Interactions of intestinal mediators in the mode of action of cholera toxin

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Summary. Cholera toxin (CT) and prostaglandin E₂ (PGE₂) increased the synthesis of 3',5'-cyclic adenosine monophosphate (cAMP) in rabbit intestinal mucosa, which appeared to be responsible for inducing the release of 5-hydroxytryptamine (5-HT) from enterochromaffin cells into the intestinal lumen. With isolated intestinal cells, CT induced the synthesis of PGE₂ more efficiently from epithelial cells than from lamina propria cells; however, the basal amount of this eicosanoid produced by lamina propria cells was approximately six-fold more than that formed by the epithelial cells. The CT-induced stimulation of arachidonate metabolism appeared to be generalised in nature, as PGF_{2α} and leukotrienes were synthesised in addition to PGE₂. Injection of dibutyryl cAMP into the intestinal lumen *in vivo* markedly reduced both basal levels of PGE₂, as well as CT-induced levels of PGE₂, released into the luminal fluid. Similarly, when biopsy samples of tissue from rabbit intestinal loops, challenged *in vivo* with dibutyryl cAMP, were washed and incubated *in vitro*, the amount of PGE₂ synthesis remained below basal levels. In contrast, when biopsy samples of normal small intestinal tissue were exposed *in vitro* to dibutyryl cAMP, PGE₂ synthesis increased. Thus, cAMP appeared to down-regulate the levels of intestinal eicosanoids *in vivo*, despite its innate capacity to evoke PGE₂ synthesis from mucosal tissue *in vitro*. Thus, the data indicate that CT-induced mediators exhibit interactive effects that alter their cellular concentrations, that in turn could affect the biological responses.

Introduction

From a clinical standpoint, cholera is a simple disease of dehydration. We have been striving to learn more about the precise sequence of molecular events and the cells that are responsible for the hyperstimulation of water and electrolyte transport in the intestinal mucosa during the course of this disease. Once these factors are known, strategies for interrupting or reversing the mechanism might be developed to promote absorption of water and electrolytes, at least until patients could receive other therapeutic regimens (e.g., water and electrolyte replacement by the parenteral or oral routes supplemented with broad-spectrum antibiotics).

Cholera toxin (CT) is known to be an enterotoxin that stimulates cells by increasing the synthesis of cAMP, a potent second messenger which is associated with many stimulatory processes occurring in many different cell types.¹ We are interested in evaluating the role of several other substances that also are increased in concentration after CT treatment. The fact that a substance is increased in CT-treated cells does not

automatically indicate that it plays an integral role in altering secretory function or in stimulating intestinal smooth muscle.² Likewise, there is concern about attributing all physiological effects to a single substance, when several have been identified. For example, numerous prostaglandins (PGs) have been identified in several types of cell after exposure to CT.³ The CT-induced secretory event is markedly reduced *in vivo*, as well as *in vitro*, by indomethacin, a drug known to block the synthesis of PGs. Similarly, it has been demonstrated that 5-hydroxytryptamine (5-HT) also may be involved in the stimulation of intestinal cell physiology.⁴ Specialised cells along the intestinal epithelium, known as enterochromaffin cells, store large amounts of 5-HT in vacuoles, presumably for use in signalling to other cell types when noxious substances are present in the intestinal lumen.⁵ These events are accomplished by a complex network of neurons and neurotransmitters that function beneath the epithelium and provide communication with smooth muscle and other epithelial cells.⁵ Close arterial injection of 5-HT results in stimulation of prostaglandin E₂ (PGE₂) synthesis and stimulation of intestinal secretion.⁶ Furthermore, a combination of 5-HT₂ and 5-HT₃ receptor antagonists (e.g., ketansarin and ICS 205-930) completely blocks CT-induced

secretion of fluid in ligated intestinal segments in the rat.⁷ Methysergide, a 5-HT₁ receptor antagonist, also blocks CT-induced secretion.⁸ Such evidence indicates that 5-HT and neural elements play a role in the pathogenesis of cholera, along with cAMP and PGE₂.

Materials and methods

Reagents

CT was purchased from List Biological Laboratories (Campbell, CA, USA). Cyclic AMP, 5-HT, and PGE₂ were purchased from Sigma.

