

# Role of Platelet-activating Factor in Chinese Hamster Ovary Cell Responses to Cholera Toxin

Nathan M. Thielman,\*† M. Marcinkiewicz,§ J. Sarosiek,§ Guodong D. Fang,\* and Richard L. Guerrant\*†

\*Division of Geographic and International Medicine, †Division of Infectious Diseases, and §Division of Gastroenterology, Department of Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908

## Abstract

Cholera toxin (CT)-induced intestinal secretion and Chinese hamster ovary cell (CHO) elongation involves cyclic adenosine monophosphate and protein synthesis-dependent prostaglandin formation. We previously reported inhibition of CT-induced intestinal secretion and CHO elongation by platelet-activating factor (PAF) receptor antagonists and secretion of PAF by human intestinal epithelial cells exposed to CT. Herein, we show that PAF is involved after cAMP and that PAF, like CT, mediates prostaglandin  $E_2$  synthesis in CHO cells. CT-induced CHO elongation was blocked by specific PAF receptor antagonists, BN52021 and SR27417. SR27417 blocked dibutyl cAMP-induced CHO elongation, but did not alter CHO elongation caused by  $PGE_2$ . Neither CT-stimulated cAMP accumulation nor  $PGE_2$  production was inhibited by SR27417. Both  $PGE_2$  and PAF caused significant CHO elongation, but the latter did not stimulate significant cAMP production. In addition, PAF, like CT and dibutyl cAMP, stimulated significant  $PGE_2$  production. Finally, the protein synthesis inhibitor cycloheximide, which completely blocks the effect of CT on prostaglandin synthesis, also blocked that of PAF, suggesting that PAF also mediates protein synthesis-dependent prostaglandin formation. We conclude that PAF is involved in CHO cytoskeletal responses to CT after the accumulation of cAMP and, like CT, PAF stimulates protein synthesis-dependent prostaglandin accumulation. (*J. Clin. Invest.* 1997; 99:1999–2004.) Key words: platelet-activating factor • cyclic adenosine monophosphate • cholera toxin • prostaglandins • cytoskeleton

## Introduction

Adenylate cyclase activation (1–4) and prostaglandin synthesis (5–8), widely believed to be of pathogenic significance in the secretory diarrhea caused by cholera toxin, correspond with characteristic morphologic changes observed in Chinese ham-

ster ovary (CHO)<sup>1</sup> cells (9–11). Recent evidence suggests that cholera toxin (CT)-induced prostaglandin synthesis and cAMP formation in CHO cells are interrelated. Peterson and co-workers have shown that dibutyl cAMP causes arachidonic acid release from CHO cells, and that this step involves synthesis of phospholipase  $A_2$  activating protein, which is ultimately responsible for prostaglandin production (11, 12).

We have shown that platelet-activating factor (PAF), a well documented secretagogue (13–17), and a potent stimulus for prostaglandin synthesis via phospholipase  $A_2$  (18, 19) is a necessary cofactor for both secretion and CHO cell elongation caused by cholera toxin (20). Moreover, we observed PAF secretion both in mucosal homogenates of rabbit ileal loops and in human intestinal epithelial cells (T84 cell line) exposed to cholera toxin. The relevance of these findings, however, to classical mechanisms of cholera toxin-mediated effects on cells has not been determined. Herein, using highly selective PAF receptor antagonists, we explore the relationship of PAF to CT-mediated CHO cell elongation, in particular as it relates to the sequence of cAMP production and prostaglandin effects.

## Methods

**Morphologic and viability studies.** Stock cultures of CHO-K1 cells (CCL61; American Type Culture Collection, Rockville, MD) were grown in F12 medium supplemented with 10% FCS in 5%  $CO_2$  at 37°C and were passaged by trypsinization. For morphologic studies, freshly trypsinized cells in 200  $\mu$ l of F12 medium with 1% FCS were added to each well of a 96-well plate and incubated overnight in 5%  $CO_2$  at 37°C as previously described (8). Cholera toxin, with or without the PAF receptor antagonists BN52021 or SR27417, prostaglandin  $E_1$ , prostaglandin  $E_2$ , or platelet activating factor were added to the designated wells, which were again incubated overnight. Cells were fixed with 100% methanol, stained with Giemsa, and the percentage of cells elongated in each well was counted. Cells were considered elongated when they were clearly bipolar, elongated three times or more their width, and had lost their knoblike projections. For each well, 100 cells were counted. To assess the effect of PAF antagonists on CHO cell viability at 24 h, additional wells containing  $10^{-5}$  to  $10^{-7}$  M SR27417, were examined for trypan blue exclusion, and the percentage of cells taking up trypan blue was counted. In addition, lactate dehydrogenase was measured in supernatants of cells exposed to  $10^{-5}$  to  $10^{-7}$  M SR27417 using a lactate dehydrogenase quantitative colorimetric determination kit.

