Localization of the Action of Cholera Toxin on Adenyl Cyclase in Mucosal Epithelial Cells of Rabbit Intestine

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ABSTRACT Brush borders and plasma membranes have been purified from mucosal epithelial cells of rabbit ileum under control conditions and after treatment for 3 hr with cholera toxin in vivo. The activity of several enzymes in these preparations was measured. It was concluded that adenyl cyclase, like NaK-ATPase, seems not to be a normal constituent of brush borders. Both these enzymes are present in plasma membrane preparations derived largely from the basal and lateral margins of the epithelial cells, both may be phospholipid dependent enzymes and both are affected by cholera toxin. Adenyl cyclase activity is increased while NaK-ATPase is decreased. The activities of alkaline phosphatase, leucine-aminopeptidase, 5'-nucleotidase, glucose-6-phosphatase, and Mg-ATPase were not found to be affected by the toxin. Cholera toxin, which makes contact with the luminal side of the epithelial cells, in the natural disease and in the experimental model, would appear to exert its pathologic effect on adenyl cyclase at the opposite (basal and lateral) side of the cells.

INTRODUCTION

The small intestine of the rabbit is a useful model for the study of the action of cholera toxin (1, 2). Application of the enterotoxin to the lumen of the intestine results, after a brief latent period, in accumulation of fluid. This occurs in the duodenum, jejunum, and ileum so that the whole of the small intestine can be involved (3-5). It has been shown that the toxin effect is mediated by a stimulation of the enzyme adenyl cyclase and increased intracellular concentrations of adenosine 3',5'-cyclic monophosphate (cyclic AMP) (6-12). The cyclic AMP causes fluid accumulation by its subsequent effects upon electrolyte transport (13-15).

One striking feature of the action of toxin is its effectiveness after only brief contact with the intestine (16). Exposure for only 5 min is followed by a gradual increase in both adenyl cyclase activity and fluid accumulation over the next several hours. Thus a rapid interaction with the tissue results in progressive and irreversible changes. This would appear to be an unusual result. The toxin is a protein macromolecule with a molecular weight of around 84,000 (17, 18) which would not be expected to enter the epithelial cells rapidly. Thus the following studies were performed in an attempt to localize the site of action of cholera toxin on adenyl cyclase in the intestinal cells. In addition, the activities of several other enzymes were measured to assess the homogeneity of purified cell membrane fractions and to determine if cholera toxin affects more than the one enzyme.

METHODS

Preparation of membranes. New Zealand white rabbits were used for the study. They were anesthetized with i.v. sodium pentobarbital, the abdomen opened and a portion of the ileum cleaned by washing the succus entericus into a more distal portion of the intestine with Ringer's solution. Two loops of intestine were prepared for use in each animal separated by a third small loop. Double sutures were used to seal off each loop and rubber catheters were inserted to drain them. Care was taken that the blood supply was not affected by the sutures. 100 mg of cholera toxin (essentially a dried crude culture filtrate, Wyeth—NIH lot 001) was placed in

"Abbreviations used in this paper: cyclic AMP, adenosine 3',5'-cyclic monophosphate; IMP, inosine-5'-monophosphate; TCA, trichloroacetic acid."

Adenosinetriphosphate-phosphohydrolase. ATPase (E.C.3.6.1.3) activity was determined by the amount of inorganic phosphate produced during the incubation of subcellular fractions with ATP. MgNaK-ATPase was determined in a medium consisting of MgCl₂, 3 mM; Tris-ATP, 3 mM; NaCl, 100 mM; KCl, 20 mM, and imidazole-Cl, 70 mM; pH 7.5. Mg-ATPase was measured by omitting NaCl and KCl. After 30 min at 37°C the reaction was stopped by the addition of ice-cold trichloroacetic acid (TCA). Inorganic phosphate was determined in the supernatant by the method of Fiske and Subbarow (22). Protein was determined by the method of Lowry, Rosenbrough, Farr, and Randall (23) using crystalline bovine albumin as the standard. The specific activity of ATPase and other phosphatases was expressed as μmoles phosphate-milligram protein⁻¹·hr⁻¹. NaK-ATPase was defined as the difference in the specific activities of MgNaK-ATPase and MgATPase.

5'-Nucleotidase. 5'-Nucleotidase (E.C.3.1.3.5) was determined with inosine-5'-monophosphate (IMP) as substrate in a medium of MgCl₂, 5 mM; IMP, 5 mM; CoCl₂, 1 mM; and bovine albumin-Tris buffer, 50 mM, pH 6.5.

