MOLECULAR PHARMACOLOGY, 20:59–67

Cholera Toxin-Stimulated Cyclic AMP Accumulation in Glial Tumor Cells

Modulation by Ca^{2+}

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Received November 7, 1980; Accepted February 2, 1981

SUMMARY

BROSTROM, M. A., C. O. BROSTROM, S.-C. HUANG, AND D. J. WOLFF. Cholera toxin-stimulated cyclic AMP accumulation in glial tumor cells: modulation by Ca^{2+}. *Mol. Pharmacol.* **20**:59–67 (1981).

Accumulation of cyclic AMP in response to cholera toxin was studied in Ca^{2+}-depleted and Ca^{2+}-restored C6 glial tumor cells. Accumulation was retarded when the cells were depleted of Ca^{2+}, but the maximal cyclic AMP content observed with toxin in Ca^{2+}-restored cells was eventually attained in Ca^{2+}-depleted cells. The concentration of cholera toxin which elicited half-maximal cyclic AMP accumulation (apparent K_{act}) was not altered by Ca^{2+}. Micromolar free Ca^{2+} concentrations in the extracellular medium were sufficient to overcome the retardation of toxin-stimulated cyclic AMP accumulation in Ca^{2+}-depleted cells. The effect of Ca^{2+} on cyclic AMP accumulation in response to toxin was rapid and was reversible upon the addition of ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA) in excess of the cation. Accumulation of cyclic AMP by intact cells correlated with increases in the adenylate cyclase activities of Ca^{2+}-depleted and Ca^{2+}-restored cells exposed to cholera toxin. Ca^{2+}, therefore, appears to enhance the rate of adenylate cyclase activation by cholera toxin. Trifluoperazine retarded cholera toxin-dependent cyclic AMP accumulation in a manner similar to cellular Ca^{2+} depletion. Cyclic AMP accumulation in both Ca^{2+}-depleted and Ca^{2+}-restored preparations was slowed by the drug at micromolar concentrations. Once maximal cell cyclic AMP was attained, however, neither trifluoperazine nor EGTA in excess of Ca^{2+} affected the nucleotide contents of cells or their extracellular fluids. Binding of ^{125}I-labeled cholera toxin by intact cells was not altered by Ca^{2+} or by trifluoperazine. The Ca^{2+} dependence of cyclic AMP accumulation in response to norepinephrine was not affected by pretreatment of cells with cholera toxin, although the extent of the response to the catecholamine was reduced. Similar effects of Ca^{2+} on cyclic AMP accumulation in cholera toxin-treated cells were identified in neuroblastoma, neuroblastoma-glioma, pituitary tumor, and Chinese hamster ovary cells.

INTRODUCTION

Cholera enterotoxin is recognized to activate adenylate cyclase of many types of vertebrate cells (1, 2). Following binding of toxin to ganglioside G_{M1} on the surface of the cell, the active subunit A_1 is released into the cytoplasm, where it subsequently produces an irreversible activation of adenylate cyclase. In cell-free systems, the A_1 fragment catalyzes an ADP-ribosylation of the guanyl nucleotide regulatory component of the enzyme (3, 4), the consequence of which is proposed to be the inhibition of GTP hydrolysis and persistence of the activated state of adenylate cyclase (5). The fully activated enzyme is highly stable *in vitro*; in intact cells the enzyme may remain activated for days (6). ADP-ribosyl transferase activity of the A_1 fragment requires NAD^+, GTP, and other cytosolic factors which have not been completely characterized (1, 2, 7). The adenylate cyclase of brain, which is regulated by Ca^{2+} and CDR[1] (8, 9), is fully activated by cholera toxin only when Ca^{2+} and CDR in addition to the above cofactors are present (10). Full expression of

This research was supported by United States Public Health Service Grants NS 10975 and NS 11340.

[1] The abbreviations used are: CDR, calcium-dependent regulator or calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid.

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the toxin-activated brain enzyme similarly requires the presence of Ca^{2+} and CDR.

The catecholamine-sensitive adenylate cyclase of C6 glial tumor cells is enhanced approximately 40% by CDR and is more sensitive to Ca^{2+} in the presence of CDR (11). Cyclic AMP accumulation in intact C6 cells in response to norepinephrine was recently shown to be influenced by intracellular Ca^{2+} (12); the results were consistent with the hypothesis that CDR is the intracellular mediator of this Ca^{2+}-modulated response. Evidence that Ca^{2+} is involved in the cyclic AMP response to cholera toxin in intact cells, however, is lacking. The present investigation was conducted to assess the role of Ca^{2+} in the cyclic AMP response of toxin-treated C6 cells. The results obtained were compatible with an intracellular Ca^{2+} requirement for, or facilitation of, adenylate cyclase activation by toxin. In addition, accumulation of cyclic AMP in response to toxin in other types of cultured cells was shown to be Ca^{2+}-modulated.

MATERIALS AND METHODS

Materials. Cholera enterotoxin was purchased from Schwarz/Mann (Orangeburg, N. Y.). *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, cyclic AMP, ATP, norepinephrine bitartrate, and cycloheximide were obtained from the Sigma Chemical Company (St. Louis, Mo.). Trifluoperazine was a gift from Smith, Kline and French Laboratories (Philadelphia, Pa.). Cell culture media were obtained from Grand Island Biological Company (Grand Island, N. Y.), and fetal bovine and horse sera were purchased from Flow Laboratories, Inc. (Rockville, Md.). ^3H cyclic AMP, 36.7 Ci/mmole, was obtained from New England Nuclear Corporation (Boston, Mass.), and [^3H]ATP, 12 Ci/mmole, from ICN Pharmaceuticals, Inc. (Plainview, N. Y.).

Cell cultures. The C6 clonal line of rat glial tumor cell was obtained from the American Type Culture Collection (Rockville, Md.). Stock monolayer cultures were maintained (13) and experimental cultures were grown (12) according to protocols previously described. Other cell cultures were grown in Falcon plastic flasks or glass roller bottles to confluency. Anterior pituitary tumor cells were grown in Ham's F-10 medium with 2.5% fetal bovine serum and 15% horse serum; neuroblastoma (neuro 2A) in Eagle's minimal essential medium with 10% fetal bovine serum; neuroblastoma-glioma (NG 10815) in Ham's F-10 medium with 10% fetal bovine serum; and Chinese hamster ovary cells in Ham's F-12 medium with 10% fetal bovine serum. The neuroblastoma-glioma cells were obtained originally from Dr. Arthur Blume of The Roche Institute for Molecular Biology (Nutley, N. J.). All other cell types were purchased from the American Type Culture Collection.

