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Phorbol Esters Enhance the Cyclic GMP Response of T84 Cells to the Heat-Stable Enterotoxin of *Escherichia coli* (STa)

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We examined the effect of protein kinase C (PKC) activation on the cyclic GMP response to heat-stable enterotoxin (STa) in a colonic carcinoma intestinal epithelial cell line, T84 cells. Our results demonstrate that the active phorbol ester analog, phorbol dibutyrate, but not the inactive alpha-phorbol dibutyrate, acts synergistically with STa to elevate cyclic GMP in intact T84 cells. The effect is observed in the absence or presence of the phosphodiesterase inhibitor, isobutylmethylxanthine, which suggests that phorbol dibutyrate modifies cyclic GMP synthesis rather than cyclic GMP degradation. In contrast to several systems in which prolonged treatment with phorbol ester desensitizes PKC-mediated responses, the cyclic GMP response in T84 cells is not diminished by prolonged treatment of T84 cells with phorbol dibutyrate. Also, transient treatment of T84 cells with phorbol dibutyrate enhances subsequent STa-stimulated cyclic GMP accumulation. These observations suggest that PKC activation produces a long-lived signal in T84 cells which enhances cyclic GMP accumulation in response to STa. Second messenger "cross talk" [T. Yoshimasa, D. R. Sibley, M. Bouvier, R. J. Lefkowitz, and M. G. Caron, Nature (London) 327:67–70, 1987] may be important in the pathogenesis of diarrheal disease.

Diarrheal diseases, most often caused by infectious pathogens or their toxins, constitute the greatest causes of mortality and morbidity on a global scale (30, 31). At least four distinct pathways appear to be involved in intestinal ion secretion stimulated by bacterial toxins or neurohumoral substances. The first two pathways are cyclic nucleotide dependent. The first is activation of adenylate cyclase by cholera toxin or the heat-labile enterotoxin of *Escherichia coli*, and the other is activation of particulate guanylate cyclase by the heat-stable enterotoxin (STa) of *E. coli* (25). In addition, two pathways are cyclic nucleotide independent. The first is secretion stimulated by a second heat-stable enterotoxin of *E. coli* (STb), the mechanism of which is unknown but which does not involve activation of adenylate or guanylate cyclase (22, 33). The second is secretion stimulated by poorly understood calcium-dependent mechanisms. Neurohumoral agonists such as carbachol may stimulate secretion by calcium-dependent mechanisms, which include elevation of intracellular calcium concentrations or activation of protein kinase C (PKC) (9, 11) or both. However, no infectious enteric pathogen has been shown to definitively act through a calcium-dependent process. The name PKC encompasses a ubiquitous family of phospholipid- and calcium-dependent enzymes which are the intracellular receptors for phorbol esters which activate these enzymes (5, 27). Treatment of intestinal mucosa with phorbol esters, which directly activate PKC, results in secretion in piglet and rat jejunum, rat and rabbit colon, and chicken ileum (1, 6, 12, 15, 34). However, despite an increasing body of knowledge about these mechanisms stimulating intestinal secretion, little is known about the relationships between cyclic nucleotide and calcium-dependent pathways in the intestinal epithelial cell.

In other systems, interactions between cyclic nucleotide metabolism and calcium-dependent enzymes, particularly PKC, have been extensively explored (20, 26). The best characterized is the adenylate cyclase system where signals stimulating cyclic AMP production may either antagonize or act synergistically with a PKC-dependent agonist. In other cases, PKC- and cyclic nucleotide-mediated cellular responses may function independently. In contrast, much less information is available regarding the interactions of cyclic GMP-dependent pathways and PKC activation. In a variety of cell types (e.g., human lymphocytes, fibroblasts, and myoblasts), phorbol esters alone increase cyclic GMP levels (7). In contrast, PKC activators attenuate the response to receptor agonists stimulating cyclic GMP production in, for example, rat adrenocortical cells and mouse neuroblastoma cells (21, 24). In vitro, soluble rat brain guanylate cyclase has been demonstrated to be a substrate for purified rat brain PKC (37).

In the present study, we explored the effect of PKC activation on the STa-stimulated cyclic GMP levels in an intestinal epithelial cell line, T84 cells. The T84 human colonic carcinoma cell line maintains electrogenic chloride transport in vitro and is responsive to agents which elevate cyclic nucleotides such as cholera toxin, STa, and prostaglandins of the E series (10, 19). In addition, a recent study demonstrated that phorbol esters inhibited the cyclic AMP response of this cell line to prostaglandin E_2 (32). Our results demonstrate that phorbol esters, direct activators of PKC but not guanylate cyclase, act synergistically with STa to enhance the cyclic GMP levels in T84 cells. These observations suggest that PKC activation may enhance guanylate

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cyclase activation by STa, which may be important in the pathogenesis of diarrheal diseases.

