tween cytoplasm and cell membrane has been demonstrated in proton-secreting renal epithelial cells (*14*). In keeping with evidence that resorptive cells do not effectively attach to unmineralized bone matrix (*15*), H^+-ATPase was absent at all sites where osteoclasts were juxtaposed to osteoid (unmineralized bone). Finally, we used immunoelectron microscopy to show that the ruffled border, the highly convoluted cell membrane characterizing the site of bone resorption, contains the H^+-ATPase (Fig. 3, C and D).

Thus, the osteoclast has a vacuolar-type proton pump that is physiologically and structurally identical to the kidney H^+-ATPase and that appears to be responsible for creation of the acidic microenvironment fundamental to bone resorption. The osteoclast's ruffled membrane has a dense array of cytoplasmic proteinaceous studs (*16*) similar to those found on the plasma membrane of renal proton-transporting epithelia, which represent the cytoplasmic domain of the vacuolar H^+-ATPase (*17*). As in the kidney intercalated cell, osteoclastic proton pumps are polarized to one plasma membrane domain, allowing vectorial proton secretion. The mechanism by which the osteoclast maintains the polarity of the proton pump at the osteoclast-bone interface is unknown and is an intriguing problem, since osteoclasts, unlike epithelial cells, do not form tight junctions. However, when epithelial cells attach to substrate, polarization begins before the formation of tight junctions and is associated with cytoskeletal reorganization (*18*). The development of the osteoclastic attachment site, which is rich in cytoskeletal elements, may represent a similar polarization process.

REFERENCES AND NOTES

1. A. Cretin, *Presse Med.* **59**, 1240 (1951).
2. M. D. Fallon, *Endocrine Control of Bone and Calcium Metabolism*, C. V. Conn, T. Fujita, J. R. Potts, Jr., R. V. Talmage, Eds. (Elsevier, Amsterdam, 1984), vol. 8A, pp. 144–146.
3. R. Baron, L. Neff, P. J. Courtnoy, *J. Cell Biol.* **101**, 2210 (1985).
4. R. Baron, L. Neff, C. Roy, A. Bolsvert, M. Caplan, *Cell* **46**, 311 (1986).
5. C. DeDuve *et al.*, *Biochem. Pharmacol.* **23**, 2495 (1974).
6. H. C. Blair, A. J. Kahn, E. C. Crouch, J. J. Jeffrey, S. L. Teitelbaum, *J. Cell Biol.* **102** 1164 (1986).
7. S. Gluck and Q. Al-Awqati, *J. Clin. Invest.* **73**, 1704 (1984).
8. J. Glickman, K. Croen, S. Kelly, Q. Al-Awqati, *J. Cell Biol.* **97**, 1303 (1983).
9. L. Faller *et al.*, *Ann. N.Y. Acad. Sci.* **402**, 146 (1982).
10. S. Gluck and J. Caldwell, *J. Biol. Chem.* **262**, 15780 (1982).
11. S. Ohkuma, Y. Moriyama, T. Tatsuy, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 2758 (1982).
12. I. Mellman, R. Fuchs, A. Helenius, *Annu. Rev. Biochem.* **55**, 663 (1986).
13. C. J. Galloway, G. E. Dean, G. Rudnick, I. Mellman, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3334 (1983).
14. S. Gluck *et al.*, *ibid.* **79**, 4327 (1982).
15. J. Jonesey, *J. Clin. Invest.* **51**, 9 (1972); Z. Bar-Shavit *et al.*, *ibid.* **72**, 526 (1983).
16. S. L. Teitelbaum, C. C. Stewart, A. J. Kahn, *Calcif. Tissue Int.* **27**, 255 (1979).
17. D. Brown, S. Gluck, J. Hartwig, *J. Cell Biol.* **105**, 1637 (1987).
18. K. Simons and S. Fuller, *Annu. Rev. Cell Biol.* **1**, 243 (1985).