Preparation of Ca^{2+}-depleted cells. Roller bottle monolayer cultures were washed twice with 40 ml of a buffered saline solution containing 137 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1 mM EGTA, and 25 mM *N*-tris(hydroxymethyl)-2-aminoethanesulfonic acid; pH 7.5. Another 40 ml of buffered saline were added, and the cells were incubated at 37° until they detached from the glass surface. The cell suspension was centrifuged at 600 × *g* for 3 min at room temperature and the supernatant fluid was discarded. Cells were resuspended in the above saline solution and recentrifuged. The pellet of cells was resuspended in 40 ml of the buffered saline containing 1 mM $MgCl_2$ and 1 mM isobutylmethylxanthine. This suspension of Ca^{2+}-depleted cells routinely contained 0.2–0.4 mg of protein per milliliter. Ca^{2+}-restored cells were prepared by adding $CaCl_2$ to a concentration of 3 mM (2 mM in excess of EGTA) to a portion of the suspension of Ca^{2+}-depleted cells. Unless indicated otherwise, aliquots of cell suspensions were pretreated at 37° in glass or polypropylene tubes with or without Ca^{2+} for 30 min. The Ca^{2+} content of Ca^{2+}-restored cells was routinely 5-fold greater than that of Ca^{2+}-depleted cells (12). Cholera toxin, drugs, or other additives were dissolved in buffered saline and were added to the cells in 10- to 20-μl volumes per milliliter of cell suspension. Viability of cell preparations was determined by dye uptake studies or regrowth procedures as described previously (12). By these criteria, cells routinely were 90–95% viable. Alternately, when extracts of Ca^{2+}-depleted or Ca^{2+}-restored cells were prepared and ATP measurements performed as previously described (12), no differences in nucleotide concentrations between the two types of cell preparations were observed.

Determination of cyclic AMP content. The cyclic AMP contents of trichloroacetic acid extracts of cell suspensions, cells alone, or extracellular fluids alone were measured as discussed previously (12). In some experiments, extracts of cells or of cells with extracellular fluids were prepared by heating samples at 90° for 10 min. Denatured protein was removed by centrifugation at 2000 × *g* for 10 min, and supernatant fluids were used directly for cyclic AMP analyses. Values obtained with this procedure were identical with those obtained by using trichloroacetic acid extracts of cells or cell suspensions.

Unless specifically indicated, results are expressed as the means ± standard error of triplicate incubations from a single preparation of cells in a single experiment. Assays of cyclic AMP for each incubation sample were performed in duplicate. Each experiment was performed at least three times to verify results, and experiments reported herein are considered representative.

Adenylate cyclase assays. Pellets of C6 cells obtained by centrifugation at 1000 × *g* for 2 min were resuspended in 10 mM imidazole, pH 7.5, containing 3 mM $MgCl_2$ and 1 mM dithiothreitol to 0.1 volume of original cell suspension and homogenized by hand in a Potter-Elvehjem homogenizer fitted with a Teflon pestle. Aliquots (25 μl) of homogenate were assayed for enzyme activity as described previously (9) with the exception that 1 mM EGTA was present in the assay buffer. Results are expressed as the average of triplicate determinations ± range of values obtained.

Preparation of ^{125}I-labeled cholera toxin. Commercial cholera toxin was chromatographed on a Sephadex G-75 column equilibrated with 10 mM sodium phosphate, pH 7.4, to remove salts and sodium azide. The chromatographed toxin was then iodinated by using the radioiodination system components obtained from New England Nuclear Corporation. Toxin (220 μg) was incubated at room temperature with a phosphate buffer containing 1 mCi of ^{125}I (Na^+ salt), 0.1% β-D glucose, and iodination

reagent beads (lactoperoxidase and glucose oxidase on uniform inert solid supports). After 30 min, the reaction was terminated with sodium metabisulfite (0.5 mg/ml). The reaction mixture was subjected to Sephadex G-75 column chromatography in order to separate iodinated protein from free iodide. Radioactive fractions in the void volume were pooled. A specific activity of 9.5×10^7 cpm per nanomole of toxin was obtained by this procedure; over-all recovery of toxin was 15%. Iodinated toxin was stored at 4° with 0.15 M NaCl, 3 mM sodium azide, and 1 mM EDTA, and used within a few days after preparation.

Determination of cholera toxin binding. Ca^{2+}-depleted and Ca^{2+}-restored cells were prepared as described above, except that the final cell pellet was resuspended in 8 ml of buffered saline. Aliquots (150 μl) were incubated at 25° for 30 min in glass tubes. Unlabeled cholera toxin (0.5 μM) was added to one-half of each preparation of cells and the incubation continued for 10 min. ^{125}I-Labeled cholera toxin and, where indicated, trifluoperazine were added, and the final volume was adjusted to 200 μl. After incubation for the indicated times at 25°, ice-cold buffered saline (2 ml), lacking or containing Ca^{2+}, was added to the appropriate cell suspensions. Suspensions were centrifuged at 1000 × *g* for 2 min at 4° and the supernatant fluids were discarded. Cells were resuspended in 2 ml of cold buffered saline with or without Ca^{2+} and recentrifuged. The suspension and centrifugation procedure was repeated for a total of three washes. Radioactivity of cell pellets was measured directly with a Packard autogamma scintillation spectrophotometer. Radioactivity in samples pretreated with unlabeled cholera toxin was taken as a measure of nonspecific binding. Specific binding was defined as the difference between the amount of radioactive toxin bound in the absence and presence of unlabeled toxin. All samples were corrected for a small amount of binding of labeled toxin to glass tubes in the absence of cells.

Miscellaneous. Protein was determined by the method of Lowry *et al.* (14). Cholera toxin was quantitated by absorbance at 280 nm by using $A^{1\%}_{1\,cm} = 11.42$ (15).