Glucose-6-phosphatase. Glucose-6-phosphatase (E.C.3.1.3.2) was measured by the method of Hubscher and West (24) with glucose-6-phosphate, 10 mM, in a 50 mM maleic acid-NaOH buffer at pH 6.0. The reaction medium included Na₂ EDTA, 4 mM; and NaF, 1 mM, to inhibit alkaline phosphatase and acid phosphatase activity. In both enzyme assays the amount of Pi liberated in a 30 min incubation period at 37°C was determined in the supernatant after stopping the reaction with TCA.

Alkaline phosphatase. Alkaline phosphatase (E.C.3.1.1.4) was determined with 5 mM p-nitrophenylphosphate as the substrate, MgCl₂, 20 mM; ZnCl₂, 0.5 mM, as activator in 50 mM glycine-NaOH buffer; pH 9.5. The reaction was stopped after 15 min by the addition of TCA. After centrifugation the supernatant was neutralized with Tris, diluted with distilled water, and the amount of liberated p-nitrophenol measured at 400 nm. Enzyme activity was expressed in μmoles p-nitrophenol-milligram protein⁻¹·hr⁻¹.

Lecineaminopeptidase. Lecineaminopeptidase (E.C.3.4.1.1) was measured according to the method of Tuppy, Wiesbauer, and Wintersberger (25). The reaction medium consisted of 10 mM leucine-p-nitroaniline as substrate in MgCl₂, 1 mM; and Tris buffer, 50 mM at pH, 7.6. The amount of p-nitroanilinede liberated at 25°C was recorded at 405 nm with a Beckman-Gilford recorder over a time period of 5 min. Enzyme activity was expressed in μmoles p-nitroanilinemilligram protein⁻¹·min⁻¹.

Sucinidehydrogenase. Sucinidehydrogenase (E.C.1.3.99.1) was measured by recording the succinate dependent reduction of dichloroindophenol to leucoidophenol at 578 nm according to the method reported by Green, Mill, and Kolout (26). The assay medium contained potassium-phosphate buf-
TABLE I

Enzyme Activities of Brush Border Preparations from Rabbit Ileum under Control Conditions and after Treatment with Cholera Toxin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control</th>
<th>Toxin</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmole·mg protein⁻¹·hr⁻¹ ± SEM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>129.5±20.7</td>
<td>131.5±21.0</td>
<td>15</td>
</tr>
<tr>
<td>Leucineaminopeptidase*</td>
<td>26.4±5.4</td>
<td>21.8±5.6</td>
<td>15</td>
</tr>
<tr>
<td>5'-Nucleotidase</td>
<td>3.3±0.6</td>
<td>3.8±0.6</td>
<td>13</td>
</tr>
<tr>
<td>Glucose-6-phosphatase</td>
<td>1.9±0.3</td>
<td>2.2±0.6</td>
<td>13</td>
</tr>
<tr>
<td>Mg-ATPase</td>
<td>11.5±2.7</td>
<td>11.3±1.9</td>
<td>9</td>
</tr>
<tr>
<td>NaK-ATPase</td>
<td>2.1±0.6</td>
<td>1.5±0.6</td>
<td>9</td>
</tr>
</tbody>
</table>

* nmole·mg protein⁻¹·min⁻¹ ± SEM.

fer, 10 mM, at pH 7.5; albumin, 500 μg/ml; potassium cyanide, 1 mM; 2,6-dichloroindophenol, 25 μg/ml; and sodium succinate, 10 mM. The extinction coefficient was taken to be 14.3 X 10⁻⁵ cm·mole⁻¹ (27).

RESULTS

Purification of brush borders

The brush borders were characterized by a high amount of alkaline phosphatase activity (see Table I) and also by leucine amino peptidase activity. At the same time moderate activities of ATPase, 85% of which was Mg-ATPase, 5'-nucleotidase and glucose-6-phosphatase were measured. In all enzyme activities listed in Table I there was no significant difference between brush borders derived from control or cholera toxin-treated loops. The successful isolation of purified brush borders is indicated by the high alkaline phosphatase activity and appearance

![Figure 1](image-url)  

**Figure 1** Electron micrograph of sample brush border preparation. These elements appear to be intact. Tags of lateral cell membrane are evident (arrows) and appear to be the only non-brush border constituents in the preparation. × 7500.
under phase and electron microscopic examination (Fig. 1).

Purification of outer cell membranes

As can be seen in Table II the purified cell membrane fraction (equivalent to the M1 band of Quigley and Gotterer [20]) exhibited low levels of alkaline phosphatase activity. Moderate leucineaminopeptidase activity was also present. Electron microscopy showed that large amounts of membrane material, relatively free of mitochondria and brush borders was present (Fig. 2). For the enzymes listed in Table II no difference in activity was observed except for NaK-ATPase. This enzyme however had its activity reduced 60% by the action of cholera toxin.