**Cyclic AMP determinations.** CHO cells were grown to near confluence in 60-mm tissue culture dishes and incubated for times ranging from 2 min to 24 h in F12 medium with 1% FCS with 10 ng/ml of cholera toxin (with or without  $10^{-6}$  M SR27417),  $10^{-5}$  M prostaglandin  $E_2$ , or  $10^{-5}$  M PAF. Incubations were terminated by aspiration of the medium and addition of 1 ml 0.1 N HCl to each plate,

Address correspondence to Richard L. Guerrant, Division of Geographic and International Medicine, Box 485, University of Virginia School of Medicine, Charlottesville, VA 22908. Phone: 804-924-5242; FAX: 804-977-5323.

Received for publication 1 November 1995 and accepted in revised form 30 January 1997.

*J. Clin. Invest.*

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0021-9738/97/04/1999/06 \$2.00

Volume 99, Number 8, April 1997, 1999–2004

1. Abbreviations used in this paper: CHO, Chinese hamster ovary; CT, cholera toxin; PAF, platelet-activating factor; PLA<sub>2</sub>, phospholipase  $A_2$ .

which remained at room temperature for 30 min. Supernatants were assayed for cAMP using a radioimmunoassay by methods previously described (21). Cellular protein was dissolved in 0.2 N NaOH and determined by the method of Lowry et al. (22). cAMP concentrations are expressed as picomoles of cAMP per milligram of cellular protein.

**PGE<sub>2</sub> measurements.** CHO cells grown to near confluence in 60-mm tissue culture dishes in F12 with 1% FCS were exposed to CT, dibutyl cAMP, or PAF. In protein synthesis inhibition experiments, cells were preincubated for 30 min with cycloheximide or actinomycin D, each at 10 µg/ml. Incubations were terminated at 4 h and medium was aspirated promptly into 1:4 water:ethanol with glacial acetic acid and citrate aspirin. PGE<sub>2</sub> was eluted over an Amprep minicolumn (C18; Amersham Corp., Arlington Heights, IL) with ethyl acetate. The eluate, after evaporating to dryness under nitrogen and reconstituting in phosphate buffered gelatin saline, underwent methyl oximation overnight. PGE<sub>2</sub> content was then quantified with a radioimmunoassay. Cellular protein for each experiment was determined by Lowry assay.