RESULTS

Cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored C6 cells in response to cholera toxin. Ca^{2+}-depleted and Ca^{2+}-restored cells were treated with 6 nM cholera toxin, and the cyclic AMP content of the cells (Fig. 1A) and of their extracellular fluids (Fig. 1B) was measured as a function of incubation time. Fifteen minutes of incubation were required to detect an increase in the cyclic AMP content of Ca^{2+}-restored cells. Thereafter, cyclic AMP content increased rapidly; maximal accumulation of cyclic AMP was obtained 90 min after toxin addition. In Ca^{2+}-depleted cells, however, cyclic AMP was accumulated more slowly with detectable increases in cyclic AMP content observable after 30 min of incubation with toxin. Maximal cyclic AMP accumulation was attained in Ca^{2+}-depleted cells after 150 min of incubation. No cyclic AMP was detected in the medium of toxin-treated cells during the first 45 min of incubation. During the next 2 hr, cyclic AMP in medium from Ca^{2+}-depleted and Ca^{2+}-restored cells was observed to increase as a function of incubation time. Presumably, the cyclic

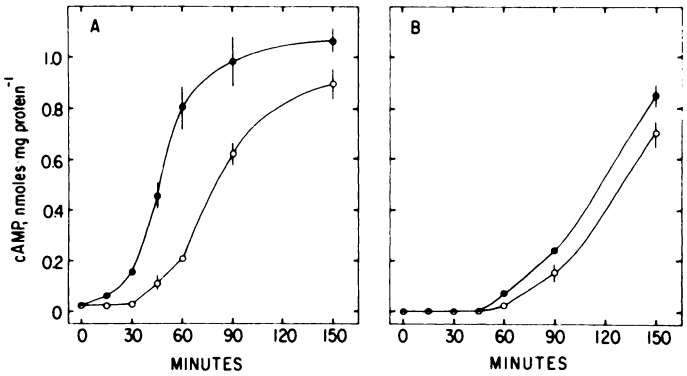

FIG. 1. *Accumulation of cyclic AMP with time in Ca^{2+}-depleted and Ca^{2+}-restored C6 cells and the respective cyclic AMP content of their extracellular fluids following addition of cholera toxin*

Ca^{2+}-depleted (○) or Ca^{2+}-restored (●) cells were challenged with 6 nM cholera toxin. Aliquots of cell suspension (1 ml) were removed at the indicated times following toxin addition and centrifuged immediately at 1000 × *g* for 2 min at 4°. Cell pellets and extracellular fluids were analyzed for cyclic AMP content. A, Cyclic AMP content of cells; B, cyclic AMP content of extracellular medium.

AMP content of cell suspensions (cells plus extracellular fluids) during the 1st hr of incubation is derived primarily from the intracellular cyclic nucleotide pool, whereas at later time points the cyclic AMP content of cell suspensions represents the sum of the nucleotide content in the cells and the accompanying extracellular fluids. The nucleotide in the medium from Ca^{2+}-restored cells was slightly greater than that from Ca^{2+}-depleted cells.

The cyclic AMP response to cholera toxin obtained in C6 cells in the presence of Ca^{2+} was similar in magnitude to that reported by Nickols and Brooker (16, 17), but faster and substantially greater in magnitude than that observed by Fishman *et al.* (18). Presumably, variables such as the absence or presence of methylisobutylxanthine in the incubation medium, cell preparation (monolayer or suspension), and growth conditions could account for such quantitative differences.

The cholera toxin concentration dependence of cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored cells is illustrated in Fig. 2. The cyclic AMP contents of

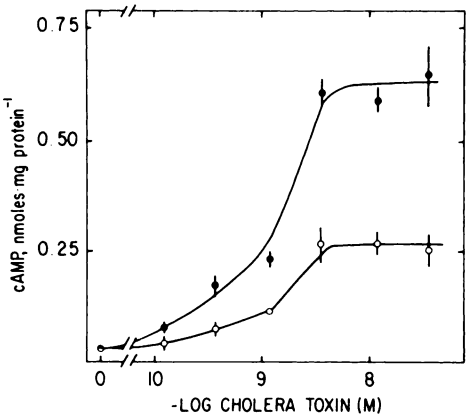

FIG. 2. *Cholera toxin concentration dependence of cyclic AMP accumulation in Ca^{2+}-depleted or Ca^{2+}-restored C6 cells*

Varying concentrations of cholera toxin were added to Ca^{2+}-depleted (○) or Ca^{2+}-restored (●) cells, and the cyclic AMP content of cell suspensions was determined after 60 min of incubation.

cell suspensions were determined after incubation with varying toxin concentrations for 60 min, a time period during which (a) the cyclic AMP contents of the extracellular medium are negligible and (b) differences in cyclic AMP contents of the two cell preparations are readily observed. With both preparations of cells, maximal and half-maximal cyclic AMP accumulation were obtained with 4 nM and 1.2 nM cholera toxin, respectively. The cyclic AMP content of Ca^{2+}-restored cells, however, was at least twice that of Ca^{2+}-depleted cells at all toxin concentrations tested, with the exception of controls without added toxin.

The effect of extracellular Ca^{2+} concentration on the cyclic AMP content of cells exposed to cholera toxin for 60 min was investigated (Fig. 3). Accumulation of cyclic AMP was dependent on external Ca^{2+} concentration and was maximal at approximately 1 mM added Ca^{2+}. Since the EGTA concentration in the external medium was 1 mM, it is probable that micromolar free Ca^{2+} concentrations provide maximal accumulation of cyclic AMP in response to cholera toxin. External Ca^{2+} concentrations of 10 mM or greater were inhibitory (data not shown).

The time required for Ca^{2+}, when added to Ca^{2+}-depleted cells, to enhance cyclic AMP accumulation in response to cholera toxin was examined (Fig. 4A). Ca^{2+}-depleted and Ca^{2+}-restored cells were challenged with toxin. At 37 min, one-half of the Ca^{2+}-depleted cell suspension was treated with Ca^{2+}; the other half was left untreated. Cyclic AMP measurements were conducted for all cell preparations through 70 min of incubation. As discussed above, Ca^{2+}-depleted cells accumulated cyclic AMP much more slowly than did Ca^{2+}-restored cells. When Ca^{2+} was restored to the Ca^{2+}-depleted cells at 37 min, the time course of cyclic AMP accumulation shifted to parallel that seen in Ca^{2+}-restored cells. Similarly, when EGTA was added in excess of medium Ca^{2+} to part of a suspension of Ca^{2+}-restored cells which had been treated with toxin for 37 min (Fig. 4B), accumulation of cyclic AMP with time became parallel with that observed in Ca^{2+}-depleted cells.

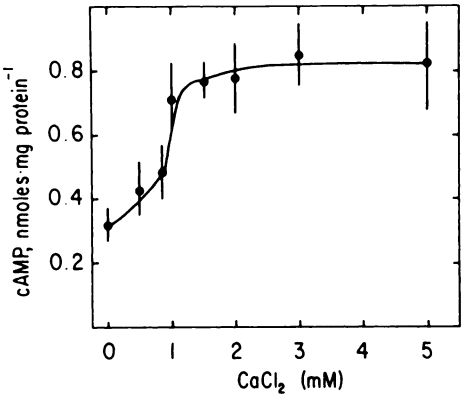

FIG. 3. *Extracellular Ca^{2+} concentration dependence of cyclic AMP accumulation in C6 cells in response to cholera toxin*

Cells were treated in medium containing the indicated concentrations of Ca^{2+} and subsequently challenged with 6 nM cholera toxin. After 60 min of incubation with toxin, cell suspensions were analyzed for cyclic AMP content. Basal cyclic AMP, 30 ± 5 pmoles·mg of protein^{-1}, was unaffected by external Ca^{2+} concentrations.

