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Inhibition of Heat-Labile Cholera and *Escherichia coli* Enterotoxins by Brefeldin A

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Cholera enterotoxin and the related heat-labile enterotoxins of *Escherichia coli* enter their target cells through noncoated vesicles, but how the toxins are processed intracellularly and how they get to their targeted enzyme, adenylate cyclase, remain to be defined. Brefeldin A, an inhibitor of the trans-Golgi network, is shown herein to transiently block the morphologic and enzymatic effects of the toxin at a step distal to the initial binding process but prior to activation of adenylate cyclase by the toxin. It is likely, therefore, that these toxins are processed by the Golgi apparatus before trafficking to the membrane adenylate cyclase.

The heat-labile enterotoxins of *Vibrio cholerae* and *Escherichia coli* bind to specific glycolipid receptors on target cells, followed by entry of the A, and possibly B, subunits into the cells (3, 10, 12, 18). The entry process is not through clathrin-coated receptosomes but involves noncoated vesicles (5, 17, 22). The subsequent processing and fate of both A and B subunits remain speculative, although evidence has been presented for the processing of the toxin by the Golgi apparatus (13–16). Because the target of the A subunit's enzymatic activity is the adenylate cyclase complex located in the cell membrane, it may seem peculiar that the A subunit does not simply traverse the membrane to find its target but is detoured through the Golgi apparatus.

The evidence that cholera toxin is processed by the Golgi apparatus is based on studies with electron microscopy and unreported data that brefeldin A, an inhibitor of the trans-Golgi network, inhibits the effects of cholera toxin (13–16, 24). It has been shown that certain toxins (e.g., *Pseudomonas* exotoxin A, ricin), which are internalized by clathrin-coated receptosomes, are processed through the Golgi apparatus, and their actions are inhibited by brefeldin A, whereas diphtheria toxin, which is also internalized by clathrin-coated receptosomes, is not processed through the Golgi apparatus, and its action is not inhibited by brefeldin A (24). To better delineate the trafficking of the cholera and *E. coli* enterotoxins, studies were conducted with both families of toxins to determine whether brefeldin A had any effects on the binding or mechanisms of action of the toxins.

MATERIALS AND METHODS

Toxins. Purified cholera toxin and cholera toxin subunit A were obtained from List Biochemicals. Purified *E. coli* (heat-labile enterotoxin) type I and type II toxins were the same as those used in previous studies (9). All toxins were diluted in a 0.01 M phosphate-buffered saline (PBS) solution.

Chemicals. Adrenocorticotropin (ACTH), dibutyryl cyclic AMP, and brefeldin A were all obtained from Sigma Co. Dilutions of ACTH and brefeldin A were made in PBS. The cyclic AMP was dissolved in glass-distilled H_2O with gentle heating, and subsequent dilutions were made in H_2O.

Tissue culture. Y1 adrenal cells were propagated and maintained in Ham's nutrient mixture F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum at 37°C under a humidified atmosphere of 95% air–5% CO_2, as previously described (7). For the morphologic studies and for studies of cyclic AMP production, cells were seeded in identical numbers in 12-well plates and were grown to near confluence over the subsequent 24 h. The medium was changed once, 18 h prior to the addition of toxins to the culture medium. Brefeldin A (1 μg/ml) was added to the culture medium at various times prior to the addition of toxins. Analyses of cyclic AMP levels in the tissue culture medium employed a radioimmunoassay (New England Nuclear), as reported previously (6).

