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EFFECTS OF CHOLERA TOXIN ON CELLULAR AND PARACELLULAR SODIUM FLUXES IN RABBIT ILEUM

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SUMMARY

The diarrhea observed in patients with cholera is known to be related to secretion of water and electrolytes into the intestinal lumen. However, the exact mechanisms involved in these secretory processes have remained unclear. Although it is clear that purified toxin acts on epithelial cell metabolism, its activity on Na^+ transport across intestinal mucosa is equivocal: reported either to prevent net Na^+ absorption or to cause net secretion of Na^+ from serosa to mucosa. Since total transmural Na^+ fluxes across "leaky" epithelia involve very significant movement via a paracellular shunt pathway, we studied the effects of cholera toxin on the cellular and paracellular pathways of Na^+ movement. Unidirectional Na^+ fluxes were examined as functions of applied potential in control tissues and in tissues from the same animal treated with purified cholera toxin. Treatment of rabbit ileum in vitro with toxin stimulated the cellular component of serosa-to-mucosa Na^+ flux (from 2.41 ± 0.49 μequiv./h per cm^2 under control conditions to 4.71 ± 0.43 μequiv./h per cm^2 after treatment with toxin, $P < 0.01$). The effect of cholera toxin on Na^+ movement through the cells from mucosa to serosa appeared to be insignificant. Finally, a marked decrease in the Na^+ permeability ($P < 0.01$) and no detectable significant changes in transference number for Na^+ of the paracellular shunt pathway were observed following treatment with cholera toxin. These results provide direct evidence for the hypothesis that purified cholera toxin stimulates active sodium secretion but has minimal effect on sodium absorption.

INTRODUCTION

The diarrhea observed in patients with cholera is known to be related to

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secretion of water and electrolytes into the intestinal lumen [1–7]. However, the precise mechanisms involved in these secretory processes have remained unclear. Considerable progress has been made in understanding the phenomena as a result of studies on experimental animals treated with either crude or purified cholera toxin but many questions still remain. Cholera toxin applied to the mucosal surface of the small intestine causes an isotonic secretion of NaCl and $NaHCO_3$ in vivo and secretion of Cl^- with either an inhibition of net Na^+ absorption [8, 9] or causes net Na^+ secretion in vitro [10–13]. Treatment with cholera toxin also causes biochemical changes in the epithelial cells [14–22]. The activity of adenyl cyclase and the level of intracellular cyclic AMP are increased by toxin treatment while the activity of $(Na^+ + K^+)$-ATPase is reported to be unchanged [1–7].

The present studies were undertaken in an effort to develop a better understanding of the effects of cholera toxin on Na^+ movements across rabbit ileum. Since total transmural Na^+ fluxes across "leaky" epithelia involve very significant movement via a paracellular shunt pathway, we were interested in exploring more fully the effects of cholera toxin on the cellular and paracellular pathways of Na^+ movement. The techniques described by Desjeux et al. [21] have therefore been used in effort to explore the relative role of these pathways in the Na^+ secretion observed in the presence of cholera toxin.

METHODS

Exposure of intestine to cholera toxin

All experiments were carried out on in vitro preparations of distal ileum from adult male New Zealand white rabbits. Two techniques were employed for exposure of the intestine to purified cholera toxin*. In the first method used most frequently, rabbits were anesthetized with sodium pentobarbital, the abdomen was opened and two loops of distal ileum 10–15 cm in length were prepared. 5 ml of Ringer solutions were placed in the control loop and a similar volume of Ringer containing 5 μg of cholera toxin were placed in the test segment. The loops were tied off and replaced in the abdominal cavity for 1 h. After that time the animal was sacrificed by injection of pentobarbital. The loops of intestine were removed and prepared for in vitro experiments as described below. In a few experiments, rabbits were killed immediately by intravenous injection of pentobarbital. The distal ileum was removed and segments mounted in chambers for measurement of transmural flux. Cholera toxin (2 μg/ml) was added to the solution, bathing both sides of the tissue and after 1 h, experiment was carried out as described below. Identical results were obtained with the two methods of exposure to cholera toxin and the results have therefore been pooled for final analysis.

Measurement of fluxes

(a) *Transmural fluxes.* Transmural sodium fluxes and electrical parameters (short-circuit current, I_{sc}; electrical potential difference, PD; conductance, G) were

* The cholera enterotoxin used in these experiments was the highly purified preparation provided by Dr. Richard Finkelstein. The toxin was prepared as described by Finkelstein and Lospalleto [3] and is referred to as cholera toxin.

determined in Ussing-type chambers [23] as described previously [10]. Briefly, 2–8 pieces of stripped intestine from the same rabbit were mounted as flat sheets between two lucite half-chambers (aperture 1.13 cm^2) equipped to measure PD and I_{sc}. The mucosal and serosal sides of the epithelium were bathed with identical solution circulated, oxygenated and maintained at 37 °C. Ringer's solution contained, in mmol per l, 140 Na^+, 5.2 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 120 Cl^-, 25 HCO_3^-, 2.4 HPO_4^{2-} and 0.4 $H_2PO_4^-$. This solution was used in all the experiments, without glucose in experiment 1 and with 10 mM glucose in experiments 2 and 3. Unidirectional Na^+ fluxes were measured by means of tracer counted by liquid scintillation at constant efficiency. In experiment 3, the unidirectional flux of Na^+ from serosa to mucosa was determined with ^{22}Na as a tracer. The net flux of Na^+, J_{net}^{Na}, across the ileum was calculated as the difference between the unidirectional transmural fluxes from mucosa to serosa, J_{ms}^{Na}, and from serosa to mucosa, J_{sm}^{Na};

