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Synthesis of Protein in Intestinal Cells Exposed to Cholera Toxin (42599)

JOHNNY W. PETERSON, WILLIAM D. BERG, JR., AND DORIAN H. COPPENHAVER

Department of Microbiology, University of Texas Medical Branch, Galveston, Texas 77550

Abstract. The mechanism by which cyclic adenosine monophosphate (AMP), formed by intestinal epithelial cells in response to cholera toxin, ultimately results in alterations in water and electrolyte transport is poorly understood. Several studies have indicated that inhibitors of transcription or translation block much of the transport of ions and water in the intestine and edema formation in tissue elicited by cholera toxin. Data presented in this study confirmed the inhibitory effects of cycloheximide on cholera toxin-induced fluid accumulation in the rabbit intestinal loop model. Neither cycloheximide nor actinomycin D altered the amount of cyclic AMP that accumulated in intestinal cells and Chinese hamster ovary cells exposed to cholera toxin. An increase in [^{3}H]leucine incorporation was readily demonstrable in intestinal epithelial cells from rabbits challenged with *Vibrio cholerae*. Similarly, intestinal epithelial cells incubated with cholera toxin for 4 hr synthesized substantially more protein than controls as determined by relative incorporation of [^{35}S]methionine. Most of the new protein synthesized in response to cholera toxin was membrane associated and of high molecular weight. The possible significance of the toxin-induced protein relative to cholera pathogenesis was discussed.

Suggestive evidence that a protein(s) might be involved in the mode of action of cholera toxin was first reported by Serebro *et al.* (1). They observed that cycloheximide treatment of rabbits reduced net fluid secretion in jejunal loops subsequently instilled with crude cholera toxin. The effective dose of cycloheximide (20 mg/kg) did not affect glucose absorption; rather, its primary effect was decreased mitotic figures in the crypts, where cholera toxin-induced secretion is believed to occur. The inhibitory effect of cycloheximide on net secretion was later confirmed by Moritz *et al.* (2), who demonstrated the inhibitory effects of cycloheximide on net water and ion fluxes as well as on transmural electric potentials in rabbit jejunum.

Finkelstein *et al.* (3) reported that cycloheximide treatment of rats suppressed the development of edema in foot pads, following injection of purified cholera toxin. Further, Lexomboon *et al.* (4), using the mouse foot edema model, surveyed a variety of drugs for possible effects on cholera toxin-induced edema formation. They noted that cycloheximide (100 mg/kg) inhibited cholera toxin-induced edema by 87.1% at 36 hr. Lower doses were less toxic for the mice and reduced edema proportionately less. Other drugs of interest and their inhibitory effects on edema formation included (i) actinomycin D (1.25 mg/kg), 80.4% reduction; and (ii) puromycin (100 mg/kg), 40.4% reduction.

While these data provide no direct evidence that protein synthesis is required in the mode of action of cholera toxin, their inhibitory effects remain unexplained. No reports of a protein intermediate have appeared, and concentrated research efforts on the direct stimulatory effects of cholera toxin on adenylate cyclase have not revealed any protein involvement. Indeed, cholera toxin has been observed to exert an inhibitory effect on macromolecular synthesis in lymphoid cells. Sultzer and Craig (5, 6) reported that purified cholera toxin suppressed lymphocyte blastogenesis by inhibiting synthesis of protein at 24 hr and nucleic acid after 48 hr in splenic lymphocytes from A/HeJ and C3H/HeJ mice.

Kimberg *et al.* (7) confirmed the inhibitory effects of cycloheximide on fluid accumulation and ion fluxes in rabbit ileal loops. It was observed that this antisecretory effect of cycloheximide observed *in vivo* could be demonstrated also *in vitro* using Na and Cl fluxes as well as short-circuit current measurements on ileal mucosa. Additionally, it was shown that adenylate cyclase activity in intestinal mucosa from rabbits treated with cycloheximide was not significantly different from that of control rabbits challenged with cholera toxin. These data suggested that involvements of protein

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synthesis probably occurred after synthesis of cyclic nucleotides, if indeed protein synthesis was involved at all.