Receptor binding and toxin distribution studies. Cholera toxin was radioiodinated with ^{125}I (New England Nuclear) by the chloramine T method, as previously described, and was used within 2 weeks of iodination (7). To study the effects of brefeldin A on ^{125}I-cholera toxin binding, Y1 cells grown in the presence or absence of brefeldin A (1 μg/ml) were harvested from tissue culture plates and were diluted in F10 medium to a final concentration of 10^5 cells per ml. ^{125}I-toxin was then added (10^5 dpm = 5 ng), with or without excess (1 μg) unlabelled cholera toxin to assess specific binding and nonspecific binding, in a total volume of 1.0 ml (7). The mixture was incubated under steady-state conditions (40 min at 37°C) prior to the trapping and washing of cells on Millipore EHWP filters, followed by counting of cells with a Beckman gamma counter. Specific binding was calculated as the difference between total bound counts and nonspecific bound counts (i.e., the counts bound in the presence of excess unlabelled toxin) and was expressed as a percentage of the total counts present in the assay mixture (7).

For the toxin distribution studies, the tissue culture medium was replaced with F10 medium without serum 1 h prior to the addition of 10^5 dpm of ^{125}I-cholera toxin, with or without brefeldin A (1 μg/ml). After a 30-min incubation period, the culture medium was removed, and the cells were washed twice with fresh F10 medium without serum and then were further incubated from 1 to 6 h. At hourly intervals, cells and medium were harvested for analyses of ^{125}I counts. For analyses of intracellular counts of ^{125}I-toxin, cells were lysed by rapid freeze-thawing in 1.0 ml of H_2O, followed by centrifugation in Eppendorf tubes for 15 min to separate cell membranes (pellet) from cell cytosol (superna-

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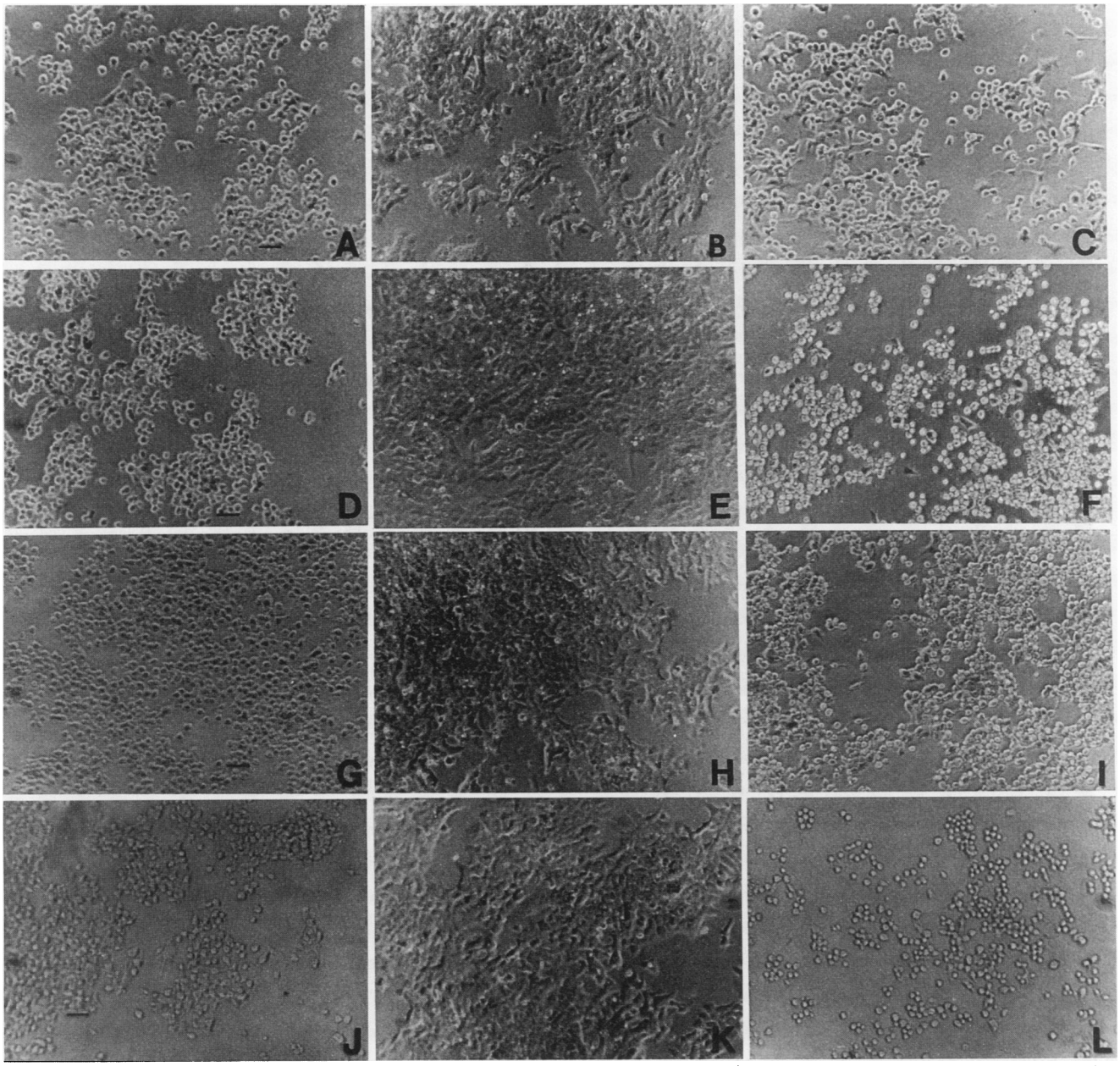

