Effect of Cholera Enterotoxin on Ion Transport across Isolated Ileal Mucosa

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ABSTRACT The effects of cholera enterotoxin on intestinal ion transport were examined in vitro. Addition of dialyzed filtrate of *Vibrio cholerae* (crude toxin) to the luminal side of isolated rabbit ileal mucosa caused a delayed and gradually progressive increase in transmural electric potential difference (PD) and short-circuit current (SCC). A similar pattern was observed upon addition of a highly purified preparation of cholera toxin, although the changes in PD and SCC were smaller. Na and Cl fluxes across the short-circuited mucosa were determined with radioisotopes 3–4 hr after addition of crude toxin or at a comparable time in control tissues. The toxin caused a net secretory flux of Cl and reduced to zero the net absorptive flux of Na. Similar flux changes were observed when either crude or purified toxin was added in vivo and tissues were mounted in vitro 3–4 hr later. Addition of D-glucose to the luminal side of toxin-treated mucosa produced a large net absorptive flux of Na without altering the net Cl and residual ion fluxes.

Adenosine 3′,5′-cyclic phosphate (cyclic AMP) and theophylline had previously been shown to cause a rapid increase in SCC and ion flux changes similar to those induced by cholera toxin. Pretreatment of ileal mucosa with either crude or purified cholera toxin greatly reduced the SCC response to theophylline and dibutyryl cyclic AMP, which, together with the flux data, suggest that both cyclic AMP and cholera toxin stimulate active secretion by a common pathway. Inhibition of the SCC response to theophylline was observed after luminal but not after serosal addition of toxin. In vitro effects of cholera toxin correlated closely with in vivo effects: heating toxin destroyed both; two *V. cholerae* filtrates which were inactive in vivo proved also to be inactive in vitro; PD and volume flow measurements in isolated, in vivo ileal loops of rabbit revealed that the PD pattern after addition of toxin is similar to that seen in vitro and also correlates closely with changes in fluid movement. The results suggest that stimulation by cholera toxin of a cyclic AMP-dependent active secretory process of the intestinal epithelial cells is a major cause of fluid loss in cholera.

INTRODUCTION

Gastrointestinal fluid loss in human and experimental cholera arises throughout the small intestine (1–3), occurring by way of an epithelium which remains intact by histologic criteria (4, 5) and also by functional criteria in that permeability to macromolecules is not increased (6) and active absorptive processes for nonelectrolytes are not impaired (2, 7–9). The effector of this intestinal secretion is a *Vibrio cholerae* exotoxin (cholera toxin) which has recently been isolated in apparently pure form (10, 11). It is a protein with a molecular weight of about 90,000 (12). The driving force for cholera toxin–induced intestinal secretion could be an active transport process of the epithelium or a hydrostatic pressure difference from interstitium to lumen. Against the latter possibility is the failure to influence the rate of intestinal fluid loss by large alterations in

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both pressures and flows of the perfusing vasculature (13). Consistent with the former possibility is the in vitro demonstration that the epithelium of the small intestine can actively secrete Cl. This has been shown to occur upon addition of adenosine 3',5'-cyclic phosphate (cyclic AMP[1]) or theophylline to isolated rabbit ileal mucosa (14, 15) and in several other circumstances (16–19).

Despite the failure of earlier studies with frog skin (20), rabbit ileum (21), and human ileum (22) to demonstrate an in vitro effect of cholera toxin, we considered it worthwhile to attempt once again to establish such an effect by using the same in vitro preparation on which the effects of cyclic AMP and theophylline had already been demonstrated. Our observations suggest that cholera toxin stimulates the same secretory process in the epithelium of the small intestine as does cyclic AMP. These in vitro effects of cholera toxin are consistent in most respects with effects of the toxin on water and ion transport in the iluem of the intact animal, suggesting that the active secretory process demonstrated in vitro is largely responsible for intestinal fluid loss in cholera.

The majority of results reported here were obtained with a relatively crude preparation of cholera toxin. When a purified preparation subsequently became available, this was also tested and found to confirm in all major respects the earlier observations.

METHODS

In vitro studies. Segments of distal ileum were obtained from New Zealand white, male rabbits that weighed between 2 and 3.5 kg and were fed a standard rabbit chow ad lib.

For in vitro addition of cholera toxin, animals were either directly killed by a blow to the neck or first anesthetized with intravenous pentobarbital and then, after a segment of ileum had been removed, killed with additional pentobarbital.