19. We thank J. Konsek and R. Henry for technical assistance. Supported by NIH grants AM01631 (to H.C.B.), AM32788 (to S.L.T.), and AM34788 (to S.G.) and a grant from the Shriners' Hospital for Crippled Children, St. Louis Unit. This work was done during the tenure of an established investigatorship award from the American Heart Association and Sandoz Pharmaceuticals Corporation (to S.G.).

28 April 1989; accepted 23 May 1989

Role of Prostaglandins and cAMP in the Secretory Effects of Cholera Toxin

JOHNNY W. PETERSON AND LAURA G. OCHOA

The role of adenosine 3′,5′-monophosphate (cAMP) and prostaglandins in the pathogenesis of experimental cholera was evaluated. Fluid accumulated in the rabbit intestinal loop model after 16 hours of incubation with cholera toxin, prostaglandin E_1, or prostaglandin E_2, but not with membrane-permeable derivatives of cAMP or forskolin. Dibutyryl cAMP triggered a small, transient intestinal fluid accumulation response by 4.5 hours; however, the fluid was completely absorbed by 9 hours. After exposure of intestinal loops to cholera toxin, prostaglandin E was released into the intestinal lumen in a concentration-dependent manner independent of cAMP. Thus, not only cAMP, but also prostaglandins may regulate water and electrolyte secretion in cholera.

THE PATHOGENIC MECHANISM OF cholera and several other intestinal enteropathies caused by *Escherichia coli*, *Salmonella* species, *Campylobacter jejuni*, and other enterotoxin-producing bacteria is thought to be mediated by cAMP (*1*). The A_1 subunit of cholera toxin possesses both nicotinamide adenine dinucleotide glycohydrolase and adenosine diphosphate (ADP)–ribosyltransferase activities. Cholera toxin catalyzes the ADP-ribosylation of the α subunit of the stimulatory G protein ($G_{s\alpha}$) of adenylate cyclase (*2*). The guanosine triphosphate–binding activity of $G_{s\alpha}$ is altered by covalent linkage to ADP-ribose, which causes the catalytic site of adenylate cyclase to increase cAMP production. The cAMP content of cholera toxin–affected cells increases markedly and in proportion to the amount of cholera toxin stimulation (*3*).

Clinically, the only important cell types naturally affected by cholera toxin reside in the intestinal mucosa. After exposure to cholera toxin, the cAMP content of the crypt epithelial cells of the small intestine increases, and water and electrolyte transport is altered (*4, 5*). Application of dibutyryl cAMP to stripped intestinal mucosa in Ussing chambers increases chloride ion transport (*4*). Thus, fluid and electrolyte loss in cholera patients is associated with increased cAMP concentrations.

We have reevaluated the cause-effect relation of cAMP formation, prostaglandin E (PGE) release, and intestinal fluid secretion. The possible participation of prostaglandins in the pathogenesis of cholera was suggested by Bennett (*6*); both PGE_1 and PGE_2 stimulate intestinal adenylate cyclase (7). The effects of cholera toxin on secretion and on cAMP accumulation are inhibited by drugs that inhibit prostaglandin formation (for example, indomethacin, aspirin, and ibuprofen) (*8–11*). The PGE_2 content of jejunal aspirates from individuals with acute cholera is significantly higher than in those from convalescent patients (*12*). Concentrations of PGE increase in the intestinal mucosa of rabbits challenged with cholera toxin, *Vibrio cholerae*, or *Salmonella* species (*13*). Cholera toxin also increases the release of PGE_2 and 5-hydroxytryptamine in rat jejunal segments (*14, 15*). A murine macrophage cell line releases PGE_2 when exposed to either cholera toxin or pertussis toxin, but not when exposed to dibutyryl cAMP (*16*). We used the rabbit intestinal loop model (*17*) to investigate the relation of cAMP and PGE to the cholera toxin–mediated secretory response.

To compare the effects of cAMP, PGE, and cholera toxin on net fluid secretion, we injected the lumina of rabbit intestinal segments with various agents (*18*) and measured the mean fluid accumulation responses of intestinal loops after an overnight (16 hours) exposure (Table 1). Cholera toxin caused approximately 1.5 ml of fluid per centimeter of intestine to accumulate. When

Department of Microbiology, University of Texas Medical Branch, Galveston, TX 77550.