The present report attempts to probe further the question of whether protein synthesis might be involved at some stage in the action of cholera toxin on the intestinal mucosa. Briefly, we have confirmed that drugs that block transcription or translation do not alter cyclic adenosine monophosphate (cAMP) levels of isolated intestinal cells. In addition, it was demonstrated that cholera toxin stimulates rather than inhibits incorporation of labeled amino acids into protein in isolated intestinal epithelial cells from adult rabbits. Some preliminary characteristics indicate that much of the radiolabeled methionine is incorporated into membrane proteins. The identity and function of this protein(s) in regulating water and electrolyte transport remain a mystery.

Materials and Methods. *Intestinal loop challenge.* Procedures for challenge of rabbits have been described previously (8). Animals were fasted for 48 hr prior to surgery. Immediately prior to surgery, an im injection of 0.05 ml/kg of xylazine hydrochloride (Haver–Lockhart, Shawnee, KS) combined with 0.35 ml/kg of ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) was administered to anesthetize the animal. A 5-cm incision was made along the abdominal midline, and the small intestine was ligated into 10-cm segments with "OO" silk suture. All injections were made through 2-cm interspaces. The intestine was returned to the peritoneal cavity and a period of either 6 or 18 hr was allowed to pass before again anesthetizing the rabbits with an iv injection of sodium pentobarbital (Abbott) in a dosage of 0.3 ml/kg. After administering an intravenous injection of air to kill the animals, the intestinal loops were exposed and the fluid:length ratio was determined. In some cases, the intestinal mucosa was scraped using glass microscope slides for the purpose of collecting mucosal cells for assaying cyclic AMP. In other rabbits, no loops were constructed and 15- to 20-cm lengths of small intestine were clamped with hemostats for isolation of intestinal epithelial cells (9). The intestinal cells contained in the early cell fractions (1–3) contained tip cells, whereas crypt cells were present in later cell fractions (5–8).

In some cases, 10-cm intestinal segments were challenged with 1 ml of an overnight culture of *Vibrio cholerae* V86 (Inaba, El Tor), grown in trypicase soy broth (Difco). The control loops were injected with 1 ml of sterile medium. Intestinal loops challenged with live *V. cholerae* were removed after 18 hr and the intestinal epithelial cells were isolated by the method of Weiser (9). In some instances, 10-cm intestinal loops were constructed and injected with 200 μg of purified cholera toxin (Schwarz/Mann Biotech). This dose of cholera toxin is in excess of that needed to elicit a secretory response in the 6-hr period. Lower doses of cholera toxin (1 μg) yield approximately the same volume of fluid, but we wanted to ensure maximal stimulation of adenylate cyclase in these studies. After an incubation period of 6 hr, the loops were removed and the intestinal mucosa was scraped to collect the cells. In other experiments, 10- to 15-cm segments of normal small intestine were scraped to collect the mucosal cells for *in vitro* exposure to cholera toxin.

Measurement of [^{3}H]leucine incorporation. The amount of protein synthesis occurring in isolated rabbit intestinal cells exposed to cholera toxin was estimated by adding [4,5-^{3}H]leucine (ICN) to 0.5-ml aliquots of the isolated cell suspension (approximately 1×10^7 cells/ml) to a final concentration of 10 μCi/ml. The cells were incubated for 4 hr at 37°C under 5% CO_2. Precipitation of total cellular protein was accomplished by addition of an equal volume of 15% trichloroacetic acid (TCA) in 0.1 *M* HCl. After overnight incubation at 4°C, the labeled protein precipitates were collected by filtration over a Brandel cell harvester and washed with 5% TCA. The precipitates in the filter paper disks were treated with 250 μl of NCS tissue solubilizer (Amersham) for 30 min at 56°C prior to addition of 4.5 ml of Soluscint 0 (National Diagnostics).

Exposure of cells to cholera toxin and drugs. Some experiments were performed with Chinese hamster ovary (CHO) cells, while others utilized mucosal cells scraped from the small intestine of adult rabbits. CHO cells were seeded at a concentration of 8×10^5/2 ml in 35-mm culture dishes in Ham's F12 medium containing 10% fetal calf serum (FCS) and 100 U/ml of penicillin combined with 100 μg/ml of streptomycin. The cells were incubated at 37°C with 5% CO_2 overnight, at which time

the culture medium was aspirated and replaced with Ham's F12 containing 1% FCS and 50 μg/ml of gentamicin. Cholera toxin (Schwarz–Mann) was added to a final concentration of 100 ng/ml (1 ED_{50}), and the cells were incubated for an additional 4 hr with or without cycloheximide (0.0005–50 μg/ml) or actinomycin D (0.0005–50 μg/ml). Some experiments were conducted with 0.05 m*M* isobutylmethylzanthine (MIX) added to inhibit cellular phosphodiesterase activity. Prior to extraction of cyclic AMP, the culture medium was aspirated and discarded.

