Stimulatory Effects of Cholera Toxin on Arachidonic Acid Metabolism in Chinese Hamster Ovary Cells (43022)

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Abstract. **Cholera toxin (CT) stimulated the release of arachidonic acid (AA) from Chinese hamster ovary cells with no apparent lag period. CT-induced release of [^{3}H]AA or its metabolites was dose dependent during a 4-hr period of toxin exposure with a minimum effective dose of 0.1 ng/ml. CT-induced release of [^{3}H]AA metabolites began within 15 min of toxin addition and became maximal after approximately 5 hr. Neither CT-A subunit nor CT-B subunit alone caused [^{3}H]AA release. Furthermore, [^{3}H]AA release was not caused by addition of dibutyryl cAMP to the culture medium, indicating that the observed effect of CT on arachidonate metabolism appeared to be independent of cAMP. The effect of CT on AA metabolism is proposed as a possible mechanism leading to the synthesis of prostaglandin E and fluid secretion during cholera.**
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The A_1 subunit of cholera toxin (CT) is known to stimulate adenylate cyclase, increasing cAMP levels in cells. Stimulation of adenylate cyclase is thought to occur by ADP-ribosylation of the regulatory protein $G_{s\alpha}$ of adenylate cyclase catalyzed by the A_1 subunit of the toxin (1). While cAMP has been considered to be the intracellular mediator of water and electrolyte transport from the intestinal mucosa during cholera, the possible role of prostaglandins in this mechanism has been reexamined recently (2–5). Prostaglandins are among the numerous metabolites of arachidonic acid (AA) and may be involved in fluid imbalances induced by several gram-negative bacteria (4). The involvement of prostaglandins in the pathogenesis of cholera was first suggested by Bennett (6), since both prostaglandin E_1 (PGE_1) and prostaglandin E_2 (PGE_2) can stimulate intestinal adenylate cyclase (7). PGE_2 levels in jejunal aspirates from acute cholera patients are significantly higher than those from convalescent patients (5). The PGE concentration increases in the intestinal mucosa of rabbits challenged with cholera toxin, *Vibrio cholerae*, or Salmonella species (4). Cholera toxin also increases the release of PGE_2 and 5-hydroxytryptamine in rat jejunal segments (3, 8). Additionally, recent research in this laboratory has shown that CT causes the synthesis and release of PGE from rabbit intestinal segments (2). Furthermore, PGE release appeared to correlate better with CT-induced fluid accumulation than with cAMP levels.

CT-induced AA release has not been characterized using Chinese hamster ovary (CHO) cells, although AA metabolism has been studied in many other cell lines including rat thyroid (FRTL5) (9), neutrophils (10, 11), bovine retina (12, 13), bovine endothelium (14), 3T3 fibroblast (15), mast (16), and platelets (17). In addition, a murine macrophage cell line, exposed to either cholera toxin or pertussis toxin, but not dibutyryl cAMP, released AA metabolites including PGE_2 (18). Release of AA metabolites, in particular PGE, from the intestinal mucosa could constitute an important mechanism in the pathogenesis of cholera and possibly other enterotoxin-mediated diarrheal diseases. We initiated this study in a cell line (CHO) that has been used extensively for the study of bacterial enterotoxins (19–24) for the purpose of studying the mechanism by which CT causes the synthesis of PGE.

Materials and Methods

Cell Culture and Labeling. Producing monolayers of CHO cells with labeled phospholipids was accomplished by the addition of 0.3 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (Amersham, Arlington Heights, IL) to a suspension of 2×10^5/ml CHO cells suspended in Ham's F-12 medium (Gibco, Gaithersburg, MD) with 10% fetal calf serum. The