FIG. 1. Phase-contrast photomicrographs of Y1 cells in monolayer culture in the presence or absence of brefeldin A. (A to C) Cholera toxin. (D to F) Heat-labile enterotoxin type I. (G to I) Heat-labile enterotoxin type IIa. (J to L) Heat-labile enterotoxin type IIb. The left panels show toxin-treated cells (10 ng/ml) after 3 h. The middle panels show toxin-treated cells after 3 h in the presence of brefeldin A. The right panels show toxin-treated cells after 24 h in the presence of brefeldin A. Control (untreated and brefeldin A-treated) cells appeared similar to those in the middle panels (see Fig. 2). Each bar in panels A, D, G, and J represents 0.01 mm.

tant). Total (whole) cell counts were determined, as were individual cell membrane and cell cytosol counts.

Statistical considerations. Where relevant, statistical analyses employed Student's *t* test.

RESULTS

Inhibition of morphologic effects by brefeldin A. Cholera and the heat-labile enterotoxins of *E. coli*, both type I and type II toxins, all cause morphologic effects (i.e., rounding) in Y1 tissue-cultured cells after a lag period of one-half hour or more. These effects are thought to be mediated by cyclic AMP, the product of the toxin's enzymatic activation of adenylate cyclase (10, 12). Brefeldin A effectively inhibited these morphologic effects if coincubated or preincubated with Y1 cells (Fig. 1). The inhibition, however, was transient, and, by 6 h, rounding of the cells was almost as prominent in the presence of brefeldin A as in its absence. In contrast, the morphologic effects of cyclic AMP could not be prevented by the presence of brefeldin A (Fig. 2), which implies that the inhibitor's effects occurred prior to the toxin's activation of adenylate cyclase. Brefeldin A was also