Incorporation of [^{35}S]methionine in cholera toxin-treated intestinal cells. Aliquots of intestinal cells scraped from the small intestinal mucosa of adult rabbits were resuspended and washed in minimum essential medium plus Earle's salts (without methionine) containing 100 μg/ml chloramphenicol. Each plate of cells received 50 μCi of [^{35}S]methionine (ICN) as well as 100 ng/ml of cholera toxin, and cultures were incubated for 4 hr at 37°C in the presence of 5% CO_2. Cells were then scraped from the plastic dishes, washed by centrifugation (250*g*) for 10 min, and resuspended in phosphate-buffered saline (PBS, composition in g/liter: NaCl, 8; KCl, 0.2; Na_2HPO_4, 1.15; KH_2PO_4, 0.2; $CaCl_2$, 1; $MgCl_2$-$6H_2O$ 1; pH 7.4). The cells were lysed by freezing and thawing (1×) followed by sonication (70 W, 30 sec). The cell membranes and subcellular particles were separated from the cytoplasm by ultracentrifugation (SW55) rotor at 325,000*g* for 18 hr. Later experiments were performed using 100,000*g* for 1 hr. Samples were dissolved in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer containing 5% 2-mercaptoethanol and chromatographed by high-performance liquid chromatography (HPLC) using a TSK-125 and a TSK-250 (Bio-Rad) column connected in series and equilibrated with phosphate diluent (same as PBS but without Ca^{2+} and Mg^{2+}). A 500-μl aliquot of each 1-ml fraction was added to 5 ml of Hydrofluor (National Diagnostics) and newly synthesized proteins were located by liquid scintillation counting. Samples were also separated on SDS–PAGE using gels ranging from 3 to 12%. The gels were treated with En^{3}Hance (New England Nuclear) prior to drying under vacuum. The dried gels were then subjected to autoradiography using XAR-5 film (Kodak).

Extraction of cyclic AMP. Cyclic AMP was extracted from intestinal mucosal scrapings and CHO cell monolayers by addition of 15 and 7.5% trichloroacetic acid (TCA) in 0.1 *M* HCl, respectively. After precipitation overnight at 4°C, 0.3 μCi of ^{3}H-cyclic AMP was added to each milliliter of supernatant. The TCA was removed by extraction (5×) with 2 ml of anhydrous ether, and percentage recoveries for each sample were determined. All cyclic AMP extracts were lyophilized to dryness in a vacuum centrifuge.

Cyclic AMP assay. Intracellular levels of cyclic AMP were assayed using methods described previously (8). Briefly, dried samples were rehydrated with 200 μl of 0.1 *M* sodium acetate buffer (pH 4.0), prior to determination of the cyclic AMP concentration using the method of Gilman (10) as modified by Bros-

TABLE I. EFFECT OF CYCLOHEXIMIDE ON FLUID ACCUMULATION IN RABBIT LIGATED LOOPS INSTILLED WITH CHOLERA TOXIN

Animal group	Loop content	Fluid accumulation, $\bar{X}$ ml/cm ± 1 SD, (range)
Normal rabbits (Nos. 131 and 133)	Cholera toxin[a]	1.1 ±0.36[b] (0.762–1.400)
	Control	0
Cycloheximide rabbits[c] (Nos. 130 and 132)	Cholera toxin	0.55 ± 0.12 (0.416–0.695)
	Control	0.04 ± 0.08 (0–0.16)

[a] Cholera toxin dose was 200 μg/loop and time of exposure was 6 hr.
[b] n = 4 (2 rabbits per group and duplicate loops per rabbit).
[c] Rabbits given cycloheximide (20 mg/kg) intravenously 1 hr prior to surgery.

trom and Kon (11). Known cyclic AMP standards ranging from 0.475 to 60.8 pmole in 20 μl of acetate buffer were combined with 20 μl of acetate buffer containing 1.0 pmole of ^{3}H-cyclic AMP. Then, 15 μl of protein kinase (500 μg/ml, Sigma), dissolved in 0.01 *M* potassium phosphate buffer (pH 6.0) containing 1 mg/ml bovine serum albumin, was added to each tube. After 90 min incubation at 4°C, 100 μl of hydroxyapatite (Sigma) diluted 1:3 in phosphate buffer (pH 6.0) was added. After 5 min incubation, the hydroxyapatite was washed twice with phosphate buffer prior to dissolving in 100 μl of 3 *N* HCl and counting in 5 ml of Hydrofluor (National Diagnostics).

