Interaction of Cholera Enterotoxin with Cultured Adrenal Tumor Cells

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In the adrenal tumor cell system ganglioside G₄₅ inhibited cholera enterotoxin (CT)-induced steroidogenesis if it was preincubated with the toxin or added to adrenal cells 10 min before CT. In the preincubation studies a molar ratio of G₄₅ to toxin of 3:1 was necessary for half-maximal inhibition of steroidogenesis. On the other hand, horse serum anticholeragenoid neutralized the steroidogenic response to cell-bound CT by 50% if it was added to adrenal monolayer cultures 15 min after the toxin. Specific antiserum was able to neutralize 20% of the toxin-induced activity even if it was added to adrenal cultures 2 h after CT. Phase contrast microscopy demonstrated that partial neutralization of the biochemical effect of CT by horse serum anticholeragenoid was accompanied by partial prevention of toxin-induced rounding of adrenal cells. Further studies showed that pretreatment of cultured adrenal cells with a maximal dose of CT increased cyclic adenosine 3′-5′-monophosphate formation in response to a maximal stimulating dose of adrenocorticotropic. This result suggested potentiation of hormonal activation of adenylate cyclase in intact adrenal tumor cells in response to CT.

Previous studies suggest that cholera enterotoxin (CT) and adrenocorticotropic (ACTH) interact with separate adrenal membrane receptors to stimulate cyclic adenosine 3′-5′-monophosphate (cAMP) formation and steroidogenesis in cultured adrenal tumor cells (3–5, 16, 17, 20). Firstly, the addition of ACTH to adrenal cells leads to a rapid increase in cAMP concentration, whereas in response to CT there is a lag phase of 45 min before any change in cAMP can be detected. Secondly, a mutant adrenal cell line unresponsive to ACTH can be maximally stimulated by CT. Thirdly, ACTH and CT have an additive effect on steroidogenesis, suggesting that they do not compete for the same membrane receptor. Furthermore, horse serum anticholeragenoid and a mixed ganglioside preparation completely block the steroidogenic effect of the toxin if added to adrenal cells before CT, but these agents have no effect on hormone-induced steroidogenesis.

The present studies show that the steroidogenic effect of CT can be partially neutralized by horse serum anticholeragenoid but not by ganglioside G₄₅ after the toxin is fixed to specific membrane receptors. Furthermore, these studies suggest that pretreatment of adrenal cells with CT potentiates ACTH activation of adrenal adenylate cyclase.

MATERIALS AND METHODS

Cell culture procedures, steroid assay, and cAMP determination. The incubation procedures and fluorometric steroid assay for Y-1 adrenal tumor cells have been described previously (20, 21). cAMP was measured in the cells and in the media after ethanol extraction by the Gilman competitive binding assay as described previously (10, 16). Adrenal monolayer morphology was observed under a phase contrast microscope (Nikon, MS).

Ganglioside experiments. In these experiments ganglioside G₄₅ was preincubated with CT, added to cells 10 min before toxin, or added 5 min after CT. Under the first conditions, 5 ng of CT plus from 25 pg to 5 ng of G₄₅ were incubated at 24 C for 20 min in calcium- and magnesium-free phosphate-buffered saline. Then the mixture was added to duplicate confluent adrenal tumor cells in 2.0 ml of serum-free Eagle minimal essential medium (GIBCO, Grand Island, N.Y.) to give a final concentration of 2.5 ng of CT per ml plus 0.0125 to 2.5 ng of GM per ml and incubated for 18 h before determining steroidogenesis. In the next series of experiments from 2.5 to 125 ng of G₄₅ per ml was added to adrenal cells 10 min before 2.5 ng of CT per ml, and steroidogenesis was determined at 18 h. In the third series of experiments, G₄₅ (25 to 125 ng/ml) was added to adrenal cultures 5 min after the addition of CT (2.5 ng/ml), and steroidogenesis was measured at 18 h.

Neutralization experiment. Confluent cultures were preincubated with CT (5 ng/ml) in Eagle minimal essential medium for 5 min, washed twice with 2.0 ml of phosphate-buffered saline, and incubated in Eagle minimal essential medium. At 15, 30, 60, 90, and 120 min of incubation horse serum anticholera genoid (4 U/ml) was added and steroidogenesis in duplicate cultures was determined at the end of 24 h. An equal volume of horse serum without anticholera genoid activity was added to duplicate cultures.
after pretreatment with CT to determine the control level of steroidogenesis. The results are expressed as a percentage of this control value. For the 0-min determination specific antiserum was added to duplicate cultures immediately after CT and steroidogenesis was determined at the end of 24 h.

