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# CHOLERA TOXIN INDUCES CHANGES IN THE ION PERMEABILITY OF INTESTINAL BRUSH BORDER MEMBRANES

FRANCISO BAVROS, PILAR DEL LE PEÑA, SANTIAGO GASCÓN, SOFÍA RAMOS and PEDRO S. LAZO

*Departamento Interfacultativo de Bioquímica, Universidad de Oviedo, Oviedo (Spain)*

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**Cholera toxin can alter the ion permeability of brush border membrane vesicles from rabbit small intestine. This alteration is reflected by differences in membrane potential-stimulated, $Na^+$-dependent, D-[$^3$H]glucose transport by these vesicles, as well as by an enhancement in the accumulation of the lipophilic cation [$^3$H]tetraphenylphosphonium in response to an artifically imposed membrane potential. Analogous effects were observed when the intact tissue was treated with the toxin and the vesicles subsequently obtained. An important implication of this finding is that cholera toxin does not need to activate adenylate cyclase to induce permeability changes in the cell membrane since the experiments were carried out in conditions where neither ATP nor cyclic AMP was present.**

Current views regarding the mechanism of action of cholera toxin invoke alteration in the concentration of adenosine 3′-5′-cyclic monophosphate (cyclic AMP) as the second event in the transfer of information to the cell [1]. Extensive studies have been carried out to clarify the mechanism by which cholera toxin activates adenylate cyclase, but all of them in systems where the toxin was allowed to interact directly with the cyclase [2–5]. Moreover, most of these studies have been carried out in erythrocytes or cells other than the enterocyte [6] where a particularly intriguing problem exists. In these cells adenylate cyclase appears to be localized in the basolateral membrane [7], while cholera toxin binds exclusively to the brush border when intact tissue is used (unpublished observation).

It has been reported that there are several similarities between cholera and tetanus toxin [8], and recent evidence has extended these analogies to some glycoprotein hormones and interferon [9,10], suggesting an analogy in the mechanism of action of these effectors [9]. Recently it has been reported that TSH [11] and tetanus toxin [12] can alter the electrochemical ion gradient across the membrane. It might be possible, therefore, that cholera toxin could exert a similar action. This hypothesis was tested using a preparation of purified brush border membrane [13] from rabbit small intestine. It is well known that the transport of D-glucose in this tissue is a $Na^+$-dependent process [14]. Thus, when a 50 mM NaCl gradient was imposed to 50 mM KCl loaded vesicles, these were able to accumulate D-[$^3$H]glucose (Fig. 1). Because of leakage of $K^+$ when valinomycin was added, an artificial membrane potential was created which was able to drive additional D-glucose transport. When the vesicles were previously incubated with cholera toxin both the $\Delta$pNa-driven D-[$^3$H]glucose transport and the $\Delta\Psi$ stimulated transport were enhanced.

The change in membrane permeability was also detected with a more direct approach. As the $K^+$ equilibrates, a membrane potential (interior negative) reflected by the uptake of the permeant lipophilic cation [$^3$H]tetraphenylphosphonium ([$^3$H]-$TPP^+$) is created. Previous treatment of the vesicles

Abbreviations and symbols: $\Delta\Psi$, the membrane potential; $\Delta$pNa, the concentration gradient of sodium; $TPP^+$, tetraphenylphosphonium.

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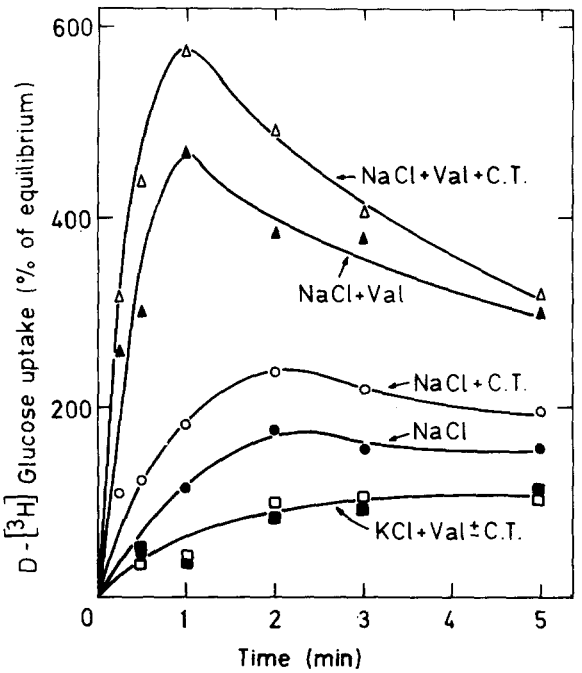