Animal model

A modification of the intestinal loop model described by De and Chatterje⁹ was used to evaluate the secretory effects of CT and various mediators. Adult New Zealand white rabbits were specific pathogen free (SPF) and were purchased from D and D Rabbitry (Tyler, TX, USA). The rabbits were housed in an SPF animal care facility and were fasted for 24 h before being anaesthetised with a combination of ketamine HCl 35 mg/kg and xylazine HCl 5 mg/kg. All surgical procedures were performed aseptically. The lumen of the small intestine was lavaged with 50–60 ml of warm phosphate-buffered saline (PBS) injected into the duodenal region and expelled through a 0.25-in latex tube inserted at the ileo-caecal junction. The small intestine was ligated into 5-cm segments with 2-cm interspaces. All substances were injected into the lumen of the loops in a volume of 1 ml, and the duration of the experiments *in vivo* was 16 h. The animals were over-anaesthetised with an intravenous 100-mg dose of Nembutal (Abbott Laboratories, North Chicago, IL, USA). At post-mortem examination, the volume of fluid accumulating within each loop was divided by the length of the respective loop.

Tissue preparation

Mucosal tissue from each loop was scraped from the serosa into trichloroacetic acid (TCA) 7.5% containing 0.1 M HCl. The TCA extracts were assayed for cAMP.¹⁰ PGE₂ levels in the intestinal fluids were measured.¹¹ Intestinal fluids or PBS lavages from negative loops were similarly extracted for cAMP. In addition, 1-ml samples of intestinal fluid, before centrifugation, were mixed with 14.5 µl of 7 N perchloric acid. The perchloric acid extracts were stored in amber microfuge tubes at –70°C in the dark before measurement of 5-HT. In loops that contained no fluid, a 10-ml lavage with PBS was performed.

Assays for 5-HT, PGE₂ and cAMP

Perchloric acid extracts of intestinal fluid (200 µl) were injected into a Waters HPLC fitted with a 5-µm C18 column (Vydac, Hespera, CA, USA). The column was pumped at a rate of 1 ml/min with Pump A (26%) containing methanol. Pump B (74%) contained a solution (pH 2.75) of 0.05 M Na₂HPO₄, 0.03 M citric acid, 0.1 mM EDTA, and sodium octyl sulphate 0.042% as described previously.¹² On this column, 5-HT eluted at 7.5 min, while other tryptophan metabolites—5-hydroxytryptophan, 5-hydroxyindole acetic acid, tryptophan and melatonin—typically eluted at 5.1, 5.5, 10.6 and 14.3 min, respectively. Standard amounts of 5-HT down to 20 pg were detected with a Waters 470 fluorescence detector programmed for an excitation wavelength of 285 nm and an emission wavelength of 340 nm.

The PGE₂, PGF_{2α} and leukotriene content of the intestinal fluids was measured by radioimmunoassay with kits (Perseptive Diagnostics Inc., Cambridge, MA, USA). Samples were extracted as recommended by the manufacturer. The level of cAMP in mucosal tissue extracts and in intestinal fluid was assayed by a radiometric assay with protein kinase, as described previously.¹⁰

Biopsy of mucosal tissue and in-vitro incubation

Mucosal biopsies were taken from the rabbit small intestine with an 8-mm biopsy tool, either with or without in-vivo exposure of 15-cm intestinal segments to CT 2 µg or dibutyryl cAMP 100 mg for 4 h. Mucosal

Fig. 1. Effect of increasing doses of PGE₂ injected into ligated intestinal loops of six adult rabbits. Panel a depicts the mean fluid accumulation values (ml/cm) at 16 h. Panels b and c indicate the respective amounts of cAMP and 5-HT accumulating in the fluid from intestinal loops. The vertical bars show one standard error above and below the mean.

Fig. 2. Effect of increasing amounts of dibutyryl cAMP on fluid accumulation in rabbit intestinal loops after 16 h. Panel a shows the fluid accumulation values (ml/cm); panels b and c illustrate the mean 5-HT and PGE₂ responses in the resulting fluid in each intestinal loop. Responses to CT are included as a positive control. The vertical bars show one standard error above and below the mean.

biopsies were washed with PBS before incubating in six-well tissue-culture plates (two tissue samples/well), and 2 ml of Ham's F-12 medium, containing fatty acid-free BSA 0.1%, was then added to each well. Mucosal biopsies from normal intestinal tissue were then incubated at 37°C *in vitro* with CT 0.5 µg/ml or dibutyryl 250 µM cAMP for a period of 18 h. Subsequently, PGE₂ levels in the culture medium and in mucosal scrapings of the biopsies were determined as described above.