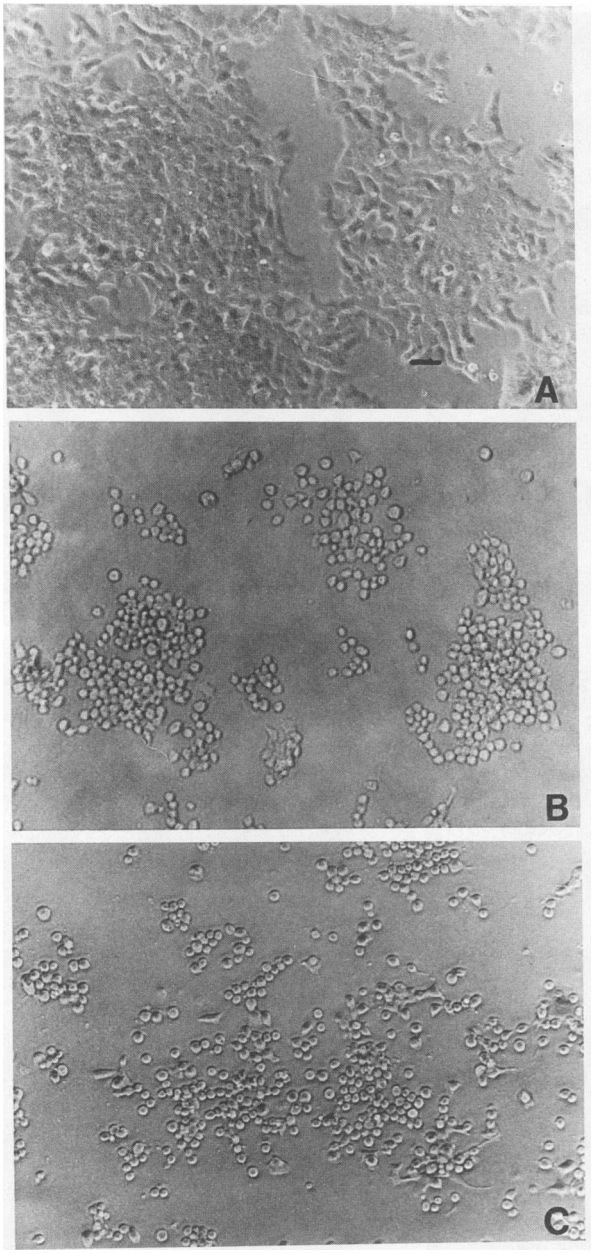

FIG. 2. Phase-contrast photomicrographs of Y1 cells treated for 3 h with 8 μM dibutyryl cyclic AMP in the absence (B) or presence (C) of brefeldin A. (A) Brefeldin A-treated cells. The bar in panel A represents 0.01 mm.

unable to inhibit the morphologic effects of ACTH (data not shown), effects which are due to transmembrane signaling of adenylate cyclase.

Inhibition of enzymatic effects by brefeldin A. In the presence of brefeldin A, the generation and release of cyclic AMP into the extracellular medium that are induced by the action of cholera toxin on membrane-associated adenylate cyclase were both delayed and reduced (Fig. 3). Brefeldin A alone had a slight inhibitory effect on basal cyclic AMP production. The effects of brefeldin A on toxin-induced cyclic AMP production could be reproduced regardless of whether it was added at the same time or up to 18 h prior to the addition of toxins to the medium.

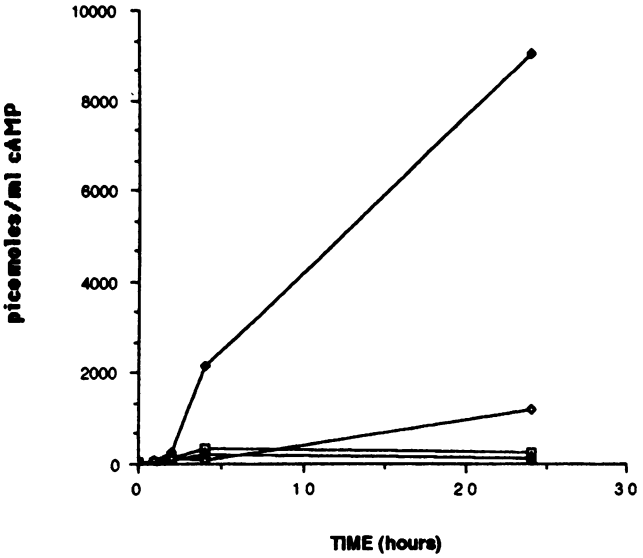

FIG. 3. Production and release of cyclic AMP by Y1 cells into the tissue culture medium induced by cholera toxin (10 ng/ml) in the presence or absence of brefeldin A (1 μg/ml). In these experiments, the brefeldin A was added 4 h prior to the addition of toxin. The results are expressed as picomoles of cyclic AMP produced per ml of culture medium. ◆, cholera toxin; ◇, cholera toxin plus brefeldin A; □, medium only; ■, medium plus brefeldin A.