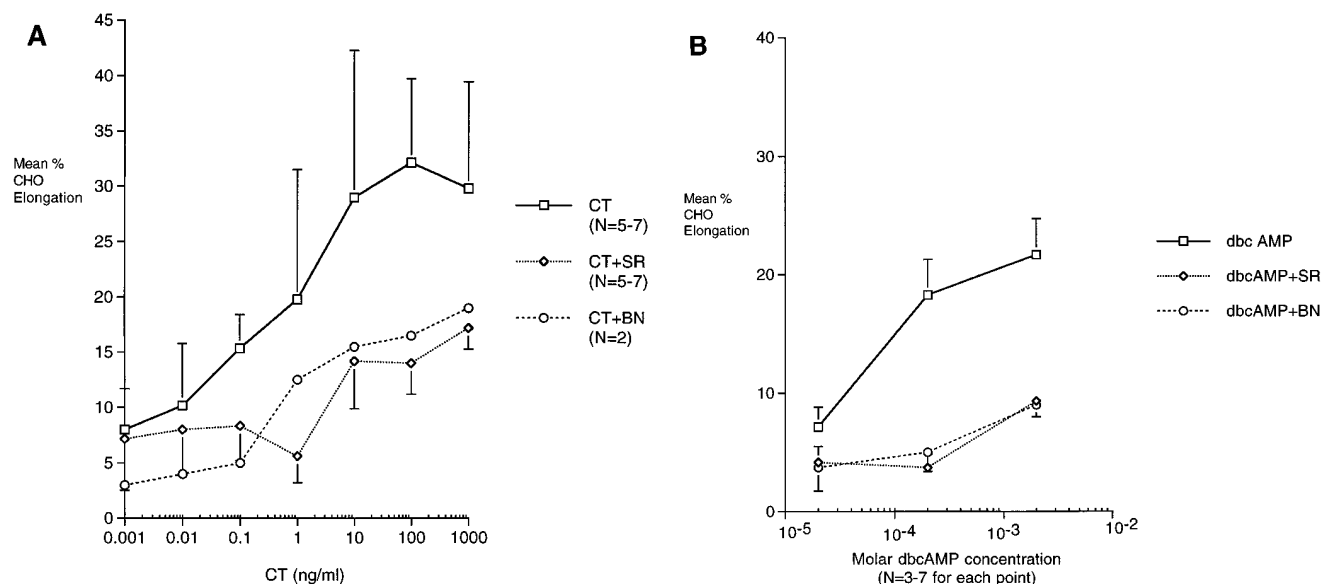
**Materials.** F12 medium was supplied by Gibco Laboratories (Grand Island, NY). Fetal calf serum, prostaglandin E<sub>2</sub>, cycloheximide, and actinomycin D were obtained from Sigma Chemical Co. (St. Louis, MO). Cholera toxin (#55135) was obtained from ICN Biomedical Inc. (Aurora, OH) and from List Biological Laboratories, Inc. (Campbell, CA) (#CVX-466). PAF was supplied by Calbiochem Corp. (La Jolla, CA). BN52021 and SR27417 were the kind gifts of Drs. Pierre Braquet and J.M. Herbert from Institut Beaufour (Paris, France) and Sanofi Recherche (Toulouse Cedex, France). Prostaglandin E<sub>2</sub> radioimmunoassay kit was purchased from Amersham Corp. Tissue cultureware was obtained from Beckton Dickinson & Co. (Lincoln Park, NJ). The lactate dehydrogenase determination kit was obtained from Sigma Diagnostics (St. Louis, MO).

**Statistics.** Statistical analysis was by Student's *t* test for analyses comparing less than three groups. One way analysis of variance procedures were used in analyses involving multiple comparisons with significant differences assessed conservatively with the Bonferroni test. Logistic regression modeling was used for comparing dose-response curves with and without antagonist present.

## Results

**Effects of PAF antagonists on CHO cell elongation.** In preliminary studies, we reproduced the dose-response CHO cell elongation to CT and demonstrated no change in baseline morphology of unstimulated CHO cells exposed to the BN52021 and SR27417 at concentrations  $\leq 10^{-6}$  M. Moreover,  $10^{-6}$  M SR27417 did not affect CHO cell viability, as measured by trypan blue uptake ( $0.3 \pm 0.6\%$  vs.  $1 \pm 1\%$  for PBS,  $n = 6$  total,  $P > 0.05$ ) and lactate dehydrogenase (LDH) release ( $5.1 \pm 8.3$  LDH activity units vs.  $54.4 \pm 94.8$  for PBS,  $n = 9$  total,  $P > 0.05$ ; Triton X positive controls with  $565.7 \pm 102.6$ ,  $n = 5$ ). Protein synthesis, as determined by <sup>35</sup>S-methionine-cysteine incorporation into trichloroacetic acid-precipitable proteins at 4 h, while inhibited by cycloheximide in the doses used, was not inhibited by SR27417. In data pooled from two observers and four experiments shown in Fig. 1 A, a significant inhibitory effect on CHO cell elongation was observed with SR27417 ( $P < 0.003$ ) and BN52021 ( $P < 0.001$ ) overall in logistic regression plots of log dose CT (nanograms per milliliter) versus percent CHO cell elongation. For PBS controls (not shown) run with each experiment, the mean percent CHO elongation was  $4\% \pm 2.9\%$ ,  $n = 30$ . As shown in Fig. 1 B, membrane permeable, dibutyl cAMP also effected dose-dependent CHO cell elongation, which was significantly blocked by the PAF receptor antagonists ( $P < 0.001$  overall for both SR27417 and BN52021 in logistic regression plots of log dose dibutyl cAMP versus percent CHO cell elongation).