Studies with adenyl cyclase

Localization and the effect of cholera toxin. In Table III is shown the specific activity of adenyl cyclase in the supernatant obtained by homogenization in the Warner blender and centrifugation at 450 g, and in the brush borders. In both supernatant and brush border fractions adenyl cyclase activity was increased by cholera toxin. The specific activity in the supernatant was four times greater than that in the brush border for control tissue and eight times greater for the toxin-treated tissue. The reason for these discrepant ratios between control and toxin-treated tissue is not known. The essential point is that the supernatant has a much higher specific activity than the brush border. As the amount of material in the supernatant is greater than that of the brush border, the absolute amounts of adenyl cyclase in each fraction show that a preponderance of the enzyme is in the supernatant. This can be seen from the results in Table IV where the cyclase is expressed in absolute terms (i.e. the total amount of cyclic AMP produced by each fraction). Total brush border activity is only 5% or less of the total

<table>
<thead>
<tr>
<th>Table III</th>
<th>Adenyl Cyclase Activity in “Supernatant” and Brush Border Preparations from Rabbit Ileum under Control Conditions and after Treatment with Cholera Toxin</th>
</tr>
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<tr>
<td></td>
<td>Adenyl cyclase activity</td>
</tr>
<tr>
<td></td>
<td>Supernatant</td>
</tr>
<tr>
<td>pmoles cyclic AMP·mg protein⁻¹·20 min⁻¹±SE</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>85.0±15.6</td>
</tr>
<tr>
<td>Toxin</td>
<td>317.9±74.2</td>
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</table>

Figure 2 Negatively stained preparation of M1 band material. This preparation consists of large smooth-surfaced elements identified as plasma membrane fragments. This fraction was relatively free of mitochondria and microsomes which were found in the M2 band (not shown). Preparation negatively stained with ammonium molybdate. × 50,000.

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Adenyl cyclase activity expressed as μmoles cyclic AMP produced in 20 min (total activity) ±SE.

Adenyl cyclase activity is present only in small amounts in brush borders, and may be present only as a contaminant introduced during homogenization.

After purification of the supernatant by centrifugation at 10,000 g, resuspension in buffer, "aging" and centrifugation over a discontinuous sucrose gradient, three fractions were tested for adenyl cyclase activity. These were the fraction put on the brush borders and the membranes found in the M₁ and M₂ bands. The results are shown in Table V. The highest specific activity was found in the M₁ band—the purified plasma membrane preparation of Quigley and Gotterer.

DISCUSSION

In histochemical studies of the intestinal epithelium, alkaline phosphatase has been located in the brush borders but not at the basal or lateral membranes (Brandes, Zetterqvist, and Sheldon [28]; Clark [29]). In the present work the brush borders were characterized by the presence of a highly active alkaline phosphatase. A comparable amount of alkaline phosphatase activity was reported by Quigley and Gotterer (20) for a brush border preparation from the rat intestine. Even higher alkaline phosphatase activities were reported by Forstner et al. (19) and by Eichholz and Crane (30) in brush border preparations of the rat and hamster intestine. The high activity of alkaline phosphatase in this fraction indicates a successful isolation of the brush borders from the rabbit intestine. This conclusion is supported by the electron microscopic appearance of the fraction. However, it is evident that there are other enzyme activities present that are not typical for brush borders. First, the low but measurable activities of NaK-ATPase indicated a contamination of the fraction with plasma membranes. This contamination might be due to adherent tags from the lateral membranes which can be seen by electron microscopy (see Fig. 1). The percentage contamination with plasma membranes, however, is low. If one takes the NaK-ATPase as a marker enzyme for plasma membranes and presumes that the measured specific activities of different subcellular fractions are quantitatively comparable, then the contamination is not greater than 15%. Contamination of the brush border preparation with subcellular fractions was the same in the control and cholera toxin-treated loops.