For in vivo addition of cholera toxin and subsequent mounting in vitro, a 10 cm loop of distal ileum was isolated under local anesthesia (lidocaine), rinsed clean of luminal contents, and cannulated at both ends. The intestines proximal and distal to the loop were closed with ligatures. The abdominal wall was then closed, the proximal and distal cannulae being brought to the outside through the wound. The loop was injected with 1–3 ml of either toxin-containing or control solution, which was initially removed and reinjected a few times to insure contact with the entire mucosal surface. Cannulae were kept open to avoid loop distension. After 3–5 hr exposure to a toxin-containing or control solution, the loop was excised.

Once excised, the ileum was stripped of muscularis and mounted in chambers as described previously (23). Usually, three to six tissues from the same animal were mounted simultaneously. In most experiments, tissues were bathed in an HCO_3-Ringer solution (pH 7.4) which was gassed with 5% CO_2 in O_2 and contained the following ions in mmoles/liter: Na, 141; K, 10; Ca, 1.25; Mg, 1.1; Cl, 127; HCO_3, 25; H_2PO_4, 0.3; and HPO_4, 1.65. In several experiments, a HCO_3-free Ringer solution (pH 7.4) was used (HCO_3 replaced by Cl) which was gassed with pure O_2. In all experiments, D-glucose 7.5–10 mmoles/liter) was added to the serosal side and an equimolar amount of mannitol was added to the luminal side.

Transmural electric potential difference (PD), short-circuit current (SCC) and DC electric resistance were determined as described previously (23). A positive PD indicates the serosal potential to be higher than the luminal potential. The sign of the SCC corresponds to the sign of the PD that was nullified.

The methods for measuring fluxes of ^{22}Na, ^{24}Na, and ^{36}Cl and the calculation of unidirectional and net Na and Cl fluxes were those described previously (23). Net fluxes from mucosa (M) to serosa (S) are recorded as positive. The exact procedure for Na and Cl flux determinations differed for in vivo and in vitro additions of toxin. Na and Cl fluxes across tissues that had been exposed to cholera toxin in vivo or across suitable control tissues were determined with ^{22}Na and ^{36}Cl. Both radioisotopes were added to the luminal side of one tissue and the serosal side of another about 30 min after mounting in vitro. After allowing 20 min to reach steady state, the unlabeled sides were sampled at 10-min intervals. Four or five successive 10-min flux determinations were then averaged.

For in vitro addition of toxin, three tissues were mounted from each animal; cholera toxin was added to two and the third served as a control. 3 hr later, ^{22}Na and ^{36}Cl were added to the luminal side of one toxin-treated tissue and to the serosal side of the other. ^{24}Na was added to the remaining sides. ^{36}Cl was not added to the control tissue. Fluxes were calculated from initial samples taken 15–20 min after addition of radioisotopes and final samples taken 30 to 45 min later. Since only Na fluxes were determined on the control tissues (paired controls), control Cl fluxes were determined on tissues from a separate group of animals (unpaired controls), which had the same mean SCC as the group of paired controls had.[2]

In vivo studies. Under local anesthesia (lidocaine), a 14 cm loop of terminal ileum was isolated, rinsed clean of luminal contents, and cannulated at both ends. The open ends of the proximal and distal intestine were ligated. The proximal end of the cannulated loop was anchored to the anterior abdominal wall and both cannulae were brought out of the abdomen through stab wounds away from the main

[1] *Abbreviations used in this paper:* cyclic AMP, adenosine 3',5'-cyclic phosphate; M, mucosa; PD, potential difference; PEG, polyethylene glycol; S, serosa; SCC, short-circuit current.

[2] Control Cl fluxes were actually measured on tissues from 27 animals. The mean SCC for all 27 animals (3.33 ±0.16 μEq/hr cm²) was greater, however, than the mean SCC (2.75 ±0.30 μEq/hr cm²) for the paired controls. In order to select a smaller group of unpaired controls that matched the paired controls with respect to SCC, the individual unpaired control experiment with the highest SCC was rejected repeatedly until the mean SCC for the remaining unpaired controls was as close as possible (2.76 ±0.12 μEq/hr cm²) to the mean value for the paired controls. This was done so that a single value could be calculated for the control residual ion flux (SCC − net Na flux + net Cl flux). The selection procedure does not affect the conclusion that cholera toxin stimulates active Cl secretion: the mean net Cl flux for all 27 controls (− 0.53 ±0.29) μEq/hr cm²) is less negative than the net Cl flux in the presence of cholera toxin ($P < 0.001$).

incision. Care was taken to avoid kinking or twisting of the loop.

For PD measurements, one Ringer-agar bridge (PE-90 tubing) was introduced through the distal end of the loop and brought out of the abdomen along with the distal cannula; the other bridge was placed on the serosal surface so that the tip was in contact with the serosa at a point opposite to that where the tip of the mucosal bridge was located; the serosal bridge was held in position by plicating serosa over it and was brought out of the abdomen along with the proximal cannula. The bridges were connected to reference electrodes and PD measurements were made as described previously (23). A continuous recording of PD was obtained.