MATERIALS AND METHODS

Cell culture. T84 cells were obtained from the American Type Culture Collection (Rockville, Md.) and were grown at 37°C in humidified 5% CO_2 in media consisting of a 1:1 mixture of Dulbecco modified Eagle medium and Ham's F12. The medium was supplemented with 13.5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4), 12.8 mM $NaHCO_3$, 90 U of penicillin per ml, 90 μg of streptomycin per ml, and 5% newborn calf serum. Cells were grown in monolayers in polystyrene flasks and were subcultured when confluent with 0.25% trypsin and 0.9 mM EDTA. For experiments, cells were plated into 24-well plates at a density of approximately 5×10^5 cells per well. A confluent 75-cm^2 flask provided cells sufficient to inoculate two 24-well plates. The cells were allowed to grow for 7 or more days at which time the wells were confluent. The T84 cells were at passage number 49 upon receipt from the American Type Culture Collection and were maintained for an additional 25 passages.

Cyclic GMP accumulation in intact cells. T84 cells grown in multiwell plates were used in experiments by removing the standard medium and replacing it with 1 ml of medium containing the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (1 mM; IBMX) and lacking calf serum unless otherwise noted in the results. STa was added to some wells at a final concentration of 1 μg/ml (0.5 μM), and the plates were incubated at 37°C in a CO_2 incubator for the specified time intervals. The dose of 1 μg/ml was determined to be a maximal dose in experiments not shown. Stock solutions of phorbol esters (10^{-2} M) in absolute ethanol were stored at −20°C and diluted to achieve the specified experimental concentrations in 0.01% ethanol. Only phorbol esters having a beta-hydroxyl group at position 4 in the parent phorbol molecule bind to and directly activate PKC. The alpha analogs are inactive and do not bind to PKC. In our studies, phorbol-12,13-dibutyrate (PDB) was used as the active (beta) analog and alpha-phorbol-12,13-dibutyrate (alpha-PDB) was used as the inactive analog of the phorbol esters. Treatment of intact T84 cells with PDB, but not alpha-PDB, increases membrane-associated phorbol ester receptor (C. S. Weikel, J. A. Lavoie, and J. J. Sando, Abstr. United States-Japan Cooperative Med. Sci. Program, 1987, p. 116–117), and specific PKC activity stimulated by PDB is measurable in T84 cell homogenates (Les Reinlib, Johns Hopkins University School of Medicine, personal communication). These data are consistent with PKC activation by PDB as in other cell types (26, 27). Incubations were terminated by aspirating the media from cells and adding of 0.25 to 0.5 ml of 0.1 N HCl for 30 min at room temperature. Previous reports have indicated that practically all of the cyclic nucleotide produced remains intracellular in T84 cells (19, 35). The cell extracts were frozen at −20°C until assayed. Several wells from each plate were used to measure cell protein instead of cyclic GMP. After removing the media from these wells, the cellular protein was solubilized by either 0.2 N NaOH or 100% formic acid and was measured by the Bradford method (2). Cyclic GMP determinations were done in at least triplicate in each experiment.

Radioimmunoassay of cyclic GMP. Cyclic GMP was measured by two methods. In the first method, cyclic GMP was measured by acetylating each sample and standard with 45 μl of a 3.5:1 mixture of triethylamine:acetic anhydride per ml of sample, followed by automated radioimmunoassay (3). Monosuccinyl cyclic GMP tyrosine methyl ester was radioiodinated and purified as previously described (29). In the second method, cyclic GMP was measured with the ^3H-cyclic GMP radioimmunoassay kit available from Amersham Corp. (Arlington Heights, Ill.). For this method, samples in 0.1 N HCl were dried with nitrogen and suspended in 0.05 M Tris (pH 7.5)–4 mM EDTA buffer prior to each assay.

Materials. Purified STa (obtained from Donald C. Robertson, University of Kansas) was prepared as described by Dreyfus et al. (13). IBMX was obtained from Sigma Chemical Co. (St. Louis, Mo.). The phorbol esters (PDB and alpha-PDB) were obtained from either Sigma Chemical Co. or LC Services Corporation (Woburn, Mass.).

Data analysis. The data presented are expressed as means ± standard errors of the means. The Student's *t* test was used to test statistical significance.

