Cholera Toxin Induces Synthesis of Phospholipase A₂-Activating Protein

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The mechanism of cholera toxin (CT)-stimulated arachidonate metabolism was evaluated. CT caused rapid *in vitro* synthesis of prostaglandin E₂ (PGE₂) in murine smooth muscle-like cells (BC₃H1), reaching maximal levels within 3 to 4 min. In comparison, cyclic AMP (cAMP) levels were unchanged, and addition of dibutyryl cAMP did not affect PGE₂ synthesis. CT-induced PGE₂ synthesis was prevented by actinomycin D or cycloheximide, indicating a need for *de novo* protein synthesis. Northern blot analysis of total RNA from BC₃H1 cells revealed that exposure to CT resulted in an increase in abundance of mRNA encoding phospholipase A₂ (PLA₂)-activating protein (PLAP). PLAP is a regulatory protein that increases the enzymatic activity of cellular PLA₂, which in turn causes increased hydrolysis of arachidonate from membrane phospholipids. Furthermore, CT evoked the accumulation of PLAP mRNA in J774 (murine monocyte/macrophage) and Caco-2 (human intestinal epithelial) cells *in vitro*, but the responses were more delayed than that of BC₃H1 cells. A protein band of approximately 35 kDa, which corresponded to the size of PLAP, was observed in sodium dodecyl sulfate extracts of Caco-2 cells by Western blot (immunoblot) analysis using affinity-purified antibodies to PLAP synthetic peptides. Synthesis of PLAP protein was increased after 2 h of exposure to CT. Exposure of mouse intestinal loops to either CT or live *Salmonella typhimurium* for 3 h increased mucosal PLAP mRNA levels. The role of PLAP in CT-induced PGE₂ synthesis provides an attractive explanation for the reported suppression of CT-induced intestinal secretion by inhibitors of protein synthesis.

Cholera toxin (CT) is widely known to affect a variety of cells by catalyzing the ADP-ribosylation of the regulatory protein G_{sα} of adenylate cyclase, which in turn increases the synthesis of the intracellular second messenger 3',5'-cyclic AMP (cAMP) (23). A second important signaling reaction that occurs in CT-treated eukaryotic cells is an increased synthesis of prostaglandins (PGs) (e.g., PGE₂ and PGF_{2α}), which results from CT-mediated release of arachidonic acid (AA) from membrane phospholipids (10). CT induction of PG synthesis has been well described (27, 31, 35), although the mechanism is poorly understood.

The cyclooxygenase pathways are the source of PGs, and the rate-limiting step in their synthesis is substrate availability (i.e., arachidonate) (32). Free arachidonate, the initial substrate required for synthesis of PGs, is limited in eukaryotic cells, but most membrane phospholipids contain esterified arachidonate in the *sn*-2 position of the glycerol moiety of the phospholipid structure. Phospholipase A₂ (PLA₂) specifically hydrolyzes fatty acids from the *sn*-2 position of phospholipids and constitutes a central point of control for PG biosynthesis. Although eukaryotic cells contain constitutive levels of PLA₂, CT increases PLA₂ activity and hence synthesis of PGs (10, 26–28, 30, 35). In search of a cellular protein that could activate PLA₂ activity, we noted that Bomalaski et al. (5–9) described the synthesis of a protein activator of PLA₂ related to melittin (13, 19), known as PLA₂-activating protein (PLAP), by inflammatory cells in inflamed joint tissues of patients with rheumatoid arthritis. We have been investigating the mechanism by which

CT increases PLA₂ activity and have postulated that CT exerts its effect on PLA₂ by increasing PLAP mRNA levels. The ultimate effect of CT-induced PLAP synthesis would be increased PLA₂ activity, which would result in increased amounts of AA and subsequently PGs.