For determination of volume fluxes, the loop was perfused with HCO_3-Ringer solution (for ionic composition, see above) which contained 1% polyethylene glycol (PEG) and which had been equilibrated with 5% CO_2 in O_2. The solution was infused from a syringe pump (Harvard Apparatus Co., Inc., Millis, Mass.) at a rate of 15 ml/hr and was maintained at 38°C by being passed through water-jacketed tubing. About 45 min after completing the surgery, the perfusion was started and 45 min thereafter, the first of a series of successive 30-min collections of effluent from the loop was obtained. After obtaining one to three initial 30-min samples, cholera toxin–containing solution was perfused for 30 min and replaced with toxin-free solution, which was perfused for another 2.5–4 hr. Volume flux was calculated as follows:

$$\text{volume flux} = R \cdot (PEG_o/PEG_f - 1)$$

where R is the infusion rate (15 ml/hr) and the subscripts o and f refer to concentrations of PEG in the infusion solution and the 30 min effluent, respectively. A negative flux indicates absorption. PEG concentration was determined by the method of Hyden (24).

Statistical calculations. Probabilities were determined by Students' *t* test for either paired or unpaired variates. Mean values are generally given as the mean ± standard error of the mean (SE).

Materials. Crude cholera toxin[3] is a lyophilized filtrate of *V. cholerae,* Ogawa strain B 1307. In most instances the crude toxin was dialyzed against HCO_3-Ringer solution for 4–24 hr before use. The amount of dialyzed filtrate used is expressed as the weight of the undialyzed filtrate to which it corresponds.

Purified cholera toxin[4] is a chromatographically isolated product from the filtrate of *V. cholerae,* Inaba strain 569 B (10). It is a single protein as determined by immunoelectrophoretic, disc electrophoretic, ultracentrifugal, and immunological criteria. *Heat-inactivated cholera toxin* is a solution containing the crude or purified toxin which had been placed in a boiling water bath for 15 min. *Preparation V-B12* was a filtrate of *V. cholerae,* Inaba strain 569 B grown for 12 hr in 0.1% sucrose, syncase medium at 37°C with low surface-to-volume ratio and no shaking. It was dialyzed under vacuum. This material had no activity in the rabbit ileal loop assay as evidenced by the failure of a 5 cm closed loop to become distended with fluid over 8 hr following introduction of 20 mg of the preparation. Preparation 02068 was a dialyzed filtrate of *V. cholerae,* strain 02068, an organism, which, when grown in vitro conditions identical with those used for producing active toxin from Inaba 569 B (syncase medium, shaken with high surface to volume ratio), produced filtrates with no rabbit ileal loop activity. ^{22}Na and ^{36}Cl were obtained from the New England Nuclear Corporation, Boston, Mass. ^{24}Na was obtained from the Cambridge Nuclear Corporation, Cambridge, Mass. Dibutyryl cyclic 3',5'-AMP (N^6-2'0-dibutyryl-adenosine, 3',5'-monophosphate, cyclic) was obtained as the monosodium salt from Boehringer Mannheim Corp., New York.

RESULTS

Effect of cholera toxin on SCC. The changes in SCC produced by addition of either dialyzed crude or purified cholera toxin to the luminal side of the ileal mucosa are shown in Fig. 1. After addition of dialyzed crude toxin, the SCC gradually increased until it reached a peak in about 2.5 hr (Fig. 1 A). The major part of this increase began about 40 min after addition of toxin. A delayed increase in SCC was also observed after the addition of purified toxin, but a plateau was reached earlier and the increment in SCC was of smaller magnitude (Fig. 1 B).[5] Lower concentrations of purified toxin did not result in a significant increment in SCC: increments in SCC after addition of 0.01 and 0.10 μg/ml were 8 ±11 and 5 ±6 μa/cm² respectively (five experiments at each concentration).

Tissues exposed to either crude or purified cholera toxin for 3–4 hr in vivo and subsequently mounted in vitro displayed a SCC only slightly higher than control (Table I).

Effect of cholera toxin on electrical resistance. The electrical resistances of tissues exposed to either crude or purified toxin in vivo and subsequently mounted in vitro were higher than the resistances of comparable controls (Table II). In contrast, the electrical resistance of tissues to which toxin had been added in vitro did not become significantly higher than that of control. A relative increase in resistance due to cholera toxin was apparent, however, when the changes in resistance that occurred between the time toxin was added in vitro and 3 or 4 hr later were compared to the resistance changes over the same time period in paired controls.