$$J_{net}^{Na} = J_{ms}^{Na} - J_{sm}^{Na} \tag{1}$$

Each unidirectional Na^+ flux has been studied as a function of applied PD at equal Na^+ concentration on both sides of the tissue, as described previously [21]. Indeed, small changes in the external driving force for Na^+ have been used successfully to evaluate two pathways involved in each unidirectional flux: a diffusional flux, J_d^{Na}, which is thought to enter a paracellular pathway and a flux independent of small PD variations, J_c^{Na}, which is thought to enter a transcellular pathways, thus, each transmural unidirectional flux of Na^+ is expressed as

$$J_{ms}^{Na} = J_{cms}^{Na} + J_{dms}^{Na} \tag{2}$$

and

$$J_{sm}^{Na} = J_{csm}^{Na} + J_{dsm}^{Na} \tag{3}$$

Within the limits of the applied PD in these experiments, +9 to −9 mV, J_c^{Na} is a constant and J_d^{Na} is measured as

$$J_{dms}^{Na} = {}_oJ_{dms}^{Na}\,[\exp\,(F\Psi_{ms}/RT)]^{-\frac{1}{2}} = {}_oJ_{dms}^{Na}\xi^{-\frac{1}{2}} \tag{4}$$

and

$$J_{dsm}^{Na} = {}_oJ_{dsm}^{Na}\,[\exp\,(F\Psi_{ms}/RT)]^{+\frac{1}{2}} = {}_oJ_{dsm}^{Na}\xi^{+\frac{1}{2}} \tag{5}$$

in which ${}_oJ_d^{Na}$ is flux through the shunt pathway under short-circuit conditions, Ψ_{ms} is potential difference across the tissue with the mucosal side taken as reference, and F, R and T have their usual meetings. Thus Eqns. 2 and 3 can be written

$$J_{ms}^{Na} = J_{cms}^{Na} + {}_oJ_{dms}^{Na}\xi^{-\frac{1}{2}} \tag{6}$$

and

$$J_{sm}^{Na} = J_{csm}^{Na} + {}_oJ_{dsm}^{Na}\xi^{+\frac{1}{2}} \tag{7}$$

Thus, in the presence of a PD, the net flux of Na^+ should take the form [21]

$$J_{net}^{Na} = J_{cms}^{Na} - J_{csm}^{Na} - \frac{{}_oJ_d^{Na}F}{RT}\Psi_{ms} \tag{8}$$

The experiments were, therefore, conducted as follows. After a 30 min equili-

bration period under short-circuit condition, samples for fluxes determinations were taken for 100 min. The transmural PD was clamped at 0 mV from 30 to 50 min, at either +9 or −9 mV from 50 to 80 min, at the opposite polarity from 80 to 110 min, and, finally, at 0 mV from 110 to 130 min. The flux determinations, under each conditions, were averaged to give a single value for flux in that part of control or cholera toxin intestine (2–4 pieces of tissue) at a particular PD. The mean flux values for each PD were then fitted by a straight line according to Eqns. 6 and 7 by the method of least squares to provide values of J_c^{Na} and ${}_oJ_d^{Na}$.

(b) *Fluxes across the brush border.* Transmural fluxes and influx from mucosal solution to tissue were determined on adjacent pieces of tissue of four rabbits. Cholera toxin was first placed, in vivo, in intestinal loops. After one hour, one part of control or cholera toxin intestine was stripped of its muscular layers and mounted in Ussing chambers to determine transmural fluxes (experiments 1). The other part was mounted with the muscular layers intact in an influx chamber, as described previously [24]. After a 30 min preincubation period in Ringer, the unidirectional Na^+ influx from mucosal solution to tissue during 1 min was measured, [^{3}H]inulin was added to correct for extracellular space. No attempt was made to control the transmural PD in the influx chambers. However, the spontaneous PD of the tissue mounted in identical solution in Ussing chambers was determined after 30 min (i.e. at the time of the influx measurement).

Statistical analysis

The means and S.E. of the fluxes thus obtained from each part of intestine (i.e. control or cholera toxin) are reported with n, equal to the number of pieces of tissue. The differences observed between the means of the groups are compared by Student's t-test.

RESULTS

Effect of cholera toxin on net Na^+ transport

The effects of cholera toxin on Na^+ fluxes across the ileum are shown in Table I for experiments carried out in the presence and in the absence of glucose. Only those experiments in which both unidirectional Na^+ fluxes were determined simultaneously on the same piece of tissue are included. In control tissues without glucose, there was no significant net Na^+ flux under short-circuit conditions but treatment with cholera toxin caused a significant ($p < 0.01$) Na^+ secretion of -1.3 ± 0.2 μequiv./h per cm^2. Both unidirectional Na^+ fluxes were reduced by cholera toxin but only the change in the mucosal-to-serosal flux was statistically significant. The short-ciruit current was slightly but not significantly higher in control tissues than in those treated with cholera toxin. In experiments carried out with 10 mM glucose in the bathing solutions, there was a significant net Na^+ absorption under control conditions which was reduced by treatment with cholera toxin to a value not significantly different from zero (-0.2 ± 0.4 μequiv./h per cm^2). Thus in both the presence and the absence of glucose, cholera toxin caused a decrease in J_{net}^{Na} of a similar magnitude under short-circuit conditions and these results are similar to those reported previously [8, 9, 11–13]. However, in the presence of glucose, mucosal-to-serosal Na^+ flux appears to be decreased and serosal-to-mucosal increased by cholera toxin although neither change