**Effects of PAF, and prostaglandins E<sub>1</sub> and E<sub>2</sub> on CHO cell elongation.** In Fig. 2 are shown the effects of PAF and prostaglandin E<sub>1</sub> and E<sub>2</sub> on CHO cell elongation. Significant elongation ( $P < 0.001$ , compared with PBS controls), morphologically similar to that produced by both CT and dibutyl cAMP, was observed at PAF and prostaglandin E<sub>2</sub> concentrations of  $10^{-7}$  to  $10^{-5}$  M and at prostaglandin E<sub>1</sub> concentrations of  $10^{-5}$



**Figure 1.** Effects of PAF receptor antagonists SR27417 and BN52021 on CHO cell elongation responses to cholera toxin (A) and dibutyl cAMP (B). Bars represent 1 SD. SR27417 and BN52021 significantly inhibited CT effects on CHO cell elongation ( $P < 0.003$  and  $P < 0.001$  overall, respectively) as well as those of dibutyl cAMP ( $P < 0.001$ , each) in plots of log dose CT (nanograms per milliliter) or dibutyl molar concentration versus percentage CHO cell elongation.

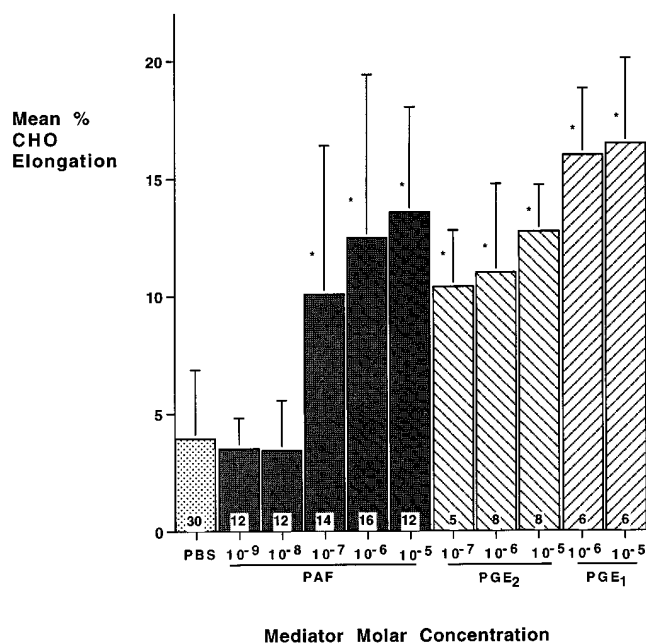


Figure 2. CHO cell elongation induced by PAF and prostaglandin  $E_1$  and  $E_2$ .  $n$  for each datum is at bottom of column; bars represent 1 SD; \* $P < 0.001$  compared with PBS control, significant by Bonferroni test where  $\alpha = 0.05$  corresponds with  $P < 0.005$  for comparisons of the 10 experimental groups vs. controls.

and  $10^{-6}$  M. The CHO cell elongation caused by  $PGE_2$  at  $10^{-9}$  to  $10^{-5}$  M (5–21%) was not altered by the presence of  $10^{-6}$  M SR27417.

**Effects of PAF antagonists, PAF, and prostaglandin  $E_2$  on cAMP accumulation.** Shown in Fig. 3 are cAMP measurements from CHO cells exposed for varying time intervals to doses of CT (10 ng/ml), PAF ( $10^{-5}$  M), and prostaglandin  $E_2$  ( $10^{-5}$  M), all at concentrations that caused significant CHO cell elongation. The increase in cAMP after exposure to CT in the presence of SR27417 was similar to that observed in CHO cells exposed to CT alone ( $P > 0.2$ ). Concentrations of PAF and prostaglandin  $E_2$  that maximally stimulated CHO cell elongation did not alter cAMP levels significantly from controls at 24 h. A transient and slight increase in cAMP formation that was statistically significant ( $P < 0.001$  with Bonferroni  $\alpha = 0.05$  significance corresponding to  $P < 0.0026$  for multiple comparisons) was noted after 4-min exposure to prostaglandin  $E_2$ ; no increase was seen after PAF exposure. At 2–4 h after exposure, both PAF and prostaglandin  $E_2$  exposure effected significant ( $P < 0.001$ ) decreases in cAMP accumulation.