RESULTS

In initial experiments, we examined the time course of the effects of STa, PDB, alpha-PDB, STa-PDB, and STa–alpha-PDB on cyclic GMP accumulation by T84 cells. For these experiments ($n = 2$), cells were incubated with 1 mM IBMX for 30 min prior to the addition of STa or the phorbol esters. STa (1 μg/ml) with or without PDB (10^{-6} M) or alpha-PDB (10^{-6} M) was added simultaneously at time zero to a minimum of four wells per condition in each experiment. To control for any possible effect of the diluent, ethanol (final concentration 0.01%) was added to the wells treated with STa alone. Basal cyclic GMP levels in the presence of IBMX were 2.7 ± 0.6 pmol of cyclic GMP per mg of protein ($n = 32$ wells in eight experiments). Treatment of the cells with PDB or alpha-PDB alone for intervals of 5 min to 18 h did not elevate cyclic GMP levels (see legend to Fig. 1; data not shown). However, PDB, but not alpha-PDB, was observed to enhance significantly the cyclic GMP accumulation stimulated by STa in T84 cells by 60 min after agonist addition (Fig. 1). At 60 and 120 min, a 1.6-fold increase and a 2.4-fold increase ($P < 0.005$ for both time points) in cyclic GMP levels over STa alone were measured. By 240 min after agonist addition, the intracellular level of cyclic GMP elicited by STa-PDB had declined but was still more than twofold higher than that in cells treated with STa alone or STa–alpha-PDB ($P < 0.01$). The cyclic GMP response to STa–alpha-PDB did not differ from the response to STa alone. These results demonstrate that PKC activators do not alter basal cyclic GMP levels but do act synergistically with STa to stimulate cyclic GMP production in T84 cells.

In additional experiments ($n = 3$), we examined whether the synergistic effect of PDB on the STa response would occur in the absence of the phosphodiesterase inhibitor, IBMX. At 60 min following treatment of the cells with PDB (10^{-6} M) and STa (1 μg/ml) simultaneously, the cyclic GMP accumulation was increased 1.5- to 4.1-fold over that accumulated in cells treated with STa–alpha-PDB ($P < 0.04$; data not shown), indicating that IBMX was not required.

In our next experiments, we determined the effect of varying doses of PDB on the cyclic GMP response to STa. Cellular cyclic GMP was extracted at 60 min after simultaneous treatment of the cells with STa and PDB. PDB (10^{-6} M) combined with STa (1 μg/ml) led to a maximal increase in cyclic GMP production (Fig. 2). The response to 3×10^{-6} M PDB was less but was still significant ($P < 0.001$). The lesser response may be secondary to an increase in the final ethanol concentration in the assay (0.03%), as reflected by the

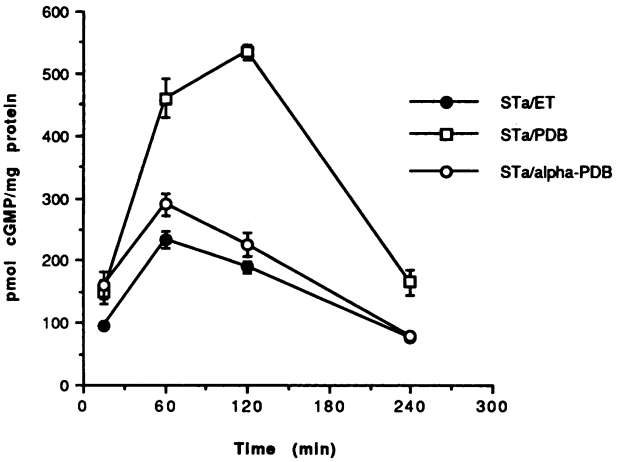

FIG. 1. Time course of STa and phorbol ester effect on T84 cells. A representative experiment in which T84 cells grown in 24 well plates were treated with STa (1 μg/ml) alone or treated simultaneously with STa (1 μg/ml) and either PDB (10^{-6} M) or alpha-PDB (10^{-6} M) beginning at time zero (n = four wells per data point). At the designated time intervals, cyclic GMP (cGMP) was extracted from the cells and measured as described in Materials and Methods. The STa condition (STa-ET) also contained the ethanol (ET) diluent (0.01% final concentration). The STa-PDB curve is significantly different from either STa-ET or STa–alpha-PDB at 60, 120, and 240 min ($P < 0.01$). Cyclic GMP levels (picomoles of cyclic GMP per milligram of protein) after treatment for 15, 60, 120, or 240 min with alpha-PDB or PDB at 10^{-6} M alone were as follows: at 15 min, 3.62 ± 1.2 for alpha-PDB and 3.04 ± 1.0 for PDB; at 60 min, 4.6 ± 1.0 for alpha-PDB and 3.9 ± 1.0 for PDB; at 120 min, 6.7 ± 1.5 for alpha-PDB and 4.8 ± 1.1 for PDB; and at 240 min, 0.58 ± 0.46 for alpha-PDB and 1.13 ± 0.63 for PDB.

decreased cyclic GMP response to STa with 0.03% ethanol (see legend to Fig. 2). PDB exhibited a narrow dose-response range with 10^{-7} M being the lowest dose of PDB at which a significant effect on the STa response was observed