TABLE I

EFFECT OF CHOLERA TOXIN ON Na^+ FLUXES ACROSS ILEUM

The two unidirectional Na^+ fluxes, J^{Na}_{ms} and J^{Na}_{sm}, the net Na^+ flux, $J^{Na}_{net} = J^{Na}_{ms} - J^{Na}_{sm}$, and short circuit current, I_{sc}, were determined simultaneously on the same pieces of ileum treated or not treated with cholera toxin in the presence and in the absence of glucose. Numbers in parentheses give number of tissues. The means and S. E.of cholera toxin-treated tissues are compared to the means and and S.E. of control tissues.

	J^{Na}_{ms}	J^{Na}_{sm}	J^{Na}_{net}	I_{sc}
No glucose				
Control (18)	8.0±0.5	7.7±0.6	0.3±0.4	1.9±0.2
Toxin (19)	5.3±0.4*	6.5±0.4	−1.3±0.2*	1.4±0.2
Glucose				
Control (12)	10.5±0.6	8.5±0.6	2.0±0.1	2.4±0.2
Toxin (15)	9.3±0.6	9.5±0.8	−0.2±0.4**	3.1±0.3

The results are expressed as μequiv./h per cm^2. Significantly different from control: * $P < 0.001$; ** $P < 0.05$.

was statistically significant. Short-circuit current was slightly but not significantly higher in tissues treated with cholera toxin.

The effects of 1 h in vivo or in vitro treatment with cholera toxin on unidirectional Na^+ fluxes and total tissue conductance are approximately identical to those from 3 h in vitro treatment with cholera toxin. In the absence of glucose, the reduction in J^{Na}_{sm}, J^{Na}_{ms} (Table I) and total tissue conductance (Table II) caused by 1 h in vivo or in vitro treatment with cholera toxin were 34, 16, and 34, % respectively. Under the same conditions after approx. 3 h in vitro treatment with cholera toxin, Powel et al. [11] have observed reductions of 32, 18 and 28 % in J^{Na}_{ms}, J^{Na}_{sm}, and conductance, respectively.

The relationship between net Na^+ flux and imposed PD observed in these

TABLE II

EFFECT OF CHOLERA TOXIN ON COMPONENTS OF Na^+ FLUX

The relationship between unidirectional Na^+ flux, J^{Na}_{ms} and J^{Na}_{sm}, and PD was evaluated using Eqns. 6 and 7 to estimate flux via the cellular, J^{Na}_{cms} and J^{Na}_{csm}, and paracellular, ${}_oJ^{Na}_d$, pathways. ${}_oJ^{Na}_d$ represents the diffusional flux in short-circuit conditions from either direction, mucosa to serosa or serosa to mucosa.

	J^{Na}_{cms} (μequiv./h per cm^2)	J^{Na}_{csm} (μequiv./h per cm^2)	${}_oJ^{Na}_d$ (μequiv./h per cm^2)	G ($m\Omega^{-1} \cdot cm^{-2}$)
No glucose				
Control	2.2±0.7 (18)	2.2±0.7 (18)	5.6±0.6 (28)	17.2±1.0 (22)
Toxin	1.8±0.6 (19)	3.2±0.4 (19)	3.3±0.4 (28)	11.4±0.6 (22)
Glucose				
Control	4.1±0.6 (12)	2.4±0.5 (28)	6.8±0.5 (40)	21.0±0.9 (28)
Toxin	3.5±0.4 (15)	4.7±0.4*(31)	5.0±0.4*(48)	18.1±0.8 (31)

* Significantly different from control, $P < 0.01$.

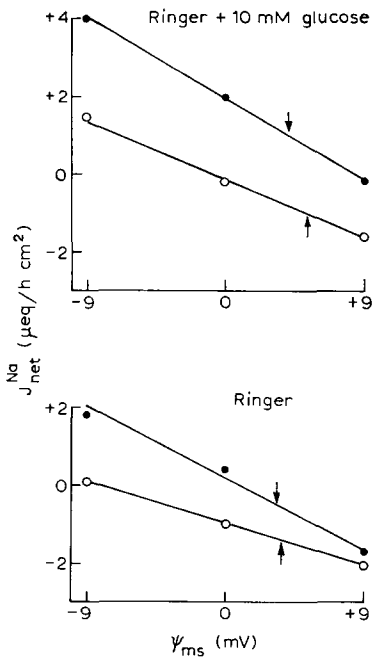

Fig. 1. Relationship between net Na^+ flux, J^{Na}_{net}, and imposed PD, Ψ_{ms}. The J^{Na}_{net} were obtained by the simultaneous determination of both unidirectional Na^+ fluxes on the same piece of tissue. Points are average values from 18 control tissues (●) and 19 cholera toxin-treated tissues (○) in Ringer and 12 control tissues (●) and 15 cholera toxin-treated tissues (○) in the presence of glucose. The arrows indicate presence of glucose. The arrows indicate the open-circuit PD measured during the experiments.

studies is shown in Fig. 1. Under all conditions examined, Na^+ flux is a linear function of PD over the range −9 to +9 mV as described by Eqn. 8. Since the relationship is linear, it is of interest to consider the net Na^+ flux expected under open-circuit conditions in these experiments since this would perhaps be more similar to in vivo condition. The open-circuit PD measured during the experiments is indicated by arrows on Fig. 1. In the presence of glucose a net Na^+ absorption of 1 μequiv./h per cm^2 would be predicted at a PD of +4.0 mV (serosa positive) while in the presence of cholera toxin, a net secretion of approx. −1 μequiv./h per cm^2 at a PD of 5.4 mV is predicted. In the absence of glucose a small net Na^+ secretion of −0.1 μequiv./h per cm^2 would be expected under control conditions and a secretion of −0.1 μequiv./h per cm^2 is predicted after treatment with cholera toxin.

