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Pharmacology

Role of platelet activating factor in the intestinal epithelial secretory and Chinese hamster ovary cell cytoskeletal responses to cholera toxin

(diarrhea/phospholipase/secretion)

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ABSTRACT **With the recent heightened concern about cholera around the world come new questions about the mechanism by which cholera toxin causes diarrhea. Peterson and Ochoa have suggested that prostaglandin synthesis is key to both the intestinal epithelial secretory and the CHO cell responses to cholera toxin [Peterson, J. W. and Ochoa, G. (1989) *Science* 245, 857–859]. Because platelet activating factor (PAF) can be a potent stimulus for prostaglandin synthesis, we examined its role in the intestinal and tissue culture effects of cholera toxin. We report that the specific PAF receptor antagonists BN 52021 and SR 27417 inhibit the effects of cholera toxin on intestinal secretion in rabbit ileal loops *in vivo* and on the cytoskeleton of Chinese hamster ovary cells *in vitro*. We also show that PAF itself can cause net fluid secretion in the rabbit model and that PAF potentiates the effects of cholera toxin on intestinal secretion. Finally, we demonstrate that cholera toxin stimulates significant PAF production (2.6-fold) in isolated T-84 intestinal epithelial cells. We conclude that cholera toxin stimulates PAF production and that PAF is involved in both the secretory and cytoskeletal responses to cholera toxin. These findings further support the involvement of additional mediators of cholera toxin effects other than mucosal cell cyclic AMP and help explain the effects of cholera toxin on prostaglandin synthesis.**

Active cholera toxin is well known to ADP-ribosylate $G_s\alpha$ protein and thereby activate mucosal adenylate cyclase and increase cyclic AMP (1, 2). This pathway of adenylate cyclase activation has been classically held responsible for the secretory diarrhea that characterizes cholera (3–5) and for the elongation of Chinese hamster ovary (CHO) cells in response to cholera toxin (6–8). However, several investigators have shown that cyclooxygenase antagonists that block prostaglandin synthesis block cholera toxin-induced secretion (9–14), and Peterson and Ochoa (15) showed that prostaglandins correlated better with cholera toxin-induced secretion than did cyclic AMP itself (15). Furthermore, Peterson *et al.* (16, 17) showed that dibutyryl cyclic AMP caused arachidonic acid release from CHO cells that was blocked by actinomycin D or by cycloheximide. They also showed that the elongation of CHO cells but not the increased cyclic AMP caused by cholera toxin was blocked by cycloheximide or by actinomycin D, thereby suggesting that prostaglandin synthesis follows cyclic AMP formation.

Because platelet activating factor (PAF), recently identified as a secretagogue (18–21), can also stimulate phospholipase A_2 (22, 23), thereby increasing the synthesis of prostaglandins, we sought to explore the potential role of PAF in the secretory and CHO cell responses to cholera toxin.

MATERIALS AND METHODS

Studies of Secretion in Ligated Rabbit Ileal Loops. To measure intestinal secretion *in vivo*, male New Zealand White rabbits (1.5–2.0 kg) were fasted overnight and anesthetized. After flushing with saline, 4- to 5-cm ileal segments were ligated and injected with 1 ml of phosphate-buffered saline (PBS) alone or containing cholera toxin (1 μg/ml) or PAF (10^{-4}–10^{-8} M), with or without 10^{-5} M BN 52021, SR 27417, or indomethacin, or containing cholera toxin (1 μg/ml) with 10^{-8} M PAF as indicated. The specificity of these antagonists has been well documented (24, 25). In experiments designed to test synergy, 10^{-8} M PAF was placed in the intestinal segments together with cholera toxin (1 μg/ml). After 8 hr, loop length and volume were measured and the volume/length ratio was calculated. All animal anesthesia and care were in accordance with the University of Virginia's institutional guidelines. Cholera toxin (lot 55135) was obtained from ICN, and cholera toxin B subunit (lot CVXG-15C) and goat anti-choleragenoid antibody (lot GAC-01C) were obtained from List Biological Laboratories (Campbell, CA). The PAF antagonists BN 52021 and SR 27417 were kindly provided by Pierre Braquet, Boris Vargaftig, and J. M. Herbert from Institut Pasteur (Paris), Institut Beaufour (LePlessis–Robinson), and Sanofi Recherche (Toulouse) in France. Indomethacin was from Sigma.

Studies of Effects on CHO Cell Elongation. Fresh trypsinized CHO cells were placed in 96-well plates in Ham's F12 medium with 1% fetal bovine serum and studied as described previously (7). Cholera toxin (100 ng/ml) with or without 10^{-5} M BN, 10^{-5} M SR, or 10^{-5} M indomethacin was added to each well. After incubation overnight at 37°C in a 5% CO_2 atmosphere, cells were fixed, stained with Giemsa, and counted for the percentage of cells elongated.