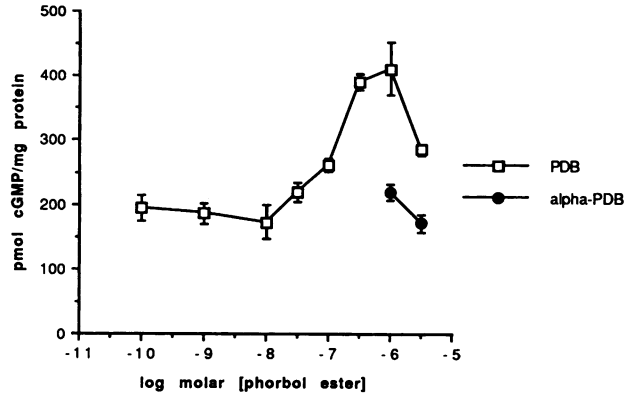

FIG. 2. Dose response of PDB on STa response in T84 cells. Cells grown in 24-well plates were incubated with STa (1 μg/ml) and either PDB in doses between 10^{-10} M and 3×10^{-6} M or alpha-PDB at 10^{-6} M and 3×10^{-6} M (n = four wells per data point). The cyclic GMP (cGMP) response to treatment of the cells with STa with 0.01% ethanol and STa with 0.03% ethanol was 198.8 ± 10.9 pmol of cGMP per mg of protein and 158.8 ± 6.5 pmol of cGMP per mg of protein, respectively (n = three wells each). These values are not statistically different from the STa–alpha-PDB conditions graphed. However, the response to STa-PDB was significantly greater than STa–alpha-PDB at 3×10^{-6} to 3×10^{-7} M ($P < 0.005$) and at 10^{-7} M ($P < 0.05$).

TABLE 1. Effect of 18-h incubation with phorbol esters on the response to STa

Treatment conditions	Mean pmol of cyclic GMP per mg of protein ± SEM[a]		Fold increase in cyclic GMP[b]
	STa–alpha-PDB	STa-PDB	
Phorbol ester 1 h/STa 1 h[c]	323.5 ± 40.5	510.7 ± 29.6[d]	1.6
Phorbol ester 18 h/STa 1 h[e]	365.7 ± 32.1	561.7 ± 27.8[f]	1.5

[a] SEM, Standard error of the mean.
[b] Fold increase in cyclic GMP, the ratio of the cyclic GMP produced in STa-PDB divided by STa–alpha-PDB.
[c] Simultaneous treatment with phorbol esters (10^{-6} M) and STa (1 μg/ml) for 1 h before cyclic GMP extraction.
[d] $P \leq 0.03$ versus STa–alpha-PDB condition.
[e] Treatment (18 h) with phorbol esters (10^{-6} M) followed by 1-h treatment with STa (1 μg/ml) in the continued presence of phorbol esters.
[f] $P \leq 0.01$ versus STa–alpha-PDB condition.

($P \leq 0.04$). On the basis of these experiments, we elected to use PDB and alpha-PDB at 10^{-6} M for our subsequent studies.

Because, in other systems, the response to active phorbol esters may vary with the dose and time of incubation with cells (e.g., as those described in 7, 23), we examined the effect of incubating the cells for either 30 or 60 min with PDB or alpha-PDB prior to the addition of STa. In these experiments, incubation of the cells with phorbol esters did not alter the time course of the subsequent responses to STa-PDB or STa–alpha-PDB (data not shown). In addition, because prolonged treatment of cells with active phorbol esters has been shown to downregulate PKC in other cell types, we examined the effect of incubating the cells for 18 h with PDB or alpha-PDB prior to the addition of STa to the cells. Surprisingly, cells treated for 18 h with PDB (10^{-6} M) responded to STa (1 μg/ml for 60 min) with a significant increase in cyclic GMP production when compared with cells treated with alpha-PDB (10^{-6} M) for 18 h and then stimulated with STa (n = 3; Table 1). In addition, as shown in a representative experiment in Table 1, the response to STa after treatment with PDB or alpha-PDB for 18 h did not differ from the cyclic GMP response to treatment of the cells with STa and either PDB or alpha-PDB simultaneously for only 60 min. Comparison of the fold increases in cyclic GMP produced in this experiment are essentially identical to the 60-min time point shown in Fig. 1. Unlike the data shown in Fig. 1, time course experiments suggested that treatment of the cells with PDB for 18 h enhanced the cyclic GMP response to treatment with STa for as little as 15 min (two of three experiments; data not shown). Similarly, treatment with PDB for 18 h significantly enhanced the cyclic GMP response to 30 min of treatment with STa in the presence or absence of IBMX (n = 2; $P < 0.01$; data not shown). Thus, prolonged treatment with PDB did not prevent a synergistic cyclic GMP response to STa. This result could be explained either by incomplete or differential downregulation of PKC(s) by 18 h of phorbol ester treatment in these cells or could indicate that there is persistent phosphorylation of the substrate(s) key to the response despite downregulation of the PKC enzyme. Experiments are in progress to determine the isoenzyme(s) of PKC present in T84 cells and to examine the time course of the effect of phorbol ester treatment on PKC enzymatic activity in T84 cells. Preliminary data (27) suggest that the isoenzymes of PKC may be differentially downregulated.