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medium also contained the following antimicrobial agents: penicillin (100 units/ml), streptomycin (100 μg/ml), gentamicin (50 μg/ml), and mycostatin (50 units/ml). One milliliter of the CHO cell suspension was added to each well of 12-well tissue culture plates (Costar) or 2 ml to 35-mm tissue culture dishes (Falcon). All plates or dishes were incubated overnight at 37°C with 5% CO_2. After incubation the monolayers were washed three times with phosphate-buffered saline (composition in g/liter: NaCl, 8; KCl, 0.2; Na_2HPO_4, 1.15; KH_2PO_4, 0.2; $CaCl_2 \cdot 6H_2O$, 1.0; pH 7.4), and a volume of fresh culture medium equivalent to the original volume was added. CT was purchased from List Laboratories (Campbell, CA) and it was diluted in Ham's F-12 medium with 10% fetal calf serum to a final concentration range of 1×10^{-2} ng/ml to 1000 ng/ml. Treatments were performed in quadruplicate and the results of three experiments were averaged, except for the 200 and 500 ng/ml levels, which were based on one experiment with measurements performed in quadruplicate. After 4 hr of incubation at 37°C in 5% CO_2, 200-μl aliquots of the medium from each well or dish were mixed with 5 ml of scintillation cocktail (Hydrofluor; National Diagnostics) and counted in a liquid scintillation counter. The results of these and subsequent experiments were expressed as the percentage of counts released from the CT-treated cells above that of the controls.

The A and B subunits of CT (List Laboratories) each were tested by labeling CHO cells as described above and adding either purified A subunit (28 kDa-250 ng/ml) or B subunit (58 kDa-500 ng/ml). For comparison, cholera holotoxin (84 kDa) was used at 750 ng/ml. Treatments were performed in triplicate in three separate 12-well plates. The plates were incubated and samples from each well were counted as described above.

The kinetic experiments were performed in triplicate in 25-cm^2 tissue culture flasks containing 25 ml of Ham's F-12 medium with fetal calf serum as described above, and the results of four experiments were averaged. Cells were labeled with [^{3}H]AA as described above and exposed to 100 ng/ml of CT. Two-hundred-microliter aliquots of medium were withdrawn periodically after addition of cholera toxin for liquid scintillation counting.

Statistics. The natural variation of a biologic assay requires analysis within a test as well as between replicate tests. We employed an analysis of variance test to determine variation within replicate tests and the Tukey test to establish significance between various concentrations of CT based on a proportional increase over control levels within each individual experiment.

Results

Dose Response. CT caused an increase in the release of [^{3}H]AA metabolites from CHO cells at all concentrations above 0.01 ng/ml (Fig. 1). The slight

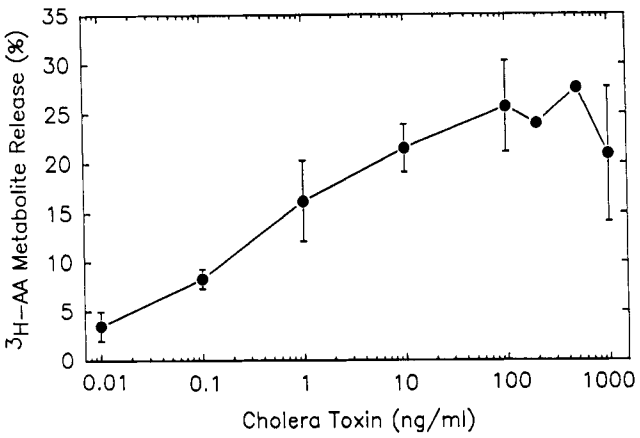

Figure 1. Dose response of CT on CHO cells measuring release of [^{3}H]AA metabolites after 4-hr incubation at 37°C. The brackets depict ±1 SE of the mean derived from three experiments performed in quadruplicate for all but the 200 and 500 ng/ml doses of CT, which represent quadruplicate measurements from one experiment.

increase (3.5%) caused by 0.01 ng/ml was considered insignificant and was used as a control level when determining statistical significance for the other concentrations tested. The 0.1 ng/ml (1.2×10^{-12} *M*) dose of CT increased the release of [^{3}H]AA and was significant by the least significant means test, but it was not considered significant by the Tukey test ($P \geq 0.05$). All subsequent statistical analyses were performed by the more conservative Tukey test. The probability values for the 1.0 ng/ml dose and all higher doses of CT indicated a significant increase ($P \leq 0.05$) in [^{3}H]AA release. The response reached a plateau at 100 ng/ml.