Localization of the effects of brefeldin A. Brefeldin A had no significant effect on the binding of ^{125}I-cholera toxin to cells (Table 1). Preincubation of cells with brefeldin A for up to 4 h prior to the binding assay did not seem to alter the results. Experiments with radiolabelled (^{125}I) cholera toxin to study the distribution of the toxin within the cell (i.e., membrane, cytosol) or release of toxin from the cell into the surrounding medium, in the presence or absence of brefeldin A, revealed no major shifts in toxin distribution from or to cell membranes and cytosol caused by brefeldin A (data not shown). In general, there was a gradual release of ^{125}I-toxin into the extracellular medium over time that was unaffected by brefeldin A. Within cells, there was no consistent pattern of shifting of ^{125}I-toxin between membrane and cytosol in the presence or absence of brefeldin A.

DISCUSSION

The pathways and mechanisms involved in the activation of membrane-bound adenylate cyclase by cholera and the

TABLE 1. Lack of effect of brefeldin A on binding of ^{125}I-cholera toxin to Y1 cells[a]

Treatment	% Total binding[b]	% Specific binding[b]
^{125}I-cholera toxin		
− brefeldin A	17.1 ± 2.9	11.9 ± 0.2
+ brefeldin A (30 min)	17.3 ± 3.7	13.1 ± 0.5
+ brefeldin A (1 h)	19.6 ± 3.9	15.5 ± 1.7
+ brefeldin A (2 h)	19.1 ± 1.9	14.9 ± 1.3
+ brefeldin A (3 h)	17.2 ± 1.1	14.5 ± 1.0
+ brefeldin A (4 h)	15.2 ± 1.4	13.7 ± 1.5

[a] Y1 cells were preincubated with brefeldin A (1 μg/ml) for 30 min to 4 h prior to harvesting of cells and assay of ^{125}I-toxin binding.

[b] Percentage of total available counts bound in the presence or absence of excess unlabelled cholera toxin (specific binding = total bound counts − nonspecific bound counts [see Materials and Methods]).

related *E. coli* (heat-labile) enterotoxins are not completely understood. These enterotoxins first bind to specific ganglioside receptors, but the subsequent events remain to be delineated. In most model systems, the B subunits are freely and reversibly bound to their cellular receptors, while the A subunit is cleaved from the B subunit by proteolysis (7). The precise localization of this proteolytic cleavage is unclear.

The major question appears to be how the enzymatically active A subunit gets to its target, the $G_{s\alpha}$ subunit of the adenylate cyclase, in the cell membrane. It has been proposed that the A subunit migrates through the membrane layer until it reaches its target, but there is little evidence to support this hypothesis (12, 23). Alternatively, it has been proposed that the toxin is processed through the Golgi apparatus, and evidence in support of this has been reported from studies with electron microscopy to examine the fate of the toxin (13–16). One problem in the interpretation of those results is whether the toxin that appears in the Golgi apparatus is nonspecifically absorbed, biologically irrelevant toxin.

The results of our studies with brefeldin A, an inhibitor of the trans-Golgi network (19), support the hypothesis that the cholera and *E. coli* toxins must first be processed by the Golgi apparatus prior to their reaching their membrane target. Brefeldin A, while having no effect on toxin binding, had a profound effect on toxin-induced morphologic changes and generation of cyclic AMP. The fact that brefeldin A had no effect on cyclic AMP-induced morphologic changes further supports the hypothesis that the A subunit is processed by the Golgi apparatus, with the brefeldin delaying, albeit not totally inhibiting, the enzymatic activity of the toxins. The results of experiments with the A subunit of cholera toxin show that it, too, as with the holotoxin, is inhibited by brefeldin A (data not shown). Why the toxins' actions are not completely inhibited is unclear. Whether the brefeldin A effects are simply temporary, or whether the toxin is rerouted through other pathways, with simply a delay in the toxin's reaching its target, remains to be defined.