Effect of cholera toxin on ion fluxes in vitro. Na and Cl fluxes across the short-circuited mucosa were measured 3–4 hr after addition in vitro of dialyzed crude toxin and were compared to fluxes measured during the same time period in control tissues. Heat-inactivated toxin was added to most controls (see legend to Table I). As indicated in Table I, cholera toxin induced a net Cl flux from S to M and reduced to zero the net Na

[3] Wyeth crude cholera exotoxin, lot No. 001, supplied by Dr. John Seal, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

[4] Supplied by Richard A. Finkelstein, University of Texas, Southwestern Medical School, Dallas, Tex.

[5] This difference cannot be attributed to a variation in the responsiveness of the animals used. In two experiments both preparations were added to tissues from the same animal. In both cases the SCC response to crude toxin was substantially greater.

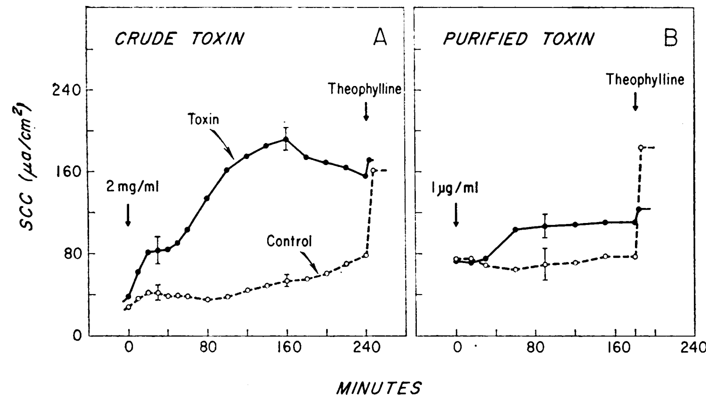

FIGURE 1 Effects of crude and purified cholera toxin preparations on SCC and SCC response to theophylline. Control tissues taken from same animals as toxin-treated tissues. Cholera toxin added to luminal bathing solutions at time 0 and theophylline, 10 mmoles/liter, added to serosal bathing solution 3 or 4 hr later. Brackets represent ±1 SE. 1A: Dialyzed crude toxin, 2 mg/ml added. Heat-inactivated toxin added to all but one control. Each point is the average of values for eight animals. 1B: Purified toxin, 1 μg/ml added. Each point is the average of values for five animals. The bracketed values are significantly different ($P < 0.01$).

flux from M to S. The residual ion flux (SCC − net Na flux + net Cl flux) did not change significantly.

Na, Cl, and residual ion fluxes across tissues exposed to toxin in vivo and subsequently mounted in vitro were similar to those measured across tissues exposed to toxin only in vitro (Table I). Fluxes were the same, furthermore, whether the in vivo exposure had been to crude or purified toxin.

TABLE I

Effects of Cholera Toxin on Ion Fluxes In Vitro

	Na			Cl				
	M → S	S → M	Net	M → S	S → M	Net	SCC	Residual*
			A. In vitro addition					
Crude toxin‡ (11)	10.28 ±0.45	10.26 ±0.44	0.01 ±0.26	6.15 ±0.19	8.74 ±0.22	−2.59 ±0.29	5.47 ±0.51	2.87 ±0.37
Controls§ (11, 16)	11.72 ±0.72	10.69 ±0.66	1.04 ±0.37	7.45 ±0.35	7.19 ±0.35	0.26 ±0.28	2.75 ±0.30‖	1.97 ±0.45¶
P	>0.05	>0.5	<0.05	<0.01	<0.01	<0.001	<0.001	>0.1¶
			B. In vivo addition					
Crude toxin‡ (12)	9.25 ±0.72	9.98 ±0.91	−0.73 ±0.58	5.69 ±0.43	7.96 ±0.56	−2.27 ±0.42	3.32 ±0.22	1.78 ±0.98
Purified toxin** (7)	11.47 ±1.08	11.55 ±0.57	−0.07 ±0.79	6.90 ±1.22	8.70 ±1.06	−1.81 ±0.19	3.34 ±0.22	1.61 ±0.81
Controls (13)	12.72 ±0.74	11.32 ±0.43	1.39 ±0.49	9.48 ±1.03	8.44 ±0.44	1.03 ±0.96	2.65 ±0.25	2.29 ±0.83
P‡‡	<0.02	>0.2	<0.02	<0.01	>0.5	<0.001	<0.05	>0.5
Crude toxin + glucose§§ (6)	18.29 ±1.93	15.35 ±1.39	2.95 ±1.00	6.76 ±0.90	8.67 ±1.28	−1.92 ±0.87	5.82 ±0.39	0.95 ±0.74
P‖ ‖	<0.001	<0.01	<0.01	>0.5	>0.5	>0.5	<0.001	>0.5