Effect of cholera toxin on components of Na^+ flux

As previously discussed [21, 26] and summarized briefly in Methods, these measurements of Na^+ fluxes as functions of applied PD can also be used to provide estimates of Na^+ movement via operationally defined "cellular" and "paracellular" pathways. For each piece of tissue studied, the relationship between unidirectional Na^+ flux and PD was evaluated to estimated flux via the cellular (J_{csm} or J_{cms}) and paracellular ($_oJ_{dsm}$ or $_oJ_{dms}$) pathways and the results were averaged for each of the

four experimental conditions (control and cholera toxin with and without glucose). Two sets of experiments were performed with glucose present. In one set, both J_{sm} and J_{ms} were determined simultaneously using ^{22}Na and ^{24}Na; in the other only J_{sm} was measured in order to check the conclusion reached on the basis of double labeling experiments without any possible complications involved in measuring both isotopes. Since the results of these single label experiments were identical to those obtained with two isotopes, we have pooled all data on serosal-to-mucosal Na^+ fluxes for presentation. In double labeling experiments, unidirectional fluxes through the shunt pathway under short-circuited conditions ($_oJ_{dms}$ and $_oJ_{dsm}$) were determined simultaneously. Under each conditions studied the average values of these fluxes in the two directions differed by less than 10% as would be expected for fluxes via a passive pathway in the absence of net driving forces (PD = 0 and identical Na^+ concentrations in the mucosal and serosal solutions). For example, in control experiments with glucose $_oJ_{dms} = -6.3 \pm 0.8$ μequiv./h per cm^2 and $_oJ_{dsm} = 6.0 \pm 0.7$ μequiv./h per cm^2. These diffusional fluxes have therefore been averaged for each experimental condition irrespective of the direction of flux measurement.

Results of these evaluations of components of Na^+ fluxes are summarized in Table II. In the presence of glucose, treatment with cholera toxin causes a significant ($p < 0.01$) increase in Na^+ flux from serosa to mucosa via a cellular pathway (J_{csm}) and a small and statistically unsignificant decrease in flux from mucosa to serosa via a cellular pathway (J_{cms}). Cholera toxin also causes a significant ($p < 0.01$) decrease in the permeability of the paracellular pathway to Na^+. Changes in the same direction and of approximately similar magnitude were observed in experiments carried out in the absence of glucose. However, in this series, only the decrease in Na^+ permeability of the paracellular pathway was statistically significant.

Since the change in J_{csm} caused by cholera toxin seems significant and is important in attempts to evaluate the effects of this agent, it is perhaps appropriate to consider the results of these experiments in an alternative manner. As previously discussed [21, 27] fluxes for each tissue can be normalized to the flux in that tissue under short-circuit conditions in order to minimize scatter due to different tissue conductances. These normalized fluxes can then be used to estimate $_oJ_{dsm}$ and J_{csm}. Fig. 2 shows the average value of normalized fluxes as functions of $\xi^{\frac{1}{2}}$ for control and cholera toxin-treated tissues in the presence of glucose. In agreement with the results shown in Table II, cholera toxin causes a significant decrease in slope ($_oJ_{dsm}$) and a significant increase in the intercept of the line on the y-axis (J_{csm}).

The present results indicate, in agreement with our previous study [21], that J_{csm} is not altered by the presence of glucose ($J_{csm} = 2.4$ and 2.2 μequiv./h per cm^2 in the presence and absence of glucose, respectively). Thus it might be appropriate to pool the results of all measurements of J_{csm}. If this is done, we find that J_{csm} is 2.41 ± 0.49 μequiv./h per cm^2 under control conditions and 4.71 ± 0.43 μequiv./h per cm^2 after treatment with cholera toxin and that the difference is statistically significant ($p < 0.01$). On the other hand, glucose causes a significant increase in the cellular component of Na^+ flux from mucosa to serosa, J_{cms}, as might be expected on the basis of the fact that actively transported sugars cause an increase in net Na^+ absorption [28] and an increase in Na^+ entry into the cells across the brush border membrane [29]. The increment in J_{cms} caused by 10 mM glucose is essentially the same in tissues treated with cholera toxin as it is in control tissues.