Early investigations into the pathogenesis of experimental cholera provided preliminary evidence suggesting that the synthesis of a protein intermediate was necessary to enable CT to stimulate the intestinal mucosa, culminating in an increased transport of water and electrolytes. These studies were based on the observations that cycloheximide (i) reduced the amount of fluid accumulating in rabbit intestinal segments challenged with CT by approximately 90% (36), (ii) blocked CT-induced Na⁺ flux into intestinal segments (18), and (iii) reduced serosal to mucosal Cl[−] secretion in rabbit intestinal patches mounted in Ussing chambers *in vitro* (20, 22). Similarly, CT-induced edema in rat and mouse foot models could be reduced at least 85% by concomitant subcutaneous injection of puromycin, actinomycin D, or cycloheximide (16, 21). Furthermore, cycloheximide and actinomycin D abrogated the stimulatory effect of CT on [³H]AA metabolite release and PG synthesis in Chinese hamster ovary cells *in vitro* and in rabbit intestinal mucosae *in vivo* (25, 31). While these studies presented intriguing hypotheses regarding the pathogenesis of cholera, no CT-induced protein was identified.

In an attempt to clarify the pathogenic mechanism of experimental cholera, we initiated experiments to identify the protein(s) mediating CT's effects on AA metabolism. The rationale for initiating our experiments was based on the discovery of a PLAP in synovial fluid from patients with rheumatoid arthritis (14). On the basis of earlier observations that CT stimulated PLA₂ activity and PGE₂ synthesis (10) and that antimetabolites (e.g., cycloheximide) blocked PGE₂ synthesis

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(31), we initiated experiments to determine whether CT increased PLAP mRNA abundance. The gene encoding PLAP was cloned from a murine smooth muscle-like cell line (BC₃H1) cDNA library by Clark et al. using antibodies to melittin (13, 14, 19). Those investigators demonstrated that the PLAP mRNA levels were increased in BC₃H1 cells within 4 min after treatment with leukotriene D₄, which is another lipid metabolite derived from arachidonate via the lipoxygenase pathway (14). We examined the effect of CT on PLAP mRNA levels in these cells, as well as in an intestinal epithelial cell line (Caco-2) and in a murine monocyte/macrophage cell line (J774). The *plap* cDNA encoded a protein with a predicted molecular mass of 36,009 Da (14). This study investigated the hypothesis that PLAP is one of the protein intermediates responsible for CT effects on AA metabolism.

MATERIALS AND METHODS

Reagents. CT was purchased from List Biological Laboratories (Campbell, Calif.). All of the reagents needed for RNA isolation and the random primer kit for labeling of the gene probes were obtained from Gibco-BRL, Gaithersburg, Md. Diethylpyrocarbonate was purchased from Sigma Chemical Co., St. Louis, Mo. Salmon sperm DNA and Quikhyb solution for prehybridization and hybridization were ordered from Stratagene, La Jolla, Calif. A kit was purchased from Pierce Chemical Co. (Rockford, Ill.) to conjugate the synthetic peptides to bovine serum albumin (BSA). Synthetic peptides were coupled to cyanogen bromide (CNBr)-activated epoxy-Sepharose (Pharmacia, Piscataway, N.J.) for preparation of peptide affinity columns. For Western blot (immunoblot) analysis, goat anti-rabbit antibodies conjugated with horseradish peroxidase were obtained from Bio-Rad Laboratories (Hercules, Calif.), and an enhanced chemiluminescence (ECL) kit was obtained from Amersham Life Science, Arlington Heights, Ill. [α -³²P]dCTP was purchased from ICN, Irvine, Calif.

Cultured cells and intestinal tissues. Tissue culture cell lines (BC₃H1, J774, and Caco-2) were obtained from the American Type Culture Collection, Rockville, Md. J774 cells were grown in flasks of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 0.45% glucose, 1% nonessential amino acids, and 0.1% penicillin-streptomycin-gentamicin with 5% CO₂. BC₃H1 cells were grown under the same conditions, except that the FCS concentration was increased to 20%. Caco-2 cells were grown in Eagle's minimum essential medium containing 20% FCS, 0.45% glucose, 1% nonessential amino acids, and 0.1% penicillin-streptomycin-gentamicin with 5% CO₂. Experiments were performed with cells plated in either 35-mm-diameter dishes or 75-cm² flasks at a concentration of 2×10^5 /ml, and FCS was not present in the culture medium during CT challenge.

