

Effects of Prostaglandins and Cholera Enterotoxin on Intestinal Mucosal Cyclic AMP Accumulation

EVIDENCE AGAINST AN ESSENTIAL ROLE FOR PROSTAGLANDINS IN THE ACTION OF TOXIN

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ABSTRACT Both cholera enterotoxin and certain prostaglandins have been shown to stimulate intestinal fluid secretion *in vivo*, to cause ion flux changes *in vitro* similar to those caused by addition of cyclic 3',5'-adenosine monophosphate (cyclic AMP), and to activate intestinal mucosal adenylyl cyclase. It has been suggested that the effects of the enterotoxin on intestinal cyclic AMP metabolism may be indirect, and that locally synthesized prostaglandins may serve as required intermediates for the effects of the enterotoxin in activating intestinal mucosal adenylyl cyclase. In order to clarify certain aspects of the mechanisms by which these two agents alter intestinal mucosal cyclic AMP metabolism and ion transport, their effects on cyclic AMP accumulation in rabbit ileal mucosa were examined *in vitro*. Addition of 5 μg per ml (75 μg per 150 mg mucosa) of purified cholera enterotoxin produced a peak increase in cyclic AMP level in 3 h but there was a time delay of at least 30 min before any effect was observed. Inhibition of cyclic nucleotide phosphodiesterase with theophylline failed to reduce this time delay. In contrast, addition of prostaglandin E_1 (PGE_1) increased the cyclic AMP level rapidly, a peak effect being observed in 2 min. The time of the peak prostaglandin-induced changes in cyclic AMP level and short-circuit current correlated closely. A maximal increment in cyclic AMP level was achieved with 5×10^{-5} M PGE_1 . When 10^{-4} M PGE_1 was added to mucosa already maximally stimulated with cholera toxin, the resulting cyclic AMP level was equal to the

sum of the levels reached when each agent was added alone. Furthermore, the effects of the enterotoxin on mucosal cyclic AMP levels were not influenced by indomethacin under conditions where mucosal prostaglandins synthesis was inhibited. The results suggest that endogenous prostaglandins do not provide an essential link in the activation of intestinal mucosal adenylyl cyclase by cholera enterotoxin. The present study also indicates that the effect of cholera enterotoxin on intestinal mucosal cyclic AMP metabolism involves a definite time delay which is not due to cyclic nucleotide phosphodiesterase activity.

INTRODUCTION

There has been a great deal of recent interest in elucidating the mechanism of action of cholera enterotoxin in the intestine (see references 1-3 for reviews). It is now known that the massive outpouring of fluid and electrolyte is of small intestinal origin (4, 5), that it occurs in the absence of significant alterations in small bowel morphology (6, 7), and that an active secretory process provides the driving force for these intestinal losses (8-10). Several recent studies strongly suggest that cholera enterotoxin produces its effects by stimulating intestinal mucosal adenylyl cyclase, thereby altering the concentration of cyclic 3',5'-adenosine monophosphate (cyclic AMP)¹ (11-13). Both cyclic AMP and cholera enterotoxin have similar effects on ion transport when added *in vitro* to isolated ileal mucosa (10-

¹Abbreviations used in this paper: cyclic AMP, cyclic 3',5'-adenosine monophosphate; PG, prostaglandin; SCC, short-circuit current.

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14). Evidence has also been presented that the two agents act upon the same secretory mechanism (10). Furthermore, cholera enterotoxin has been shown to increase adenylyl cyclase activity and cyclic AMP levels in the intestinal mucosa with a time pattern similar to that of its effect on fluid transport (13, 15).

The mechanism by which cholera enterotoxin activates intestinal mucosal adenylyl cyclase is largely unknown. After addition of toxin, adenylyl cyclase activity increases slowly over 2-4 h (11, 15). There may also be an initial time lag of 15-45 min before any change in adenylyl cyclase activity, cyclic AMP level, or ion transport occurs (1-4, 10-13, 15). Because of the inevitable difficulty in measuring initial small changes, however, both the existence of such a time lag and its duration are uncertain. If an irreducible interval between contact of toxin with the mucosa and initiation of a cyclic AMP-related event does indeed exist, this fact has important implications with respect to the mechanism by which the toxin acts. One possible explanation for a time lag is the presence of sufficient cyclic nucleotide phosphodiesterase activity to prevent the cyclic AMP level from increasing in response to a small increment in adenylyl cyclase activity. If that is the case, it should be possible to shorten the time lag by inhibiting phosphodiesterase activity with theophylline. Another possible explanation for a time lag is that cholera enterotoxin may affect the synthesis or degradation of another compound which interacts more directly with adenylyl cyclase. The cellular level of this compound would first either have to increase or decrease sufficiently before a change in adenylyl cyclase activity could occur. Aside from bacterial toxins, the prostaglandins are the only other group of compounds which has been shown to enhance intestinal mucosal adenylyl cyclase (11, 12) and to stimulate small bowel water and electrolyte secretion. The latter has been demonstrated both *in vivo* (16, 17) and *in vitro* (18).