Values are mean fluxes (μEq/hr cm²) ±1 SE. Number of animals in parentheses.
* SCC − net Na flux + net Cl flux.
‡ Dialyzed crude toxin, 2 mg/ml luminal bathing solution added in vitro and 2–10 mg/10 cm loop added in vivo.
§ Heat-inactivated toxin added to 8 of 11 controls for Na flux determinations (paired controls) and 12 of 16 controls for Cl flux determinations (unpaired controls). Results were the same for controls to which heat-inactivated toxin was not added.
‖ Based on 11 paired controls.
¶ SE and *P* value based on pooled variance.
** 10 μg/10 cm loop.
‡‡ Results for crude and purified toxin additions pooled and compared to controls.
§§ D-glucose, 7.5–20 mmoles/liter added to luminal side in vitro after preincubation in vivo with dialyzed crude toxin.
‖ ‖ Comparison of crude toxin results with and without glucose.

Table II

Effect of Cholera Toxin on Electrical Resistance In Vitro

	R* ohms·cm²	ΔR‡ ohms·cm²
A. In vitro addition		
Crude toxin§ (9)	41 ±2	+4.6 ±1.1
Paired controls	40 ±2	+0.2 ±1.6
	$P > 0.5$	$P < 0.002$
Purified toxin§ (5)	48 ±5	+6.9 ±4.1
Paired controls	39 ±2	−4.7 ±2.6
	$P > 0.2$	$P < 0.05$
B. In vivo addition		
Crude toxin§ (12)	58 ±3	—
Purified toxin§ (7)	50 ±2	—
Controls (13)	41 ±2	—
	$P < 0.001$‖	

Values are means ± 1 SE. Number of animals in parentheses.
* Electrical resistance measured approximately 3.5 hr after addition of toxin.
‡ Change in resistance from time toxin added to 3.5 hr later.
§ See legend of Table I for toxin concentrations.
‖ Results for crude and purified toxin additions pooled and compared to controls.

Also shown in Table I are ion fluxes across tissues from six animals which had been exposed to crude toxin in vivo and then to 7.5 or 20 mM glucose in vitro. Glucose increased the net Na flux by approximately 3 μEq/hr cm² but did not significantly alter the net Cl and residual ion fluxes.

Effect of cholera toxin on the SCC response to theophylline and dibutyryl cyclic AMP. The stimulation of active Cl secretion by theophylline and cyclic AMP is associated with a rapid increase in SCC that reaches a peak within a few minutes of their addition to the serosal side of the stripped ileum (14, 15). The dibutyryl analogue of cyclic AMP produces a similar rise in SCC (15). The relation of these ion transport changes to those produced by cholera toxin was examined by determining the SCC responses to theophylline and dibutyryl cyclic AMP when these compounds were added 3 to 6 hr after cholera toxin. Results are shown in Fig. 1 and Table III. Both crude and purified toxin preparations, whether added in vivo or in vitro, markedly reduced the SCC responses to theophylline and dibutyryl cyclic AMP. An effect of purified toxin was probably already present at a concentration of 0.01 μg/ml ($P < 0.1$).

Two *V. cholerae* filtrates known to be inactive in vivo (for details see under *Methods*) failed, when added in

Table III

Effect of Cholera Toxin on SCC Response to Theophylline and Dibutyryl Cyclic AMP

	Per cent increase in SCC relative to control (control increment* = 100%)	
	+ theophylline (10 mM)	+ dibutyryl cyclic AMP (0.5 mM)
A. In vitro addition of cholera toxin‡		
Purified toxin, 0.01 μg/ml	61 ±18 (5)	—
" " , 0.10 μg/ml	26 ±7 (5)	—
" " , 1.00 μg/ml	14 ±6 (6)	26 ±8 (4)
Crude toxin, 2–10 mg/ml	22 ±3 (9)	33 ±11 (4)
Preparation 02068,§ 2–10 mg/ml	98 ±8 (9)	—
" V-B12,§ 2–10 mg/ml	79 ±10 (9)	—
B. In vivo addition of cholera toxin‖		
Crude toxin	3 ±1 (4)	—
Purified toxin	6 ±1 (6)	—

Values are means ± 1 SE. Number of experiments in parentheses.
Experiments done with HCO_3-containing (5) and HCO_3-free (4) Ringer were pooled.
* Mean increments of SCC among control groups varied from 58 to 135 μa/cm².
‡ Theophylline or dibutyryl cyclic AMP added to serosal side 3 to 4 hr after addition of cholera toxin or control preparations to luminal side.
§ *V. cholerae* filtrates found to be inactive in vivo. See under Methods for details.
‖ Exposed to cholera toxin for 3 to 4 hr in vivo. Theophylline or dibutyryl cyclic AMP added to serosal side 2 hr after mounting in vitro. SCC increments related to those obtained for five unpaired controls.

vitro, to reduce significantly the SCC response to theophylline added 3 hr later[6] (Table III).