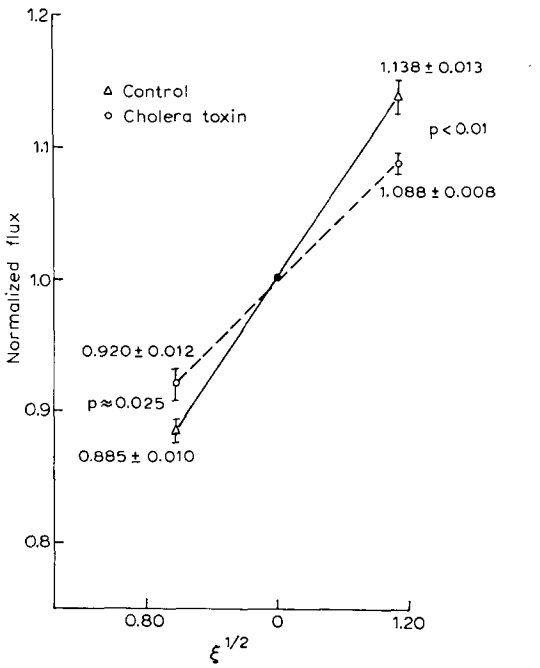

Fig. 2. Relationship between fluxes normalized to flux under short-circuit conditions and $\xi\ddagger$. $\xi\ddagger = \exp(F\Psi_{ms}/RT)$ where Ψ_{ms} is the potential difference; F, R, T are constant. Points are average values (±S.E.) from 28 control tissues and 31 cholera toxin-treated tissues in the presence of glucose.

As indicated in Table II treatment of the tissue with cholera toxin causes a decrease in total tissue conductance and in Na^+ flux via the shunt pathway. In the presence of glucose, the change in ${}_oJ_d^{Na}$ accounts for 62 % of the change in conductance while in the absence of glucose the change in ${}_oJ_d^{Na}$ is only 44 % of the corresponding change in tissue conductance. These results might suggest that the relatively large change in conductance caused by cholera toxin might involve a change in the Na^+ selectivity of the shunt pathway as suggested by Powell [30]. This possibility can be examined more closely by evaluating the relationship between ${}_oJ_d^{Na}$ and tissue conductance. As discussed by Desjeux et al. [21], there is a linear relationship between these two quantities and the slope of the line relating them should provide an estimate of the transference number for Na^+ (t_{Na}) in the shunt pathway. Results obtained in the present control studies with glucose were identical to those obtained earlier [21] so we have pooled the two sets of data to evaluated the relationship between ${}_oJ_d^{Na}$ and conductance, G, under this condition. The least squares line is given by

$${}_oJ_d^{Na} = 0.45G - 1.91$$

and is shown as the solid line in Fig. 3. Experimental points obtained in the presence of cholera toxin are also shown with each point representing the values observed for tissue from a single rabbit (obtained by averaging values for each individual tissue from that animal). Although there is appreciable scatter of the points, they do not depart systematically from the control line and in fact all but one point fall within the

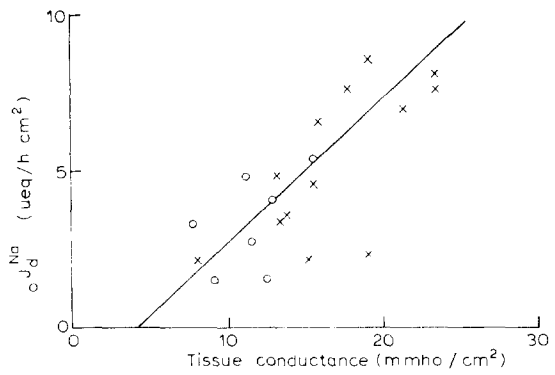

Fig. 3. Relationship between Na^+ fluxes through the shunt pathway under short-circuit condition, ${}_oJ_d^{Na}$, and total tissue conductance. The line relating these two quantities was calculated from data obtained in the present control studies with glucose and in earlier identical experiments [21]. Experimental points obtained in the presence of cholera toxin are shown with each point representing the values for tissue from a single rabbit (obtained by averaging values for each individual tissue from that animal).

95 % confidence limits for the control line. Thus these results suggest that there is not a significant change in the cation selectivity of the shunt pathway following treatment with cholera toxin.

The results summarized in Table II, indicate that cholera toxin has a minimal effect on the flux of Na^+ from mucosal to serosal solution via the cellular pathway. Since one of the main determinants of this flux is the unidirectional flux across the brush border membrane from mucosal solution to cell [3, 1] and since this flux can be measured directly [24, 26, 27, 32] a partial test of the above conclusion is possible. Direct measurements of Na^+ influx across the brush border were carried out on tissues from four rabbits with the results summarized in Table III. There was a small but statistically significant reduction in Na^+ influx following treatment with cholera toxin. However, as shown by Frizzell and Schultz [26], a portion of the measured influx is due to Na^+ movement into the shunt pathway. Since cholera toxin reduces Na^+ movement through the shunt (Table II) we have corrected the influx measurements for this effect in the following manner. Pieces of tissue from each of the four animals were also mounted in transmural flux chambers and ${}_oJ_d^{Na}$ determined

TABLE III

EFFECT OF CHOLERA TOXIN ON Na^+ INFLUX, J_{in}^{Na}, ACROSS BRUSH BORDER

J_d^{Na} was determined on adjacent pieces of tissue mounted in Ussing-type chambers as described above. Influx into the cells was then calculated as the difference between total measured influx, J_{in}^{Na}, and ${}_oJ_d^{Na}$. The results are expressed as μequiv./h per cm^2.

	J_{in}^{Na}	${}_oJ_d^{Na}$	J_{mc}^{Na}
Control (32)	18.8±0.9	6.2±0.5	12.6±1.0
Cholera toxin (31)	15.5±1.1*	3.6±0.7*	11.9±1.0

* Significantly different from control: $P < 0.001$. See Addendum A, page 364.

as described above at approximately the same time as influx measurements were made. As shown in column 2 of Table III, cholera toxin was again found to decrease ${}_oJ_d^{Na}$. Influx into the cells was then calculated as the difference between total measured influx and ${}_oJ_d^{Na}$. As shown in column 3, most of the decrease in influx could be accounted for by reduction in influx into the shunt and there appeared to be no significant reduction in influx into the cells.