Results. *Effect of cycloheximide on fluid secretion and cyclic nucleotide levels.* Cycloheximide decreases cholera toxin-induced fluid secretion from small intestinal segments, when the drug is administered prior to toxin challenge. Table I illustrates that intravenous administration of cycloheximide (20 mg/kg) to a pair of adult rabbits 1 hr prior to surgery results in a reduction in fluid accumulation in intestinal segments instilled with purified cholera toxin from 1.1 ml/cm to 0.55 ml/cm compared to that of a matched pair of normal rabbits. The dose of cholera toxin used in these studies was relatively large (200 μg/loop) so that the animal responses could be examined after only 6 hr. These observations are similar to that reported by Serebro *et al.* (1).

Although cycloheximide decreased cholera toxin-induced fluid secretion of intestinal segments, the mechanism did not appear to involve effects on cyclic AMP levels in samples of mucosal tissue from the treated rabbits compared to tissue from normal rabbits responding to cholera toxin. Table II shows the concentrations of cyclic AMP in intestinal tissue collected from representative animals used in the present study. Note that cholera toxin caused a two- to threefold increase in the intracellular concentration of cyclic AMP regardless of whether cycloheximide had been administered to the animal. Despite equivalent increases in concentrations of this cyclic nucleotide in the intestinal tissues in response to cholera toxin, fluid accumulation in cycloheximide-treated animals was impaired.

Effects of cycloheximide and actinomycin D on cyclic nucleotide levels in cultured cells. The *in vivo* data described above strongly indicated that the effects of cycloheximide on the intestinal mucosa involved a mechanism occurring after cyclic AMP formation. Studies with cycloheximide and actinomycin D were extended to an *in vitro* model in which parameters could be carefully controlled. Initially, we determined by titration that cycloheximide, when added to the culture medium in a concentration of 5–50 μg/ml, would block essentially all protein synthesis in monolayers of Chinese hamster ovary (CHO) cells for a period of 4 hr, a time selected for these studies (data not shown). Figure 1A depicts a typical experiment in which cycloheximide was examined for its effect on the capacity of CHO cells to respond to cholera toxin. The lower curves show the basal level of cyclic AMP in normal CHO cells and in cells exposed to increasing concentrations of cycloheximide,

TABLE II. EFFECT OF CYCLOHEXIMIDE ON CYCLIC AMP CONCENTRATIONS IN RABBIT LIGATED LOOPS INSTILLED WITH CHOLERA TOXIN

Animal	Location	Content	Cyclic AMP (pmole/mg cell protein, $\bar{X} \pm 1$ SD)	Fold increase
Rabbit No. 133 Normal rabbit	Ileal loops	Cholera toxin[a]	31 ± 9	1.7
		Control	18 ± 8	
	Duodenal loops	Cholera toxin	83 ± 1.9	2.4
		Control	35 ± 1.6	
Rabbit No. 130 Cycloheximide-treated rabbit[b]	Ileal loops	Cholera toxin	41 ± 2.2	3.4
		Control	12 ± 0.63	
	Duodenal loops	Cholera toxin	59 ± 13.5	2.5
		Control	24 ± 9.8	

[a] Cholera toxin dose = 200 μg/loop and time of exposure was 6 hr.
[b] Rabbit given cycloheximide (20 mg/kg) intravenously 1 hr prior to surgery.