C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and housed in a specific-pathogen-free animal facility at the University of Texas Medical Branch. The mice were anesthetized with ether, and a single intestinal segment per mouse was ligated with 00 silk suture. CT (1 μ g/100 μ l) or live *Salmonella typhimurium* (17) (2×10^7 CFU/100 μ l) in phosphate-buffered saline (PBS) was injected into the lumen of the intestinal segments. After 3 h, the animals were sacrificed by cervical dislocation, and the intestinal tissue specimens were processed for RNA isolation. All animal experiments were performed under a protocol approved by the University of Texas Medical Branch Animal Care and Use Committee in compliance with National Institutes of Health guidelines.

Assays for PGE₂ and cAMP. The PGE₂ content of the culture media was measured by radioimmunoassay, using kits purchased from Perseptive Diagnostics Inc. (Cambridge, Mass.). PGE₂ was extracted from samples of culture media as recommended by the manufacturer. cAMP was released from the cultured cells by adding 1 ml of 7.5% trichloroacetic acid to each plate. cAMP was measured with a radiometric assay based on competitive binding to protein kinase, as described previously (29).

Isolation of RNA. Total RNA was extracted from approximately 2×10^6 cultured cells or 3- to 5-cm segments of mouse intestine as described previously (2). Cultured cells or intestinal tissues were suspended in a solution of 4 M guanidine isothiocyanate–25 mM sodium citrate–0.5% sodium sarcosyl–0.1 M 2-mercaptoethanol at 4°C before a sodium acetate-phenol-chloroform-isoamyl alcohol mixture was added. After centrifugation at 10,000 $\times$ g for 20 min, the RNA dissolved in the top aqueous phase layer was collected. The RNA was precipitated with isopropanol, washed with 80% ethanol, and stored at –70°C in 0.1% diethylpyrocarbonate. Alternatively, the RNA in guanidine isothiocyanate solution was placed on top of a cesium chloride cushion (5.7 M) and ultracentrifuged at 20°C for 18 to 20 h at 100,000 $\times$ g.

Northern (RNA) blot analysis. Aliquots of total RNA (10 to 15 μ g) were subjected to electrophoresis using a 1.2% formaldehyde agarose gel (2). The RNA was transferred onto a nitrocellulose membrane, which then was baked at 80°C for 2 h, prehybridized for 1 h at 68°C in Quikhyb, and then hybridized for 2 h in the presence of 100 μ g of salmon sperm DNA per ml and the ³²P-labeled

plap cDNA probe (10⁷ cpm/ml). The pUC9 recombinant plasmid (obtained from J. Bomalaski, Philadelphia, Pa.) contained a 2.5-kb *EcoRI* DNA fragment encoding the *plap* gene. Since the sizes of the vector and insert were very similar, we digested the recombinant pUC9 plasmid with *EcoRI*-*BglI*. Vector pUC9 has one *BglI* site, whereas no *BglI* restriction site exists in the insert. These two enzymes (*EcoRI* and *BglI*) generated three fragments of 2.5, 1.8, and 1.0 kb. The last two fragments were from the vector. The 2.5-kb DNA fragment containing the *plap* gene was isolated from the gel and purified before being labeled with [α -³²P]dCTP (2). After hybridization, the filters were washed with 2 $\times$ SSC (1 $\times$ SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate, pH 7.0)–0.1% sodium dodecyl sulfate (SDS) (2) at 68°C for 1 h and then with 1 $\times$ SSC–0.1% SDS for another hour. The filters were dried and exposed to X-ray film at –70°C for 5 h to 3 days. Subsequently, the ³²P-*plap* gene probe was stripped from the filters by heating the filters at 65°C for 30 min in a solution containing 2 $\times$ SSC and 50% formamide. Then the filters were reprobed with a ³²P-labeled β -actin gene probe, which was used as an internal control to quantitate the RNA load in each lane of the gel.