Fig. 1. Effect of cholera toxin (C.T.) on D-glucose transport by brush border membrane vesicles. Brush border vesicles were prepared from fresh tissue as in Ref. 13 except that they were resuspended in 150 mM KCl instead of mannitol as osmotic stabilizer. Vesicles (100 $\mu$l of 20 mg of protein/ml) loaded with 50 mM KCl were incubated for 30 min at 0°C with 0.25 $\mu$g (1 $\mu$l) of cholera toxin (Schwarz/Mann) which was added in 0.25 M sodium phosphate buffer, pH 7.5. The control vesicles were incubated with the same buffer without the toxin. After treatment the uptake of D-[$^3$H]-glucose by control vesicles (filled symbols) and cholera toxin-treated vesicles (open symbols) was measured as follows: to two series of tubes containing 100 $\mu$l of 10 mM Tris-HCl buffer (pH 7.5) plust 50 mM NaCl and a third series containing the same buffer plus 50 mM KCl was added 1 $\mu$l of D-[$^3$H]glucose (13.5 Ci/mmol; 1 mCi/ml). The reaction was initiated by addition of 5 $\mu$l of vesicles (approx. 100 $\mu$g of protein) and 1 $\mu$l of 0.5 mM valinomycin (Val) in ethanol (or an equal volume of ethanol when required). At indicated times the reaction was stopped by dilution of the sample with 2 ml of cold 10 mM Tris-HCl buffer (pH 7.5) plus 150 mM NaCl and filtered through Whatman GF/C filters which were then washed with an additional 2 ml of the dilution buffer. Dilution, filtration and wash steps were carried out within 5 s. After drying the filters were counted in a Beckman liquid scintillation spectrophotometer. The equilibrium (100%) corresponds to the uptake obtained with 50 mM KCl in the external medium in the presence of valinomycin, which was identical to the one obtained with NaCl plus 2 mM phlorizin or 5 $\mu$M monensin. Glucose uptake was previously measured in untreated vesicles and found to be 2200 pmol/mg of protein when the

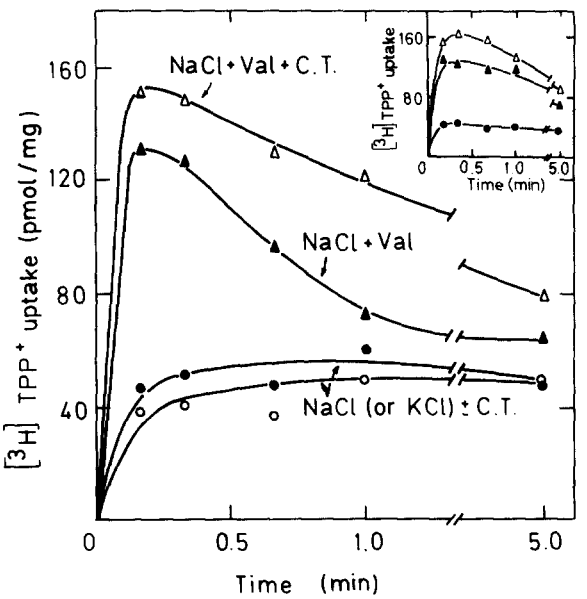


Fig. 2. Effect of cholera toxin (C.T.) on the accumulation of the lipophilic cation [$^3$H]TPP$^+$ by brush border membrane vesicles. Vesicles were prepared and treated with cholera toxin as in Fig. 1. The uptake of [$^3$H]TPP$^+$ was determined as that of D-glucose except that 2 $\mu$l of [$^3$H]TPP$^+$ (0.25 mM; 200 Ci/mol) and 150 $\mu$g of membrane protein per tube was used. The inset shows the results obtained when choline chloride was used instead of NaCl. When NaCl and valinomycin (Val) were in the external medium [$^3$H]TPP$^+$ uptake in cholera toxin-treated vesicles with respect to untreated vesicles was 133% ± 31% (mean ± S.D., $n$ = 10). When choline chloride substituted for NaCl the uptake was 123% ± 14% ($n$ = 5). Symbols are as in Fig. 1.

with cholera toxin resulted in an enhanced uptake of the cation which in addition was maintained inside the vesicles for a longer period of time than in the control vesicles, reflecting that cholera toxin-treated vesicles were in a more stable hyperpolarized state (Fig. 2). Since [$^3$H]TPP$^+$ uptake was equally affected by the toxin when choline chloride was substituted for NaCl (Fig. 2, inset) it follows that $Na^+$ is not essential for this phenomenon to take place,

external concentration of the sugar was 0.5 mM. When NaCl was in the external medium D-[$^3$H]glucose uptake in cholera toxin-treated vesicles between 0.5 and 3 min with respect to untreated vesicles during the same period of time was 154% ± 33% (mean ± S.D., $n$ = 11) in the absence of valinomycin and 132% ± 19% (mean ± S.D., $n$ = 8) when the ionophore was present.