We also sought to explore the reversibility of the synergistic effect of PDB on cyclic GMP production in response to

TABLE 2. Effect of time of incubation with PDB on the cyclic GMP response to treatment with STa for 5 or 60 min[a]

Conditions (min)	STa treatment time (min)	Mean pmol of cyclic GMP per mg of protein ± SEM
Nothing (no wash)	60	170.2 ± 11.7
alpha-PDB (5)	60	142.8 ± 6.9
PDB (5)	60	219.5 ± 17.2[b]
PDB (30)	60	210.5 ± 18.5[c]
PDB (60)	60	280.5 ± 39.4[c]
Nothing (no wash)	5	17.8 ± 0.8
alpha-PDB (5)	5	19.2 ± 1.3
PDB (5)	5	25.1 ± 1.5[d]
PDB (30)	5	27.0 ± 1.6[d]
PDB (60)	5	24.7 ± 1.1[d]

[a] Each condition indicates a 5-min to 60-min treatment with phorbol ester (10^{-6} M), followed by three washes and subsequent incubation for 5 or 60 min with STa (1 μg/ml). The data for the 60-min STa treatment are representative of three or four experiments. The data for the 5-min STa treatment are from a single experiment with four replicates for each value shown. SEM, Standard error of the mean.

[b] $P \leq 0.006$ versus 5-min alpha-PDB (60-min STa treatment).

[c] $P \leq 0.02$ versus 5-min alpha-PDB (60-min STa treatment).

[d] $P \leq 0.03$ versus 5-min alpha-PDB (5-min STa treatment).

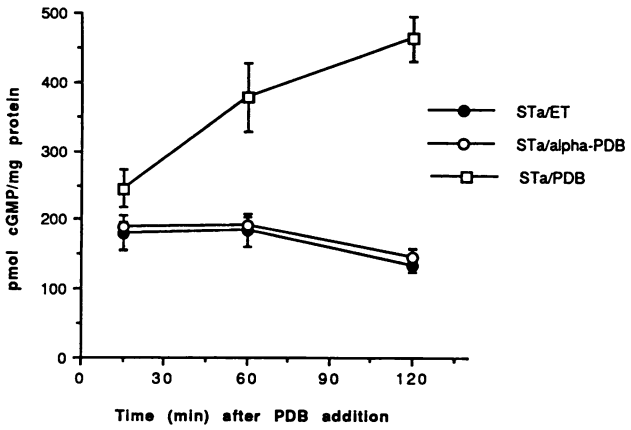

FIG. 3. Effect of PDB on STa-stimulated cyclic GMP (cGMP) response. For this experiment, T84 cells grown in 24-well plates were stimulated with STa (1 μg/ml; 0.01% ethanol [ET]) for 60 min prior to the addition of either PDB (10^{-6} M) or alpha-PDB (10^{-6} M) (n = five wells per data point). At the designated time intervals, cyclic GMP was extracted and measured as described in Materials and Methods. The cyclic GMP produced was significantly greater at 60 and 120 min in the STa-PDB condition compared with either STa alone or STa–alpha-PDB ($P \leq 0.007$).

STa. For these experiments, cells were treated with PDB (10^{-6} M) for 5, 30, or 60 min, washed three times with cell media, and then incubated with STa (1 μg/ml) for 60 min. To control for the effect of cell washing, cells were also treated for 5 min with alpha-PDB (10^{-6} M), washed, and incubated with STa for 60 min. In experiments in our laboratory examining tritiated phorbol ester binding capacity of T84 cells, three washes have been adequate to remove greater than 90% of the PDB from these cells (C. S. Weikel et al., Abstr. United States-Japan Cooperative Med. Sci. Program, 1987). As shown in Table 2 (60-min STa treatment), only a 5-min treatment with PDB was necessary to elicit the synergistic effect of PDB on cyclic GMP accumulation stimulated by STa (three of four experiments). This synergistic effect was maintained after both a 30-min (n = 3) and a 60-min incubation (two of three experiments) with PDB. In an additional experiment (Table 2; 5-min STa treatment), the cyclic GMP response to a shorter exposure to STa was also significantly enhanced by PDB in each condition. These experiments suggest that phosphorylation of the substrate(s) mediating the response occurs quickly (by 5 min), consistent with a PKC-mediated event, and persists for at least 60 min in the absence of the continual presence of PDB.