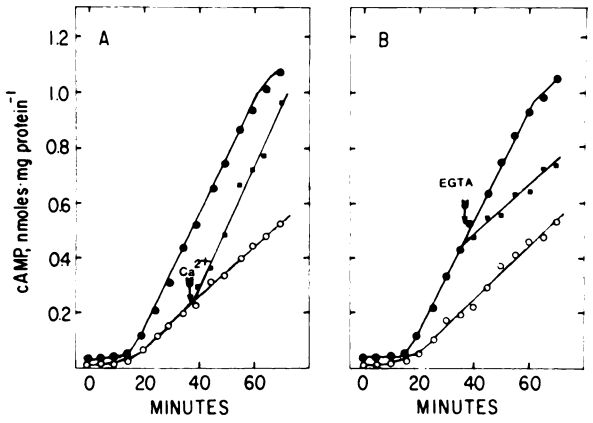

FIG. 4. *Enhancement of cholera toxin-stimulated cyclic AMP accumulation in C6 cells by Ca^{2+}*

A preparation of Ca^{2+}-depleted cells was suspended in medium lacking Ca^{2+} and EGTA and was used for experiments of A and B.

A. Time dependence of Ca^{2+} effect. Suspensions of cells were adjusted to contain either 1 mM EGTA (Ca^{2+}-depleted; ○) or 1 mM EGTA and 3 mM Ca^{2+} (Ca^{2+}-restored; ●) and were challenged with 6 mM cholera toxin. At 5-min intervals, aliquots of cell suspensions were removed for cyclic AMP measurements. At 37 min following the addition of cholera toxin, the suspension of Ca^{2+}-depleted cells was divided in half; one half was treated with 3 mM $CaCl_2$ (■) and the other half with an equal volume of buffered saline. Sampling of all preparations was continued to 70 min.

B. Time dependence of EGTA effect. Suspensions of cells in medium containing 1 mM EGTA (○) or 0.2 mM Ca^{2+} (●) were each challenged with 6 nM cholera toxin. At 5-min intervals, aliquots of cell suspensions were removed for cyclic AMP measurements. At 37 min following the addition of cholera toxin, the suspension of cells in Ca^{2+}-containing medium was divided in half; one half was treated with 1 mM EGTA (■) and the other half with an equal volume of buffered saline. Sampling of all preparations was continued to 70 min. Each point in A and B represents a cyclic AMP determination on a single cell sample.

Adenylate cyclase activity of Ca^{2+}-depleted and Ca^{2+}-restored cells treated with cholera toxin. It was desirable to know whether the effect of Ca^{2+} on cyclic AMP accumulation was attributable to an increased rate of activation of adenylate cyclase by cholera toxin or to modification of another factor modulating cyclic AMP accumulation in intact cells. In Table 1, the adenylate cyclase activities and cyclic AMP contents of Ca^{2+}-depleted and Ca^{2+}-restored cells treated with toxin for varying times are compared. The adenylate cyclase activities of Ca^{2+}-depleted cells treated with toxin for either 30 or 60 min were significantly lower than those of Ca^{2+}-restored cells treated with toxin for the same time periods. However, after 2 hr of incubation with toxin, the enzyme activity from both cell preparations was identical. Adenylate cyclase activities were found to increase qualitatively in parallel with the cyclic AMP contents of Ca^{2+}-depleted and Ca^{2+}-restored cells treated with toxin for the same time periods. It is probable, therefore, that Ca^{2+} is necessary for or enhances in some manner the activation of adenylate cyclase of intact C6 cells by cholera toxin.

Effect of trifluoperazine on cholera toxin-stimulated cyclic AMP accumulation. Stimulation of brain adenylate cyclase by CDR is inhibited competitively by phenothiazines (19) and evidence has been provided that these agents bind to CDR in a Ca^{2+}-dependent manner

TABLE 1

Adenylate cyclase activity and cyclic AMP content of Ca^{2+}-depleted and Ca^{2+}-restored cells treated for varying time periods with cholera toxin

Ca^{2+}-depleted and Ca^{2+}-restored cells were challenged with 6 nM cholera toxin. At the indicated times, aliquots of cell suspensions were withdrawn for adenylate cyclase and cyclic AMP measurements. Cells were separated from their extracellular fluids by centrifugation at 1000 × *g* for 2 min at 4°. Cell pellets were analyzed for cyclic AMP content. For adenylate cyclase measurements, cells were resuspended in buffered saline lacking Ca^{2+} and EGTA and recentrifuged to remove traces of EGTA or Ca^{2+} from the original extracellular medium. The pellet of cells was resuspended in 10 mM imidazole, pH 7.5, containing 3 mM $MgCl_2$ and 1 mM dithiothreitol to 0.1 volume of original cell suspension. Cells were homogenized and aliquots of homogenate were assayed for enzyme activity.

Time with toxin	Adenylate cyclase activity		Cyclic AMP content	
	Ca^{2+}-depleted cells	Ca^{2+}-restored cells	Ca^{2+}-depleted cells	Ca^{2+}-restored cells
	pmoles cyclic AMP·min^{-1}·mg protein^{-1}		*pmoles·mg protein^{-1}*	
0	40 ± 2	41 ± 1	23 ± 6	32 ± 4
30	49 ± 2	62 ± 1	47 ± 2	98 ± 6
60	87 ± 3	120 ± 2	130 ± 3	530 ± 20
120	130 ± 3	130 ± 5	1200 ± 10	1400 ± 90

producing an ineffective Ca^{2+}·CDR·phenothiazine complex (20). Norepinephrine-dependent cyclic AMP accumulation in intact C6 cells is inhibited by trifluoperazine in the presence, but not in the absence, of Ca^{2+} (12). It was of interest, therefore, to ascertain whether trifluoperazine affected cyclic AMP accumulation in response to cholera toxin in these cells. The effect of increasing concentrations of trifluoperazine on cyclic AMP accumulation following 45 min of incubation with toxin in Ca^{2+}-depleted and Ca^{2+}-restored cells is depicted in Fig. 5. Accumulation of the nucleotide was progressively inhibited in both cell preparations as drug concentration