Preparation of antipeptide antibodies. On the basis of a hydrophilicity plot of the PLAP amino acid sequence (14), two synthetic peptides, F-31 (¹⁸⁵NSKD-FVTTSEDRSLR¹⁹⁹) and F-32 (²³⁸RVFTESEERTASAEIK²⁵⁴), were synthesized and purified to 95% purity by the Protein Chemistry Laboratory at M. D. Anderson Cancer Center, Houston, Tex. The peptides were conjugated with BSA by using an Inject SuperCarrier System (Pierce), mixed with Freund's complete adjuvant (Sigma Chemical Co.), and injected into multiple subcutaneous sites in rabbits (2 mg per rabbit). Blood samples were taken from the animals before immunization, and the serum from this initial bleeding was used as a negative control. The animals were immunized with the respective conjugated peptides in Freund's incomplete adjuvant every 4 weeks, and blood was drawn every 3 weeks. The antibody titers were determined by enzyme-linked immunosorbent assays using the synthetic peptides (50 ng per well) as the source of antigen. Subsequently, the antibodies to peptides F-31 and F-32 were purified by affinity chromatography after the synthetic peptides were conjugated to CNBr-activated epoxy-Sepharose as described by the manufacturer (Pharmacia). The antibodies were eluted from the column with 0.5 M glycine, pH 2.5. After dialysis against 10 mM phosphate buffer (pH 7.0), the antibody solution was concentrated to a final protein concentration of 1 mg/ml and stored at –20°C.

Western blot analysis. Caco-2 cells were stimulated with CT (1 μ g/ml) for various times, and the monolayer was detached from the tissue culture flask by using Eagle's minimum essential medium containing 0.25% trypsin. The cell suspensions were sonicated and centrifuged at 100,000 $\times$ g for 1 h. The protein concentrations of the samples were estimated by A₂₈₀, and approximately 50 μ g of each cell lysate was mixed with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) buffer (2), boiled for 5 min, and separated on an SDS–12% polyacrylamide gel. After completion of electrophoresis, the protein bands were transferred onto a nitrocellulose membrane, probed with affinity-purified antibodies to PLAP synthetic peptides, reacted with affinity-purified goat anti-rabbit immunoglobulin G (IgG) conjugated with horseradish peroxidase (Bio-Rad Laboratories), and developed by the ECL technique (Amersham Life Sciences).

Quantitation of Northern and Western blot signals. The signals detected on all X-ray films used in Northern and Western blotting were quantitated by densitometric scanning with a computer-controlled video device (Applied Imaging, Pittsburgh, Pa.). After subtraction of background, the PLAP mRNA levels on Northern blots were normalized in proportion to the maximum density of the β -actin signal on each filter. Representative blots were selected for use in the study.

RESULTS

CT-induced PGE₂ synthesis in BC₃H1 cells. The kinetic effect of CT on PGE₂ synthesis in BC₃H1 murine smooth muscle-like cells is illustrated in Fig. 1. The data indicate that maximum synthesis of PGE₂ occurred within 3 to 4 min ($P < 0.05$). Similar PGE₂ kinetic responses have been reported for leukotriene D₄ stimulation of BC₃H1 cells (14). No change in cAMP levels ($P > 0.05$) was detected at this early time (Fig. 2B), and addition of membrane-permeable cAMP derivatives (e.g., dibutyryl cAMP) had no detectable effect on PGE₂ synthesis in a 5-min period (data not shown).

