cell-free membrane preparations did not activate the cyclase. As has been reported for ACTH in normal adrenal cells (25), the cyclase response to choleragen is much less sensitive to stimulation than is the steroid response. Half-maximal stimulation of adenylate cyclase occurred at 36 ng/ml or about 30-times the concentration required for steroid secretion (see Fig. 1). As in the case of ACTH, presumably only a small fraction of the cyclic AMP that can be generated is sufficient to stimulate steroid production maximally (26).

Since it has been reported that certain entero- and endotoxins also stimulate adenylate cyclase (15, 27, 28), their effect on steroid production in Y-1 cells was investigated. Crude lipopolysaccharides from *E. coli*, *Salmonella typhosa* and *Serratia marcescens* stimulated steroid production in these cells (Table 3). The potency (on a weight basis) was lower than that of choleragen and varied considerably among the endotoxins. There appeared to be no difference if the lipopolysaccharide was produced by the Boivin or Westphal methods, and only data for the former are shown. To investigate possible effects of the carbohydrate portion of these toxins, we studied various polysaccharides. A polysaccharide from a Gram-positive organism (pneumoccus type I) was ineffective at the concentrations studied, whereas gum xanthan from *Xanthomonas campestris* stimulated steroid production (Table 3), with a half-maximal stimulation of steroid output at the rather high concentration of 120 μg/ml. Plant gums failed to stimulate steroid production in the adrenal tumor cells.

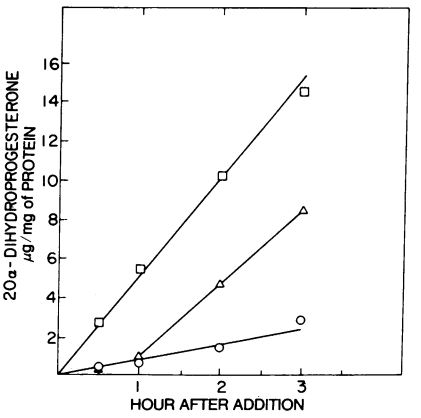

FIG. 3. Time course of steroid secretion by Y-1 cells after addition of ACTH (10 mU/ml) or choleragen (200 ng/ml). 1-ml Samples were withdrawn from the dishes at the intervals indicated and assayed fluorometrically. All values are means of triplicate determinations. □, ACTH; △, choleragen; ○, control.

During attempts to identify the choleragen receptor material, van Heyningen *et al.* (29) discovered that gangliosides interact with choleragen and prevent the effects of the toxin. This activity appears to reside in the GM1 fraction (30). Similar findings have been obtained by Davies *et al.* (31). Addition of mixed gangliosides *before* addition of choleragen prevented stimulation of steroid secretion (Table 4). Once choleragen is bound to cells the effect is not reversed by ganglioside, and even 12,000 ng/ml caused only a 20% decrease in the maximal steroid production resulting from choleragen treatment (Table 4). On the basis of a molecular weight of about 1500 for the ganglioside (ceramide monosialyl tetrahexoside), this amounts to a molar ratio of about 22,000. Polyanions such as polyvinyl sulfate, dextran sulfate (molecular weight 500,000), or poly(L-aspartate) (molecular weight 7000–8000) at concentrations of 50 μg/ml failed to prevent choleragen-induced stimulation of steroid secretion. The inhibitory effect is thus not due simply to charge–charge interactions. Moreover, inhibition of ganglioside is specific to the extent that the response to ACTH is not affected.

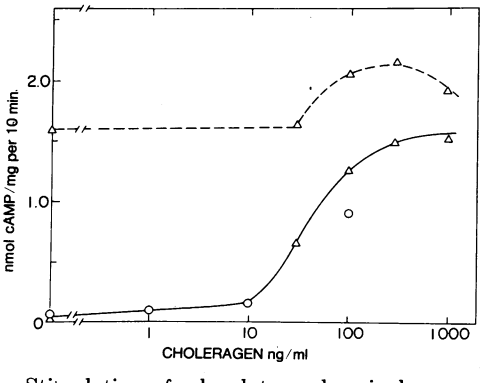

FIG. 4. Stimulation of adenylate cyclase in homogenates of Y-1 cells by choleragen. Cells were exposed to choleragen for 2 hr, harvested in buffered 0.25 M sucrose containing the same concentration of choleragen, and homogenized in a loose-fitting Dounce homogenizer. The 8000 × *g* pellet was washed twice in the same medium and then used to start the reaction, which went for 10 min. The assay was done in the presence of 50 μM ITP (21). F^- concentration was 10 mM (*dashed curve*); basal response (*solid curve*); △ and ○ are quadruplicate determinations in separate experiments.

TABLE 4. *Effect of ganglioside on the steroid response to choleragen*

Treatment and order of addition	Concentration	Steroid secretion† (μg/mg of protein per hr)
Control		0.8
Choleragen	30 ng/ml	3.2
Ganglioside	12 μg/ml	0.5
Ganglioside → choleragen*	12 μg/ml → 30 ng/ml	0.5
Choleragen → ganglioside*	30 ng/ml → 12 μg/ml	2.5

* Added after 10 min.

† Spectrophotometric assay. Incubation time was 12 hr.

In summary, it is clear that various macromolecular bacterial products are able to stimulate steroid production in Y-1 adrenal tumor cells. Whether or not all of these work by similar mechanisms has not been determined. It has been suggested that, for liver adenylate cyclase, choleragen selectively affects the epinephrine-sensitive response whereas the glucagon response is unaffected (10). It is also clear that the choleragen effect can occur independently of any involvement of prostaglandin synthesis (12). To what extent the above stimulators overlap the receptor domain of ACTH in these adrenal tumor cells is unknown. If the adenylate cyclases of membranes normally operate under constraints that are relieved upon combination of the hormone with its receptor, then it is possible that these agents may be useful in studying the nature of such constraints. Moreover, the great sensitivity of the Y-1 cell for choleragen may make these cells useful for detection of the enterotoxin.

Note Added in Proof. Since submission of this paper, similar findings have been reported by Donta, S. T., King, M. & Sloper, K. (1973) *Nature New Biology* **243**, 246–247.

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