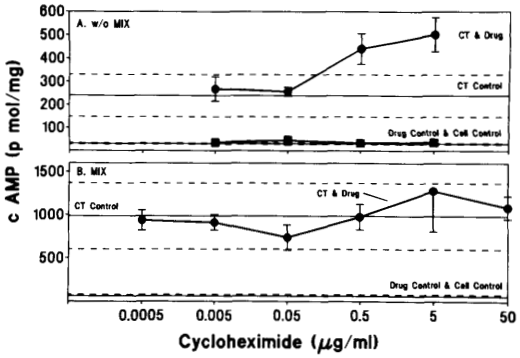

FIG. 1. Effect of cycloheximide on cyclic AMP formation in CHO cells exposed to cholera toxin (100 ng/ml). The two panels depict the results observed when CHO cells were incubated in the presence (B) or absence (A) of 0.05 m*M* MIX. CHO cell monolayers were treated with various concentrations of cycloheximide 1 hr prior to addition of cholera toxin, and the drug remained during the ensuing 4-hr incubation period. The cell control consisted of CHO cell monolayers exposed only to a 200-μl aliquot of PBS, while the drug control cells were exposed only to the drug contained in the same volume. The upper curves represent the amount of cyclic AMP accumulating in response to cholera toxin in normal cells (CT) and cycloheximide-treated cells (CT and drug). Each point represents the mean of the responses of two plates assayed in triplicate, and the standard deviation values are shown as either vertical bars or dashed horizontal lines.

while the upper horizontal bar indicates the extent of the increase in cyclic AMP observed in response to cholera toxin in the absence of cycloheximide. The upper curve labeled "CT & Drug" reveals that concentrations of cycloheximide sufficient to block protein synthesis in these cells did not reduce the cyclic AMP levels elicited by cholera toxin. Concentrations of cycloheximide between 0.5 and 5 μg/ml actually enhanced the cholera toxin effect on cyclic AMP levels. The latter phenomenon was explained when the experiment was repeated in the presence of isobutylmethylxanthine (MIX), an inhibitor of phosphodiesterase. Figure 1B depicts essentially identical results, except that the accumulation of cyclic AMP in response to cholera toxin was larger than that observed without MIX and no additional increase in cyclic AMP was apparent at and above 5 μg/ml of cycloheximide. Clearly, cycloheximide did not decrease the capacity of cells to respond to cholera toxin, since cyclic AMP levels rose to predicted levels following exposure to the toxin. Further, blocking protein synthesis with cycloheximide (≥5 μg/ml) appeared to inhibit synthesis of phosphodiesterase and parallels the effects of specific inhibitors of phosphodiesterase (e.g., MIX).

In a similar set of experiments, we examined the potential effects of actinomycin D on cholera toxin-induced cyclic AMP concentrations in CHO cells. Since prior studies by Lexomboon *et al.* (4) demonstrated that actinomycin D, a potent inhibitor of transcription, decreased cholera toxin-induced edema in the mouse foot edema model, we were interested in determining if the drug altered cell responses by decreasing their cyclic AMP concentrations. We first determined by titration that actinomycin D in a concentration of 50 μg/ml did not affect protein synthesis in a 4-hr period, but effectively blocked the incorporation of [^{3}H]uridine into RNA by 80% (data not shown). As in the case of cycloheximide, actinomycin D did not reduce the capacity of CHO cells to form cyclic AMP in response to cholera toxin. Figure 2 illustrates a typical experiment in which CHO cell monolayers were treated with cholera toxin in the presence or absence of actinomycin D. The data indicate clearly that the cyclic AMP responses of the cholera toxin-treated CHO cells were unaffected by actinomycin D. When the experiment was repeated in the presence of MIX, the results were essentially identical except that the accumulated cyclic AMP levels were higher.

Stimulatory effect of Vibrio cholerae infection and cholera toxin on protein synthesis in intestinal cells. When intestinal segments were challenged with *V. cholerae,* fluid accumulation (at 2 ml/cm) occurred in the intestinal lumen as expected, but not in the control seg-

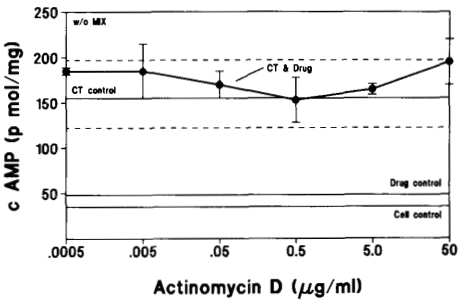

FIG. 2. Effect of actinomycin D on cyclic AMP levels in CHO cells exposed to cholera toxin (100 ng/ml). Labels for the curves are the same as in Fig. 1. MIX was not added to the cells in this experiment.

ments (data not shown). The epithelial cells were isolated from both the *V. cholerae*-infected and control loops, as previously described (9). The epithelial cells were then incubated for 4 hr at 37°C with 5% CO_2 in EMEM (without leucine) containing [^{3}H]-leucine. Figure 3 shows that the amount of [^{3}H]leucine incorporation (cpm/μg of cell protein) occurring in isolated epithelial cells from the *V. cholerae*-infected loops was larger than that in control cell fractions, particularly in the crypt cell region. Therefore, during experimental cholera, stimulation of protein synthesis occurred in addition to an increase in secretion of water and electrolytes.