Inhibition of PGE₂ synthesis in BC₃H1 cells. To confirm the involvement of a protein intermediate in the mechanism of action of CT (25), we determined whether RNA or protein synthesis was necessary for PGE₂ synthesis in BC₃H1 cells exposed to CT. We observed that either actinomycin D or cycloheximide abrogated the CT-mediated PGE₂ response in BC₃H1 cells ($P < 0.05$), in effect maintaining the same PGE₂ levels as those of the controls (Fig. 2A). Furthermore, the stimulatory effect of CT on PGE₂ synthesis was blocked by

FIG. 1. Synthesis and release of PGE₂ into the culture medium by BC₃H1 murine smooth muscle-like cells at various times after addition of CT (1 μ g/ml). PGE₂ levels between 3 and 10 min were significantly higher than those of the control or those during the 1- to 2-min periods of exposure to CT ($P < 0.05$, as determined by analysis of variance [ANOVA] and the Scheffé test using a log scale to provide a more normal distribution of data).

coinubation with G_{M1} ganglioside ($P < 0.05$), an indication that CT's effect on PG synthesis depended on receptor specificity. These data confirmed that a protein(s) was involved in the stimulatory effect of CT on PGE₂ synthesis in BC₃H1 cells. Thus, we postulated that CT induction of PLAP synthesis might be important in the biological activity of the toxin, because CT increased PLA₂ activity (10, 35). In preliminary experiments with adult rabbit intestinal loops (26, 28), we noted that intraluminal injection of actinomycin D or cycloheximide (100 μ g per loop) reduced the CT-induced fluid accumulation by 23 and 42%, respectively, in a 16-h observation period (data not shown).

Northern blot analysis of CT-stimulated BC₃H1 cells. By using methods similar to those employed to study the role of PLAP in rheumatoid arthritis (6, 7), we probed total RNA from BC₃H1 cells with a 2.5-kb ³²P-labeled cDNA probe encoding the *plap* gene (2, 14). Northern blot analysis, as shown in Fig. 3a, revealed an increased quantity of mRNA transcripts (2.5 to 3.0 kb) for PLAP at 4 min, which correlated with the time of increased PGE₂ synthesis in BC₃H1 cells (Fig. 1). By 5 min, the amount of PLAP transcript had decreased to control levels, as described previously for leukotriene D₄ stimulation

FIG. 3. Northern blot analysis of total RNA from BC₃H1 murine smooth muscle-like cells exposed to CT (1 μ g/ml). (a) BC₃H1 cells were treated with CT for 0 (lane 1), 4 (lane 2), 5 (lane 3), and 10 (lane 4) min. The position of the 2.5- to 3.0-kb transcript is indicated (arrow). (b) The densities of the PLAP mRNA signals were quantitated and normalized with regard to the amount of total RNA loaded into each lane, as determined by hybridization of the blot with the β -actin probe (5×10^5 cpm/ml). The abundance is synonymous with fold increase over the control.

of these cells (14). Figure 3b indicates the relative amount of PLAP mRNA in each lane (Fig. 3a), as determined by scanning the blot with a densitometer and normalization of total RNA reacting with a ³²P-labeled probe specific for β -actin. The PLAP message was fourfold more abundant after 4 min of exposure to CT (Fig. 3b). The constitutive level of PLAP mRNA in the absence of CT is shown in Fig. 3a (lane 1). By 5

FIG. 2. (A) Inhibition of PGE₂ synthesis by actinomycin D (Actino) (10 μ g/ml), cycloheximide (Cyclo) (20 μ g/ml), or G_{M1} ganglioside (200 μ g/ml) in BC₃H1 murine smooth muscle-like cells during 5-min exposure to CT (1 μ g/ml). Only the PGE₂ content of the CT group was significantly higher than that of the control ($P < 0.05$, as determined by ANOVA and the Scheffé test using a log scale to provide a more normal distribution of data). (B) cAMP levels in the cells and the culture medium were not significantly different from those of the respective controls ($P > 0.05$, as determined by ANOVA and the Scheffé test using a log scale to provide a more normal distribution of data).