Serosal addition of cholera toxin. As shown in Table IV, the peak SCC attained and the increment in SCC produced by theophylline were the same in tissues to which crude toxin (10 mg/ml) had been added on the serosal side as in tissues to which toxin had not been added. Cholera toxin appears to be effective, therefore, only when added on the luminal side.

Effect of cholera toxin on transmural PD and volume flux in vivo. The pattern of PD change in vivo after 30 min perfusion with dialyzed crude toxin (2–10 mg/

[6] The peak SCC attained after addition of these filtrates was also less than that attained after addition of equal weights of dialyzed crude cholera toxin. In nine experiments the highest values of SCC attained at any time after addition of filtrates were as follows (amperes per square centimeters ±1 SE): no addition, 97 ±14; after No. 02068, 100 ±17; after No. V-B12, 126 ±16; after cholera toxin, 181 ±17.

ml) was similar to the pattern of SCC change observed in vitro (compare Figs. 1 A and 2). Since electrical resistance changed only slightly in vitro, this similarity also extends to the in vitro PD pattern. Changes in volume flux in vivo paralleled the changes in PD, except for a transient increase in fluid absorption which occurred immediately after the addition of toxin and which was not associated with a PD change. In two additional experiments with heat-inactivated toxin, there was a transient initial increase in fluid absorption but no subsequent development of secretion, indicating that the earlier change must be due to a factor in the crude preparation other than cholera toxin.

TABLE IV
Serosal Addition of Cholera Toxin

Side to which cholera toxin added*	Peak SCC‡	ΔSCC, theophylline§
	$\mu a/cm^2$	$\mu a/cm^2$
Luminal	217 ±21‖	16.5 ±6.7
Serosal	110 ±12	68.5 ±10.3
Neither	109 ±18	64.7 ±18.2

Values are means ± 1 SE for six animals.
* Crude cholera toxin, 10 mg/ml, added to tissue within 30 min of mounting.
‡ Highest SCC at any time after addition of toxin.
§ 10 mmoles/liter added to serosal side 3 to 4 hr after toxin.
‖ Corrected for the increase in SCC which occurred immediately after adding crude toxin.

DISCUSSION

The present study established that cholera enterotoxin induces ion transport alterations in rabbit ileal mucosa which are secretory in nature. The ileal epithelium was found to secrete Cl in the absence of an electrochemical gradient after being exposed to cholera toxin for 3–5 hr. Under the same circumstances, the normally present net absorptive flux of Na was found to disappear. These changes were observed after direct addition of cholera toxin to the in vitro preparation, as well as after exposing ileal mucosa to the toxin in vivo before mounting it in vitro.

The cholera toxin–induced alterations of ion transport that have been observed in vitro correlate well in several respects with toxin-induced ion transport changes observed in ileal loops of intact animals. The in vitro changes proved specific for those *V. cholerae* extracts which are active in vivo. Both crude and highly purified preparations of toxin were active in vitro as they are in vivo (25). Heating the toxin preparations destroyed their in vitro as well as their in vivo activities (see legend to Table I). As indicated in Table III, two *V. cholerae* filtrates which were inactive in vivo proved also to be inactive in vitro.

The delayed and gradual increase in PD observed after addition of dialyzed crude toxin in vitro was also observed when this toxin preparation was added to the isolated ileal loop in vivo. Furthermore, the PD pattern in vivo paralleled the development of fluid secretion, suggesting their relation to be more than incidental. A