In further support of the hypothesis that the cholera and heat-labile *E. coli* enterotoxins are processed through the trans-Golgi network is the fact that both cholera and *E. coli* toxin A subunits have carboxy-terminal K(R)DEL amino acid sequences, which appear to determine further processing of proteins through the endoplasmic reticulum (1, 20). Those sequences are present in *Pseudomonas* exotoxin A and ricin but are absent in diphtheria toxin.

The final pathways after processing of the cholera and *E. coli* A subunits through the Golgi apparatus and endoplasmic reticulum that are involved in the localization of the A subunits to the membrane adenylate cyclase remain to be determined. Whether specific vesicles or organelles are involved, or whether the A subunit is simply released into the cytosol followed by random trafficking to its target in the membrane, remains to be clarified.

The lack of effects of brefeldin A on ACTH-induced morphologic effects is not surprising. ACTH's effects on membrane adenylate cyclase are almost immediate and are thought to involve transmembrane signaling, similar to other transmembrane activations of adenylate cyclase (e.g., β-adrenergic agonists) (2, 21). The rapidity of ACTH's effects also appears to be inconsistent with the time involved for processing through the Golgi apparatus and endoplasmic reticulum. In preliminary experiments with *Clostridium difficile* toxins in tissue culture (8), brefeldin A was unable to inhibit the morphologic effects of these toxins. The effects of *C. difficile* toxins A and B appear to involve rapid shifts of calcium pools after the exposure of cells to the toxins, followed by activation of the microfilament network (4). It has been proposed that *C. difficile* toxin A is processed by the Golgi apparatus (11), but our preliminary results with brefeldin A do not support that hypothesis.

Future studies of processing and trafficking of the cholera and *E. coli* enterotoxins will involve identification of the genetic and amino acid sequences, especially those in the A_2 subunit, requisite for toxin activity.