FIG. 4. (a) Northern blot analysis of total RNA (15 μ g of RNA per lane) from Caco-2 cells exposed to CT (1 μ g/ml) for 0 (lane 1), 10 (lane 2), 30 (lane 3), 60 (lane 4), and 180 (lane 5) min. The position of the transcript (2.5 to 3.0 kb) (arrow) is indicated. (b) The densities of the PLAP mRNA signals were quantitated and normalized with regard to the amount of total RNA loaded into each lane, as determined by hybridization of the blot with the β -actin probe (5×10^5 cpm/ml). The abundance is synonymous with fold increase over the control.

min, the level of PLAP message was comparable to that of the control (Fig. 3a and b, lanes 3 and 4). These data indicate to us that CT increases PLAP mRNA abundance, which subsequently could be translated into PLAP protein and result in stimulation of PLA₂. Subsequent synthesis of PGs (e.g., PGE₂) depends upon the availability of AA released from membrane phospholipids by PLA₂ activity.

Northern blot analysis of CT-treated Caco-2 and J774 cells. Exposure of Caco-2 intestinal epithelial cells to CT caused up regulation of PLAP mRNA levels, similar to that observed with BC₃H1 cells, except that the kinetics of the response was more delayed (Fig. 4a). In fact, no rapid response occurred, but the PLAP mRNA signal was evident by 3 h (Fig. 4a, lane 5), which was approximately sevenfold higher than the control level (Fig. 4a, lane 1). No significant increase in the PLAP message was apparent up to 1 h (Fig. 4a and b, lanes 2 to 4). In a similar manner, a fourfold increase in mRNA levels was noted with the J774 monocyte/macrophage cell line after 10 min of CT stimulation (Fig. 5a and b, lane 4). By 30 min (Fig. 5a and b, lane 5), PLAP mRNA abundance was approximately sevenfold greater than the control level and reached a plateau of 10-fold above the control by 2 h (Fig. 5a and b, lane 7). In general, the basal level of PLAP in J774 and Caco-2 cells was low compared with that of BC₃H1 cells (Fig. 3 to 5).

Detection of PLAP protein by immunoblot analysis. Caco-2 cells were treated with CT (1 μ g/ml) for various times, and sonic extracts of the cells were examined by SDS-PAGE and Western blot analysis. It was evident from the immunoblot

FIG. 5. (a) Northern analysis of total RNA (15 μ g per lane) from J774 monocyte/macrophage cells exposed to CT (1 μ g/ml) for 0 (lane 1), 2 (lane 2), 4 (lane 3), 10 (lane 4), 30 (lane 5), 60 (lane 6), 120 (lane 7), and 240 (lane 8) min. The PLAP transcript (arrow) is indicated. (b) The densities of the PLAP mRNA signals were quantitated and normalized with regard to the amount of total RNA loaded into each lane, as determined by hybridization of the blot with the β -actin probe (5×10^5 cpm/ml). The abundance is synonymous with fold increase over the control.

shown in Fig. 6a that a single 35-kDa protein reacted with the affinity-purified antibodies to PLAP peptide (F-32). Synthesis of the PLAP protein was increased by 2.5-fold after 2 h of exposure to CT (Fig. 6a and b, lane 5), which corresponded with the amount of PLAP mRNA detected at 3 h (Fig. 4). We did not examine the level of mRNA in Caco-2 cells after 2 h of CT treatment.

Northern blot analysis of mouse intestinal mucosae. Since the normal site of action of CT is the small intestine, we tested whether CT would enhance PLAP mRNA abundance in mouse intestinal mucosae. Previous studies have indicated that CT stimulates PGE₂ synthesis in intestinal tissue, suggesting a possible role for PGE₂ in its mechanism of action (30). The Northern blot analysis shown in Fig. 7a and b (lanes 2 to 4) shows an 8- to 12-fold increase in the amount of PLAP mRNA present in small-intestinal segments of three C57BL/6 mice after a 3-h luminal challenge with CT compared with that of RNA from normal intestinal tissue exposed to PBS (lane 1). No significant level of PLAP message was detected up to 2 h. The PLAP transcript appeared to decrease after 4 h when the loops were challenged with CT (data not shown).