To examine further the synergistic response to STa-PDB, we asked whether initial treatment of the T84 cells with STa would alter the response to PDB. In these experiments (n = 2), T84 cells were treated with STa (1 μg/ml) for 60 min and then were treated with PDB or alpha-PDB (10^{-6} M) for intervals of up to 120 min. As shown in a representative experiment in Fig. 3, it is striking that the magnitude of the cyclic GMP effect and the lag time to obtain a significant effect on cyclic GMP are identical to those of cells treated simultaneously with phorbol esters and STa (Fig. 1). The increase in cyclic GMP accumulation in response to STa-PDB treatment is significant at both 60 and 120 min ($P \leq 0.007$ for both time points versus cells treated with STa–alpha-PDB). These results indicate that the order of agonist addition is not critical to the synergistic cyclic GMP response measured.

DISCUSSION

The influence of the enteric nervous or immune systems or infection with more than one enteric pathogen on the biochemical response to the heat-stable enterotoxin of *E. coli*, STa, is unknown. Our results demonstrate that treatment of T84 cells with STa, which activates only particulate guanylate cyclase (17), and an active phorbol ester analog, PDB, used as a probe to activate PKC, elicits a synergistic cyclic GMP response to this toxin. Phorbol esters alone do not alter cyclic GMP levels in T84 cells. The potential importance of this observation is further supported by our finding that $CaCO_2$ cells, a human colonic carcinoma cell line which secretes chloride (16), also have a synergistic cyclic GMP response to STa and PDB (C. S. Weikel, unpublished observations). We hypothesize that this "cross talk" between secretory pathways may affect the clinical outcome of the infection. In other systems, cross talk between second messenger systems is felt to be important in the regulation of, for example, hormonal secretion (4, 8, 36).

The effect of this biochemical synergy on intestinal physiology remains to be examined. The secretory response to STa, both in vivo and in T84 cells in vitro, has been well established (14, 18, 19). In contrast, the effect of PKC activation on intestinal physiology is a relatively new area for study. In intact animals (rats and piglets) and in certain epithelial preparations in vitro (e.g., chicken ileum and rat and rabbit colon, but not rabbit ileum), treatment of the mucosa with PDB has led to net secretion of sodium and chloride (1, 6, 12, 15, 34). To determine the physiologic importance of the synergism measured in our experiments, we have examined the effect of the acetylcholine analog, carbachol, on chloride secretion stimulated by STa. Preliminary data suggest that carbachol may be acting, in part, through PKC in T84 cells (9). Our preliminary results indicate that treatment of T84 cells with STa and carbachol induces a synergistic chloride secretory response dependent on the order and timing of agonist addition. Notably, addition of STa to the cells followed by carbachol results in a greater synergistic response than that elicited by simulta-

neous treatment with STa and carbachol (S. A. Levine, J. K. Crane, M. Donowitz, G. W. G. Sharp, and C. S. Weikel, Abstr. Am. Gastroenterol. Assoc., 1990). These preliminary results suggest the biochemical synergy noted in the experiments in this report may be physiologically relevant.

The enhancing effect of PDB on the cyclic GMP response to STa occurred in a narrow dose range. Although in many systems, phorbol esters exert significant effects at nanomolar concentrations, in all studies to date with intestinal epithelium higher concentrations (10^{-5} to 10^{-7} M) of phorbol esters have been required to exert measurable physiologic effects (1, 6, 12, 15, 34). One explanation has been that intestinal cells may exhibit enhanced nonspecific binding compared with other cell types. Such nonspecific binding might deplete phorbol esters from the solution, making them unavailable to bind to PKC. Another possibility is that the intestinal epithelial cell contains an isotype of PKC that is relatively insensitive to phorbol ester, a diacylglycerol analog. Three major PKC isoenzymes are recognized which exhibit different sensitivities to calcium and phospholipid but which are stimulated equally by phorbol esters (27). However, several additional types of PKC have been identified by molecular cloning and sequence analysis (28). The calcium, phospholipid, and phorbol ester requirements of these enzymes have yet to be examined in detail. Identification of the type or types of PKC present in different regions and cell types of the intestinal epithelium and the subsequent purification of these enzymes may provide some insight into these issues.

The mechanism of the synergy observed between PKC activators and STa is an important area for future study. Several possible mechanisms can be entertained, including direct phosphorylation of particulate guanylate cyclase or an alteration in the STa receptor by PKC activation. Data in our laboratory suggest that PDB treatment both induces a stable increase in guanylate cyclase activity in T84 cell membranes and increases the number of binding sites for ^{125}I-STa (J. K. Crane, R. L. Guerrant, J. Linden, and C. S. Weikel, submitted for publication). Indirect mechanisms by which phorbol may act include altering the coupling of the ST receptor with particulate guanylate cyclase (e.g., by phosphorylation of regulatory proteins) or even by stimulating gene activation and new protein synthesis. Lastly, although phorbol ester has been reported to inhibit phosphodiesterase activity (and thus raise cyclic nucleotide levels) in human lymphocytes (7), an effect on phosphodiesterases is an unlikely explanation for our results because the enhanced cyclic GMP response to STa and phorbol esters could be measured both in the presence or absence of 1 mM IBMX, a dose reported to inhibit maximally phosphodiesterase activity in T84 cells (32).