Recently, it was suggested that the effects of the enterotoxin on intestinal cyclic AMP metabolism may be indirect, with an increase in locally synthesized prostaglandins serving as the required intermediates responsible for activating mucosal cyclase (19, 20). The previously reported additive effect of prostaglandin E₁ (PGE₁) and cholera enterotoxin on intestinal mucosal adenylyl cyclase (11, 12) argues against this hypothesis, but, taken alone, this evidence is less than conclusive. Effects on the adenylyl cyclase activity of broken-cell preparations may not be representative of events in the intact cell. Furthermore, neither of the two prior studies concerned with adenylyl cyclase activity demonstrated that a maximally stimulating amount of toxin had been employed (11, 12).

The present study was undertaken in order to clarify

some of these issues. More specifically, experiments were designed to (a) determine the time and dose-response patterns of mucosal cyclic AMP levels after *in vitro* additions of cholera enterotoxin and PGE₁; (b) determine whether the time interval between toxin addition and the first increase in cyclic AMP level can be shortened by adding a larger amount of toxin or by inhibiting cyclic nucleotide phosphodiesterase activity with theophylline; (c) determine the relationship between the effects of PGE₁ on mucosal cyclic AMP levels and on short-circuit current (SCC), the latter being used as a measure of cyclic AMP-dependent secretory response; and (d) explore further the relationship between prostaglandin and toxin-induced increases in mucosal cyclic AMP levels, by establishing whether their effect in combination is competitive, additive, or synergistic, and by determining whether inhibition of prostaglandin biosynthesis will interfere with the action of toxin.

METHODS

Incubation of tissues. New Zealand white male rabbits that weighed between 2 and 3 kg were killed by a blow to the head, and the distal ileum was removed, opened longitudinally, and rinsed in a Krebs-Ringer solution (pH 7.4) which was gassed with 95% O₂-5% CO₂. The Krebs-Ringer solution contained the following ions in millimoles per liter: Na, 141; K, 10; Ca, 1.25; Mg, 1.1; Cl, 127; HCO₃, 25; H₂PO₄, 0.3; and HPO₄, 1.65. The ileum was stripped of muscularis, and three to six segments of mucosa, each approximately 50 mg in wet weight, were added to 50-ml Erlenmeyer flasks containing between 7.2 and 7.5 ml of the Krebs-Ringer solution and additions of purified cholera enterotoxin,² PGE₁,³ indomethacin, acetylsalicylic acid (aspirin), or the appropriate solvents as indicated below. Flasks were incubated at 37°C in a metabolic water bath oscillating at a rate of 80 rpm while being continuously gassed with 95% O₂-5% CO₂ which was first bubbled through water to minimize evaporative water loss.

Cyclic AMP measurements. At the termination of incubation, individual segments of mucosa were rapidly removed from the flasks, and each was placed in a conical centrifuge tube containing 5 ml of ice-cold 5% trichloroacetic acid with 0.8 nCi of [³H]cyclic AMP (24 Ci per mmol) as a recovery marker. The tissues were homogenized with a Teflon pestle in a glass homogenizer, and the trichloroacetic acid precipitates were then centrifuged at 4,000 *g* for 20 min at 4°C. The supernates were decanted into 40-ml conical centrifuge tubes, and 0.5 ml of 1 N HCl was then added. The trichloroacetic acid was extracted five times with 10 ml of diethyl ether, after which the samples were evaporated to dryness at 50°C using an Evapo-Mix evaporator (Buchler Instruments Div., Nuclear-Chicago

² Purified cholera enterotoxin, prepared according to the procedure described in *J. Infect. Dis.*, **121** (Suppl.): 563, 1970 and under contract for the National Institute of Allergy and Infectious Diseases by R. A. Finkelstein, Ph.D., The University of Texas Southwestern Medical School, Dallas, Texas.

³ We thank Dr. John Pike of the Upjohn Company, Kalamazoo, Michigan for a generous supply of prostaglandin E₁.

Corp., Fort Lee, N. J.). The residues were then dissolved in 2.0 ml of 50 mM sodium acetate buffer, pH 4.0. Recoveries of cyclic AMP extracted from the tissue were determined by counting 0.5-ml aliquots in 10 ml of Bray's solution (21) comparing the results to those obtained by counting aliquots of the original recovery marker diluted in 0.5 ml of the acetate buffer. Each sample was assayed in duplicate for cyclic AMP levels by the protein-kinase binding method described by Gilman (22). The trichloroacetic acid precipitates were dissolved overnight in 5 ml of 1 N NaOH and the protein content was measured by the method of Lowry, Rosebrough, Farr, and Randall (23), using bovine serum albumin as a standard.