While performing the *in vivo* experiments with CT, we challenged other mouse intestinal loops with *S. typhimurium* TML-R66. As shown in Fig. 7, the *plap* gene probe hybridized more to RNA (5- to 12-fold) from intestinal loops challenged with live *S. typhimurium* TML-R66 (lanes 5 and 6) than to RNA from normal intestinal tissue exposed to PBS (lane 1). Preliminary intestinal loop results have indicated that minimal mes-

FIG. 6. (a) Western blot analysis of Caco-2 cells after treatment with CT (1 µg/ml) for the following times: 0 (lane 1), 15 (lane 2), 30 (lane 3), 60 (lane 4), and 120 (lane 5) min. After sonication, the cytosolic fraction (50 µg of protein per lane) was electrophoresed in SDS-12% polyacrylamide gels, transblotted, and developed by using affinity-purified antibodies to PLAP synthetic peptide (F-32). The Western blots were developed by the ECL technique. (b) The densities of the PLAP protein signals were quantitated to illustrate the relative amount of PLAP detected. The abundance is synonymous with fold increase over the control.

sage for PLAP was observed before 3 h. These data illustrate that PLAP is synthesized in intestinal tissue during *Salmonella* infection.

DISCUSSION

In this report, we document that PLAP mRNA and PLAP protein are increased in cells stimulated by CT, and the data indicate that PLAP could be responsible in part for stimulating AA metabolism, which leads to PG synthesis. The roles of PGs and other mediators in the pathogenesis of cholera have been reviewed elsewhere (26) and along with the enteric nervous system are thought to participate in the transmission of signals among various intestinal cell types (4, 15). Injection of PGE₂ into the mesenteric arteries of the rat small intestine causes secretion of water and electrolytes into the intestinal lumen (3). The PGE₂ content of luminal fluids from patients with acute cholera is markedly increased compared with that of convalescent patients (37, 38). In addition, the luminal fluid

FIG. 7. (a) Northern blot analysis of total RNA (30 µg of RNA per lane) from intestinal segments from mice challenged with CT (1 µg) or live *S. typhimurium* (2 × 10⁷ CFU/100 µl) for 3 h before the animals were sacrificed. The lanes contain RNA from loops injected with PBS (lane 1), CT (1 µg) (lanes 2 to 4), or *S. typhimurium* TML-R66 (lanes 5 and 6). The PLAP transcript (arrow) is indicated. (b) The densities of the PLAP mRNA signals were quantitated and normalized with regard to the amount of total RNA loaded into each lane, as determined by hybridization of the blot with the β-actin probe (5 × 10⁵ cpm/ml). The abundance is synonymous with fold increase over the control. S.t., *S. typhimurium*.

secreted into intestinal segments of rats and rabbits after CT challenge contains increased amounts of PGE₂ (26, 28, 30). Similarly, in vitro studies with cultured cells have indicated that CT elicits a generalized increase in PG synthesis (27, 31, 35), which appears to result from increased PLA₂ activity (10). This report addresses the mechanism by which CT increases PG synthesis.