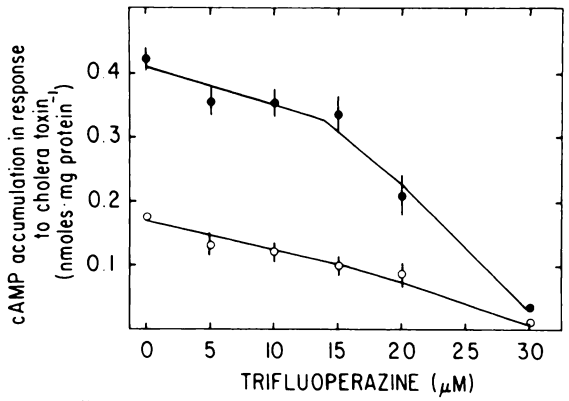

FIG. 5. *Effect of trifluoperazine concentration on cholera toxin-stimulated cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored cells*

Ca^{2+}-depleted (○) and Ca^{2+}-restored (●) cells were treated for 10 min with the indicated concentrations of trifluoperazine. Cholera toxin (6 nM) was then added and cyclic AMP contents of cell suspensions were determined after 45 min of further incubation. Basal cyclic AMP content (27 ± 2 pmoles·mg of protein^{-1}) was unaffected by Ca^{2+} or by trifluoperazine and has been subtracted from cyclic AMP contents of toxin-treated cells.

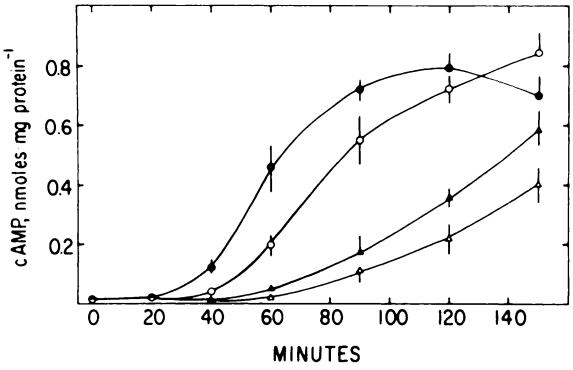

FIG. 6. *Effect of trifluoperazine on the time course of cholera toxin-elicited cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored C6 cells*

Cell suspensions were treated for 10 min with or without 30 μM trifluoperazine. Cholera toxin (6 nM) was then added to all preparations, and aliquots of suspensions were removed at 20-min intervals. Cell suspensions were centrifuged immediately and cell pellets prepared for cyclic AMP measurements. Ca^{2+}-depleted cells (○; △); Ca^{2+}-restored cells (●; ▲); without trifluoperazine (○; ●); with trifluoperazine (△, ▲).

was increased; at 30 μM trifluoperazine, the response to toxin was virtually eliminated. The inhibition by high concentrations of drug was, however, dependent on the time of incubation with toxin. When Ca^{2+}-depleted or Ca^{2+}-restored cells were treated with 30 μM trifluoperazine and incubated with toxin for varying time periods (Fig. 6), it was observed that at least 60 min of incubation were required before increases in cyclic AMP content were detected. Thereafter, the cyclic AMP content of both cell preparations increased, and maximal cyclic AMP content was approached between 2 and 3 hr of incubation. The extent of the shift in the time course of cyclic AMP accumulation by trifluoperazine appeared to be similar for Ca^{2+}-depleted and Ca^{2+}-restored cells.

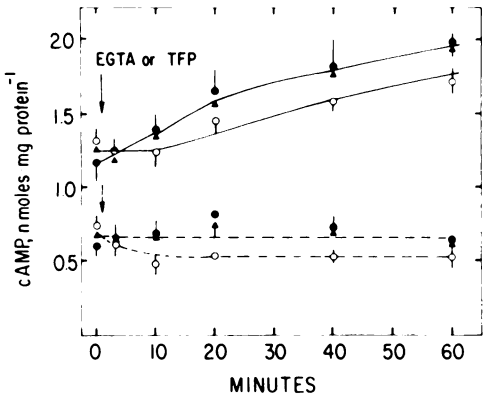

FIG. 7. *Influences of EGTA and trifluoperazine (TFP) on cyclic AMP content of C6 cells pretreated with cholera toxin*

One roller bottle culture of cells was washed twice with 40 ml of Ham's F-10 medium to remove serum proteins and incubated for 90 min in 40 ml of Ham's F-10 medium containing 6 nM cholera toxin. Ca^{2+}-restored cells in medium containing 1 mM EGTA and 3 mM Ca^{2+} were then prepared as described under Materials and Methods. Cell suspensions were warmed to 37° and then treated with either 5 mM EGTA (○), 30 μM trifluoperazine (▲), or saline (●). Samples of cells (- - -) or of cell plus medium (——) were prepared for cyclic AMP measurements at the indicated times of incubation.

Activation of adenylate cyclase by cholera toxin is widely believed to be irreversible (1, 2). It was therefore of interest to test the effects of EGTA and trifluoperazine on the cyclic AMP content of cells containing adenylate cyclase which is fully activated by toxin. Cells in monolayer cultures were incubated for 90 min in Ham's F-10 medium with cholera toxin. Ca^{2+}-containing cells in suspension were then prepared from the pretreated culture. The cells alone or cells with their extracellular fluids were assessed for cyclic AMP content throughout a 60-min incubation period with no additions, or with EGTA in excess of medium Ca^{2+} or with 30 μM trifluoperazine added at the start of the incubation period (Fig. 7). The cyclic AMP content of cells was constant throughout the incubation. Trifluoperazine had no effect on cyclic AMP content; EGTA addition resulted in a minor reduction in nucleotide content which was reproduced consistently in other experiments. The cyclic AMP content of cell suspensions rose slightly during the incubation period. Trifluoperazine was without effect, and the effect of EGTA was minimal. It appears, therefore, that Ca^{2+} and trifluoperazine influence the process of activation of adenylate cyclase by toxin, but do not significantly affect expression of enzyme which is fully toxin-activated.

Cholera toxin binding studies. Interactions of ligands of diverse classes with their receptor sites on isolated membranes or on intact cells are recognized to be influenced by divalent cations. Consequently, it was important to determine whether Ca^{2+} affected the cyclic AMP response to cholera toxin by increasing either the affinity or the number of cholera toxin binding sites. To investigate these possibilities, binding of ^{125}I-labeled cholera