Prostaglandin measurements. In certain experiments described below, prostaglandin levels were measured in the medium recovered at the end of the incubation. These determinations were performed by Clinical Assays, Inc., Cambridge, Mass.⁴ by the radioimmunoassay procedure described by Levine, Gutierrez Cernosek, Morrill, and Levine (24) and Gutierrez Cernosek, Morrill, and Levine (25). Antibodies to PGF_{2α} were produced by conjugation of this prostaglandin to poly-L-lysine with carbodiimide, followed by complexing with succinylated hemocyanin (25, 26). The conjugates, emulsified in complete Freund's adjuvant were used for immunization of rabbits. As previously reported, immunization with PGE₁ conjugates produced antibodies with PGB₁ specificity (24). Prostaglandins in 5 ml of incubation medium were extracted for 1 h at room temperature with an equal volume of a methylal-ethanol mixture (5:1). After separation, the methylal-ethanol mixture was

⁴We are grateful to Mr. Pierre-Yves Cathou and Mr. Thomas Eller of Clinical Assays, Inc. for performing the prostaglandin radioimmunoassays.

air dried at 40°C, and the residue was suspended in 1.0 ml of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.14 M NaCl, 1.5×10^{-4} M CaCl₂, 5.0×10^{-4} M MgCl₂, and 0.1% gelatin. The extract was dialyzed overnight against an equal volume of the buffered gelatin solution. 0.5 ml of the dialysate was treated by addition of NaOH (0.02 N final concentration), and this was heated at 100°C for 5 min in order to convert any PGEs and PGAs in the sample to PGBs. Duplicate 0.1-ml aliquots of the NaOH-treated material were assayed for PGBs using a PGB₁-specific antibody that cross reacts 1:3 with PGB₂. These measurements, therefore, reflect the presence of PGEs, PGAs, and PGBs, and they do not distinguish the relative quantities of each of these prostaglandins initially present in the samples. The prostaglandins measured by this method are referred to as "PGBs" in the results. Duplicate 0.1-ml aliquots of the nonalkali-treated dialysate were also assayed specifically for PGF_{2α} as previously described (24-26).

SCC measurements. The method for determining the SCC in segments of rabbit ileal mucosa stripped of muscularis and mounted in Ussing chambers has been previously described (27).

Materials. Purified cholera enterotoxin² was prepared in isotonic sodium chloride such that the stock solution contained 500 μg per ml. The effectiveness of the enterotoxin in increasing cyclic AMP levels seemed to vary with different lots of toxin or with the same lot upon storage. In order to minimize the effects of this variation on our results, the groups of experiments shown in each table and figure were performed with the same lot of toxin and within a relatively short span of time. PGE₁ was prepared as a stock solution at a concentration of 2.86×10^{-3} M in 9.5% ethanol, containing 1.7×10^{-3} M Na₂CO₃ (final pH

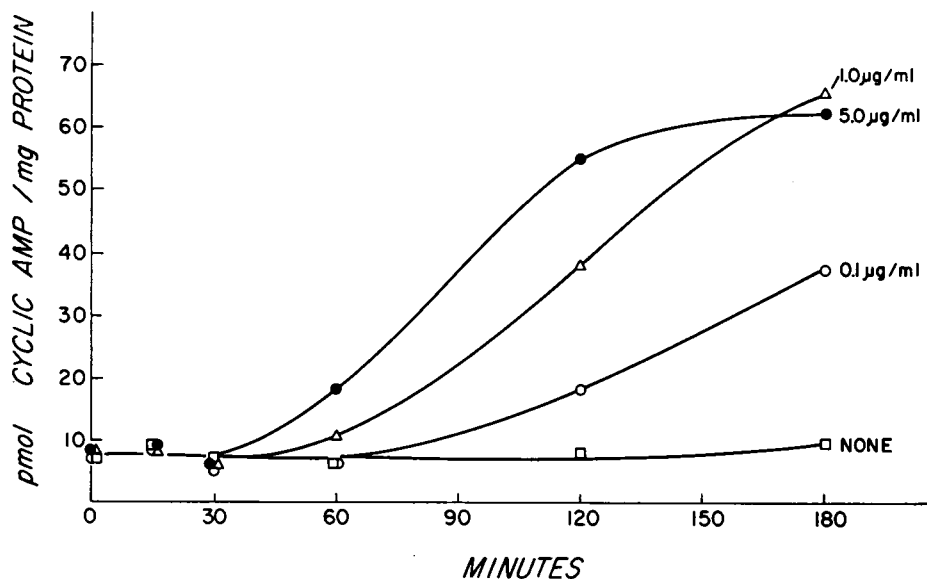


FIGURE 1 Effects of varying concentrations of cholera enterotoxin and of varying duration of incubation on cyclic AMP levels in rabbit ileal mucosa. Tissues were preincubated for 40 minutes before the addition of cholera enterotoxin or solvent. The remaining experimental conditions are described in the text. Each point represents the mean of values obtained in four experiments performed in a similar manner. Mean values ± 1 SE at 180 minutes were: 11.3 ± 2.7 for controls, 37.8 ± 18.2 for $0.1 \mu\text{g}$ toxin, 66.5 ± 34.3 for $1.0 \mu\text{g}$, and 62.6 ± 32.2 for $5.0 \mu\text{g}$. SEs are large because of a large variation in the amplitude of response in individual experiments.