Isolation of intestinal cells

In an attempt to determine the type of intestinal cell(s) producing PGE₂, the intestinal epithelial cells were separated from the intestinal mucosa by the method of Weiser.¹³ This method consisted of timed washings in PBS, containing 0.5 mM dithiothreitol and 1.5 mM EDTA. The remaining intact tissue consisted of denuded villi and lamina propria, which were scraped into PBS. After washing three times with PBS, the cells were suspended in 2 ml of Ham's F12 medium with fetal bovine serum 10%. Cell suspensions were adjusted to 100 Klett units and were incubated overnight (18 h) with CT 0.5 µg/ml. At that time, the culture medium, as well as the TCA 7.5% extracts of the cells, were assayed for PGE₂.

Statistics

Data were analysed by an analysis of variance and subsequently by the Tukey test for multiple group comparisons. Student's *t* test was used where only two independent groups were compared (e.g., fig. 5).

Results

Fig. 1a illustrates the effect of increasing doses of PGE₂ on fluid accumulation in small intestinal loops in the adult rabbit model. The lowest concentration of PGE₂ that could elicit any fluid accumulation after intraluminal injection was 25 µg/ml ($p > 0.05$), and the maximal fluid accumulation response occurred with 50 µg/ml ($p < 0.05$). Corresponding increases in cAMP and 5-HT concentrations in the luminal fluid are shown in fig. 1b and 1c, respectively. The small increase in 5-HT at the 25-µg dose of PGE₂ ($p > 0.05$) correlated better than the corresponding increase in cAMP ($p < 0.05$) with the amount of fluid accumulating in the intestinal loop. Fig. 2a shows the amount of fluid accumulation elicited by increasing doses of dibutyryl cAMP. Fluid accumulation was considered significant at the 100-mg dose ($p < 0.05$). Fluid accumulation caused by 200 mg of dibutyryl cAMP was

Table. In-vivo effect of CT and dibutyl cAMP on synthesis and release of selected eicosanoids into intestinal fluid with the adult rabbit ligated loop model

Treatment	Mean (SD) eicosanoid concentration (pg/loop)		
	PGE ₂	PGF _{2α}	LTC ₄
PBS	15 139 (5936)*	21 325 (7373)	38 440 (7656)
CT 2 µg	29 899 (9870)	57 520 (12 621)	74 040 (2070)
cAMP 100 µg	4461 (1062)	13 157 (7260)	20050 (5760)

* Arithmetic means (and SD of the mean) were derived from six rabbits.

similar to that caused by 200 ng of CT (fig. 2a). Luminal injection of dibutyl cAMP also caused a dose-dependent increase in the release of 5-HT from the enterochromaffin cells that was significant at the 75-mg dose ($p < 0.05$) (fig. 2b). In contrast, injection of dibutyl cAMP into the loops did not increase PGE₂ levels ($p > 0.05$) in the intestinal fluid above that of

the control (fig. 2c). PGE₂ levels decreased as the dose of dibutyl cAMP was increased (highest dose; $p < 0.05$).

In a previous study we reported that exposure of Chinese hamster ovary cells to CT resulted in a generalised stimulatory effect on arachidonate metabolism, such that several PGs were synthesised.³ The table indicates that CT induced the synthesis and release of PGF_{2α}, PGE₂ and leukotrienes into the intestinal fluid; however, cAMP depressed the amount of each eicosanoid below basal levels. Thus, the intestinal cell response to CT challenge *in vivo* is similar to that of non-intestinal cells in invoking a generalised eicosanoid response.³

To learn more about the responsiveness of the intestinal mucosa to stimulation by combinations of CT-induced substances, doses of each mediator that elicited maximal fluid accumulation were selected. PGE₂ and dibutyl cAMP were injected into ligated rabbit intestinal loops alone and in combination to assess their effects on the intestinal response (fig. 3). The data in fig. 3a indicate that both 100 µg of PGE₂

Fig. 3. Effect of combinations of CT with dibutyl cAMP and PGE₂ after injection into rabbit intestinal loops at 16 h. Panel a depicts the mean fluid accumulation values (ml/cm) from a total of six rabbits. Panels b and c show the amount of cAMP and PGE₂ accumulating in the intestinal fluid.

Fig. 4. Amount of PGE₂ secreted into the culture medium from small intestinal biopsies. Panel a shows the 18-h in-vitro PGE₂ responses of mucosal biopsies taken from rabbit intestinal loops inoculated with PBS, CT or dibutyryl cAMP for 4 h. In contrast, panel b depicts the in-vitro effects of PBS, dibutyryl cAMP or CT on PGE₂ synthesis in mucosal biopsies from normal rabbit small intestine. Levels of PGE₂ in tissue culture medium and in the cell extracts were determined. The vertical bars show one standard error above and below the mean. ▨, intracellular PGE₂; ▩, culture medium PGE₂; ■, total PGE₂.