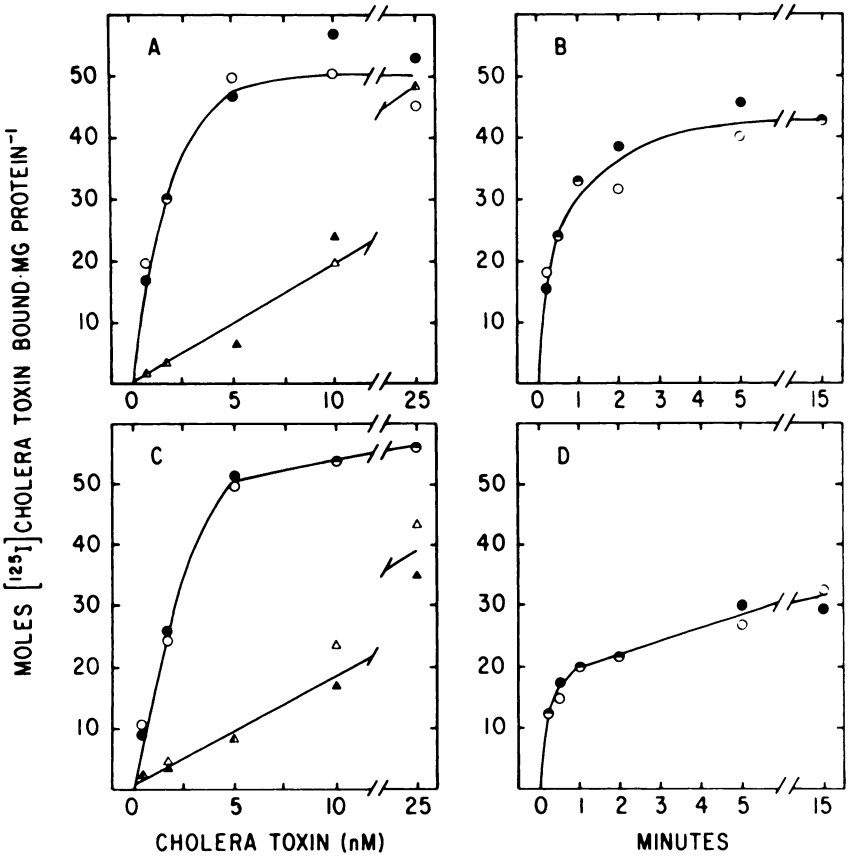

FIG. 8. *Binding of ^{125}I-labeled cholera toxin to Ca^{2+}-depleted, Ca^{2+}-restored, and trifluoperazine-treated C6 cells*

Ca^{2+}-depleted and/or Ca^{2+}-restored cells were pretreated for 30 min at 25°. Unlabeled cholera toxin (0.5 μM) was added to half of each preparation and the pretreatment was continued an additional 10 min.

A. Binding of ^{125}I-labeled cholera toxin to Ca^{2+}-depleted and Ca^{2+}-restored cells as a function of cholera toxin concentration. ^{125}I-labeled cholera toxin in increasing concentrations was added, and binding to cells was assessed after 20 min of incubation at 25°. Nonspecific binding (△; ▲) is defined as the residual binding obtained after pretreatment of cells with unlabeled toxin. Specific binding (○; ●) is taken to be the difference between total binding and nonspecific binding. Ca^{2+}-depleted cells (○; △); Ca^{2+}-restored cells (●, ▲).

B. Binding of ^{125}I-labeled cholera toxin to Ca^{2+}-depleted and Ca^{2+}-restored cells as a function of time. ^{125}I-Labeled cholera toxin (2.5 nM) was added and specific binding was assessed at the indicated times. Ca^{2+}-depleted cells (○); Ca^{2+}-restored cells (●). Nonspecific binding, 8 fmoles·mg of protein^{-1}, was constant throughout the incubation period.

C. Effect of trifluoperazine on the cholera toxin concentraton dependence of ^{125}I-labeled cholera toxin binding. Ca^{2+}-restored cells were divided in half; 30 μM trifluoperazine was added to one half and saline to the other half. After 10 min of incubation, ^{125}I-labeled cholera toxin in increasing concentrations was added and binding was assessed following 20 min of incubation with toxin. Specific binding (○, ●); nonspecific binding (△, ▲); without trifluoperazine (○, △); with trifluoperazine (●, ▲).

D. Effect of trifluoperazine on the time dependence of ^{125}I-labeled cholera toxin binding to C6 cells. Ca^{2+}-restored cells were divided in half; one half was treated with 30 μM trifluoperazine and the other half with an equivalent volume of saline. After 10 min of incubation with drug, ^{125}I-labeled cholera toxin (2.5 nM) was added and specific binding was assessed at the indicated times. Without trifluoperazine (○); with trifluoperazine (●). Nonspecific binding, 6 fmoles·mg of protein^{-1}, was constant throughout the incubation period.

toxin to Ca^{2+}-depleted and Ca^{2+}-restored cells was measured (Fig. 8). Cholera toxin binding was found to be specific and saturable (Fig. 8A). Ca^{2+} depletion of cells had no effect on either specific or nonspecific binding of iodinated toxin. Maximum specific binding (50 fmoles·mg of protein^{-1}) was observed with 5 nM toxin, with an apparent dissociation constant of 1.5 nM being obtained by using either Ca^{2+}-depleted or Ca^{2+}-restored cells. Maximal toxin binding was obtained in 5 min at 25° (Fig. 8B). The time course of toxin binding was similarly unaffected by Ca^{2+}. It was also of interest to ascertain whether trifluoperazine influenced cholera toxin binding. It was found that trifluoperazine had no effect on the cholera toxin concentration dependence of specific toxin binding or nonspecific binding (Fig. 8C). The time course of cholera toxin binding was also unaffected by the drug (Fig. 8D).

Effect of norepinephrine on cyclic AMP accumulation in cholera toxin-treated glial tumor cells. Accumulation of cyclic AMP by glial tumor cells in response to *beta* adrenergic agonists has been extensively characterized. C6 cells (17) and human astrocytoma cells (21) have been shown to accumulate cyclic AMP in response to isoproterenol after pretreatment with cholera toxin, although cyclic AMP accumulation was reduced. Cholera toxin pretreatment decreased the apparent K_{act} for agonists but did not alter *beta*-receptor number or antagonist binding. It therefore appears that toxin increases the efficiency of the coupling of the hormone receptor to the catalytic moiety of adenylate cyclase (21). In view of the observations (a) that norepinephrine stimulation of cyclic AMP accumulation in C6 cells is Ca^{2+}-modulated (12) and (b) that cyclic AMP accumulation in cholera toxin-pretreated cells is mostly Ca^{2+}-independent (Fig. 7), it was of interest to determine whether the further cyclic AMP accumulation elicited from toxin-pretreated cells in response to catecholamine was influenced by Ca^{2+}. Cells were pretreated for 90 min in Ham's F-10 medium containing cholera toxin. Following the pretreatment period, Ca^{2+}-depleted and Ca^{2+}-restored cells were prepared. Suspensions of cells were incubated for 20 min with increasing concentrations of norepinephrine, and the cyclic AMP contents of cells alone and of cells with their extracellular fluids were measured (Fig. 9). Norepinephrine increased the cyclic AMP content of the cells over that initially present. In agreement with previous reports, the response to norepinephrine was reduced when compared with that of nonpretreated cells, and the apparent K_{act} was decreased approximately 10-fold (for the nonpretreated controls, see ref. 12, Fig. 1). However, the accumulation of cyclic AMP by Ca^{2+}-restored cells in response to a range of norepinephrine concentrations was twice that of Ca^{2+}-depleted cells. Cholera toxin pretreatment, therefore, did not appear to modify the effect of Ca^{2+} on *beta*-receptor-mediated cyclic AMP accumulation.