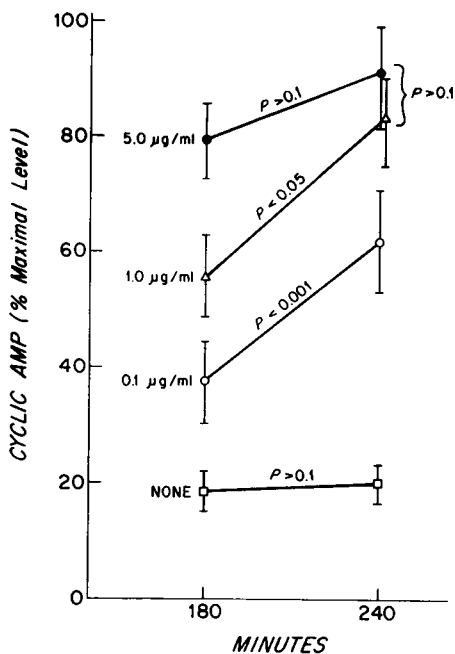


FIGURE 2 Effects of duration of incubation on cyclic AMP levels in rabbit ileal mucosa incubated in the presence and absence of varying concentrations of cholera enterotoxin. Tissues were preincubated for 40 minutes before the addition of cholera enterotoxin or solvent. The remainder of the experimental conditions are described in the text. Tissues were incubated in the absence or in the presence of varying concentrations of cholera enterotoxin for 3 and 4 h. In each experiment, the maximal level of cyclic AMP reached, whether at 3 or 4 h, with 1.0 or 5.0 $\mu\text{g/ml}$ of toxin, was taken as representing 100%, and every other value was expressed as a percent of this maximal level. Shown are the means ± 1 SE for values obtained in four experiments performed in a similar manner.

of 7.0). Indomethacin was dissolved in absolute ethanol to provide a stock solution at a concentration of 2.5×10^{-2} and was used in experiments at a final concentration of 5.0×10^{-6} M (17.89 μg per ml). Acetylsalicylic acid was prepared as an aqueous stock solution at a concentration of 2.5×10^{-3} M and was present in incubation flasks at a final concentration of 5.0×10^{-6} M (9.0 μg per ml). Appropriate solvents to control for the presence of cholera enterotoxin, PGE₁, indomethacin, and acetylsalicylic acid were added to each flask in which they were indicated.

Statistics. Statistical analyses were performed by Student's *t* test for paired variates (28).

RESULTS

Effects of cholera enterotoxin on cyclic AMP levels.

In initial experiments, ileal mucosa was incubated in the presence of varying concentrations of cholera enterotoxin for periods up to 3 h in order to establish the characteristics of the mucosal cyclic AMP response. As shown in Fig. 1, no increase in cyclic AMP level developed for the first 30 min after addition of toxin, even with the highest toxin concentration employed (5.0 μg

per ml). Thereafter, the rate of cyclic AMP accumulation with time varied directly with toxin concentration. By the end of 2 h of incubation, the cyclic AMP concentration appears to have reached a plateau in the presence of 5.0 μg per ml of toxin, but was clearly still rising at the lower two toxin concentrations.

In separate experiments, the effects of varying enterotoxin concentrations on cyclic AMP levels were compared at 3 and 4 h (Fig. 2). The cyclic AMP concentration was not significantly higher at 4 than at 3 h in the presence of 5.0 μg per ml of toxin, but rose significantly during the 4th h in the presence of 1.0 and 0.1 μg per ml of toxin. Also of note is the observation that by the end of 4 h of incubation there was no significant difference in the cyclic AMP levels achieved by incubation with 5.0 vs. 1.0 μg per ml of enterotoxin.

Since no detectable increase in cyclic AMP level developed during the first 30 min of exposure to cholera toxin, experiments were undertaken to determine whether inhibition of cyclic nucleotide phosphodiesterase activity with theophylline would result in an earlier detectable increase in cyclic AMP level. These experiments are summarized in Table I. It can be seen that 2½–3 h after addition of toxin *in vitro*, the cyclic AMP level was three- to fourfold greater in mucosa treated with both toxin and theophylline than in mucosa treated with either agent alone. 30 min after toxin addition, however, the increase in cyclic AMP level produced by theophylline was no greater in toxin-treated than in control mucosa. Therefore, inhibition of cyclic nucleotide phosphodiesterase does not reduce the observed 30-min time lag.

Effects of PGE₁ on cyclic AMP levels and SCC. As shown in Fig. 3, PGE₁ (10^{-5} M) produces a detectable increase in ileal mucosal cyclic AMP concentration within 2 min, and this effect remains near maximal between 2 and 5 min. The results in Fig. 3 are also of interest, in that they show that the peak change in SCC occurs almost at the same point in time. Fig. 4 shows the effects of varying concentrations of PGE₁ on cyclic AMP levels. A maximal effect appears to have been achieved at a concentration of 5.0×10^{-5} M.

Additive effects of cholera enterotoxin and PGE₁.