**Effects of CT, dibutyryl cAMP, and PAF on prostaglandin  $E_2$  production.** As noted in Table I, a graded increase in  $PGE_2$  production was measured after a 4-h incubation with 0.1 ng/ml CT ( $25 \pm 21\%$ ,  $n = 4$ , significant by Bonferroni test where  $\alpha = 0.05$  significance corresponds to  $P < 0.008$  for multiple comparisons), 1 ng/ml CT ( $36 \pm 75\%$ ,  $n = 4$ ), 10 ng/ml CT ( $62 \pm 25\%$ ,  $n = 5$ ,  $P < 0.008$ ), and 50 ng/ml CT ( $79 \pm 13\%$ ,  $n = 2$ ).  $10^{-3}$  M dibutyryl cAMP also caused significant  $PGE_2$  accumulation compared with controls ( $78 \pm 47\%$  increase,  $n = 4$ ,  $P < 0.008$ ). PAF stimulated  $PGE_2$  production from CHO cells at  $10^{-6}$  M ( $20 \pm 26\%$  increase,  $n = 5$ ) and  $10^{-5}$  M ( $47 \pm 37\%$  in-

Table I. Prostaglandin Production Stimulated by Cholera Toxin, Dibutyryl cAMP, and PAF

Condition	Percent increase over controls	n	Significance
<i>P &lt;</i>			
Cholera toxin (ng/ml)			
0.1	25±21	4	0.008*
1	36±75	4	NS*
10	62±25	6	0.008*
50	79±13	2	—
10 + cycloheximide	−30±32	3	0.002‡
10 + actinomycin D	−45±28	2	—
Dibutyryl cAMP (molar concentration)			
$5 \times 10^{-4}$	−18±21	2	—
$10^{-3}$	78±47	4	0.008*
PAF (molar concentration)			
$10^{-6}$	20±26	5	NS*
$10^{-5}$	47±37	12	0.008*
$10^{-5}$ + cycloheximide	−25±25	5	0.001§
$10^{-5}$ + actinomycin D	−10±8	2	—

Each value is the mean percentage increase over experimental controls  $\pm$  standard deviation. \*Analysis of variance was done for groups compared with controls;  $P < 0.008$  achieves statistical significance by Bonferroni test (corresponding to  $\alpha = 0.05$  for comparison of the six experimental groups with  $n > 2$ ) versus controls; ‡significant by Student's  $t$  test versus CT 10 ng/ml; §significant by Student's  $t$  test versus PAF  $10^{-5}$  M. Data shown span four experiments.

crease,  $n = 12$ ), the latter being statistically significant ( $P < 0.008$ ). SR27417 at  $10^{-6}$  M added 30 min before the addition of CT did not block the effects of CT on  $PGE_2$  synthesis (335 pg/mg CHO with CT 10 ng/ml,  $n = 3$  vs. 439 pg/mg CHO with CT 10 ng/ml with SR27417,  $n = 3$ ).

**Effects of protein synthesis inhibitors on CT- and PAF-mediated prostaglandin  $E_2$  production.** Summarized in Table I along with other prostaglandin production experiments are the effects of cycloheximide and actinomycin D on prostaglandin  $E_2$  production. Cycloheximide, an inhibitor of translation, and actinomycin, an inhibitor of transcription, completely blocked PAF- and CT-mediated prostaglandin  $E_2$  production. In addition, constitutive prostaglandin synthesis was decreased by exposure to these agents.

## Discussion

Since its description by Guerrant et al. (9), the morphologic changes of CT-stimulated CHO cells have been widely used as a means to detect the presence of cholera and cholera-like enterotoxins in stool (23–27) and have proven a useful tool to probe cholera toxin mechanisms of action on eukaryotic cells (12, 28). In the present report, we have used these cytoskeletal responses, as well as prostaglandin and cAMP production of CHO cells to define more precisely the sequence of molecular events in cellular responses to CT.

As previously reported, the inhibition of CT-induced secretion in ligated rabbit ileal loops by selective PAF antagonists and of CT-induced CHO cell elongation, as well as detection of PAF in the supernatants of human epithelial cells exposed