The data indicated that CT has the potential of increasing the levels of PLAP mRNA in cultured cells and in an experimental animal model. Likewise, we acknowledge that a role for PLAP in cholera is not known, and additional studies would be required to establish the role of PLAP, PLA₂, or PGs in cholera. However, we have considered the possibility for the sake of subsequent discussion. We know that cholera is a secretory diarrhea that does not involve the influx of inflammatory cells into the lamina propria. PLAP, on the other hand, is considered a PLA₂-regulatory protein associated with inflammation in rheumatoid arthritis patients (7). Therefore, it is unclear how CT-induced PLAP could stimulate intestinal PLA₂ activity, resulting in increased PG synthesis and hypersecretion of water and electrolytes, without mediating an inflammatory cell response, as seen in rheumatoid arthritis (7). A partial explanation may relate to the amount of PLAP synthesized or to the site of production. During cholera, *Vibrio cholerae* colonizes the small-intestinal mucosa and secretes CT, which exerts a localized stimulatory effect on secretion (23). In contrast, in rheumatoid arthritis patients, PLAP elicits the accumulation of synovial fluid accompanied by the influx of inflammatory cells (7). Furthermore, the type of cell(s) affected by PLAP may determine the specific phospholipid that serves as the substrate

for the PLAP-activated PLA₂ (6, 10). Although the mechanism by which PLAP stimulates PLA₂ is not known, amino acid sequence homology between four tandem repeats in PLAP and the β -subunits of various regulatory G proteins (e.g., transducin) has been noted (24). It has been hypothesized that PLAP may constitute a new class of effector proteins having G-protein-like activity in signal transduction (24).

We determined PLAP mRNA levels in the intestinal loops of mice challenged with *S. typhimurium* (Fig. 7). This invasive bacterium produces a variety of virulence factors, such as lipopolysaccharide, a CT-related enterotoxin, and other products that could be responsible for increasing PLAP mRNA levels. The bacteria induce either secretory or inflammatory-type diarrheas, and an enterotoxin has been reported to stimulate PGE₂ release (11, 33, 34). The basis for increased PLAP mRNA abundance in the *S. typhimurium*-infected mouse intestinal loops should not be assumed to result from the same mechanism as that elicited by CT. Since intestinal infection with *S. typhimurium*, unlike experimental cholera, elicits an inflammatory response, multiple mechanisms could alter PLAP abundance. In fact, Arnold et al. (1) have shown that the lesions induced in the small intestine during *Salmonella* infection are dependent upon tumor necrosis factor alpha (TNF- α) production by macrophages. Further, TNF- α and interleukin-1 can induce PLAP synthesis in endothelial cells (9, 12), and PLAP can induce synthesis of TNF- α and interleukin-1 (8). This cytokine interplay subsequent to PLAP activation may be important in regulating the development of an inflammatory response.

It was noted that the kinetics for PLAP mRNA accumulation following CT stimulation *in vivo* (Fig. 7) and in Caco-2 and J774 cells *in vitro* (Fig. 4 and 5) were remarkably similar. In contrast, BC₃H1 cells showed a rapid and transient increase in abundance of PLAP mRNA in response to CT (Fig. 3). It must be emphasized that the basal level of PLAP message in these cells also was much higher than those in Caco-2, J774, and murine intestinal cells. Although the cAMP levels in BC₃H1 cells were not increased by CT within 5 min when PLAP mRNA and PGE₂ levels were increased (Fig. 2), the role of cAMP in stimulation of PLAP in intestinal cells cannot be ruled out.

In summary, the data suggest that molecular events initiated by CT in several eukaryotic cells result in increased abundance of PLAP mRNA, which in turn could increase PLAP production and lead to increased PG synthesis. Our data indicated the need for G_{M1} ganglioside receptor binding in order for CT to stimulate PGE₂ synthesis. Although the message for PLAP in BC₃H1 cells was increased within 4 min of addition of CT, the kinetics for PLAP mRNA accumulation were more delayed in J774 and Caco-2 cells and in the intestinal mucosa. Although PG synthesis has been correlated with the pathogenic mechanism of cholera, the physiological effects of CT appear to consist of multiple mechanisms, also involving the enteric nervous system and direct effects of cAMP on ion transport, as reviewed elsewhere (26, 28). The data described in this report provide new insight into the molecular events leading up to PG synthesis in cells exposed to CT. This information may be useful in designing future studies of the pathogenic mechanism of cholera and may offer new direction for the study of other diarrheal diseases, including those that involve intestinal inflammation.

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