Specificity for CT. The [^{3}H]AA release phenomenon was shown to be specific for the cholera holotoxin (Table I). The A subunit of CT (250 ng/ml) alone failed to produce detectable increases in the release of [^{3}H]AA metabolites (−3.2%). The B subunit (500 ng/ml) produced a slight (3.1%) but nonsignificant increase ($P \geq 0.05$) in release of [^{3}H]AA metabolites. In contrast, the cholera holotoxin (750 ng/ml) produced a significant increase ($P \leq 0.05$) in the release of [^{3}H]AA (31.0%) that was equivalent to the 500 ng/ml concentration of CT in Figure 1.

Addition of dibutyryl cAMP, at concentrations of 0.1 and 1.0 m*M*, did not cause the release of [^{3}H]AA metabolites from CHO cells (data not shown). Thus, CT effects on AA metabolism were not a function of CT-induced cAMP formation.

Kinetics. We observed that the time course of CT's stimulatory effect on [^{3}H]AA release was characterized by a rapid and prolonged response beginning as early as 15 min after addition of the toxin (Fig. 2). A rapid increase in [^{3}H]AA release occurred by 15 min, after which it diminished to a low level by 1 hr. Maximum [^{3}H]AA release was observed approximately 5 hr after addition of CT. Thereafter, the response gradually diminished and, by 24 hr, no difference between CT-treated and control cultures was apparent. Based upon

Table I. Effect of CT Subunits on Release of [^{3}H]AA Metabolites from CHO Cells in a 4-hr Period

Substance[a]	Concentration (ng/ml)	[^{3}H]AA released	
		Average cpm ± SD	% Release
Cholera holotoxin	750	3296 ± 172	31.0
Cholera toxin A subunit	250	2436 ± 216	−3.2
Cholera toxin B subunit	500	2596 ± 138	3.2
Control	—	2516 ± 113	—

[a] Treatments were performed in triplicate in three separate 12-well plates.

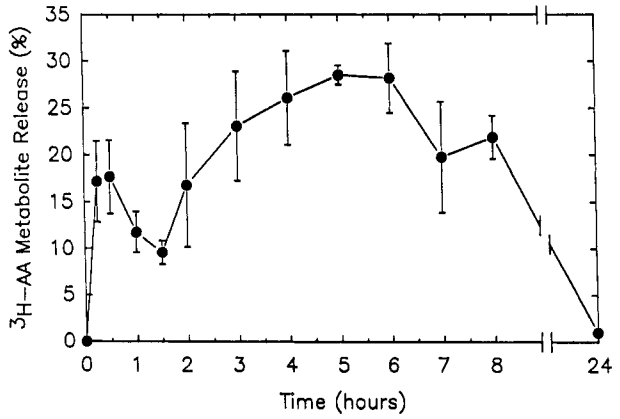

Figure 2. Kinetics of the CT effect on [^{3}H]AA release from CHO cells after exposure to 100 ng/ml of the toxin. The brackets depict ±1 SE of the mean from four experiments in which the measurements were performed in triplicate.

the Tukey test, all values above 11% [^{3}H]AA release were significant ($P \leq 0.05$).

We also have observed in a preliminary experiment that only a brief period of exposure of the CHO cells to CT was necessary to cause increased [^{3}H]AA metabolite release. For example, CHO cells exposed to CT (100 ng/ml) for periods as brief as 5 min, before replacing the medium, yielded a mean [^{3}H]AA release value (27.5%), after 4 hr of incubation, that was equivalent to that observed when the toxin remained in the medium for 4 hr (30.1%). This observation is consistent with a receptor-mediated mechanism. In the case of CT, it represents binding to G_{M1} ganglioside in the cell membrane.