Table 1. Effect of cholera toxin, cAMP, and prostaglandins on the responses of rabbit intestinal loops. Values of mucosal tissue cAMP are mean ± SEM derived from intestinal loops of seven rabbits. After cutting open the intestinal segments and washing in three changes of ice-cold PBS, the cAMP content per milligram of mucosal tissue protein was determined. Luminal PGE values are also means ± SEM derived from the same animals. Values reflect the amount of PGE in intestinal fluid or washings of the intestinal lumen per milligram of mucosal tissue protein.

Substance	Fluid accumulation (ml/cm)	Mucosal tissue cAMP (pmol/mg)	Mucosal tissue cAMP (fold increase)	Luminal PGE (pg/mg)	Luminal PGE (fold increase)
PBS-MIX* control	0	12.8 ± 0.6		7.5 ± 1.5	
Cholera toxin (350 ng/ml)	1.5 ± 0.3	29.1 ± 2.3†	2.3	46.2 ± 5.8†	6.2
Dibutyryl cAMP (100 m*M*)	0	39.4 ± 4.5†	3.1	8.3 ± 2.8‡	1.1
8-Bromo cAMP (10 m*M*)	0	24.0 ± 4.2†	1.9	4.5 ± 1.5‡	−0.6
PGE_1 (250 μg/ml)	1.0 ± 0.4	21.9 ± 3.5‡	1.7	93.1 ± 10.7†	12.4
PGE_2 (250 μg/ml)	1.0 ± 0.4	25.6 ± 3.4†	2.0	47.5 ± 5.4†	6.3
Forskolin (10 m*M*)	0	26.2 ± 2.1†	2.1	13.8 ± 4.4‡	1.8

*All substances injected into the intestinal lumen in 1 ml of PBS containing 0.1 m*M* MIX. †Value significantly different from control. Analysis of variance procedures were used separately for the cAMP and PGE data. The data were analyzed by the Scheffé test by using a square root scale to better homogenize variability. ‡Value not significantly different from control. Statistical method was as described above.

dibutyryl cAMP, over the concentration range of 0.1 to 100 m*M*, was instilled into intestinal segments there was no fluid accumulation at 16 hours. Similarly, dibutyryl cAMP from several different commercial sources and 8-bromo cAMP did not cause fluid accumulation. When another potent stimulator of adenylate cyclase, forskolin, was injected into the rabbit intestinal loops, it failed to elicit fluid accumulation (at 16 hours). We have also observed that pertussis toxin (10 μg/ml) failed to elicit fluid accumulation at 16 hours. In contrast, PGE_1 and PGE_2 caused marked fluid accumulation approaching 1 ml/cm; PGE_2 was effective at 10 μg/ml. Much lower doses of PGE_2 can cause fluid secretion when administered through the mesenteric vasculature (*15*). The gross appearance of the fluid from loops treated with PGE was identical to that of loops exposed to cholera toxin. Further, histological sections of the loops exposed to PGE were indistinguishable in appearance from those of the control.

To determine the effects of each of the above substances on tissue cAMP concentrations, we prepared mucosal scrapings from each intestinal segment (*19*). The agents were diluted in 0.1 m*M* 1-methyl-3-isobutylxanthine (MIX) (to inhibit mucosal phosphodiesterase activity) and then injected into the intestinal segments. Dibutyryl cAMP, 8-bromo cAMP, and forskolin significantly increased tissue cAMP concentrations to values achieved with cholera toxin, but did not induce fluid accumulation in the intestinal loops, in contrast to cholera toxin and PGE. Both PGE_1 and PGE_2 increased cAMP concentrations, although the increase was statistically significant only for PGE_2. Thus, tissue cAMP concentrations can be increased in the mucosal cells by several means without eliciting a sustained secretory response.