In order to identify the factor in *V. cholerae* responsible for stimulation of macromolecular synthesis, epithelial cells were isolated from the small intestine of normal rabbits, using the method of Weiser (9) and exposed *in vitro* to purified cholera toxin. Figure 4 summarizes the results of a typical experiment using small intestinal epithelial cells isolated by this method. The epithelial cells were exposed to various concentrations of purified cholera toxin, ranging from 0.45 to 91 ng/ml, for a period of 4 hr. It is clear from the data that cholera toxin exerted a stimulatory effect on the incorporation of [^{3}H]leucine (cpm/mg) in these cells. Note that the [^{3}H]leucine incorporation rates of Fig. 3 are expressed as cpm/μg of cell protein, while that in Fig. 4 is expressed as cpm/mg of cell protein. Furthermore, the cells contained in the first fractions responded proportionately better and incorporated more [^{3}H]leucine. The cells that typically are present in the early fractions are derived from the tips of the villi, whereas cells contained in the later fractions are isolated from the crypt region (8, 9). The tip cells are typically more mature and do not divide, whereas the crypt cells divide rapidly (9). The apparent difference between the results observed with live cell challenge (Fig. 3) and toxin exposure of the isolated cells *in vitro* (Fig. 4) could be explained by the propensity of *V. cholerae* to colonize the intestinal crypts (12, 13), where most secretion is believed to occur. On the other hand, nontoxin factors produced during live cell infection could also stimulate [^{3}H]leucine incorporation.

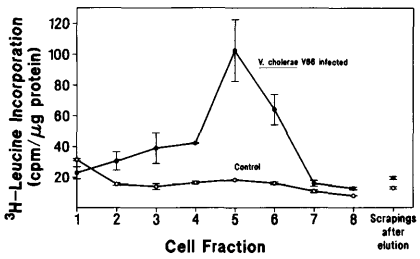

FIG. 3. Stimulatory effect of *Vibrio cholerae* V86 Inaba infection on [^{3}H]leucine incorporation in intestinal loops of adult rabbits. Each point represents the mean of two determinations, and incubation with [^{3}H]leucine was for 4 hr *in vitro*. The closed circles depict [^{3}H]leucine incorporation in cells scraped from rabbit intestinal mucosa following 18 hr of live cell challenge, while the open circles show control levels of [^{3}H]leucine incorporation in an adjacent uninfected segment.

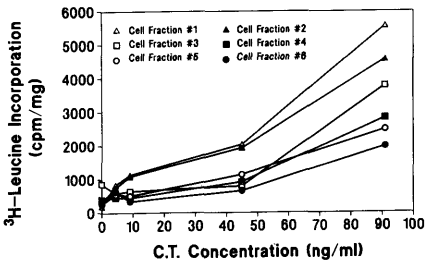

FIG. 4. Stimulatory effect of cholera toxin on [^{3}H]leucine incorporation in isolated intestinal epithelial cells from a normal rabbit. All cells exposed to cholera toxin responded with increased incorporation of [^{3}H]leucine into TCA-precipitable protein; however, cells in the tip cell region (fractions 1–3) were most responsive. Incubation with cholera toxin and [^{3}H]leucine was for 4 hr.

Chromatographic and electrophoretic profile of cellular proteins synthesized in response to cholera toxin. Since cholera toxin evoked the synthesis of new protein(s) in isolated intestinal epithelial cells, attempts were made to examine their relative size. Figure 5 shows a typical HPLC-TSK 125/250 chromatographic profile of newly synthesized proteins detected in SDS + 2-ME solubilized (unheated) ultracentrifuged pellets (325,000*g*, 18 hr) of mucosal cell scrapings pulsed with [^{35}S]methionine and exposed to cholera toxin *in vitro* for 4 hr. Note that a peak of labeled protein, migrating in the void volume (300 kDa), was much higher in cells exposed to cholera toxin than in the control. Repetition of this experiment in the presence of boiled ribonuclease A (40 μg/ml) or centrifugation at 100,000*g* for 1 hr yielded identical results (data not shown), suggesting that the high molecular weight region, containing cholera toxin-induced pro-