Studies of Cholera Toxin Effects on Epithelial Cell PAF Production. To determine whether cholera toxin could directly stimulate intestinal epithelial cells to secrete PAF, we assayed PAF in supernatants and cells of the human intestinal T-84 cell line (obtained from the laboratory of K. Dharmsathaphorn at the University of California in San Diego). This cell line has been shown to respond to cholera toxin and other secretagogues with electrogenic chloride secretion (26, 27). Cell monolayers cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 (Gibco, Grand Island, NY) with 5% fetal bovine serum were exposed to cholera toxin (1 μg/ml) for 2 hr, the cells were harvested and centrifuged, and the culture supernatants were frozen in 10% acetic acid. PAF was measured by radioimmunoassay with ^{125}I-labeled PAF (DuPont/NEN), after extraction using solid-phase C_{18} extraction columns (Varian), with chloroform,

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Abbreviation: PAF, platelet activating factor.

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methanol, and water. The chloroform layer was evaporated and the resulting solid was reconstituted in a working buffer (0.1% sodium azide/0.05% Tween 20/50 mM sodium citrate, pH 6.3). PAF was quantified by ^{125}I competitive radioimmunoassay (DuPont) specific for hexadecyl and octadecyl PAF. Recovery rates, calculated by spiking samples with tracer amounts of [^3H]PAF, averaged 70% (range, 60–78%). Final concentrations of PAF (pg per 10^7 cells) reflect the means ± SD of three measurements for each condition from two separate experiments.

RESULTS

Inhibition of Cholera Toxin-Induced Secretion. Shown in Fig. 1 is the inhibition of cholera toxin-induced secretion in rabbit ligated segments by PAF antagonists and by indomethacin. Cholera toxin (1 μg/ml)-treated segments secreted nearly 400 μl/cm by 8 hr, while the PBS controls had only 85 μl/cm. The PAF antagonists BN 52021 and SR 27417 (each at 10^{-5} M) and the cyclooxygenase inhibitor indomethacin (10^{-5} M) all significantly decreased cholera toxin-induced secretion, by 47%, 84%, and 40% respectively ($P < 0.01$ for each). Even in loops adjacent to the PAF antagonist SR 27417, 76% of the secretory response to cholera toxin was inhibited. Finally, the combination of 10^{-5} M indomethacin with 10^{-5} M BN 52021 showed no greater inhibition of cholera toxin-induced secretion than either inhibitor alone (30% reduction).

Effects of PAF on Secretion. We next examined the direct effects of PAF itself on fluid secretion. PAF at 10^{-6} to 10^{-4} M caused significant graded secretory responses in the ligated rabbit ileal loops (each $P < 0.03$) (Fig. 2*A*). Although alone it had no effect at $\leq 10^{-7}$ M, PAF at 10^{-8} M significantly potentiated cholera toxin-induced secretion ($P < 0.05$, Fig. 2*B*).

Inhibition of Cholera Toxin-Induced CHO Cell Elongation by PAF Antagonists. In Fig. 3 are shown the effects of the PAF antagonists and indomethacin on cholera toxin-induced elongation of CHO cells. While cholera toxin (100 ng/ml) caused ≈25% of CHO cells to elongate, the elongation with cholera toxin in the presence of 10^{-5} M BN 52021, 10^{-5} M SR 27417, or 10^{-5} M indomethacin was only 12%, 9%, and 9%, respectively, representing 56–70% inhibition of the CHO elongation response to cholera toxin (each $P < 0.001$). Saline controls had only 2% of cells elongated.

Cholera Toxin Induces PAF Production by Epithelial Cells. We next directly measured PAF produced by T-84 cells exposed to cholera toxin. As shown in Fig. 4, we measured a 2.6-fold increase in PAF production by T-84 cells *in vitro* 2 hr after exposure to cholera toxin at 1 μg/ml (257.7 pg per 10^7 cells for controls vs. 664.9 pg per 10^7 cells with cholera toxin; $P < 0.03$, $n = 3$ in each group). Additional experiments (data not shown) showed comparable, 1.4- to 2.7-fold (mean, 1.9-fold) increases in PAF produced by T-84 cells exposed to cholera toxin at 0.1–10 μg/ml, with a slight decrease (mean, 1.47-fold) for cholera toxin at 0.01 μg/ml. In contrast, neither inactive cholera toxin B subunit (1 μg/ml) nor cholera toxin (1 μg/ml) premixed for 30 min with 1:100 anti-choleragenoid antibody had any significant effect on T-84 cell PAF production (<1.2-fold that of PBS-treated controls; $n = 2$ for each).