The purity of the plasma membrane fraction, which was collected at the density interface of d = 1.08/1.13 was checked similarly by measurement of marker enzyme activity and by electron microscopy. The presence of NaK-ATPase in plasma membranes of the intestinal epithelial cells has been shown by Quigley and Gotterer (20). In the present study the highest NaK-ATPase activity was found after sucrose density centrifugation of the 10,000 g sediment at the density interface, d = 1.08/1.13. The pattern of other enzyme activities present in this fraction indicated that some membranes derived from the brush borders, from mitochondria and endoplasmic reticulum are present in this fraction. However, the activity of these other "marker enzymes" was considerably lower than in their fraction of origin. The alkaline phosphatase activity was only 7% related to the enzyme activity in the brush borders, the succinodehydrogenase activity was only 12% of the mitochondrial activity in membranes collected at the density interface, d = 1.13/1.18. Also, the glucose-6-phosphatase activity was approximately 50% lower than in a crude microsomal fraction. The presence of glucose-6-phosphatase activity in the plasma membranes is difficult to interpret because it is known from a recent report of Emmelot and Bos (31) that the glucose-6-phosphatase of the plasma membrane fraction from rat liver showed different properties than that derived from the endoplasmic reticulum. Thus, this enzyme might be present also as a constituent of the plasma membrane. From the evidence presented in the electron microscopic appearance as well as from the low contamination by other "marker enzymes" it can be concluded that the membranes of the density, d = 1.08/1.13, represent a part of the plasma membranes. The contamination of plasma membranes by other subcellular fractions was similar in both the control and cholera toxin-treated loops.

After the treatment with cholera toxin there was no

Table IV

Adenyl Cyclase Activity in "Supernatant" and Brush Border Preparations from Rabbit Ileum under Control Conditions and after Treatment with Cholera Toxin

<table>
<thead>
<tr>
<th>Membrane Fraction</th>
<th>Supernatant</th>
<th>Brush border</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.2±0.1</td>
<td>0.06±0.01</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>3.5±0.7</td>
<td>0.14±0.02</td>
<td>7</td>
</tr>
</tbody>
</table>

Adenyl cyclase activity expressed as μmoles cyclic AMP produced in 20 min (total activity) ±SE.

Table V

Adenyl Cyclase Activity in the F II, M₁, and M₂ Fractions of Mucosal Cells of Rabbit Ileum under Control Conditions and after Treatment with Cholera Toxin

<table>
<thead>
<tr>
<th>Membrane Fraction</th>
<th>F II</th>
<th>M₁</th>
<th>M₂</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>21.2±4.1</td>
<td>43.2±11.0</td>
<td>12.0±3.1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>69.1±13.5</td>
<td>112.6±23.3</td>
<td>44.2±10.2</td>
<td>9</td>
</tr>
</tbody>
</table>

significant change in the activity of enzymes originating in the brush border. In the plasma membranes, however, the specific activities of adenyl cyclase increased threefold while that of NaK-ATPase decreased to 40% of the control value.

An inhibition of mucosal NaK-ATPase in cholera patients who had undergone intestinal biopsy was reported earlier by Hirschhorn and Rosenberg (32). The same enzyme inhibition was found in experiments in which a microsomal preparation of the intestinal mucosa was incubated in vitro with unpurified Vibriocholerae culture filtrates (Richardson [33]). In the present work it has been demonstrated that it is the NaK-ATPase in the plasma membranes that is inhibited after the in vivo application of cholera toxin to the intestine of rabbits.

There are three main conclusions from this work: (a) Adenyl cyclase, like NaK-ATPase seems not to be present in the brush borders of intestinal epithelial cells. This point has been made previously by Hollingsworth (34) who studied the activities of several enzymes in the chick small intestine. He found no adenyl cyclase activity in the brush borders.

(b) It follows from the first conclusion that cholera toxin, which is present at the luminal surface of the intestinal epithelial cells during naturally acquired cholera and experimentally, cannot affect adenyl cyclase in the brush border with which it is in contact, but in these cells must affect the enzyme in the lateral and basal plasma cell membranes. This is of importance to our understanding of the detailed mechanism of the action of the toxin. It follows that the toxin either affects the brush borders to trigger changes subsequently affecting the lateral and basal plasma cell membranes, or that the brush borders are involved in the rapid binding and uptake of cholera toxin and that it subsequently migrates to the site of its action. One reservation to this conclusion would apply if cholera toxin affected only the crypt cells which do not have a brush border, the luminal membrane of which is presumably present in the Mit fraction. While not eliminating this possibility it should be pointed out that (1) all cell types that have been exposed to cholera toxin thus far have responded with an increase in adenyl cyclase activity. Thus, those cells in the intestine which do not have brush borders would presumably be affected by cholera toxin, and (2) that the crypt cells make up only a small proportion of the intestinal cells.

Changes in transport and permeability which are mediated by cyclic AMP after stimulation of adenyl cyclase by the toxin, may occur at one or more loci on brush border, basal, and lateral membranes.

(c) Cholera toxin does not specifically affect adenyl cyclase. It has in addition an effect upon NaK-ATPase. As evidence is available that both these membrane bound enzymes are phospholipid dependent the toxin could act via an effect on the membrane phospholipids. Investigations are continuing into a possible effect of cholera toxin on membrane lipids.

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