Ca^{2+} dependence of cholera toxin-stimulated cyclic AMP accumulation in diverse cell types. Activation of adenylate cyclase or enhancement of cellular cyclic AMP content by cholera toxin has been documented for a variety of vertebrate cells or tissues. CDR-stimulated adenylate cyclase in mammalian tissue, however, has thus far been reported only for brain (8, 9, 11), regenerating rat liver (22), pancreatic islets (23), and heart (24). It was unclear whether the Ca^{2+} dependence of cholera toxin-stimulated cyclic AMP accumulation described here was restricted to the C6 cell or could be observed in other cell types. For this reason, four other cultured cell types were examined for a stimulatory effect of Ca^{2+} on cyclic AMP accumulation in response to cholera toxin (Table 2). Cells of each culture type were pretreated with 1 mM EGTA or with 1 mM EGTA and 3 mM Ca^{2+}, and cyclic AMP accumulation in response to toxin was examined. EGTA-pretreated preparations of glial tumor, neuroblastoma, neuroblastoma-glioma, anterior pituitary tumor, and Chinese hamster ovary cells accumulated cyclic AMP more slowly than did Ca^{2+}-pretreated cells. The Ca^{2+} dependence of the initial cyclic AMP response to cholera toxin, therefore, is not restricted to a single cell type and may be of general significance.

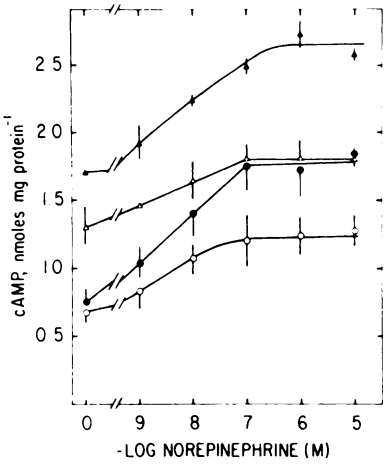

FIG. 9. *Norepinephrine concentration dependence of cyclic AMP accumulation in Ca^{2+}-depleted and Ca^{2+}-restored cells prepared from a cholera toxin-pretreated culture*

Cholera toxin-pretreated cells were prepared as described in the legend to Fig. 7. Ca^{2+}-depleted and Ca^{2+}-restored cells were then prepared from the pretreated culture and were challenged with the indicated concentrations of norepinephrine. After 20 min of incubation with catecholamine, cells alone (○, ●) or cells and extracellular fluids (△, ▲) were analyzed for cyclic AMP content. Ca^{2+}-depleted cells (○, △); Ca^{2+}-restored cells (●, ▲).

DISCUSSION

The results provided in this report demonstrated that cyclic AMP accumulation in response to cholera toxin in intact C6 cells is a Ca^{2+}-modulated process. The effects of Ca^{2+} on toxin-stimulated cyclic AMP accumulation are in many respects comparable to Ca^{2+} effects on norepinephrine-stimulated cyclic AMP accumulation in the same cell line (12). For example, equimolar concentrations of Ca^{2+} and EGTA in the external medium are sufficient to restore to Ca^{2+}-depleted cells the rates of cyclic AMP accumulation in response to toxin or to norepinephrine found for Ca^{2+}-restored controls. Assuming a K_d for Ca^{2+}·EGTA at pH 7.5 of 0.1 μM (25), free Ca^{2+} concentrations under these conditions should be in the micromolar range which is typical of free Ca^{2+} concentrations believed to exist in intracellular fluids. Second, Ca^{2+} does not alter the concentrations of toxin or of catecholamine which promote maximal or half-maximal cyclic AMP responses. Third, Ca^{2+} effects on the cyclic

TABLE 2

Effect of Ca^{2+} on cholera toxin-stimulated cyclic AMP accumulation in cultured cells

All cells detached readily from growing surfaces upon incubation at 37° with buffered saline containing EGTA. Consequently, suspensions in EGTA were prepared from each cell type according to the protocol described for C6 cells under Materials and Methods. Cells were pretreated with or without Ca^{2+} and then challenged with 6 nM cholera toxin. Aliquots of cell suspensions were withdrawn at regular time intervals and analyzed for cyclic AMP content. After examination of time courses of cyclic AMP accumulation following cholera toxin, time points were selected at which the cyclic AMP content of Ca^{2+}-pretreated cells was observed to be half of the maximal cyclic AMP content obtained with toxin in such cells. Basal cyclic AMP contents (pmoles·mg of protein^{-1}) for EGTA-pretreated and Ca^{2+}-pretreated cells, respectively, were: glial tumor cells, 20 ± 2 and 25 ± 3; neuroblastoma, 48 ± 3 and 45 ± 3; neuroblastoma-glioma, 84 ± 4 and 70 ± 3; anterior pituitary tumor, 100 ± 4 and 94 ± 4; and Chinese hamster ovary cells, 30 ± 1 and 27 ± 2.

Cell type	Time of exposure to toxin	Cyclic AMP content	
		Cells pretreated with EGTA	Cells pretreated with Ca^{2+}
	min	*pmoles·mg protein^{-1}*	
Glial tumor (C6)	50	140 ± 10	510 ± 50
Neuroblastoma (neuro 2A)	45	270 ± 20	470 ± 20
Neuroblastoma-glioma (NG 10815)	40	630 ± 100	1150 ± 30
Anterior pituitary tumor (GH_3)	20	1000 ± 50	1750 ± 100
Chinese hamster ovary (CHO)	90	150 ± 20	710 ± 10

AMP response to either agent are rapid and are quickly reversed by EGTA concentrations in excess of the Ca^{2+} in the medium, implying that protein synthesis is unlikely to mediate these effects. Finally, the norepinephrine response of cells pretreated with either toxin or norepinephrine is Ca^{2+}-modulated. The similarities in Ca^{2+} effects on cyclic AMP accumulation in response to these two agents suggest that Ca^{2+} influences cyclic AMP formation in a manner which is not agonist-specific.