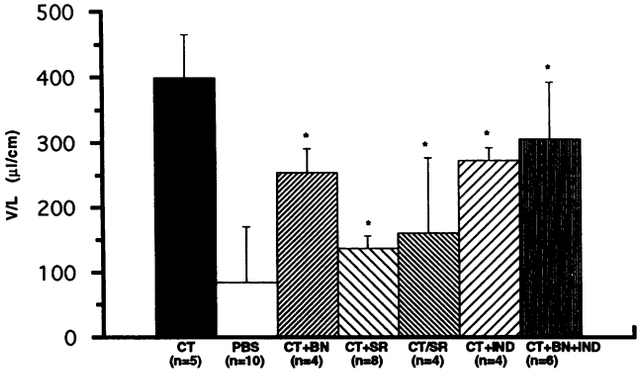

FIG. 1. Effects of PAF antagonists [BN 52021 (BN) and SR 27417 (SR)], indomethacin (IND), and indomethacin plus BN 52021 (each at 10^{-5} M) on cholera toxin (CT)-induced secretion (V/L, volume/length) in ligated rabbit ileal segments. CT/SR indicates inhibition of secretion induced by cholera toxin in a segment adjacent to a segment with SR 27417. Error bars represent 1 SD. Stars indicate $P < 0.01$ vs. CT.

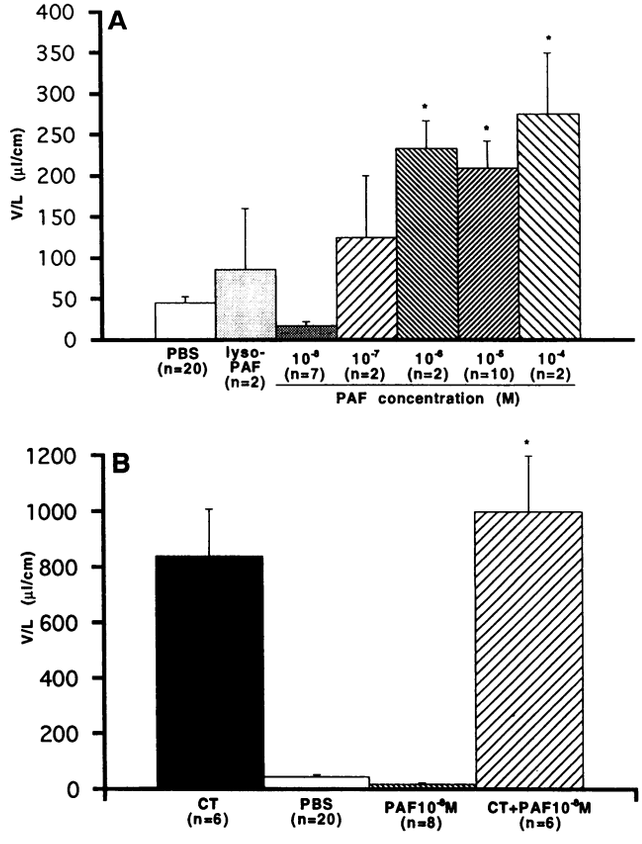

FIG. 2. Effects of PAF and lyso-PAF (10^{-5} M) (*A*) and of PAF (10^{-8} M) with cholera toxin (CT) (*B*) on secretion in rabbit ileal segments. Error bars represent 1 SD. Stars represent $P < 0.01$ vs. PBS (*A*) or $P < 0.02$ vs. CT.

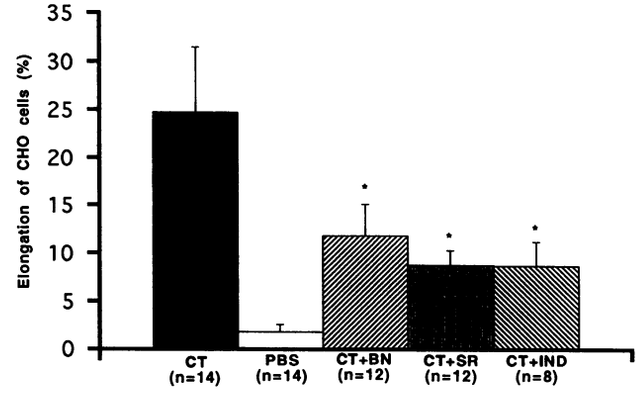

FIG. 3. Effects of PAF antagonists (BN 52021 and SR 27417, 10^{-5} M) and indomethacin (IND, 10^{-5} M) on cholera toxin (CT, 100 ng/ml)-induced elongation of CHO cells. Stars represent $P < 0.001$ vs. CT.