Cholera toxin plus ACTH incubation. Confluent monolayers were preincubated with CT (50 ng/ml) for 3 h and then ACTH (1 mU/ml) was added for 1 h, and intracellular and extracellular cAMP were determined in duplicate. In addition, duplicate cultures were preincubated with ACTH (1 mU/ml) for 1 h, and then CT (50 ng/ml) was added for 3 h and cAMP was determined. Control cultures were incubated with CT alone for 3 or 4 h and ACTH alone for 1 or 4 h.

Reagents. ACTH (Armour, Chicago, Ill.), GM1, brain ganglioside (gift of W. E. van Heyningen, Oxford, England), purified cholera enterotoxin, and horse serum anticholeragenoid (8, 9) prepared under contract for National Institute of Allergy and Infectious Diseases by R. A. Finkelstein) were used in the tissue culture incubations. [3H]cAMP (24 Ci/mmol) (New England Nuclear Corp., Boston, Mass.) was used for cAMP determinations.

RESULTS

Effect of ganglioside GM1 on toxin-induced steroidogenesis. Purified ganglioside GM1, effectively prevented CT stimulation of steroidogenesis when it was preincubated with the toxin for 20 min (Fig. 1). Under these conditions, half-maximal inhibition of the toxin effect occurred at 0.15 ng of GM1 per ml. In this experiment 2.5 ng of GM1 per ml maximally inhibited an equal weight of CT. The purified ganglioside itself had no effect on steroid production. Approximately 100 times more GM1 was required for half-maximal inhibition if the ganglioside was added to cultured adrenal cells 10 min before CT (Fig. 2). In this experiment 13.0 ng of GM1 per ml was required for half-maximal inhibition of CT-induced steroidogenesis, and 50 ng of GM1 per ml maximally inhibited the toxin effect. However, if CT was allowed to attach to its adrenal receptor for 5 min before the addition of GM1, then the ganglioside was ineffective in inhibiting CT activity. Under these incubation conditions, 125 ng of GM1 per ml reduced steroid production by 10%.

Effect of horse serum anticholeragenoid on CT-induced steroidogenesis. Previously, we have shown that 1.5 U of horse serum anticholeragenoid per ml completely neutralized CT stimulation of steroidogenesis when it was added to adrenal tumor cells before the toxin became attached to its specific membrane receptor (20). In this experiment the ability of antitoxin to neutralize the stimulatory effect of membrane-bound CT was demonstrated. Figure 3 shows that 4 U of specific antiserum per ml neutralized the steroidogenic effect of the simultaneous addition of toxin. When antitoxin was added to adrenal cells 15 min after CT there was a 50% neutralization of toxin induced steroidogenesis. This figure also shows that specific antiserum was able to partially neutralize the steroidogenic response if it was added 2 h after the toxin. Phase contrast microscopy demonstrated that neutralization of the toxin-induced biochemical effect was accompanied by a partial neutralization of the toxin-induced morphological effect. In the toxin-treated control cultures more than 95% of the cells changed from flattened to spherical shaped cells at 4 h of incubation. However, if antitoxin was added at 15 min only 20% of the cells became spherical, and if antitoxin was added at 60 min approxi-
The Gm1 brain ganglioside appears to be the natural membrane receptor for CT (12–14). Previously, a mixed ganglioside preparation has been shown to inhibit toxin-induced steroidogenesis (20). The present results show that the specific ganglioside Gm1 can only block steroidogenesis if it is preincubated with CT or if it is added to adrenal cells before the addition of enterotoxin. Once enterotoxin binds to its adrenal membrane receptor, Gm1 can no longer inhibit toxin-induced steroidogenesis. In the preincubation experiment 0.15 ng of Gm1 per ml inhibited toxin-induced steroidogenesis by 50%. If one assumes a molecular weight of 1,600 for Gm1 and 84,000 for CT, then a molar ratio of ganglioside to toxin of about 3:1 is necessary for half-maximal inhibition of CT in the adrenal cell assay system. Our results are very similar to the molar ratio of Gm1 to CT necessary to half-maximally inhibit CT-induced accumulation of cAMP in thymocytes (22). As in the adrenal system, preincubation of thymocytes with CT for 5 min prevented the ganglioside inhibitory effect. Donta and Viner have recently examined the effect of several gangliosides, including Gm1, GD1a, and GT1 on CT-induced steroidogenesis and has shown inhibition only by Gm1 (6).