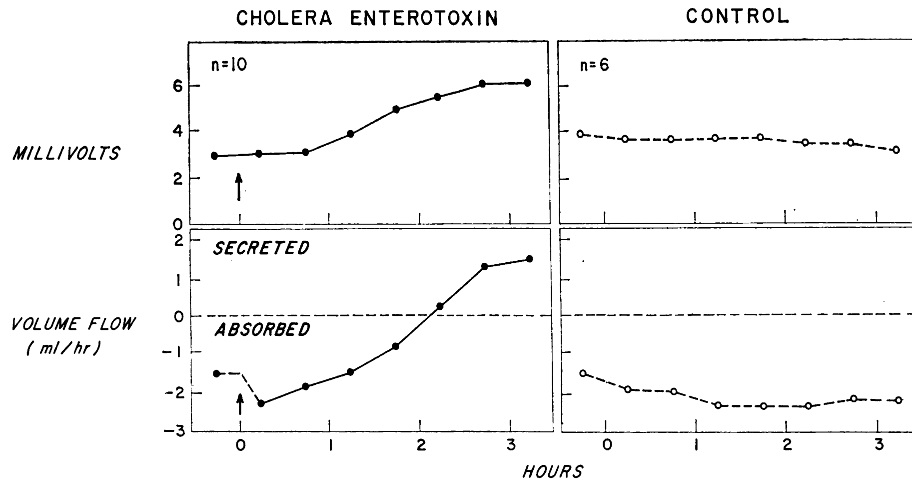

FIGURE 2 Effects of cholera toxin on transmural PD and volume flow in the isolated, in vivo rabbit ileal loop. Dialyzed crude toxin, 2–10 mg/ml, perfused through the lumen for 30 min, beginning at time 0. n = number of animals. In toxin-treated animals, the mean PD increased by 3.1 ±0.8 mv ($P < 0.005$) and the mean volume flux shifted in the direction of secretion by 2.83 ±0.50 ml/hr ($P < 0.001$). In control animals, the mean PD decreased by 0.8 ±0.6 ($P > 0.2$) and the mean volume flux changed in the absorptive direction by 0.74 ±0.37 ($P > 0.1$).

cholera toxin–induced increase in PD in vivo has been reported also for canine ileum (26) and for rabbit jejunum (27).

The changes in Na and Cl fluxes produced by cholera toxin in vitro are consistent with changes observed in vivo. Cl secretion against an electrochemical gradient has been demonstrated in the isolated, perfused ileal loop of the intact dog after addition of cholera toxin (26). In contrast, the Na secretion that developed in those circumstances could be accounted for by passive ion movements.

The responses of in vitro and in vivo preparations may differ with respect to HCO_3 transport.[7] Although HCO_3 fluxes have not been directly measured in vitro, there is reason to believe that the net HCO_3 flux can be equated with the residual ion flux, at least under control conditions (23). Since the residual ion flux did not increase significantly after cholera toxin (see Table I), the in vitro results suggest that cholera toxin does not have a major effect on the rate of active HCO_3 secretion. In the intact animals, on the other hand, most of the anion secreted into the ileum in response to cholera toxin is HCO_3 and not Cl (3, 26, 28). Although HCO_3 is also secreted by the normal ileum (29–32), its rate of secretion is greatly increased by cholera toxin (1, 3, 26). Speculation about a difference between in vivo and in vitro effects of cholera toxin on active HCO_3 transport is premature, however, until more precise information is obtained about HCO_3 transport in the two circumstances. HCO_3 fluxes need to be directly measured in vitro and the separate contributions of passive and active forces to the net HCO_3 fluxes seen in vivo need to be assessed in a quantitative manner.

The ion flux changes produced in rabbit ileal mucosa by cholera toxin are nearly identical with those that occur after addition of cyclic AMP (15). The close relation of these two agents is emphasized further by the decreased SCC responses of toxin-treated mucosa to theophylline and dibutyryl cyclic AMP. The effects of the latter agents on SCC appear to be competitive with those of cholera toxin. Since these SCC changes are easily measured, they provide a convenient bioassay for cholera toxin and other secretory stimuli. They are not by themselves sufficient evidence for the presence of secretion, however, as agents that poison the secretory process might also block the SCC responses to cyclic AMP and theophylline.

Recent biochemical studies have further clarified the relation of cyclic AMP and cholera toxin. Both crude and purified cholera toxin preparations have been shown to increase cyclic AMP levels of small intestinal mucosa in the rabbit (33) and dog (34) by enhancing the activity of intestinal mucosal adenyl cyclase (33, 35). The effects of cholera toxin on both adenyl cyclase activity and cyclic AMP concentration are delayed in onset and gradually become accumulative.

Stimulation of adenyl cyclase appears to be the principal means, therefore, by which cholera toxin alters intestinal ion transport. The steps by which cholera toxin causes this enzymatic change have not been clarified. The toxin, either directly or indirectly, may cause a delayed stimulation of existing cell membrane cyclase; it may stimulate synthesis of new cyclase; or, once combined with a cell membrane component, it may itself catalyze the conversion of ATP to cyclic AMP in a manner analogous to the way in which diphtheria exotoxin catalyzes the cleavage of nicotinamide adenine dinucleotide to nicotinamide and adenosine diphosphoribose (36).