In summary, we have shown that treatment of T84 cells with STa, a direct activator of particulate guanylate cyclase, and active analogs of the phorbol esters, direct activators of PKC, results in a synergistic stimulation of cyclic GMP production. Although many questions remain to be answered including the mechanism(s) of this biochemical response and the relevance to intestinal physiology, it is a novel observation suggesting the possibility that "cross talk" occurs between PKC and the cyclic GMP-dependent second messenger systems in the intestinal epithelium. We hypothesize that such interactions between intracellular signaling mechanisms known to alter intestinal water and electrolyte transport may be important in understanding the pathogenesis of diarrheal illnesses resulting from mixed intestinal infections or in understanding the influence of the enteric nervous or immune systems on toxin-mediated enteric disease.

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LITERATURE CITED

1. **Ahn, J., E. B. Chang, and M. Field.** 1985. Phorbol ester inhibition of Na-H exchange in rabbit proximal colon. Am. J. Physiol. **249**:C527–C530.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **Brooker, G., W. L. Terasaki, and M. G. Price.** 1976. Gamma-flow: a completely automated RIA system. Science **194**:270–276.
4. **Brostrom, M. A., C. O. Brostom, L. A. Brotman, and S. S. Green.** 1983. Regulation of Ca-dependent cyclic AMP accumulation and Ca metabolism in intact pituitary tumor cells by modulators of prolactin production. Mol. Pharmacol. **23**:399–408.
5. **Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka.** 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. J. Biol. Chem. **257**:7847–7851.
6. **Chang, E. B., W. Ning-Sheng, and M. C. Rao.** 1985. Phorbol ester stimulation of active anion secretion in intestine. Am. J. Physiol. **249**:C356–C361.
7. **Coffey, R. G., and J. W. Hadden.** 1983. Phorbol myristate acetate stimulation of lymphocyte guanylate cyclase and cyclic guanosine 3′:5′-monophosphate phosphodiesterase and reduction of adenylate cyclase. Cancer Res. **43**:150–158.
8. **Cronin, M. J., S. T. Summers, M. A. Sortino, and E. L. Hewlett.** 1986. Protein kinase C enhances growth hormone releasing factor (1-40)-stimulated cyclic AMP levels in anterior pituitary. J. Biol. Chem. **261**:13932–13935.
9. **Dharmsathaphorn, K., J. Cohn, and G. Beuerlein.** 1989. Multiple calcium-mediated effector mechanisms regulate chloride secretory responses in T_{84} cells. Am. J. Physiol. **256**:C1224–C1230.
10. **Dharmsathaphorn, K., J. A. McRoberts, G. Mandel, L. D. Tisdale, and H. Masui.** 1984. A human colonic tumor line that maintains vectorial electrolyte transport. Am. J. Physiol. **246**: G204–G208.
11. **Dharmsathaphorn, K., and S. J. Pandol.** 1986. Mechanism of chloride secretion induced by carbachol in a colonic epithelial cell line. J. Clin. Invest. **77**:348–354.
12. **Donowitz, M., H. Y. Cheng, and G. W. Sharp.** 1986. Effects of phorbol esters on sodium and chloride transport in rat colon. Am. J. Physiol. **251**:G509–G517.
13. **Dreyfus, L. A., J. C. Fratz, and D. C. Robertson.** 1983. Chemical properties of heat-stable enterotoxins produced by enterotoxigenic *Escherichia coli* of different host origins. Infect. Immun. **42**:539–548.
14. **Field, M., L. H. Graf, W. J. Laird, and P. L. Smith.** 1978. Heat-stable enterotoxin of *Escherichia coli*: in vitro effects on guanylate cyclase activity, cyclic GMP concentration, and ion