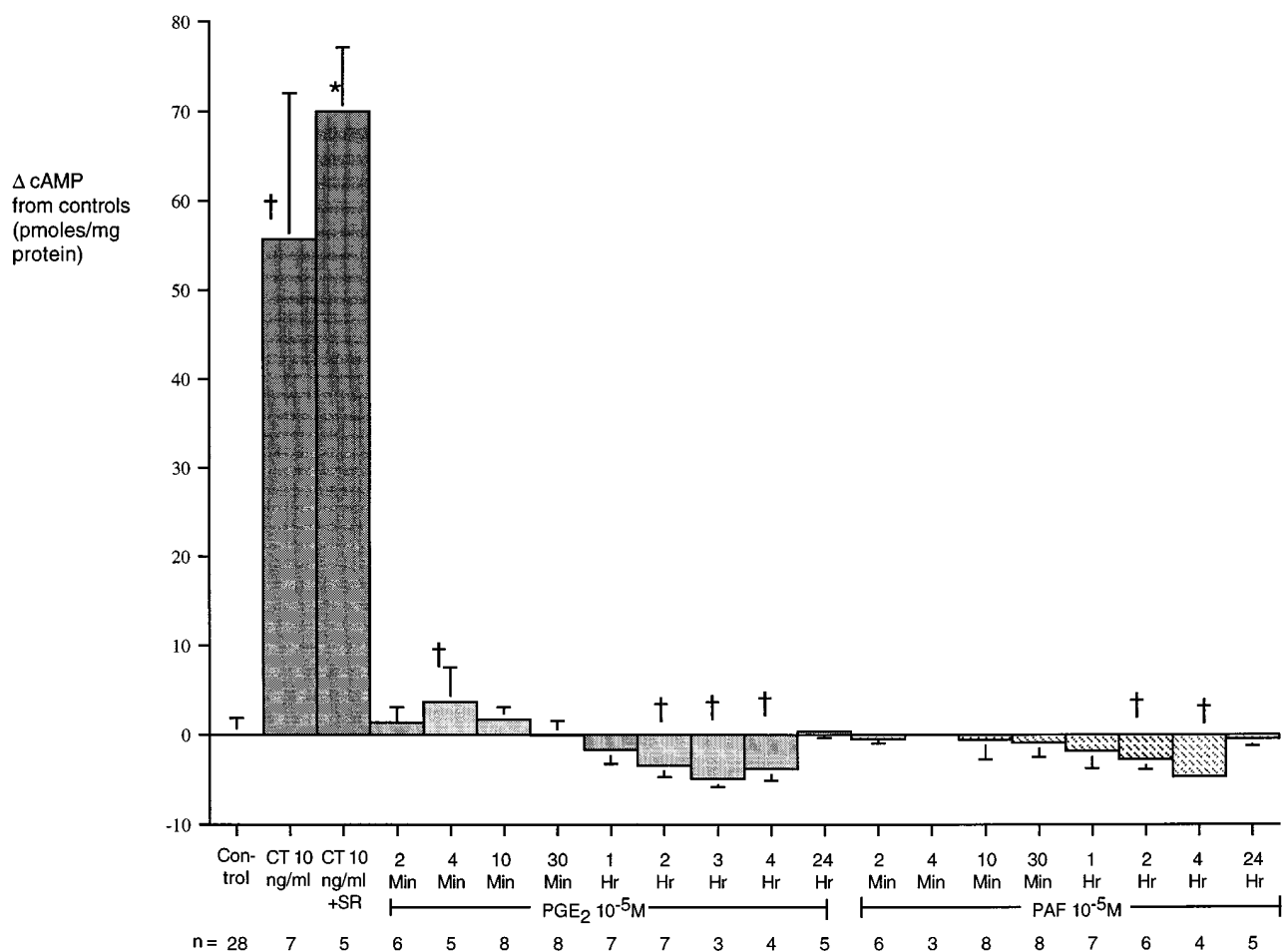


Figure 3. Cyclic AMP production by CHO cells exposed to CT, prostaglandin  $E_2$ , and PAF. †Statistically significantly different from controls by Bonferroni test ( $P < 0.0026$ ); \* $P > 0.05$  by Student's  $t$  test vs. CT 10 ng/ml without SR27417. Data are pooled from six experiments; bars represent 1 SD.

to CT, all suggest that PAF is involved in biological responses to CT (20). Although impressive, the lack of complete inhibition by the antagonists offers the possibility that other mechanisms, in addition to PAF effects, may also be involved. In this study, several different experiments demonstrate that PAF is involved after the formation of cAMP. First, the PAF receptor antagonists blocked dibutyl cAMP-mediated CHO cell elongation, just as they did with cholera toxin. Second, CT-induced cAMP production in CHO cells was completely unaffected by PAF receptor antagonists. Third, although a transient early increase in cAMP was noted in response to prostaglandin  $E_2$ , PAF (at doses that maximally stimulated CHO cell elongation) did not cause significant cAMP accumulation. Hence, cAMP accumulation appears to be a proximal event in CHO cell responses to CT, rendering PAF and PGE<sub>2</sub> likely candidates for mediating cAMP effects of CT on cells.