Fig. 5. Synthesis of PGE₂ in epithelial cells compared to that in lamina propria cells isolated from normal rabbit small intestine. Levels of PGE₂ in tissue culture medium and in the cell extracts were determined after 18 h. The vertical bars show one standard error above and below the mean. ▨, intracellular PGE₂; ▩, culture medium PGE₂; ■, total PGE₂.

and 200 mg of dibutyryl cAMP evoked substantial amounts of fluid accumulation of the same magnitude as 200 ng of CT ($p < 0.05$). When PGE₂ was combined with a dose of CT, sufficient to cause a maximal fluid accumulation response, no significant increase ($p > 0.05$) in fluid accumulation was evident (fig. 3a). One interpretation of these data was that CT and PGE₂ might share a common action, or else additional fluid

accumulation should have been formed through an alternate mechanism. Clearly, both CT and PGE₂ elevated cAMP levels ($p < 0.05$) in the intestinal fluid (fig. 3b), although the amount of mucosal cAMP was not additive when CT and PGE₂ were combined (fig. 3b). We noted again that dibutyryl cAMP diminished basal levels of mucosal PGE₂ synthesis ($p < 0.05$), as well as PGE₂ levels in any loop exposed to CT (fig. 3c).

Fig. 4 depicts the results of experiments to evaluate PGE₂ synthesis in intestinal mucosal biopsy specimens in response to CT or dibutyryl cAMP. Fig. 4a shows the in-vitro synthesis of PGE₂ from mucosal biopsies taken from intestinal loops exposed previously *in vivo* to CT, dibutyryl cAMP or PBS for 4 h. Each tissue sample was then washed and incubated for 18 h *in vitro*, after which the amount of PGE₂ inside the cells, as well as in the culture medium, was measured. The data (fig. 4a) indicate that prior in-vivo exposure to CT resulted in increased synthesis of PGE₂ ($p < 0.05$), but most was not released from the mucosa. In contrast, in-vivo exposure to dibutyryl cAMP caused an apparent decrease in PGE₂ synthesis below basal levels (fig. 4a). These in-vitro data complemented our observation that dibutyryl cAMP failed to stimulate PGE₂ synthesis *in vivo* (figs. 2c, 3c and table). Fig. 4b illustrates the effect of in-vitro exposure of mucosal biopsies from normal rabbit intestinal tissue to CT, dibutyryl cAMP or PBS on PGE₂ synthesis. It was apparent that dibutyryl cAMP elicited the synthesis of PGE₂ in mucosal biopsies from normal rabbit intestine ($p < 0.05$). The stimulatory effect of CT on PGE₂ synthesis was apparent but not considered significant by the Tukey test ($p > 0.05$).

Fig. 5a illustrates the typical response of isolated intestinal epithelial cells from normal rabbit small intestine to in-vitro challenge with CT compared to the PBS control. The data indicated that PGE₂ was formed by the isolated epithelial cells and released into the culture medium (fig. 5a) after incubation *in vitro* with CT ($p < 0.05$). A similar, but lower PGE₂ response ($p > 0.05$), was observed when lamina propria cells were exposed to CT (fig. 5b). Although the basal PGE₂ levels in the lamina propria cells were approximately six-fold higher than that in the isolated intestinal epithelial cells (fig. 5a and b), both cell types appeared to have the capacity to synthesise PGE₂ *in vitro* in response to CT.

Discussion

This study has examined the role of selected mediators in the pathogenesis of experimental cholera. Dibutyryl cAMP and PGE₂ elicited fluid accumulation responses that were typical of CT. Massive doses (200 mg/ml) of dibutyryl cAMP (fig. 2a) were required to cause fluid accumulation equivalent to CT, although such concentrations were not physiological. Our earlier experiments¹¹ had indicated that dibutyryl cAMP did not cause fluid accumulation; however, in

the current experiments the rabbit intestinal loop was modified by extensive lavage of the intestinal lumen with warm PBS to remove residual debris from the intestinal lumen before construction of the loops. In contrast, the effective dose of PGE₂ was 25–50 µg/ml (fig. 1a). Considering that luminal administration of this paracrine hormone was not the normal physiological site of action, this dose was relatively low. Beubler *et al.*⁶ demonstrated with the rat intestinal loop model that close arterial infusion of ng amounts of PGE₂ caused fluid secretion. Because PGE₂ induced the synthesis and release of cAMP and 5-HT (fig. 1b and c), it could be presumed that the PGE₂-induced increase in cAMP levels caused the release of 5-HT from the enterochromaffin cells, just as cAMP induced 5-HT release (fig. 2b).