This conclusion is based on two assumptions. First, we have assumed that estimates of ${}_oJ_d^{Na}$ from measurements of transmural fluxes are identical to influx into the shunt pathway in influx measurements. This assumption seems reasonable since, under comparable conditions, estimate of shunt permeability to Na^+ by influx measurements [26] and by transmural flux measurements [21] give essentially identical results. Second, we have ignored the effect of a small PD on Na^+ influx into the shunt. We do not know the PD in the influx chambers, but measurements on tissues from these animals in the transmural flux chambers gave average PD values of 2.9 mV in control tissues and 3.2 mV in those treated with cholera toxin. If we assume that the same PD values are present in the influx chambers, the values of ${}_oJ_d^{Na}$ corrected for PD would be 5.9 and 3.4 μequiv./h per cm^2 in control and cholera toxin-treated tissues and the calculated fluxes into the cells would become 12.9 and 12.1 μequiv./h per cm^2, respectively. Thus the above conclusion are not seriously affected by ignoring possible effects of PD.

In the tissues mounted in transmural flux chambers, net fluxes of 0.2 μequiv./h per cm^2 and -1.2 μequiv./h per cm^2 were observed in control and cholera toxin-treated tissues, respectively. If we assume that the tissue is in a steady state so that net flux across the brush border must equal net flux across the whole tissue, we can calculate that Na^+ flux from cell to mucosal solution, J_{cm}, would be 12.4 μequiv./h per cm^2 under control conditions and 13.1 μequiv./h per cm^2 following cholera toxin. Although several assumptions are involved in analysis of these results, they do appear to indicate that treatment of the tissue with cholera toxin does not cause major changes in Na^+ movement across the brush border membrane. It should be noted that a small decrease in influx and a small increase in efflux across the membrane may reverse the J_{net}^{Na} from absorption to secretion; but other approaches will be necessary to establish this clearly.

DISCUSSION

Purified cholera toxin, and compounds such as theophylline which increase intestinal cellular cyclic AMP concentrations, induce the intestinal secretion of water and electrolytes by mechanisms which are not totally understood. Many studies both in vivo and in vitro, have been directed toward an understanding of the mechanisms of transport involved in cholera toxin's action, but the data resulting from those studies have often been conflicting and this has led to differing interpretations. For example, the early studies by Field et al. [8, 33] suggested that cyclic AMP (the intermediate that mimics for the action of both cholera toxin or theophylline) simply inhibited sodium absorption while stimulating anion secretion through a presumably electrogenic process since these changes in transport were accompanied by an increase in short-circuit current. Subsequently, others [11, 25] showed that instead of just inhibiting sodium absorption, cholera toxin and theophylline actually stimulated the

net serosa to mucosa transport, or secretion, of sodium as well as chloride. The difference in these results may be explained by the presence of glucose in the bathing solutions in Field's studies. Glucose may have stimulated sodium transport via the coupled sodium-glucose phenomena [28] thus offsetting part of the secretory effect of cyclic AMP. This effect of glucose on cholera toxin-stimulated sodium transport is again demonstrated in the present studies (Table I). Thus, cholera toxin does appear to cause sodium secretion in glucose-free solutions, but this effect certainly can be modulated by the presence of glucose [34] a fact that is well known clinically [35].

The mechanism of cyclic AMP's effect on sodium transport has been further defined in Schultz's laboratory, where Nellans et al. [32] have shown that theophylline appears to inhibit coupled sodium-chloride influx across the brush border of rabbit ileum. This inhibition of coupled sodium-chloride entry is sufficient to explain a reduction of sodium absorption to zero, but not sodium secretion. With further investigation using techniques similar to those used in the present report, Nellans et al. [27] demonstrated that the entire serosal to mucosal flux of sodium in the presence or absence of theophylline in the in vitro rabbit ileum was entirely passive. That is to say, that none of the serosal to mucosal sodium flux across the epithelium takes place via transcellular route, again as would be expected if sodium transport were reduced and not reversed.

However, investigations in this laboratory [21] using techniques essentially identical to those of Nellans et al. have demonstrated that the sodium flux from the serosal to mucosal solutions involved two pathways. A diffusional flux through a paracellular shunt path and a flux, independent of applied electrical potential, which presumably involved transcellular, active transport of sodium. This latter pathway comprised approx. 20 % of the sodium flux in the serosa to mucosa direction, and was significantly stimulated by theophylline, unaffected by the removal of glucose or the addition of cardiac glycosides, and essentially completely inhibited by anoxia, dinitropherol and replacement of chloride and bicarbonate by the presumably non-transported anion isethionate. This appeared to be additional evidence for the presence of a cyclic AMP-stimulated, electrically silent sodium-anion secretory process in the rabbit ileum similar to that which accounts for the spontaneous electrolyte secretion seen in guinea pig small intestine [10]. The present experiments corroborate these findings with theophylline, in that cholera toxin stimulated the cellular component of serosal to mucosal sodium flux from 2.41 to 4.71 μequiv./h per cm^2 (Figs. 1 and 2 and Table II).