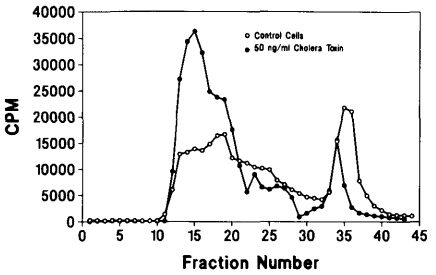

FIG. 5. Chromatography of SDS 2-ME-solubilized, ultracentrifuged pellets (unheated) from cell extracts containing [^{35}S]methionine-labeled proteins using HPLC fitted with TSK 125/250 columns connected in series. The closed circles represent the mean of two chromatography patterns from cells exposed to 50 ng/ml of cholera toxin, while the open circles show the basal level of [^{35}S]methionine incorporation in control cells. The last peak of radioactivity near fraction 36 contains free methionine. The first peak (fraction 15) elutes in the void volume stated to be 300 kDa.

tein(s), did not contain labeled peptides attached to intact ribosomes but did contain large molecular weight proteins exceeding 300 kDa that were associated with membranes. Preliminary denaturing SDS–PAGE experiments with void volume fractions (13–18) from the TSK 125/250 column revealed that the large molecular sized protein(s) did not penetrate the separating gel (data not shown).

Figure 6 illustrates the synthesis of the high molecular weight protein by intestinal mucosal cells. The lanes in this 12% SDS–PAGE gel contain SDS + 2-ME solubilized, ultracentrifuge pellets from lysates of intestinal mucosal cells exposed to 50 and 100 ng/ml cholera toxin for 6 and 12 hr. First, as would be anticipated from the previous TSK-125/250 chromatography pattern, the protein did not penetrate the 12% SDS–PAGE gel. At 6 and 12 hr, more labeled protein was apparent on top of the stacking gel as well as the separating gel interface in those cells treated with cholera toxin than in control cells. Our ability to distinguish between the intensity of the protein bands from toxin-treated and control cells decreased as the incubation time decreased. Consequently, it has not been feasible to detect reliable differences in synthesis in less than 3–4 hr. The largest molecular weight marker (myosin heavy chain) entered 0.8 mm into the gel. Thus, the molecular size of the protein by SDS–PAGE exceeds 200 kDa. Attempts to determine the precise molecular size of the large protein(s) have not yet been successful. Additional data (not shown) have indicated that the labeled protein does not readily penetrate 3–5% SDS–PAGE gradient gels. Future experiments will attempt to characterize this protein(s) and determine its function in these toxin-treated cells.

Discussion. The data presented have shown that exposure of intestinal cells to cholera toxin clearly resulted in increased synthesis of cellular protein(s) both *in vivo* and *in vitro*. The identity of the protein(s) has not yet been determined and its potential importance to ion transport has not been investigated. In comparison, it is interesting to note that substances that elevate cyclic AMP concentrations in Chinese hamster ovary cells result in increased synthesis of a 133-kDa cell surface glycoprotein whose function is unknown (14, 15). Similarly, the synthesis of a 200-kDa surface glycoprotein was enhanced by cyclic AMP in a CHO cell clone (J1) containing human chromosome 11. Both of these macromolecular synthetic events appear to involve cyclic AMP modulation of transcriptional events. Agents that elevate intracellular cyclic AMP concentration promote differentiation of leu-

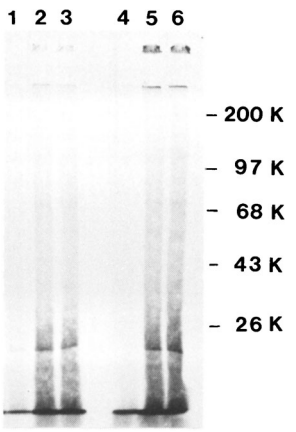

FIG. 6. SDS–PAGE analysis on 12% gels of SDS 2-ME-solubilized ultracentrifuged pellets (heated) derived from cells grown in the presence of [^{35}S]methionine with or without exposure to 50 and 100 ng/ml cholera toxin. Lanes 1 and 4 contain control cell extracts, while lanes 2 and 5 (50 ng/ml) as well as 3 and 6 (100 ng/ml) were derived from cells exposed to cholera toxin. Cells in lanes 1, 2, and 3 were incubated for 6 hr, and those in lanes 4, 5, and 6 were incubated for 12 hr. Gels were exposed to X-ray film for 2 days.

kemic cells and induce the synthesis of a variety of enzymes and surface proteins, depending on the cell type (16). Thus, cyclic AMP has been shown to be responsible for a wide variety of membrane effects that appear to be mediated by newly synthesized proteins.