In the studies just described, it had been determined that a 3–4-h incubation with cholera enterotoxin at a concentration of 5.0 μg per ml consistently produced a maximal cholera enterotoxin-related cyclic AMP response. Similarly, a 5-min exposure of ileal mucosa to 10^{-4} M PGE₁ produced a maximal PGE₁-related response. In order to determine the effect of prior addition of cholera enterotoxin on the subsequent response to PGE₁, tissues were preincubated in four flasks for 30 min at 37°C in Krebs-Ringer buffer, after which cholera enterotoxin (5.0 μg per ml) was added to two of the

TABLE I
Effects of Cholera Enterotoxin and Theophylline on Cyclic AMP Levels in Rabbit Ileal Mucosa

Time after toxin added 30 min	Cyclic AMP levels							
					150-180 min			
	-Theophylline		+Theophylline		-Theophylline		+Theophylline	
	Control	Toxin	Control	Toxin	Control	Toxin	Control	Toxin
<i>pmol/mg protein</i>								
Cholera enterotoxin,								
1 $\mu\text{g/ml}$ (15 $\mu\text{g}/150\text{ mg mucosa}$)								
Exp. 1	5.6	4.2	13.6	10.5	6.9	25.7	13.0	54.0
Exp. 2	3.8	4.3	11.2	10.4	3.8	11.1	9.2	40.1
Exp. 3	2.9	2.0	12.8	9.0	3.8	14.8	15.6	72.1
\bar{X}	4.1	3.5	12.5	10.0	4.8	17.2	12.6	55.4
Cholera enterotoxin,								
5 $\mu\text{g/ml}$ (75 $\mu\text{g}/150\text{ mg mucosa}$)								
Exp. 4	4.6	5.8	12.2	8.9	6.0	28.1	20.9	84.3
Exp. 5	8.8	7.6	16.7	18.8	7.9	34.2	28.8	109.1
\bar{X}	6.7	6.7	14.5	13.8	7.0	31.2	24.8	96.7

Results shown are for five complete and separate experiments. Three pieces of mucosa, each weighing about 50 mg, were incubated in 15 ml of the standard Krebs-Ringer solution in each of eight flasks. After a 30 min preincubation period, cholera enterotoxin was added to four flasks and 5 min thereafter theophylline (5mM) was added to four flasks.

four flasks. After an additional 3-4 h of incubation, PGE₁ (1.1×10^{-4} M) or PGE₁ solvent was added to each flask and the incubations were terminated 5 min thereafter. Results are given in Table II. PGE₁ alone caused a greater than threefold rise in cyclic AMP level, and prolonged incubation with cholera enterotoxin produced a 10-fold rise. The PGE₁-related cyclic AMP response was neither inhibited nor potentiated by prior incubation with cholera enterotoxin. Since the concentrations of toxin and PGE₁ and the durations of exposure were sufficient for maximal responses to each agent separately, the results suggest the absence of competitive interaction between the two agents.

Effects of indomethacin and acetylsalicylic acid on cyclic AMP levels. Recent studies by Vane and his colleagues (26, 29) and others (30) demonstrated that the anti-inflammatory drugs, indomethacin and acetylsalicylic acid, can effectively interfere with the synthesis of prostaglandins in guinea pig lung, canine spleen, and human platelets. In the lung homogenate system, the 50% inhibitory concentrations for indomethacin and acetylsalicylic acid were 7.5×10^{-7} M and 3.5×10^{-8} M, respectively (26). In the platelet system, the concentration of acetylsalicylic acid causing 50% inhibition of prostaglandin production was somewhat lower (1.7×10^{-8} M), and indomethacin was only 10 times more effective than acetylsalicylic acid (30).

Endogenous prostaglandin biosynthesis occurs in the

small intestine (31, 32). To explore further the possibility that cholera enterotoxin's effects on intestinal mucosal adenyl cyclase may be mediated by an enterotoxin-induced enhancement in endogenous prostaglandin synthesis, the effects of indomethacin and acetylsalicylic acid were examined in this system. In order to establish incubation conditions for these studies, it was first necessary to demonstrate that neither aspirin nor indomethacin inhibited the cyclic AMP response to exogenous PGE₁ under the conditions to be employed. As shown at the top of Table III, incubation of ileal mucosa for 2½ h in the presence of either 5.0×10^{-5} M indomethacin or 5.0×10^{-5} M acetylsalicylic acid failed to exert a significant effect on cyclic AMP levels when compared to the appropriate solvent control. Furthermore, neither acetylsalicylic acid nor indomethacin inhibited the cyclic AMP response to PGE₁ (10^{-4} M) added 5 min before the end of incubation.