The amount of PGE released into the intestinal lumen after injection of rabbit intestinal loops with cholera toxin was determined (Fig. 1). Cholera toxin increases the PGE content of intestinal tissues (*13*); however, in our study we observed that intestinal fluids yielded more reliable estimates of PGE concentration than did tissues. This may be due to the rapid export of prostaglandins from cells after synthesis (*20*). The amount of PGE present in the fluid secreted in response to cholera toxin increased approximately in proportion to the amount of fluid that accumulated in the loops and was related to the dose of cholera toxin injected. The release of PGE required the A subunit of cholera toxin; injection of an equivalent amount of B subunit failed to cause PGE release or fluid accumulation (Fig. 2). Thus, mere binding of the B subunit to the host cell membranes did not initiate nonspecific release of prostaglandins, indicating that the holotoxin is necessary for PGE release and fluid accumulation.

We conducted experiments to determine whether cAMP derivatives could cause PGE release. Neither dibutyryl cAMP nor 8-bromo cAMP significantly increased the PGE content of washings from the negative intestinal loops (Table 1). Thus, the PGE that was released when intestinal loops were exposed to cholera toxin did not appear to be dependent on the synthesis of cAMP. This finding confirmed a report (*16*) in which cholera toxin and pertussis toxin induced the release of PGE from a macrophage cell line, whereas dibutyryl cAMP was ineffective. In our study forskolin did not significantly increase the amount of PGE released, although it did significantly increase the intestinal tissue content of cAMP. Our data (Table 1) indicate that only cholera toxin caused PGE release into the intestinal lumen. The PGE detected in the PGE_1 and PGE_2 loop washes merely reflects the residual PGE initially injected into the loops. In general, intestinal fluid accumula-

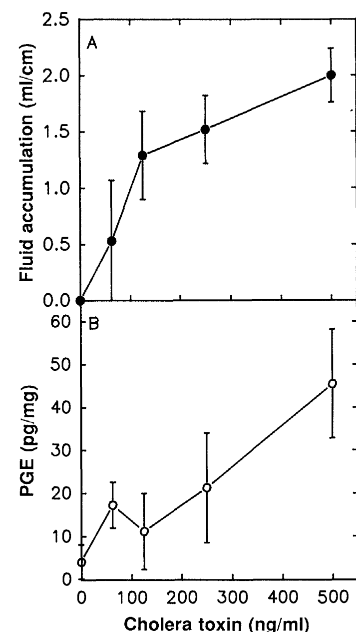

Fig. 1. (**A**) Fluid accumulation and (**B**) amount of PGE released into the intestinal fluid after exposure to cholera toxin. Each intestinal loop was injected with 1 ml of each cholera toxin dose. Measurements were made at 16 hours. Values are means ± SD of duplicate loops from two rabbits.

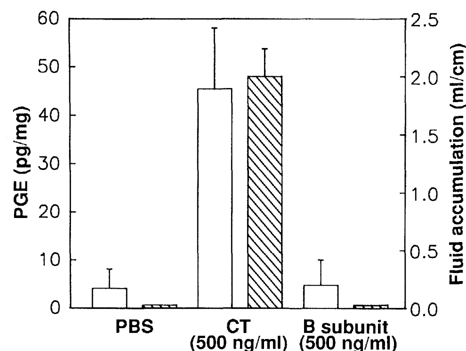

Fig. 2. Comparison of the effects of cholera toxin (CT) and cholera toxin B subunit on net fluid secretion (hatched bars) and PGE concentrations (open bars) in intestinal loop fluid. Values are means ± SD of duplicate loops from two rabbits. Measurements of PGE were made on accumulated intestinal fluid or PBS washes of the negative loops.

tion correlated better with the PGE content of the intestinal fluids or washes than to the tissue concentration of cAMP.

We performed preliminary kinetic experiments to determine the effects of dibutyryl cAMP on fluid accumulation at earlier time intervals with two rabbits per time point. After 4.5 hours, 100 m*M* dibutyryl cAMP caused 0.2 ml of fluid accumulation per centimeter, whereas cholera toxin (350 ng/ml) caused 0.4 ml/cm. After 9 hours, the fluid in the dibutyryl cAMP–treated loop was completely absorbed, whereas fluid in loops treated with cholera toxin, PGE_1, or PGE_2 progressively increased (to ≥ 1 ml/cm). The stimulatory effects of dibutyryl cAMP on early secretory events in vivo is known (*21, 22*), but the above reversal to net absorption did not coincide with the sustained secretory effects of cholera toxin.