The precise ion transport mechanisms which are regulated by cyclic AMP and which result in the observed changes in net ion fluxes also have not been clarified. Defining these mechanisms will be difficult because more than one active ion transport process may be operating simultaneously and because large portions of the unidirectional fluxes probably represent flow over an extracellular shunt pathway (37). Thus a change in unidirectional flux cannot automatically be attributed to a change in flux over an active transport pathway. At present it is not clear to what extent the secretory state produced by cyclic AMP results from inhibition of an absorptive process (or processes) and to what extent it results from stimulation of a secretory process (or processes). An exclusive effect on absorption would of course imply that there is a substantial rate of active secretion normally which is unmasked by addition of cyclic AMP, cholera toxin, or related agents. There is no available evidence to suggest that this is the case, but the possibility has not been excluded.

It was shown in the present study that glucose produces a net M-to-S Na flux across cholera toxin-treated ileal mucosa when added on the luminal side. Net Cl and residual ion fluxes were not significantly changed, suggesting that glucose specifically stimulates Na transport and does not decrease the rate of active anion secretion. Similar results have been obtained upon adding L-alanine to the theophylline-treated mucosa (38). These results are also consistent with a number of in vivo observations which indicate that intestinal fluid secretion in cholera can be decreased and sometimes abolished by addition of glucose (2, 7, 8) or glycine (9) to the lumen. The active processes for absorption of sugars and amino acids and the associated enhancement of Na absorption

[7] The terms "HCO_3 transport," "HCO_3 secretion," and "net HCO_3 flux from S to M" are used for convenience and are not meant to imply a precise mechanism of luminal alkalinization. Perhaps hydrogen ion is really transported from M to S.

do not appear to be substantially affected by secretory stimuli such as cyclic AMP and cholera toxin. Since these secretory stimuli have been shown to reduce the net Na flux across the short-circuited mucosa, there must be at least two pathways for Na transport in the ileum that can be varied independently.

Two characteristics of the in vitro interaction of cholera toxin–containing preparations with the ileal mucosa deserve additional comment: the failure of serosal addition of toxin to stimulate secretion and the different SCC responses to crude and purified toxin preparations. The failure of a high concentration of cholera toxin to stimulate any secretion when present only on the serosal side of the stripped ileum suggests that cholera toxin, in order to exert its secretory effect, must initially interact with a receptor present only on the luminal border of the epithelium. However, failure of toxin penetration to the basal surface of the epithelium could also explain the absence of response after serosal addition. This latter possibility remains to be tested but seems unlikely as the thin layer of muscularis mucosae that stands between the epithelium and the serosal bathing solution (23) is probably disrupted in places by the stripping procedure.

The results shown in Fig. 1 suggest a difference in the physiological responses of the ileal mucosa to crude and purified toxin preparations. The in vitro addition of dialyzed crude toxin at a concentration of 2 mg/ml of luminal bathing solution (20 mg in total) produced a substantially larger increment in SCC than did the addition of purified toxin at a concentration of 1 μg/ml. Lower concentrations of purified toxin did not produce a larger increment in SCC. The possibility that still higher concentrations of purified toxin might have produced a larger increment in SCC has not been excluded but it is worth noting that a concentration of 1 μg/ml was sufficient to nearly abolish the subsequent SCC response to both theophylline and dibutyryl cyclic AMP and that the relative amount of crude and purified toxin used in the present study (1 purified to 2000 crude) exceeds their relative potency (1 to 10,000) in the in vivo rabbit ileal loop (25). Since tissues exposed to the two toxin preparations in vivo behaved identically after being mounted in vitro (and bathed in fresh Ringer solution), it appears that the difference in SCC observed after in vitro addition of the two preparations is dependent on the continued presence in the bathing medium of some factor contained only in the crude preparation.

What effect this factor has on solute secretion is unclear. It is important to bear in mind that the relation between change in SCC and change in net solute flux cannot be predicted a priori. Addition of cyclic AMP or cholera toxin to short-circuited ileum decreases the net M-to-S Na flux and produces a net S-to-M Cl flux. These flux changes have opposite effects on SCC. The small magnitude of SCC change after in vitro addition of purified toxin suggests that the Na and Cl flux changes produced by this preparation were nearly equal in magnitude. The higher SCC produced by crude toxin could have resulted from a smaller reduction in net Na flux or a larger secretion of anion. Only in the latter case would the higher SCC be associated with a higher rate of solute secretion. Whatever the correct explanation is, the different SCC responses to in vitro additions of different cholera toxin preparations suggest that cyclic AMP accumulation may not be the only means by which these preparations alter intestinal ion transport. Crude and perhaps even purified toxin preparations may have additional effects which augment or reduce the secretory response to cyclic AMP.

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