In a separate series of experiments summarized at the bottom of Table III, mucosa was preincubated for 30 min in the presence of either acetylsalicylic acid, indomethacin, or the solvents. Cholera enterotoxin (5.0 $\mu\text{g per ml}$) or its solvent was then added to appropriate flasks as indicated in the Table, and the incubation was continued for an additional 2 h. 5 min before the termination of the 2½-h experiment, PGE₁ (10^{-4} M) or its solvent was added as indicated. The results of these experiments indicate that neither acetylsalicylic acid nor

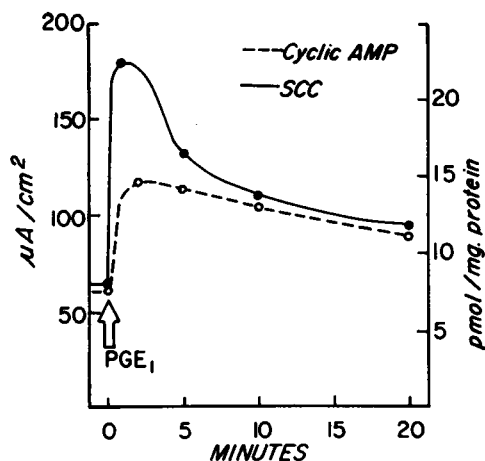


FIGURE 3 Effects of duration of incubation with 10^{-5} M PGE_1 on cyclic AMP levels and SCC in rabbit ileal mucosa. Tissues were preincubated for 40 minutes before the addition of PGE_1 or its solvent. The remaining experimental conditions are described in the text. Each cyclic AMP and SCC point represents the mean of values obtained in four experiments. Mean values ± 1 SE for cyclic AMP levels were 7.5 ± 0.34 just before adding PGE_1 , 14.8 ± 0.68 at 2 minutes, 14.2 ± 0.63 at 5 minutes, and 10.7 ± 0.57 at 20 minutes. All values except the base line were significantly higher than levels (not shown) measured at the same times on control tissues ($P < 0.01$). Furthermore, the 20-minute value was significantly lower than the 2- and 5-minute values ($P < 0.05$ and $P < 0.025$, respectively). Mean values ± 1 SE for SCC were 66 ± 8.4 just before adding PGE_1 , 177 ± 26.4 at the peak (about 1 minute), 134 ± 16.0 at 5 minutes and 94 ± 8.7 at 20 minutes. All values except the base line were significantly higher than levels (not shown) measured at the same times on control tissues. Furthermore, the 20 minute value was significantly lower than the peak and 5 minute values ($P < 0.025$).

indomethacin in the relatively high concentrations employed interfered with the cyclic AMP response to cholera enterotoxin nor with the previously demonstrated additive effects of the enterotoxin and PGE_1 . The additive effects of PGE_1 and the enterotoxin, coupled with the failure of substantial concentrations of either acetylsalicylic acid or indomethacin to inhibit the effects of the enterotoxin on cyclic AMP metabolism, suggest that endogenously synthesized prostaglandins may not be required intermediates for the enterotoxin's effects on adenylyl cyclase.

Effects of indomethacin, acetylsalicylic acid, and cholera enterotoxin on prostaglandin levels. In a number of tissues studied, the release of prostaglandins into the medium proved to be a good measure of the rate of synthesis (29, 30, 33). In order to explore more directly the effects of cholera enterotoxin, indomethacin, and acetylsalicylic acid on prostaglandin biosynthesis in ileal mucosa, experiments were performed in which the effects of these agents on prostaglandin accumulation in

the medium were examined. Effects on tissue cyclic AMP levels were determined simultaneously. In these experiments, three pieces of ileal mucosa were preincubated for 30 min at $37^\circ C$ in each of six flasks, pairs of which contained either indomethacin, acetylsalicylic acid, or the appropriate solvents. On the basis of studies in other tissues, a 30-min preincubation should have been sufficient for the tissue prostaglandin initially present to have been released and for the anti-inflammatory drugs to have exerted an inhibitory effect on de novo prostaglandin synthesis (29). After preincubation, the tissues were transferred to other flasks containing fresh medium with the same additions. At this time, cholera enterotoxin was added to one control flask, one indomethacin-containing flask, and one acetylsalicylic acid-containing flask. After an additional 2 h of incubation, the experiment was terminated, tissues were rapidly removed for cyclic AMP assay, and media were frozen for subsequent determination of prostaglandin levels. Results are given in Table IV. Neither of the inhibitors of prostaglandin biosynthesis prevented the cholera enterotoxin effect on cyclic AMP levels, findings consistent with those already presented. Base line levels of "PGB" and $PGF_{2\alpha}$ were lowered by incubation in the presence of indomethacin, whereas acetylsalicylic acid in the concentration employed did not seem to exert a consistent effect. Of principal importance for the present study is the observation that indomethacin, at a concentration which consistently and markedly inhibits prostaglandin biosynthesis by the