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REFERENCES

1. **Andres, D. A., J. D. Rhodes, R. L. Meisel, and J. E. Dixon.** 1991. Characterization of the carboxyl-terminal sequences responsible for protein retention in the endoplasmic reticulum. J. Biol. Chem. **22**:14277–14282.
2. **Cerione, R. A., B. Strulovici, J. L. Benovic, R. J. Lefkowitz, and M. G. Caron.** 1983. Pure β-adrenergic receptor: the single polypeptide confers catecholamine responsiveness to adenylate cyclase. Nature (London) **306**:562–566.
3. **Donta, S. T.** 1976. Interactions of choleragenoid and G_{M1} ganglioside with enterotoxins of *Vibrio cholerae* and *Escherichia coli* in cultured adrenal cells. J. Infect. Dis. **133**:S115–S119.
4. **Donta, S. T.** 1988. Mechanism of action of *Clostridium difficile* toxins, p. 168–181. *In* R. D. Rolfe and S. M. Finegold (ed.), *Clostridium difficile*: its role in intestinal disease. Academic Press, New York.
5. **Donta, S. T., P. Damiano-Burbach, and N. J. Poindexter.** 1988. Modulation of enterotoxin binding and function *in vitro* and *in vivo*. J. Infect. Dis. **157**:557–564.
6. **Donta, S. T., and A. D. Haddow.** 1978. Cytotoxic activity of *Aeromonas hydrophila*. Infect. Immun. **21**:989–993.
7. **Donta, S. T., N. J. Poindexter, and B. Ginsberg.** 1982. Comparison of the binding of cholera and *E. coli* enterotoxins to Y1 adrenal cells. Biochemistry **21**:660–664.
8. **Donta, S. T., N. Sullivan, and T. D. Wilkins.** 1982. Differential effects of *Clostridium difficile* toxins on tissue-cultured cells. J. Clin. Microbiol. **15**:1157–1158.
9. **Donta, S. T., T. Tomicic, and R. K. Holmes.** 1992. Binding of class II *Escherichia coli* enterotoxins to mouse Y1 and intestinal cells. Infect. Immun. **60**:2870–2873.
10. **Finkelstein, R. A., and F. Dorner.** 1985. Cholera enterotoxin (Choleragen). Pharmacol. Ther. **27**:37–47.
11. **Fiorentini, C., W. Malorni, S. Paradisi, M. Giuliano, P. Mastrantonio, and G. Donelli.** 1990. Interaction of *Clostridium difficile* toxin A with cultured cells: cytoskeletal changes and nuclear polarization. Infect. Immun. **58**:2329–2336.
12. **Gill, D. M.** 1977. Mechanism of action of cholera toxin, p. 85–118. *In* P. Greengard and G. A. Robinson (ed.), Advances in cyclic nucleotide research, vol. 8. Raven Press, New York.
13. **Janicot, M. J., J. P. Clot, and B. Desbuquois.** 1988. Interactions of cholera toxin with isolated hepatocytes: effects of low pH, chloroquine and monensin on toxin internalization, processing and action. Biochem. J. **253**:735–743.
14. **Janicot, M. J., and B. Desbuquois.** 1987. Fate of injected ^{125}I-labeled cholera toxin taken up by rat liver *in vivo*: generation of the active A_1 peptide in the endosomal compartment. Eur. J. Biochem. **163**:433–442.
15. **Janicot, M., F. Fouque, and B. Desbuquois.** 1991. Activation of rat liver adenylate cyclase by cholera toxin requires toxin internalization and processing in endosomes. J. Biol. Chem. **20**:12858–12865.
16. **Joseph, K. C., A. Stieber, and N. K. Gonatas.** 1979. Endocytosis of cholera toxin in GERL-like structures of murine neuroblastoma cells pretreated with GM_1 ganglioside. J. Cell Biol. **81**:543–554.
17. **Montesano, R., J. Roth, A. Robert, and L. Orci.** 1982. Non-

coated membrane invaginations are involved in binding and internalization of cholera and tetanus toxins. Nature (London) **296:**651–653.

18. **Moss, J., and M. Vaughan.** 1991. Activation of cholera toxin and *Escherichia coli* heat-labile enterotoxins by ADP-ribosylation factors, a family of 20 kDa guanine nucleotide-binding proteins. Mol. Biol. **5:**2621–2627.
19. **Orci, L., M. Tagaya, M. Amherdt, A. Perrelet, J. G. Donaldson, J. Lippincott-Schwartz, R. D. Klausner, and J. E. Rothman.** 1991. Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. Cell **64:**1183–1195.
20. **Pelham, H. R. B.** 1989. Heat shock and the sorting of luminal ER proteins. EMBO J. **8:**3171–3176.
21. **Schimmer, B. P., K. Ueda, and G. H. Sato.** 1968. Site of action of adrenocorticotropic hormone (ACTH) in adrenal cell cultures. Biochem. Biophys. Res. Commun. **32:**806–810.
22. **Tran, D., J. L. Carpentier, F. Sawano, P. Gorden, and L. Orci.** 1987. Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. Cell Biol. **84:**7957–7961.
23. **Wisnieski, B. J., and J. S. Bramhall.** 1981. Photolabelling of cholera toxin subunits during membrane penetration. Nature (London) **289:**319–321.
24. **Yoshida, T., C. Chen, M. Zhang, and H. C. Wu.** 1991. Disruption of the Golgi apparatus by Brefeldin A inhibits the cytotoxicity of ricin, modeccin, and pseudomonas toxin. Exp. Cell Res. **192:**389–395.