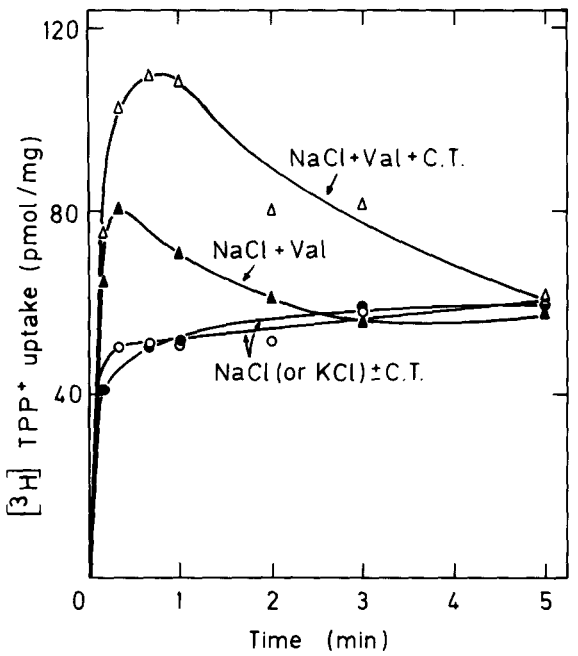


Fig. 3. [$^3$H]TPP$^+$ uptake by brush border membrane vesicles obtained from untreated and cholera toxin-treated rabbit small intestine. Two equivalent samples (5 g) of a mixture of intact tissue from several animals were everted and resuspended in 30 ml of 10 mM Tris-HCl buffer (pH 7.5) plust 150 mM KCl. To one of them was added 30 $\mu$l of cholera toxin (0.5 mg/ml) in 0.25 M sodium phosphate buffer (pH 7.5) and to the other the corresponding amount of buffer. They were incubated for 60 min at 0°C and subsequently vesicles were prepared as in Fig. 1. [$^3$H]TPP$^+$ uptake was determined then as for Fig. 2. The difference in the specific activity of alkaline phosphatase between vesicles obtained from treated and untreated tissues had an average value of 13% ± 10% (mean ± S.D., $n$ = 5). Val, valinomycin; C.T., cholera toxin.

although it would be compatible with the toxin's causing an enhanced anion permeability. The leakiness of the membranes (reflected by the stability of the steady state) varied from preparation to preparation, but in general there was good agreement between [$^3$H]TPP$^+$ uptake and D-[$^3$H]glucose transport. It is particularly important to point out that these effects can be obtained after the membranes have been incubated with cholera toxin for only 10 min, shorter than any of the described lag phases for the activation of adenylate cyclase.

Similar changes in the membrane properties were also observed when the intact tissue was treated with

TABLE I

CHARACTERIZATION OF BRUSH BORDER MEMBRANE VESICLES

Alkaline phosphatase [18], sucrase [13], protein [19], ATP [20] and cyclic AMP [21] were measured by established procedures. ATPase assay was as in Ref. 18 except that 0.75 mM ouabain was present in the tubes in which $Na^+$ and $K^+$ were absent. All the enzymatic activities are expressed in micromol of substrate hydrolyzed per min per mg of protein. ATP and cyclic AMP are expressed as pmol/mg of protein.

| Enzyme or compound | Homogenate (H) | Vesicles (V) | Relative activity (V/H) |
|---|---|---|---|
| Alkaline phosphatase | 0.037 | 0.474 | 12.8 |
| Sucrase | 0.046 | 0.616 | 13.4 |
| ($Na^+ + K^+$)-ATPase | 0.050 | 0.130 | 2.6 |
| ATP | 95 | <1.0 | – |
| Cyclic AMP | 25 | <0.5 | – |

cholera toxin for 60 min and the vesicles subsequently obtained compared to those obtained from untreated tissue in their ability to accumulate [$^3$H]TPP$^+$. Fig. 3 shows that the uptake of the cation was higher in the vesicles obtained from cholera toxin-treated tissue than in those obtained from untreated tissue.

The reported observations suggest that cholera toxin can induce changes in the brush border membrane not mediated by cyclic AMP since: (i) the contamination by basolateral membranes was very small as indicated by the specific activity of marker enzymes (Table I), (ii) neither ATP nor cyclic AMP was present in the preparation of the vesicles used (Table I), and (iii) the observed changes take place in periods of time shorter than that required for activation of adenylate cyclase. At this point it is worth mentioning the failure of Forsyth et al. [15] to reduce the net fluid secretory effect of cholera toxin eventhough they reduced the mucosal cyclic AMP concentration and the results of Ilundain and Naftalin [16] using sheets of rabbit ileum, who concluded that the steady-state concentration of cyclic AMP seems not to be an important parameter in the control of secretion. Our observation using purified brush border membranes points out the important ionophore role of cholera toxin on its natural target,

although a precise identification of the ion(s) permeability affected by the toxin requires further research. The ionophore role of cholera toxin has previously been demonstrated using bilayers containing gangliosides [17]. Thus, one has to invoke a mechanism by which cholera toxin causes changes in the permeability properties of the membrane, which would be followed by a sequential or simultaneous increase in intracellular cyclic AMP. The precise role of this cyclic AMP would have to be determined but it would certainly not be the obligatory 'second messenger'. It should also be pointed out that tetanus toxin, which is similar in several respects to cholera toxin [8,9], is not known to stimulate adenylate cyclase although it is able to alter the electrochemical ion gradient across the synaptosomal membrane [12].

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