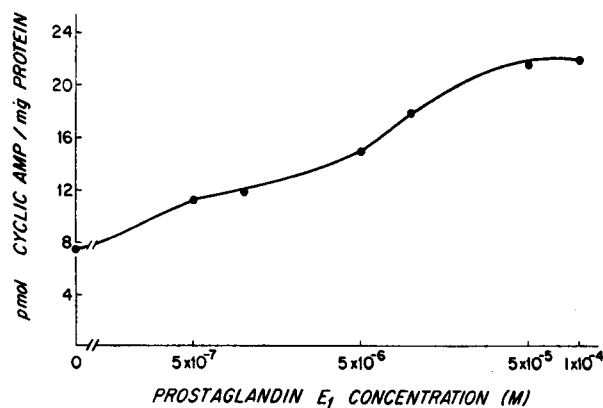


FIGURE 4 Effects of incubation with varying concentrations of PGE_1 on cyclic AMP levels in rabbit ileal mucosa. The duration of incubation with PGE_1 or solvent was 5 minutes, and these agents were added to the flasks after a 40-minute preincubation of the tissues. The remainder of the experimental conditions are described in the text. Each point represents the mean of values obtained in five experiments performed in a similar manner. Mean values ± 1 SE were: 7.5 ± 1.1 in the absence of PGE_1 , 11.4 ± 1.0 for 5×10^{-7} M PGE_1 , 12.0 ± 0.4 for 10^{-6} M, 15.0 ± 1.2 for 5×10^{-6} M, 17.9 ± 0.9 for 10^{-5} M, 21.7 ± 1.7 for 5×10^{-5} M, and 22.3 ± 2.1 for 10^{-4} M.

TABLE II
Effects of Cholera Enterotoxin and PGE₁ on Cyclic AMP Levels in Rabbit Ileal Mucosa

Experimental conditions	Cyclic AMP levels
	pmol/mg protein
Control	12.2 ± 3.3
PGE ₁	44.0 ± 7.3*
Cholera enterotoxin	114.8 ± 11.9
Cholera enterotoxin + PGE ₁	147.4 ± 13.8‡

Four complete and separate experiments were performed under the conditions described in the text. Shown are the means ± 1 SEM.

* Greater than control, $P < 0.01$.

‡ Greater than cholera enterotoxin alone, $P < 0.025$.

intestinal mucosa, fails to inhibit the enterotoxin-induced cyclic AMP rise.

DISCUSSION

There are certain observations regarding the intestinal effects of cholera enterotoxin and the interrelationship between hormones and prostaglandins in other tissues which suggest a possible role for endogenously synthesized prostaglandins as mediators for the enterotoxins' effects on intestinal mucosal adenylyl cyclase activity. Thus, as other studies have suggested (1, 11-13) and the present study confirms, there is an unexplained delay in the onset of the enterotoxin's effect on adenylyl cyclase activity in the intestine. It has also been suggested that prostaglandins may serve as modulators of hormone action in the toad bladder (34) and that endogenous prostaglandin synthesis or release may provide an essential link in the effects of luteinizing hormone on adenylyl cyclase activity in mouse ovary (35).

Recently, Jacoby and Marshall (36) reported that the administration of acetylsalicylic acid, indomethacin, phenylbutazone, sodium salicylate, or glucocorticoids is associated with a decrease in the amount of fluid accumulated in small intestinal loops in rats 4 h after intraluminal challenge with cholera enterotoxin. Subsequently, Finck and Katz (37) demonstrated in cats that the intravenous administration of acetylsalicylic acid prevents the accumulation of fluid in jejunal loops in vivo after exposure of the mucosa to cholera enterotoxin. Although the in vivo administration of anti-inflammatory agents capable of inhibiting prostaglandin biosynthesis may be associated with inhibition of cholera enterotoxin-induced intestinal fluid accumulation, the results of the present in vitro studies strongly suggest that the prostaglandins do not provide an essential link in the activation of intestinal mucosal adenylyl cyclase by cholera toxin. Thus, indomethacin markedly inhibited prostaglandin biosynthesis in rabbit ileal mucosa, but

failed to reduce the magnitude of the cyclic AMP response to toxin. Furthermore, when maximally stimulating amounts of both cholera toxin and PGE₁ were added to the mucosa, the effect on mucosal cyclic AMP level was additive.

Additional evidence supporting the hypothesis that prostaglandins do not mediate the effects of the enterotoxin on intestinal mucosal cyclic AMP metabolism is provided by the recent studies of Bourne (38), dealing with the effects of the toxin on leukocyte cyclic AMP levels, as well as other experiments concerned with the effects of the toxin on cyclic AMP metabolism in turkey erythrocytes. Bourne (38) has shown that neither acetylsalicylic acid nor indomethacin interferes with the ability of cholera enterotoxin to increase cyclic AMP levels in human leukocytes. His observations are entirely con-

TABLE III
Effects of Indomethacin and Acetylsalicylic Acid on PGE₁ and Cholera Enterotoxin-Related Increases in Cyclic AMP Levels in Rabbit Ileal Mucosa

Experimental conditions	Cyclic AMP levels
	pmol/mg protein
A. Prostaglandin addition	
Control	9.9 ± 1.3
Indomethacin	8.4 ± 1.5
Acetylsalicylic acid	8.1 ± 1.8
PGE ₁	22.4 ± 1.0*
Indomethacin + PGE ₁	32.3 ± 3.0*
Acetylsalicylic acid + PGE ₁	23.0 ± 4.1*
B. Cholera enterotoxin addition	
Control	7.8 ± 0.3
Indomethacin	8.2 ± 1.0
Acetylsalicylic acid	7.2 ± 0.5
Cholera enterotoxin	25.8 ± 3.4‡
Indomethacin + cholera enterotoxin	29.8 ± 4.2‡
Acetylsalicylic acid + cholera enterotoxin	24.8 ± 3.2‡
Cholera enterotoxin + PGE ₁	52.4 ± 2.5§
Indomethacin + cholera enterotoxin + PGE ₁	65.1 ± 16.3
Acetylsalicylic acid + cholera enterotoxin + PGE ₁	53.1 ± 3.2§

Results shown represent the means ± 1 SEM for three experiments (Part A) or four experiments (Part B) conducted in the same manner. Experimental conditions are described in the text.