transport in small intestine. Proc. Natl. Acad. Sci. USA **75:** 2800–2804.
15. **Fondacaro, J. D., and L. S. Hendersen.** 1985. Evidence for protein kinase C as a regulator of intestinal electrolyte transport. Am. J. Physiol. **249:**G4322–G4326.
16. **Grasset, E., J. Bernabeu, and M. Pinto.** 1985. Epithelial properties of human colonic carcinoma cell line CaCo-2: effect of secretagogues. Am. J. Physiol. **248:**C410–C418.
17. **Guerrant, R. L., J. M. Hughes, B. Chang, D. C. Robertson, and F. Murad.** 1980. Activation of intestinal guanylate cyclase by heat-stable enterotoxin of *Escherichia coli*: studies of tissue specificity, potential receptors, and intermediates. J. Infect. Dis. **142:**220–228.
18. **Hughes, J. M., F. Murad, B. Chang, and R. L. Guerrant.** 1978. Role of cyclic GMP in the action of heat-stable enterotoxin of *Escherichia coli*. Nature (London) **271:**755–756.
19. **Huott, P. A., W. Liu, J. A. McRoberts, R. A. Giannella, and K. Dharmasathaphorn.** 1988. Mechanism of action of *Escherichia coli* heat-stable enterotoxin in a human colonic cell line. J. Clin. Invest. **82:**514–523.
20. **Hussen, H.** 1980. Calcium and cyclic AMP in stimulus-response coupling. Ann. N.Y. Acad. Sci. **356:**346–353.
21. **Jaiswal, R. K., N. Jaiswal, and R. K. Sharma.** 1988. Negative regulation of atrial natriuretic factor receptor coupled membrane guanylate cyclase by phorbol ester. FEBS Lett. **227:** 47–50.
22. **Kennedy, D. J., R. M. Greenberg, J. A. Dunn, R. Abernathy, J. S. Ryerse, and R. L. Guerrant.** 1984. Effects of *Escherichia coli* heat-stable enterotoxin, STb, on intestines of mice, rats, rabbits, and piglets. Infect. Immun. **46:**639–643.
23. **Kiss, A., and R. A. Steinberg.** 1985. Phorbol ester-mediated protein phosphorylation in S49 mouse lymphoma cells. Cancer Res. **45:**2732–2740.
24. **Lai, W. S., and E. E. El-Fakahany.** 1987. Phorbol ester-induced inhibition of cyclic GMP formation mediated by muscarinic receptors in murine neuroblastoma cells. J. Pharmacol. Exp. Ther. **241:**366–373.
25. **Moss, J., D. L. Burns, J. A. Hsia, E. L. Hewlett, R. L. Guerrant, and M. Vaughan.** 1984. Cyclic nucleotides: mediators of bacterial toxin action in disease. Ann. Intern. Med. **101:**653–666.
26. **Nishizuka, Y.** 1984. Turnover of inositol phospholipids and signal transduction. Science **225:**1365–1370.
27. **Nishizuka, Y.** 1988. The molecular heterogenity of protein kinase C and its implications for cellular regulation. Nature (London) **334:**661–665.
28. **Ono, Y., T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, and Y. Nishizuka.** 1988. The structure, expression, and properties of additional members of the protein kinase C family. J. Biol. Chem. **263:**6927–6932.
29. **Patel, A., and J. Linden.** 1988. Purfication of ^{125}I-labeled succinyl cyclic nucleotide tyrosine methyl esters by high performance liquid chromatography. Anal. Biochem. **168:**417–420.
30. **Snyder, J. D., and M. H. Merson.** 1982. The magnitude of the global problem of acute diarrheal disease: a review of active surveillance data. Bull. W.H.O. **60:**605–613.
31. **Walsh, J. A., and K. S. Warren.** 1979. Selective primary health care. N. Engl. J. Med. **310:**967–974.
32. **Warhurst, G., N. B. Higgs, M. Lees, A. Tonge, and L. A. Turnberg.** 1988. Activation of protein kinase C attenuates prostaglandin E_2 responses in a colonic cell line. Am. J. Physiol. **255:**G27–G32.
33. **Weikel, C. S., H. N. Nellans, and R. L. Guerrant.** 1986. In vivo and in vitro effects of a novel enterotoxin, STb, produced by *Escherichia coli*. J. Infect. Dis. **153:**893–901.
34. **Weikel, C. S., J. J. Sando, and R. L. Guerrant.** 1985. Stimulation of porcine jejunal ion secretion in vivo by protein kinase C activators. J. Clin. Invest. **76:**2430–2435.
35. **Weymer, A., P. Huott, W. Liu, J. A. McRoberts, and K. Dharmsathaphorn.** 1985. Chloride secretory mechanism induced by prostaglandin E_1 in a colonic epithelial cell line. J. Clin. Invest. **76:**1828–1836.
36. **Yoshimasa, T., D. R. Sibley, M. Bouvier, R. J. Lefkowitz, and M. G. Caron.** 1987. Cross-talk between cellular signalling pathways suggested by phorbol-ester-induced adenylate cyclase phosphorylation. Nature (London) **327:**67–70.
37. **Zwiller, J., M.-O. Revel, and A. N. Malviya.** 1985. Protein kinase C catalyzes phosphorylation of guanylate cyclase in vitro. J. Biol. Chem. **260:**1350–1353.