In experiments predating the discovery of the central role of cAMP, cholera toxin-induced intestinal fluid accumulation was shown to be inhibited by cycloheximide, suggesting a protein synthesis dependence (29, 30). Since then, the pivotal role for prostaglandins in the mechanism of action of CT was suggested by studies demonstrating: (a) increases in prostaglandins in intestinal mucosa in animal models (8, 31) as well as

CHO cells (32), (b) increased PGs in jejunal aspirates from patients with cholera (33), and (c) animal model studies demonstrating decreased CT-induced secretion with cyclooxygenase inhibitors (5, 6). Like CT, dibutyl cAMP stimulates prostaglandin  $E_2$  release (32); moreover, the arachidonic acid release caused by both CT and dibutyl cAMP is blocked by cycloheximide and actinomycin D (12). Although prostaglandin  $E_2$  is considered to represent a "classic" receptor-mediated cAMP stimulus, cAMP levels in animal models are not influenced by indomethacin despite inhibition of CT-mediated secretion (34, 35), and the PG doses required to stimulate cAMP generation in vitro are two to three logs greater than those needed to stimulate intestinal secretion (36). The present data, taken in the context of these earlier observations, suggest that CT-stimulated cAMP accumulation mediates protein synthesis-dependent prostaglandin production both directly and indirectly, via a pathway involving PAF.

Among the diverse biological activities of cAMP, recent evidence suggests that, in some cell types, this nucleotide is capable of stimulating phospholipase  $A_2$  (37–39), a key enzyme involved in the synthesis of both PAF and prostaglandins (18, 37). In CHO cells, cAMP has been found to promote transcription of mRNA encoding phospholipase or a phospholi-

pase-activating protein, thereby leading to prostaglandin synthesis (11), and Peterson et al. has recently described CT induction of phospholipase A<sub>2</sub>-activating protein synthesis in another mammalian cell line (12). The critical role of PAF in this pathway is demonstrated by the findings we report here. Our observations that PAF antagonists block elongation effects of CT and cAMP but not that of prostaglandins suggest that PAF may be involved before prostaglandin synthesis. This is supported by the additional experiments demonstrating that PAF, like CT and dibutyl cAMP, directly stimulates prostaglandin E<sub>2</sub> production. That this PAF-mediated prostaglandin production, like that of CT, is protein synthesis dependent further strengthens our suggestion that PAF induces a protein synthesis-dependent activation of PG synthesis, presumably via phospholipase A<sub>2</sub> (PLA<sub>2</sub>) or a PLA<sub>2</sub>-activating protein. PAF-induced synthesis of prostaglandins via PLA<sub>2</sub> activation has been well described in human polymorphonuclear leukocytes (38), rat Kupffer cells (19), rat liver cells (39), and other cell lines (18). Since the SR27417 did not block CT-induced PG synthesis, a direct effect of CT on PG formation (probably via PLA<sub>2</sub>) also seems likely.

In addition, PAF production may further augment cAMP activation of PLA<sub>2</sub>, or a cAMP-independent effect of CT on PAF and PLA<sub>2</sub> may occur. Interestingly, such an effect of CT on PAF has been suggested by Gandhi et al., who found that in rat Kupffer cells CT synergistically stimulated PAF-induced glycerophosphoinositol release and prostaglandin synthesis, without apparent cAMP involvement (19). We have successfully measured PAF from human intestinal epithelial cells and homogenates of rabbit ileal mucosa exposed to cholera toxin (20). However, attempts to measure PAF from CT-stimulated CHO cells have not been successful. Limited data (not shown) in our laboratory suggest that PAF may be rapidly bound or degraded in this system, making direct measurements of PAF accumulation difficult.

In summary, the present data suggest that CT effects on CHO cells involve PAF-dependent pathways after adenylate cyclase activation and cAMP formation and that PAF, like CT, stimulates synthesis-dependent prostaglandin formation. These findings may explain the previously reported effects of CT and cAMP on prostaglandin synthesis. An understanding of these molecular events in the action of cholera toxin on eukaryotic cells may lead to improved understanding of cholera toxin effects on cytoskeleton and intestinal secretion and potentially to new approaches to antisecretory therapy.

## Acknowledgments

We thank Mrs. Yatta Jacob and Mrs. Mary Loving for assistance with preparing the manuscript.

This work was supported in part by National Institutes of Health training grant T32AI07046 and by the Rockefeller Foundation.

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