* Greater than appropriate control in absence of PGE₁, $P < 0.05$.

‡ Greater than appropriate control in absence of toxin, $P < 0.025$.

§ Greater than appropriate control in absence of PGE₁, $P < 0.01$.

|| Greater than appropriate control in absence of PGE₁, $P < 0.1$ (paired t analysis), $P < 0.05$ (unpaired t analysis).

TABLE IV
Effects of Indomethacin, Acetylsalicylic Acid and Cholera Enterotoxin on Cyclic AMP
and Prostaglandin Levels in Rabbit Ileal Mucosa

Experimental conditions	Prostaglandin levels								
	Cyclic AMP levels	"PGB's"				PGF _{2α}			
	Exp. 1 → 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 1	Exp. 2	Exp. 3	Exp. 4
	<i>pmol/mg protein</i>					<i>ng/mg protein</i>			
Control	10.2 ± 1.6	0.46	0.31	0.42	0.74	0.83	0.64	0.64	0.93
Indomethacin*	9.0 ± 1.2	<0.04	<0.04	0.10	0.18	<0.40	<0.40	<0.40	<0.40
Acetylsalicylic acid	8.6 ± 0.6	0.43	0.45	0.24	0.44	0.86	0.70	0.56	0.79
Cholera enterotoxin	32.0 ± 7.2	1.20	0.92	0.30	0.50	1.23	1.51	0.62	0.96
Indomethacin + cholera* enterotoxin	27.0 ± 3.0	<0.04	<0.04	0.06	0.16	0.55	0.44	<0.40	<0.40
Acetylsalicylic acid + cholera enterotoxin	35.9 ± 6.6	0.58	0.64	0.26	0.57	1.36	1.64	0.69	1.05

Results shown represent those obtained in four similar experiments. Experimental conditions are described in the text. "PGB's" represent the aggregates of PGE's, PGA's and PGB's.

* "PGB's" and PGF_{2α} each less than control, $P < 0.025$.

sistent with the results of the present study. Whether the amounts of acetylsalicylic acid and indomethacin employed actually inhibited prostaglandin biosynthesis in leukocytes was not determined. The failure of acetylsalicylic acid to inhibit prostaglandin synthesis in the present study highlights the necessity for such determinations. In a recent study concerned with the effect of cholera toxin on turkey erythrocytes, it has been shown that the toxin is a potent stimulator of adenylyl cyclase activity in the erythrocyte membrane (M. Field, unpublished data). It has been known for some time however, that these cells contain little if any prostaglandin and that there is no evidence for biosynthesis or metabolism of prostaglandins by these cells (39). The present study, together with other studies (11, 12, 38), suggest, therefore, that cholera enterotoxin and prostaglandins activate adenylyl cyclase by independent means. In view of the studies of Jacoby and Marshall (36) and Finck and Katz (37), however, one cannot exclude the possibility that the effects of the anti-inflammatory agents on intestinal fluid transport may be related to cyclic AMP-independent, prostaglandin-mediated effects of the toxin that occur in vivo.

A few additional observations resulting from the present study deserve comment. The effect of PGE₁ on SCC appears to be a consequence of its effect on cyclic AMP metabolism. Both cyclic AMP level and SCC increase to a peak level in the first 2 min after addition of prostaglandin. A rapid increase in SCC also occurs upon addition of theophylline, cyclic AMP, or dibutyryl cyclic AMP (14). The fact that purified cholera toxin produces a much smaller increase in SCC (10) can be attributed to its delayed and gradual effect on cyclic AMP level. The initial large increases in SCC pro-

duced by cyclic AMP and theophylline decrease considerably with time (14). This is also true for PGE₁ (see Fig. 3). The exact ionic components of the early SCC response to the prostaglandins, to theophylline, and to cyclic AMP itself have not been established. The SCC increase may reflect an active secretion of anion (Cl⁻ or HCO₃⁻), but a paradoxical early stimulation of active Na absorption, which is later replaced by secretory changes, has not been excluded.

The present study also provides evidence that there is an unavoidable delay of at least 30 min between addition of cholera toxin and a demonstrable increase in cyclic AMP level. This proved to be the case even upon addition of the comparatively huge dose of 5 μg per ml of toxin (or 75 μg per 150 mg of mucosal wet weight). Furthermore, inhibition of phosphodiesterase activity with theophylline did not shorten this delay. The reason for the delay remains to be established. It is clearly not due to a primary effect on prostaglandin biosynthesis. It is probably also not due to an induction of protein synthesis, since cycloheximide does not block the effect of cholera toxin on adenylyl cyclase (40).

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