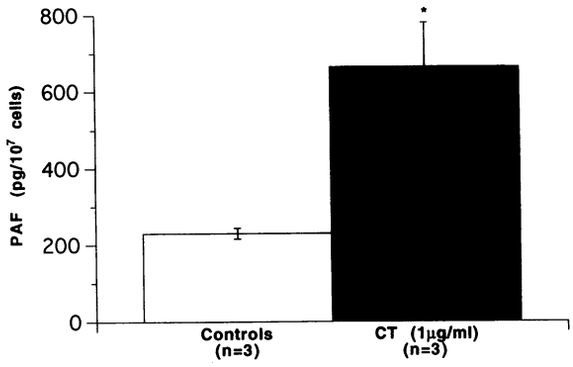

FIG. 4. Cholera toxin stimulation of PAF secretion in T-84 cells. T-84 cells grown to confluence in tissue culture were exposed to cholera toxin (CT, 1 μg/ml) for 2 hr. After cell harvest and centrifugation, the supernatants were frozen in 10% acetic acid. PAF was extracted from the samples with a solid-phase C_{18} extraction column as described in *Materials and Methods*. Error bars represent 1 SD. Star represents $P < 0.03$ vs. controls.

DISCUSSION

The significant inhibition of cholera toxin-induced secretion in ligated rabbit ileal loops by the PAF antagonists BN 52021 and SR 27417 and by the cyclooxygenase inhibitor indomethacin suggests that PAF may be involved in the secretory response to cholera toxin. The lack of additive inhibition of cholera toxin-induced secretion by indomethacin with the PAF antagonist BN 52021 suggests that both may be inhibiting the same pathway and that this pathway is responsible for part, but not all, of the secretory response to cholera toxin. The residual secretory response to cholera toxin may represent a direct effect of cholera toxin on mucosal adenylate cyclase activation, with cyclic AMP formation and stimulation of cyclic AMP-dependent protein kinase and chloride secretion (28). Alternatively, the greater potency of SR 27417 suggests that the predominant effect of cholera toxin and cyclic AMP may occur via PAF. The secretory effects of cholera toxin were also inhibited in ligated segments adjacent to those with the more potent PAF antagonist, SR 27417. Furthermore, the elongation of CHO cells by cholera toxin was significantly inhibited by BN 52021, SR 27417, or indomethacin. These findings suggest that PAF is involved in both the secretory and cytoskeletal responses to cholera toxin, and may explain the reported effects of cholera toxin on the synthesis of prostaglandins.

In further support of a potential role for PAF in causing secretion, PAF alone causes significant secretion in this model. Although the effective PAF concentrations are $\geq 10^{-6}$ M, these concentrations may have been required to gain access to epithelial receptors which, from previous studies, including one with T-84 cell monolayers (29), appear to effect secretion predominantly on the serosal side (18–21). Although other submucosal cell types may also produce PAF, as has been suggested by Berschneider and Powell (29, 30), our finding that T-84 cells can directly respond to cholera toxin with increased PAF production documents that human epithelial cells may serve as an autocrine source of PAF in response to cholera toxin. The lack of effects of cholera toxin B subunit and the inhibition of cholera toxin effects by anti-choleragenoid antibody further document the specificity of the effects of active cholera toxin on PAF production in T-84 cells. In addition, preliminary experiments suggest that PAF is increased in both intestinal fluid and mucosal scrapings in rabbit ileal loops 3 hr after exposure to cholera toxin. Since PAF also acts synergistically at 10^{-8} M with cholera toxin, we postulate that PAF also may participate as a key cofactor in cholera toxin-induced secretion. The synergy of PAF with cholera toxin could be explained in part by an enabling effect of cholera toxin on G-protein dependent PAF activation of phospholipase A_2, as suggested by Gandhi *et al.* (22, 23). This would further explain the capacity of cholera toxin to augment both prostaglandin and PAF synthesis, perhaps in response to endogenous levels of PAF. Alternatively, cholera toxin may provide a necessary cofactor, either by augmenting access of PAF to its relevant receptor or by otherwise enhancing its signal transduction pathway. Not shown are similar data on the enhancement of CHO cell responses to either cholera toxin or to dibutyryl cyclic AMP by 10^{-7} M PAF (31).

These findings further our understanding of the mechanism(s) by which cholera toxin induces its characteristic secretory and tissue culture effects and suggest another mechanism by which cholera toxin may induce secretion and CHO cell elongation, involving PAF. These results provide a potential explanation of the previously reported effects of cholera toxin on prostaglandin synthesis. Furthermore, they suggest that both PAF-dependent and cyclic AMP-dependent pathways are involved in cholera toxin-induced secretion and CHO cell elongation. These findings also suggest potential approaches to antisecretory therapy of cholera and cholera-like diarrhea.

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