We question whether the magnitude and duration of the effect of cAMP alone can account for the massive loss of water and electrolytes in cholera. If cAMP were the sole intracellular mediator of the diarrhea elicited by cholera toxin, then it might be expected that a sustained fluid accumulation response would have been observed with cAMP derivatives and adenylate cyclase stimulators. Instead, only a small, transient secretory effect was seen with dibutyryl cAMP after 4.5 hours. Neither membrane-permeable derivatives of cAMP nor forskolin caused fluid accumulation in rabbit intestinal loops after overnight exposure despite increased mucosal tissue concentrations of cAMP equal to that of tissues exposed to cholera toxin.

Although one possible interpretation of our data questions the role of cAMP in the cholera toxin–mediated secretory response, an alternative explanation is that the cAMP derivatives or forskolin may not have reached the appropriate secretory cells, presumably the crypt cells. To address this latter possibility, we used inordinately large concentrations of these substances to maximize their diffusion into the crypts. We did not attempt to isolate and measure the cAMP content of the crypt cells because of technical problems (for example, diffusion of dibutyryl cAMP from isolated crypt cells). However, in these experiments, cholera toxin, PGE_1, and PGE_2 reached the appropriate cells and elicited a net secretory response.

We conclude that enhanced production of both cAMP and PGE occurred in cholera toxin–treated cells and that cAMP did not cause PGE release. The mechanism leading to alterations in prostaglandin synthesis remains to be elucidated. The hypothesis that cAMP is the sole mediator of intestinal water and electrolyte transport in cholera should be reexamined. We observed a poor correlation between tissue cAMP concentrations and net fluid secretion in this model. In contrast, PGE release from cholera toxin–treated mucosal cells correlated with cholera toxin–induced fluid accumulation and increased in a dose-related manner. Thus, cAMP alone might not be responsible for cholera toxin–mediated fluid accumulation in this in vivo model. Cholera toxin–induced fluid secretion does not parallel tissue cAMP concentrations in rat jejunum, ileum, and colon (*23*). We propose that PGE could be an important factor in the secretory process leading to the loss of water and electrolytes during cholera, which could explain the effectiveness of several prostaglandin synthesis inhibitory drugs in reducing cholera toxin–mediated fluid accumulation (*8–10*).