Cyclic AMP previously has been shown to regulate important metabolic pathways in both eukaryotic and bacterial cells (17). Pastan and Perlman (18) demonstrated that the synthesis of β-galactosidase was markedly increased in *Escherichia coli* by cyclic AMP, which forms a complex with the cyclic AMP binding protein. This effector protein is believed to increase the binding of RNA polymerase to the promoter region resulting in increased transcription. The stimulatory effects of cholera toxin on intestinal cells could be explained by a similar mechanism, in which the efficiency of transcriptional processes normally occurring in the cells is enhanced by the elevated levels of cyclic AMP. Thus, the intestinal epithelial cells, being neither synchronized nor in the same stage of development, might be forced to synthesize larger amounts of several proteins depending on the specific cyclic AMP-regulated operons routinely transcribed during normal cellular functions. The increased synthesis of ion channel proteins (19, 20) in particular could have substantial effects on water and electrolyte transport. Additional studies are needed to determine if the newly synthesized proteins are important in modulating secretory mechanisms in the intestinal cells, as might be suggested by previous studies with inhibitors of transcription and translation (1–4).

Agents that inhibit RNA or protein synthesis tend to block fluid secretion in the intestine and edema formation in tissue as described in the introduction (1–4). It now seems clear that these agents do not alter cholera toxin's biological function by reducing cyclic AMP levels. Both *in vivo* and *in vitro* data reported here show that cycloheximide and actinomycin D do not decrease the intracellular levels of cyclic AMP. Actually, high doses of cycloheximide were shown to cause an increase in the amount of cyclic AMP accumulating in the cells. This effect was reversed by adding MIX to block phosphodiesterase activity. Potentiation of cyclic AMP formation by inhibitors of RNA and protein synthesis has been observed previously. Nichols and Brooker (21) reported that cycloheximide, like MIX, markedly increased cyclic AMP formation in C6-2B astrocytoma cells exposed to cholera toxin. Thus, it is unlikely that these drugs alter fluid and electrolyte transport by decreasing cyclic nucleotide levels.

Conclusive data are now available showing that any effect of cycloheximide or actinomycin D on cholera toxin-induced ion transport in the intestine or in tissue must result after cyclic AMP is formed. Since few details of the mechanism by which cyclic AMP controls fluid and electrolyte balance are understood, we can only postulate that a protein(s) may regulate the process. This report described the increased synthesis of as yet unidentified protein(s) formed in intestinal cells from adult rabbits exposed *in vitro* to cholera toxin for 4 hr. The newly synthesized protein(s) could be sedimented in the ultracentrifuge (100,000*g*, 1 hr), suggesting that they were membrane associated. The TSK 125/250 fractions exhibiting elevated levels of [^{35}S]methionine revealed the presence of a large molecular size protein in autoradiographs of SDS–PAGE gels.

The identity and function of the cholera toxin-induced protein(s) in the cell could not be ascertained from these studies. It is possible that the synthesis of this protein(s) is an anabolic event unrelated to the mode of action of cholera toxin. Nevertheless, it is interesting to speculate that this protein(s), synthesized following exposure to cholera toxin, could be integral to the process of cellular regulation of ion transport. Such a process would be consistent with previous studies in which drugs that inhibit RNA or protein synthesis also diminish cholera toxin-induced fluid secretion in the intestine or edema formation in tissue (1–4).

Cholera toxin may induce the synthesis of other proteins with regulatory functions. Lonnroth and Lange (22, 23) recently identified a 60-kDa protein from rats that was elevated in animals exposed to cholera toxin. This antisecretory factor is believed to downregulate secretion in an attempt to suppress fluid secretion in intestinal tissue stimulated by cholera toxin. The newly synthesized protein(s) observed in cholera toxin-treated cells in this study could contain a protein(s) that controls the process of ion transport. Additional studies are needed to probe the identity

and functions of these epithelial cell protein(s) synthesized following exposure to cholera toxin.

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