An additional puzzling aspect of cyclic AMP-stimulated intestinal secretion has been the fact that the secretion of sodium and chloride as noted in vitro appears to come about through an inhibition of the mucosal to serosal fluxes of these ions rather than stimulation of serosal to mucosal fluxes [8, 9, 11, 13, 25, 27, 33]. One explanation for such a finding is that cyclic AMP simply inhibits coupled sodium-chloride entry into the mucosal cell [32]. However, Powell et al. [11, 25] have suggested that these changes in unidirectional flux may be due to a reduced passive movement of sodium-chloride through the paracellular shunt as a result of cyclic AMP-induced changes in the resistance of this path, coupled with stimulated serosal to mucosal transcellular active transport of sodium and chloride.This view gains some support in the present experiments where we have measured in the tissues from the same animal, both the mucosal influxes of sodium as well as the transmucosal diffusional movement

of sodium. Since the influx and diffusion measurements were performed with different techniques, one might not be able to combine the data to completely rule out any cyclic AMP-mediated inhibition of sodium-chloride entry, but these data do support the idea that a great percentage of the decrease in mucosa to serosa influx after theophylline or cholera toxin is due to a decrease in the passive sodium flux across the paracellular shunt path.

How might one explain these striking differences in the results and, therefore, in conclusions regarding the mechanism of cyclic AMP effects on sodium transport? As indicated by Nellans et al. [27], there are some significant differences in the experimental conditions used in the two laboratories. Most importantly, Nellans et al. [27] have used a relatively high potassium (12.6 mM) low bicarbonate (10 mM) Ringer solution with a pH of 7.1–7.2, whereas we have utilized a pH 7.4 Ringer solution with a bicarbonate and potassium concentration of 25 and 5.2 mM, respectively. Sheerin and Field [36] have shown that a low pH and bicarbonate concentration the in vitro rabbit ileum had high absorptive rates of both sodium and chloride in the short-circuited state, suggesting the presence of a coupled sodium-chloride influx mechanism. In such low pH solutions theophylline reduced sodium transport to zero. In contrast, in high pH and bicarbonate solutions (pH 7.8 and 50 mM, respectively), the activity of any brush border located electrically neutral sodium-chloride influx mechanism appeared to be minimal, and in fact, there was essentially zero sodium transport and significant chloride secretion. Theophylline in the pH 7.8 solutions caused a significant sodium secretion as well as anion secretion. At physiological pH values and bicarbonate concentrations (pH 7.4 and 25 mM, respectively) transport in the control state seems to be midway between these two extremes. In light of these effects of bicarbonate and pH on ion transport by the in vitro rabbit ileum, the results described by Nellans et al. [27] and those described by ourselves in the previous paper [21], and in the present investigation, are exactly as would be expected. That is to say, at low pH values the coupled sodium-chloride influx mechanism is prominent in the control state, and can be inhibited by cyclic AMP. Sodium secretion is not stimulated and there is no corresponding transcellular movement of sodium from serosa to mucosa. At this pH the serosa to mucosa transport of sodium would be entirely passive and would occur via the paracellular shunt path. However, at more physiologic or higher pH values, the coupled sodium-chloride influx mechanism is less prominent and inhibition of this process by cyclic AMP less easily demonstrated. However, at these pH values theophylline and cholera toxin stimulate the active secretion of both sodium as well as anion. An active secretion of Na^+ requires that this process take place transcellularly, and therefore some component of the serosal to mucosal flux would be via the transcellular route and would be stimulated by theophylline and cholera toxin. This has been the finding in our previous and present investigation. It is entirely possible that at these different pH values the effect of cyclic AMP is still on a single mechanism. That is, at low pH cyclic AMP may simply inhibit coupled sodium-chloride entry into the cell, whereas at higher pH values cyclic AMP may reverse this process and actually cause active secretion across the apical cell border. The limiting factor with regard to which of these effects cyclic AMP might have on a potentially reversible sodium-anion carrier located at the apical cell membrane may well be mediated by a pH and/or bicarbonate-related change in sodium permeability at the serosal cell membrane. Sodium entry across the serosal cell membrane may govern

whether cyclic AMP simply inhibits or actually reverses a brush border-located transport process for sodium and chloride. If this present synopsis is correct, then the differences in the data and the interpretations regarding the mechanism of action of cholera, theophylline and cyclic AMP are more apparent then real.

Our studies also indicate a significant reduction in electrical conductance (34 % reduction) with a corresponding decrease in the mucosa to serosa sodium flux of a magnitude similar to that reported by Powell et al. [25]. Since there is a linear relationship between the total tissue conductance and ${}_oJ_D^{Na}$ in the range studied (Fig. 3), as discussed by Desjeux et al. [21], the slope of the line relating them provides a means of estimating the transference number for sodium (t_{Na}) in the shunt pathway. Significant changes in t_{Na} will reflect changes in the sodium selectivity of the shunt path. Since it has been reported that the relative permeabilities of sodium with respect to chloride, P_{Na}/P_{Cl} in the shunt are decreased following cholera toxin treatment, as determined by dilution potentials in ouabain-treated rabbit ileum [30], we have looked at this relationship (Fig. 3) for additional evidence of this effect. Unfortunately, the regression line through all the cholera toxin points does not yield a degree of correlation with enough significance to allow comparison of the two slopes representing t_{Na} in the shunt of normal and cholera toxin-treated tissues. The fact that the cholera toxin points do not depart systematically from the control line, suggests that there is not a significant change in sodium selectivity in the shunt pathway following treatment of cholera toxin. However, it can be shown that only a small percent decrease (10 %) in the transference number of sodium can result in a relatively larger increase (43 %) in the P_{Cl}/P_{Na} (See Addendum B, page 365).