The reversibility of the stimulation of adenylate cyclase by catecholamines made it technically difficult to obtain evidence that the effect of Ca^{2+} on the enhancement by norepinephrine of cyclic AMP accumulation in intact cells was expressed at the level of adenylate cyclase. Cholera toxin treatment, however, provides an irreversible activation of adenylate cyclase *in vivo*; consequently, the enzyme activity in homogenates of Ca^{2+}-depleted and Ca^{2+}-restored cells treated with toxin for varying time periods could be assessed. Increases in adenylate cyclase activity in cell-free preparations correlated with the increases observed in the cyclic AMP content of the cells used in preparing the enzyme (Table 1). It appears, therefore, that Ca^{2+} acts on adenylate cyclase in a fashion which enhances the rate at which the enzyme is activated by toxin in intact cells.

The exact mechanism, however, by which Ca^{2+} facilitates the effects of cholera toxin on adenylate cyclase remains in doubt. It appears that at least two potential mechanisms may be ruled out. First, neither the extent of toxin binding nor the affinity for toxin of its cell surface receptor is increased by Ca^{2+} (Fig. 8). Second, since the ATP contents of Ca^{2+}-depleted and Ca^{2+}-restored cells are identical (12), the adenylate cyclase activity of Ca^{2+}-depleted cells is not limited by substrate concentration. There are alternative mechanisms by which activation could be influenced. For example, the rate of entry of the A_1 fragment of toxin into the cytoplasm could be delayed in Ca^{2+}-depleted cells. The classic "lag phase" in the time of appearance of the cyclic AMP response to toxin (15–20 min in the case of Ca^{2+}-restored C6 cells) is believed to be due to penetration of the A_1 fragment (1, 2). However, addition of Ca^{2+} to Ca^{2+}-depleted cells treated with toxin for 37 min produced a rapid increase in cyclic AMP accumulation (Fig. 4A), suggeting that the active fragment of toxin was already present in nonlimiting concentrations in the cytoplasm. Furthermore, addition of EGTA to toxin-treated, Ca^{2+}-restored cells at a time when active fragments should have gained access to the cytoplasm (37 min) retarded cyclic AMP accumulation (Fig. 4B). A second hypothesis to be considered is that ADP ribosylation of the guanine nucleotide regulatory subunit of adenylate cyclase requires Ca^{2+}. Although Ca^{2+} increases the rate of irreversible enzyme activation by toxin, these data do not distinguish between facilitation of ADP ribosylation of the regulatory subunit of adenylate cyclase or an irreversible modification by Ca^{2+} of the ADP-ribosylated enzyme complex. The NAD glycohydrolase and ADP ribosyltransferase activities of cholera toxin have been studied by Moss and co-workers (26, 27) under carefully defined *in vitro* conditions in which NAD^+ and arginine, respectively, were used as substrates; nothing in these studies would suggest that Ca^{2+} is required for either activity. These observations, however, do not necessarily preclude a Ca^{2+} involvement in ADP ribosylation of the guanine nucleotide regulatory component of adenylate cyclase of intact cells by toxin. Clearly, more detailed studies with the components of the adenylate cyclase system are needed to determine whether such mechanisms exist. Third, the hypothesis that Ca^{2+} enhances the coupling efficiency of cell surface receptors to adenylate cyclase seems improbable, since maximally effective toxin (Fig. 2) or norepinephrine (12) concentrations are not reduced by Ca^{2+}. It is also of interest that cholera toxin pretreatment, which is proposed to increase the efficiency of coupling of *beta*-receptors to catalytic units (20), did not change the Ca^{2+} dependence of the cyclic AMP response to catecholamine (Fig. 9), implying that changes in coupling efficiency do not necessitate changes in Ca^{2+} requirements.

Moss and Vaughan (10) described the conditions under which a detergent-solubilized and partially purified adenylate cyclase of brain is activated by cholera toxin. Both the process of enzyme activation and full expression of the activated enzyme required CDR. Since CDR is the only intracellular Ca^{2+} receptor recognized to enhance adenylate cyclase activity, this protein must be seriously considered in a discussion of the effects of Ca^{2+} described in this report. Three pieces of evidence favor a role for CDR in mediating these effects. First, the association of the Ca^{2+}·CDR complex with enzymes is believed to be rapid and reversible upon removal of Ca^{2+} (28). The effect of Ca^{2+} on cholera toxin-stimulated cyclic AMP accumulation in C6 cells was rapid and quickly reversed

by EGTA. Second, the basal and norepinephrine-stimulated adenylate cyclase activities of particulate fractions of C6 cells are enhanced by CDR (11). Third, cyclic AMP accumulation in response to toxin was retarded by micromolar concentrations of trifluoperazine, an agent which binds to CDR in a Ca^{2+}-dependent manner and competitively inhibits the activation of enzymes by CDR (20). In the previous study (12), it was observed that norepinephrine-stimulated cyclic AMP accumulation in C6 cells behaved as if two components contributed to the process: one Ca^{2+}-dependent and the other Ca^{2+}-independent. Only the Ca^{2+}-dependent component of the norepinephrine response was inhibited by trifluoperazine, in accord with the observation that phenothiazines competitively inhibit CDR-dependent activation by formation of the ineffectual Ca^{2+}·CDR·phenothiazine species. In contrast, cyclic AMP accumulation in response to toxin was slowed by trifluoperazine both in Ca^{2+}-depleted and in Ca^{2+}-restored cells. This finding implies either that the drug effect is Ca^{2+}-independent or, more likely, that toxin-stimulated cyclic AMP accumulation occurs at the very low free Ca^{2+} concentrations found in Ca^{2+}-depleted cells.

All of the five types of cultured cells examined for increased cyclic AMP accumulation following cholera toxin treatment required Ca^{2+} for a rapid onset of activation. It seems likely, therefore, that the enhancement of cyclic AMP accumulation in intact cells by Ca^{2+} may be a general phenomenon. Although a CDR-dependent inhibition of Chinese hamster ovary cell adenylate cyclase has been reported (29), the effects of Ca^{2+} and CDR on cyclic nucleotide metabolism have not been extensively investigated for these cell types. Although the role of CDR, if any, in mediating the effects of Ca^{2+} on cholera toxin activation in these cell types cannot be presently defined, the possibility exists that their adenylate cyclase moieties contain a nondissociable CDR component by analogy with phosphorylase *b* kinase (30). If so, the affinity of such complexed CDR for Ca^{2+} could be much higher than that of free CDR.

Because the adenylate cyclase activity of C6 cell homogenates is enhanced to a small extent by CDR (11), it will be of interest to investigate the effects of Ca^{2+} and CDR on cholera toxin activation of the enzyme directly. It is anticipated that such investigations will shed light on the question of whether the Ca^{2+} effects on the cholera toxin responses of intact cells reported here are mediated via a CDR-dependent mechanism.

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