The relationship between cAMP and PGE₂ was intriguing in that in-vivo challenge of intestinal loops with dibutyryl cAMP decreased the basal levels of PGE₂ in intestinal fluid. Addition of dibutyryl cAMP to loops challenged with CT also diminished the PGE₂ concentration to levels below that of the controls. These data did not support the concept that cAMP signalled the synthesis of PGE₂ in cells exposed to CT, even though in-vitro experiments with Chinese hamster ovary cells and a murine macrophage/monocyte cell line demonstrated that dibutyryl cAMP could evoke PGE₂ synthesis.³ The in-vitro stimulation of PGE₂ synthesis by dibutyryl cAMP (fig. 4b) was in striking contrast to that observed *in vivo* (figs. 2c and 3c) and the in-vitro responses of mucosal biopsies from dibutyryl cAMP-treated loops (fig. 4a). These observations suggested that the intestinal mucosal response to dibutyryl cAMP, in synthesising PGE₂, depended on whether the mucosal tissue was intact and connected to the vasculature. In addition, our data suggested that intestinal cells behaved differently from other cells with regard to the production of CT-stimulated mediators. A possible interpretation of the data shown in figs. 2c, 3c, 4a and the table is that cAMP served to decrease PGE₂ synthesis or enhance its degradation *in vivo*. Considering these data, it is difficult to perceive cAMP as an intermediate leading to the synthesis of this eicosanoid *in vivo*, were it not for the innate capacity of the isolated intestinal cells to synthesise PGE₂ in response to CT *in vitro*.

As indicated by earlier studies with non-intestinal cells,³ the intestinal eicosanoid response to CT appeared to be generalised, as CT stimulated the synthesis of PGF_{2α} and leukotrienes, as well as of PGE₂ (table). Very little information is available about leukotriene synthesis in response to CT. Triadafilopoulos *et al.*¹⁴ determined that the cytotoxic

enterotoxin from *Clostridium difficile* induced the synthesis of PGE₂, as well as leukotriene B₄ (LTB₄) in the rabbit intestinal loop model. They included CT as one of their controls, which increased the synthesis of PGE₂, but not LTB₄. We observed that CT stimulated the synthesis of leukotrienes, although the RIA kits used could not determine the specific type, because of their structural similarity. The RIA kit from Perseptive Diagnostics reacted with LTA₄, LTB₄, LTC₄ and LTD₄, but not with PGs. Based on these data, CT might be expected to elicit the synthesis of several arachidonate metabolites, due to the availability of arachidonate released from phosphatidylinositol.¹⁵ In the latter study, it was established that CT increased the hydrolysis of this minor phospholipid component, releasing free arachidonate and glycerophosphatidyl inositol from a murine macrophage/monocyte cell line.

In conclusion, the data in this report provide a basis for understanding the mechanism of action of CT. Clearly, CT increases cAMP levels in virtually any cell to which it is exposed,¹ and earlier reports indicate that cAMP levels in the epithelial cell monolayer increase with little increase in cAMP in the lamina propria.¹⁰ Cyclic AMP elicits the release of 5-HT (fig. 2) from enterochromaffin cells, which are located in the epithelium, thereby activating the enteric nervous system.^{4–8, 16, 17} This concept is based, in part, upon the inhibitory effect that neural blockade with lidocaine, tetrodotoxin and hexamethonium have on CT-induced secretion.^{5, 18} Further, 5-HT antagonists diminish the secretory response.^{7, 8} Evidence indicates that the 5-HT released from the basal portion of the enterochromaffin cells initiates a signal that is transmitted *via* the enteric neurons to the crypt epithelial cells. The physiological effects include increases in intestinal ion transport, that ultimately result in loss of water and electrolytes into the intestinal lumen. The role of PGE₂ is implied by the capacity of indomethacin to block the CT-induced fluid accumulation response and the correlation of the eicosanoid synthesis (figs. 4 and 5) in intestinal cells with that of secretion.^{3, 6, 11, 18, 19} It is possible that eicosanoids could be involved in the post-neural signalling leading to secretion of fluid from crypt epithelial cells. Finally, the elevation in epithelial cell cAMP can result from the ADP-ribosylating enzymatic activity characteristic of CT, that can directly stimulate chloride secretion in the epithelial cells.¹ The precise role of each of these possible mechanisms in the pathogenesis of cholera is still very difficult to delineate, and it is likely that multiple factors and cell types are involved following exposure to CT.

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