Discussion

Release of [^{3}H]AA metabolites from a murine macrophage cell line exposed to CT has been reported by Burch *et al.* (18). However, the dose of CT required to stimulate AA metabolite release in murine macrophages was larger (30 ng/ml) than that required to stimulate release from CHO cells (0.1 ng/ml). Thus, it appears that CHO cells are very sensitive to the toxins of gram-negative bacteria. We observed, as did Burch *et al.* (18), that neither the isolated A subunit nor the B subunit of CT stimulated the release of [^{3}H]AA metabolites. Likewise, dibutyryl cAMP did not cause [^{3}H]AA release. Thus, the effect of CT on AA metabolism was specific for the holotoxin and not a function of CT-induced cAMP formation. It also appears that CHO cells, which comprise a valuable test system for evaluating the effects of enterotoxins (i.e., cAMP formation, cell elongation, cytotoxicity, etc.), will also be useful in studying the role of arachidonic acid metabolites, including PGE, in regulation of cellular functions. Some of the molecular events initiated by CT may add to our understanding of the pathogenic mechanisms of cholera and other enterotoxin-mediated diarrheal diseases.

Cholera toxin alters the arachidonate metabolism of CHO cells in a manner suggesting that it affects phospholipase activity, that is, it causes [^{3}H]AA release from membrane phospholipids. The identity of the specific phospholipase and the mechanism of stimulation remains to be determined. This activity may constitute an important aspect of the mechanism by which CT stimulates PGE synthesis (2). However, it should be pointed out that the relatively small amount of [^{3}H]AA released from CT-stimulated cells indicates limited hydrolysis of the membrane phospholipids. This observation correlates with the cytotonic, rather than the cytotoxic, action of CT on eukaryotic cells. The ultimate effect that CT-induced alterations in the phospholipid composition of the cell membrane would have on the permeability of the cell membrane to ions is unclear. Loss of phospholipid components from the lipid bilayer might increase the permeability of cell membranes to low molecular weight ions (e.g., Cl^-, Na^+, K^+, and HCO_3^-), independent of the protein ion channels. Loss of ions from epithelial cells into the intestinal lumen, followed by water, might provide another possible explanation for the severe dehydration characteristic of clinical cholera.

Virtually all biologic effects of CT have involved a "lag" period of approximately 30-min duration, during which it is believed, the toxin penetrates the cell membrane and catalyzes the ADP-ribosylation of $G_{s\alpha}$ of adenylate cyclase (1). We have described the kinetics of the CT effect on arachidonate metabolism (Fig. 2) as having essentially no lag period. Such a rapid effect on [^{3}H]AA release suggests that CT may be altering the activity of membrane phospholipase(s) by some transmembrane signaling mechanism. Whether the G proteins of intestinal cell phospholipase(s) become ADP-ribosylated like that of adenylate cyclase is unclear.

The initial peak of [^{3}H]AA release that occurred

during the first 15–30 min of incubation with CT appeared to be toxin specific, since the percentage of release values were reproducible increases above that of the controls. The significance of this early peak response was unclear but may reflect the rapid release of [^{3}H]AA that subsequently was assimilated and converted to various [^{3}H]AA metabolites. Alternatively, the initial peak of [^{3}H]AA release could suggest that some of the released AA metabolites (e.g., PGE) may bind to the cells, causing further stimulation.

The lowest dose of CT required to induce [^{3}H]AA release was 0.1 ng/ml, which is equivalent to 1.2×10^{-12} *M*. Considering this inordinately low concentration, it does not seem feasible that CT could be functioning as an enzyme, many of which require concentrations in the order of 1×10^{-6} *M*. Perhaps CT owes much of its exquisite potency to hormone-like behavior, enabled by its ability to enhance phospholipase activity resulting in AA release. Subsequently, arachidonate released from membrane phospholipids could be converted to prostaglandins (e.g., PGE), which would account for the dose-dependent release of PGE observed in rabbit intestinal segments injected with CT (2). Therefore, it is important to recognize that during the so-called lag phase, potentially important stimulatory effects of CT on arachidonate metabolism are occurring. Consequently, synthesis of cAMP in CT-treated cells could be increased by the early synthesis of arachidonate metabolites, such as PGE, which itself stimulates adenylate cyclase (7). Additional studies are in progress to characterize the AA metabolites released from CT-treated cells and to determine their overall significance in the pathogenesis of cholera and other enterotoxin-mediated diarrheal diseases.

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