The ability of specific antiserum to neutralize toxin-induced steroidogenesis by 50% at 15 min and 20% at 2 h suggests that in the adrenal tumor cell system one can partially neutralize CT after it becomes fixed to a specific membrane receptor site but possibly before the active subunit traverses the membrane (19). The fact that anticholeragenoid can partially prevent the CT-induced morphological alterations of adrenal cells supports this view since spherical adrenal morphology correlates with elevated intracellular cAMP levels (15). Previously, Donta reported a 46% inhibition of steroidogenesis if antitoxin was added 1 h after CT (5). In ileal loop experiments investigators were unable to inhibit fluid accumulation if antitoxin was added after CT (18). On the other hand, preincubation of the cells with ACTH for 1 h followed by exposure to CT for 3 h did not enhance cAMP formation. In this experiment intracellular and extracellular cAMP were determined separately, and at the end of the incubation approximately 90% of the total cAMP was in the culture media.

**DISCUSSION**

The Gm1 brain ganglioside appears to be the natural membrane receptor for CT (12–14). Previously, a mixed ganglioside preparation has been shown to inhibit toxin-induced steroidogenesis (20). The present results show that the specific ganglioside Gm1 can only block steroidogenesis if it is preincubated with CT or if it is added to adrenal cells before the addition of enterotoxin. Once enterotoxin binds to its adrenal membrane receptor, Gm1 can no longer inhibit toxin-induced steroidogenesis. In the preincubation experiment 0.15 ng of Gm1 per ml inhibited toxin-induced steroidogenesis by 50%. If one assumes a molecular weight of 1,600 for Gm1 and 84,000 for CT, then a molar ratio of ganglioside to toxin of about 3:1 is necessary for half-maximal inhibition of CT in the adrenal cell assay system. Our results are very similar to the molar ratio of Gm1 to CT necessary to half-maximally inhibit CT-induced accumulation of cAMP in thymocytes (22). As in the adrenal system, preincubation of thymocytes with CT for 5 min prevented the ganglioside inhibitory effect. Donta and Viner have recently examined the effect of several gangliosides, including Gm1, GD1a, and GT1 on CT-induced steroidogenesis and has shown inhibition only by Gm1 (6).

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**Fig. 3.** Effect of horse serum anticholeragenoid on CT-induced steroidogenesis. Adrenal cells were exposed to CT (5 ng/ml) for 5 min and washed, and antitoxin was added 15 to 120 min later. Each point represents the mean steroid determination, expressed as a percentage of CT-treated control cultures from two separate experiments.

**Fig. 4.** Effect of CT plus ACTH on cAMP accumulation. Duplicate adrenal cultures were preincubated with a maximal dose of CT for 3 h and then ACTH (1 mU/ml) was added for 1 h and the total cAMP concentration was determined. The predicted value is the sum of cAMP determinations from cultures exposed to CT or ACTH alone.
hand, Craig was able to partially neutralize CT activity in vivo (2). He used the vascular permeability factor assay to show that if antitoxin was administered intracardially 6 hours after CT it could neutralize 20% of the activity of CT.

Adrenal cells pretreated with a maximal dose of CT showed an increased sensitivity to a maximal dose of ACTH. Turkey erythrocyte ghost and rat liver membrane adenylate cyclase also shows potentiation of hormonal stimulation after pretreatment with CT (1, 7). It is possible that the irreversibly binding of ¹²⁵I-labeled toxin and its persistent biological effect may lead to stabilization of an activated adenylate cyclase complex. On the other hand, pretreatment of adrenal cells with a maximal dose of ACTH for 1 hour followed by the addition of CT for 3 hours did not potentiate the toxin's effect. This result could be related to the rapid dissociation of ACTH from its membrane receptor or possibly to hormonal stimulation of an adenylate cyclase inhibitor (11).

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LITERATURE CITED