In summary, in Ringer solution with a pH of 7.4 and a bicarbonate and potassium concentration of 25 and 5.2 mM respectively, a significant portion of the serosa to mucosa unidirectional sodium flux occurs via a high resistance pathway which is presumably transcellular. This component of sodium flux is increased by cholera toxin just as we have previously shown that it is increased by theophylline, another stimulant of cyclic AMP. Our studies suggest that at pH 7.4 cyclic AMP stimulates sodium secretion via an electrically silent transcellular process which requires an increased sodium entry at the serosal cell membrane. In view of the known effects of pH (and/or bicarbonate) on ion transport in the in vitro rabbit ileum [35] our studies are not necessarily contrary to those reported by Nellans et al. [27] which were performed at lower ambient pH values. Stimulation of cyclic AMP levels in the intestinal cell seems to have a dramatic effect on the intestinal conductance perhaps by decreasing the magnitude of the shunt path as discussed previously [21, 25]. There may also be a change in permselectivity of the shunt path with cholera toxin but we are unable to confirm that with our present studies.

ADDENDA

Addendum A. In Table III ${}_oJ_d^{Na}$ represents the flux measured across the entire shunt, i.e. tight junction and lateral space. Since the tight junction (TJ) and the lateral space (LS) are in series, the overall permeability of the shunt (s) should be given by

$$\frac{1}{P_s} = \frac{1}{P_{TJ}} + \frac{1}{P_{LS}}$$

Rearranging the above equation gives

$$\frac{P_s}{P_{TJ}} = 1 - \frac{P_s}{P_{LS}}$$

Two things can be noted: P_{TJ} is always greater than P_s and the closeness between them is measured by P_s/P_{LS}. Estimates of P_{LS} can be made as outlined by Smulders et al. [37] from the relation $P_{LS} = DA/l$ in which D is the diffusion coefficient of Na^+ (taken as the free solution value), A is the area of lateral space per unit serosa area, and l is the length of the space. Assuming the width of the lateral space in control tissues is 170 Å [21], assuming a decrease of 70 % in the width of lateral space in cholera toxin tissues (50 Å), and using the calculations similar to those of Desjeux et al. [21], we find that $P_{LS} = 15.6 \cdot 10^{-5}$ cm/s in control tissues and $4.6 \cdot 10^{-5}$ cm/s in control and cholera toxin-treated tissues, respectively. The ratio of P_s/P_{LS} is 0.074 in control and 0.15 in cholera toxin tissues. Therefore, the transmural P_s is 92 % in control and 85 % in cholera toxin tissues of the P_{TJ}. These calculations indicate that an assumed 70 % decrease in the width of the lateral space caused by cholera toxin results in only 7 % change of deviation of P_s from P_{TJ}. Using P_s to estimate the influx across the tight junction, J_{TJ}^{Na}, yield a value for J_{mc}^{Na} ($= J_{in}^{Na} - J_{TJ}^{Na}$) of 11.2 μequiv./h per cm^2 in control tissues. It appears that taking into account a change of 70 % of the lateral space does not affect significantly the calculation $J_{mc}{}^{Na}$. (DiBona et al. [38] found a decrease in lateral space after cholera toxin treatment but did not give any measurement of the lateral space).

Addendum B. In fact, Powell [30] has reported that the relative permeabilities of Na^+ with respect to Cl^-, P_{Na}/P_{Cl}, in the shunt is decreased following cholera toxin treatment based on measurements of dilution potentials in ouabain-treated rabbit ileum. Since a decrease in t_{Na} causes approximately an equal increase in t_{Cl} assuming the changes in the transference numbers of minor ions are small, the change in t_{Na} will then be relatively much smaller than the change in P_{Na}/P_{Cl}. The ratio of P_{Cl}/P_{Na} can be expressed in terms of the ratio of t_{Cl}/t_{Na} as

$$\frac{P_{Cl}}{P_{Na}} = \frac{[Na]}{[Cl]} \frac{t_{Cl}}{t_{Na}} \simeq 1.22 \frac{t_{Cl}}{t_{Na}}$$

in which [Na] and [Cl] are the Na^+ and Cl^- concentrations in the medium. When P_{Cl}/P_{Na} is 0.40 and 0.57 in the normal and cholera toxin-treated tissues, respectively, as observed by Powell [30], we get $t_{Cl}/t_{Na} = 0.33$ and $t'_{Cl}/t'_{Na} = 0.47$ where t'_{Cl} and t'_{Na} refer to the transference numbers for Cl^- and Na^+ in cholera toxin-treated tissues. Since $\Delta t_{Na} = t'_{Na} - t_{Na} \simeq -\Delta t_{Cl} = t'_{Cl} - t_{Cl}$, we get $\Delta t_{Na} = -0.095\ t_{Na}$. As t_{Na} found in control tissues is 0.45, Eqn. 9, the t'_{Na} in the cholera toxin-treated tissues becomes 0.41. An approximate 10 % decrease in t_{Na} results from a 43 % increase in P_{Cl}/P_{Na} [30] following treatment with cholera toxin. It is, therefore, reasonable to suggest that the Na^+ selectivity of the shunt is not significantly altered by cholera toxin treatment.

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