REFERENCES AND NOTES

1. R. A. Finkelstein, in *Handbook of Natural Toxins*, vol. 4, *Bacterial Toxins*, M. C. Hardegree and A. T. Tu, Eds. (Dekker, New York, 1988), pp. 1–38.
2. J. Moss and M. Vaughan, *ibid.*, pp. 39–87.
3. R. L. Guerrant, L. L. Brunton, T. C. Schnaitman, L. I. Rebhun, A. G. Gilman, *Infect. Immun.* **10**, 320 (1974).
4. M. Field, *Am. J. Physiol.* **221**, 992 (1971).
5. ———, D. Fromm, Q. Al-Awqati, W. B. Greenough III, *J. Clin. Invest.* **51**, 796 (1972).
6. A. Bennett, *Nature* **231**, 536 (1971).
7. D. V. Kimberg, M. Field, J. Johnson, A. Henderson, E. Gershon, *J. Clin. Invest.* **50**, 1218 (1971).
8. H. I. Jacoby and C. H. Marshall, *Nature* **235**, 163 (1972).
9. A. D. Finck and R. L. Katz, *ibid.* **238**, 273 (1972).
10. R. E. Gots, S. B. Formal, R. A. Giannella, *J. Infect. Dis.* **130**, 280 (1974).
11. J. W. Peterson, L. G. Ochoa, W. D. Berg, *Fed. Eur. Microbiol. Soc. Microbiol. Lett.* **56**, 139 (1988).
12. P. Speelman, G. H. Rabbani, K. Bukhave, J. Rask-Madsen, *Gut* **26**, 188 (1985).
13. I. E. Duebbert and J. W. Peterson, *Toxicon* **23**, 157 (1985).
14. E. Beubler, G. Kollar, A. Saria, K. Bukhave, J. Rask-Madsen, *J. Physiol. (London)* **382**, 46P (1987).
15. ———, *Gastroenterology* **96**, 368 (1989).
16. R. M. Burch, C. Jelsema, J. Axelrod, *J. Pharmacol. Exp. Ther.* **244**, 765 (1988).
17. S. N. De and D. N. Chatterjee, *J. Pathol. Bacteriol.* **66**, 559 (1953).
18. New Zealand White rabbits (2 to 2.5 kg) were anesthetized by intramuscular injection of ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg) before surgery (*19*). Intestinal segments (5 to 10 cm long) were ligated, beginning approximately 20 cm proximal to the cecum-appendix, and 2-cm interspaces were tied between the loops. After 16 hours, all animals were again anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg) and killed by the injection of 10 ml of air into the marginal ear vein. The cAMP content of mucosal tissue extracts was measured by a protein kinase competitive binding assay (*19*). The PGE concentration of intestinal fluids or phosphate-buffered saline (PBS) washes of negative loops was measured with a PGE radioimmunoassay kit (Dade Baxter Travenol Diagnostics, Miami, FL) (*13*). All chemicals were purchased from Sigma (St. Louis, MO) except cholera toxin, which was acquired from List Biological Laboratories (Campbell, CA).
19. J. W. Peterson, N. C. Molina, C. W. Houston, R. C. Fader, *Toxicon* **21**, 761 (1983).
20. C. J. Hawkey and D. S. Rampton, *Gastroenterology* **89**, 1162 (1985).
21. S. Eklund, J. Cassuto, M. Jodal, O. Lundgren, *Acta Physiol. Scand.* **120**, 311 (1984).
22. I. Lonnroth and S. Lange, *J. Cyclic Nucleotide Res.* **7**, 247 (1981).
23. U. M. Farack, R. Gerzer, T. M. Keravis, K. Loeschke, *Dig. Dis. Sci.* **33**, 1153 (1988).

24. We thank L. Reuss, S. Baron, A. Kurosky, A. Chopra, J. Mathias, O. Steinsland, and M. Sussman for suggestions leading to improvements in the manuscript and E. Whorton for performing statistical analyses.

9 January 1989; accepted 22 June 1989

Buprenorphine Suppresses Cocaine Self-Administration by Rhesus Monkeys

NANCY K. MELLO, JACK H. MENDELSON, MARK P. BREE, SCOTT E. LUKAS

Cocaine abuse has reached epidemic proportions in the United States, and the search for an effective pharmacotherapy continues. Because primates self-administer most of the drugs abused by humans, they can be used to predict the abuse liability of new drugs and for preclinical evaluation of new pharmacotherapies for drug abuse treatment. Daily administration of buprenorphine (an opioid mixed agonist-antagonist) significantly suppressed cocaine self-administration by rhesus monkeys for 30 consecutive days. The effects of buprenorphine were dose-dependent. The suppression of cocaine self-administration by buprenorphine did not reflect a generalized suppression of behavior. These data suggest that buprenorphine would be a useful pharmacotherapy for treatment of cocaine abuse. Because buprenorphine is a safe and effective pharmacotherapy for heroin dependence, buprenorphine treatment may also attenuate dual abuse of cocaine and heroin.

COCAINE ABUSE IS WIDESPREAD IN the general population (*1*) and has also increased among heroin-dependent persons, including those in methadone maintenance treatment programs (*2*). The many adverse medical consequences of cocaine abuse (*3*) are augmented by the combined use of cocaine and heroin (*4*). For example, dual addiction to intravenous

Alcohol and Drug Abuse Research Center, Harvard Medical School–McLean Hospital, Belmont, MA 02178.