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SPIN PROBE STUDY OF THE ACTION OF CHOLERA TOXIN ON ENTEROCYTE BRUSH BORDER MEMBRANES

V. A. Yurkiv, V. N. Chebotarev,
V. A. Kuznetsov, and V. A. Livshits

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The enterotoxin produced by *Vibrio cholerae*, if introduced into the lumen of the small intestine, causes diarrhea due to a sharp increase in permeability of the epithelium for water and electrolytes [6]. The mechanism of this process has not yet been adequately studied. It is not yet known, for instance, whether any structural changes take place in the membranes of the brush border (BB) of the enterocytes as a result either of direct interaction with cholera toxin (CT) or in the subsequent stages of its action as a result of activation of adenylate cyclase (AC), or how these possible changes are connected with disturbance of ionic transport through the epithelium.

The lysolecithin content in BB membranes is known to be increased in rabbits by the action of cholera toxin. Meanwhile, according to electronmicroscopic data, no significant changes are found in these membranes [11]. The object of this investigation was to make a spin probe study [3] of the physical state of BB membranes in experimentally induced cholera diarrhea. To characterize the state of the BB membranes under normal conditions and under the influence of CT, spin probes localized in different regions of the lipid bilayer of the membranes and also a spin label covalently bound with protein SH-groups were used.

EXPERIMENTAL METHOD

A model of cholera diarrhea *in vivo* was produced in rabbits aged 1-2 months. Under intravenous thiopental (1-2 ml of a 2% solution) anesthesia laparotomy was performed and one or two segments of small intestine 15-20 cm long were isolated by means of tied ligatures. CT (from Schwarz/Mann, West Germany) was then injected into the lumen of the intestine in a dose of up to 100 μg per animal, in medium containing 150 mM NaCl, 10 mM HEPES, pH 7.5 or (in the control experiment), the same volume of medium without toxin was injected. The abdominal incision was closed. The rabbits were killed after an exposure of 3 h.

A suspension of enterocyte BB fragments was obtained by the method in [10]. The preparation was electron-microscopically homogeneous and contained no characteristic extraneous inclusions. Activity of marker enzymes sucrose and alkaline phosphatase [10] was close to the values given in the literature.

Laboratory of the Molecular Basis of Pathogenesis of Infectious Diseases, Central Research Institute of Epidemiology, Ministry of Health of the USSR, Moscow. Sector of Kinetics of Chemical and Biological Processes, Institute of Chemical Physics, Academy of Sciences of the USSR, Moscow. [Presented by Academician of the Academy of Medical Sciences of the USSR N. N. Zhukov-Verezhnikov (deceased).] Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 92, No. 7, pp. 17-20, July, 1981. Original article submitted March 30, 1981.

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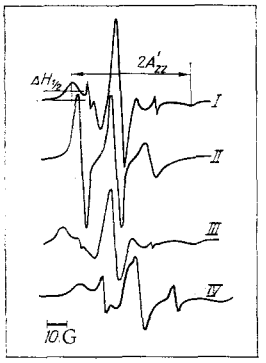

Fig. 1. Typical EPR spectra of probes I, II, and III and label IV.

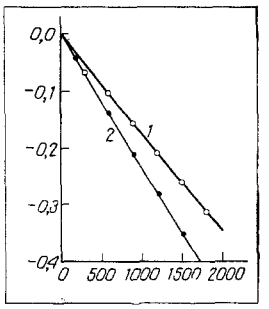

Fig. 2. Kinetics of reduction of probe I in BB membranes isolated from enterocytes by sodium ascorbate 1:10. 1) Control; 2) experimental specimen. Abscissa, time (in sec); ordinate, $\ln(I/I_0)$.

The following probes were used: 1-oxyl-2,2,5,5-oxazolidone derivative of 6-ketopalmitic (probe I), N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-N-palmitylamide (probe II), and 2,2,4,4-tetramethyl-1,2,3,4-tetrahydro-5,6-benzo-γ-carbolin-3-oxyl (probe III).

BB membrane proteins were modified with N-(1-oxyl-2,2,5,5-tetramethyl-4-piperidinyl)-maleimide (label IV) by incubation with the BB preparation for 12 h at 4°C in the ratio of 5 M label to 1 M protein.

The spin probes were added from alcoholic solutions (1 μl 10^{-2} M to 100 μl of membrane suspension) in the molar ratio of 1:100 to phospholipids. All measurements were made on the E-104 EPR spectrometer (from Varian, USA) (accuracy of thermostatic control ±0.5°C).

Microviscosity and orderliness of the surroundings of probes I and III and the rotational mobility of label IV were characterized by the parameter $2A_{zz}$ (Fig. 1); the correlation time of rotation of probe II was determined by the equation for rapid rotations, with introduction of a correction factor for time $r > 10^{-9}$ sec [3]. To characterize the local surroundings of probe I, its reduction reaction by the ascorbate ion was used [1].

EXPERIMENTAL RESULTS

Typical spectra of probes I-III and label IV in vesicles of BB membranes are illustrated in Fig. 1. Probes I and III were distributed between the lipid and aqueous phases. The spectrum of label IV also corresponded to two regions of binding with widely different rotational mobility.

In the experiments of series I *in vivo* the experimental animals were different from the controls. As a rule, at autopsy on the experimental animals a well-marked diarrheal effect was observed. In the experimental specimens parameter $2A_{zz}$ was reduced compared with the controls on average by 0.9 G and the half-width of the low-field peak $\Delta H_{1/2}$ was increased on average by 0.4 G. Similar differences were observed for probe III. However, these differences did not exceed variations of the parameters $2A_{zz}$ and $\Delta H_{1/2}$ in the control specimens of different pairs; consequently, despite the fact that experimental and control animals in each pair were chosen to be as close as possible in size, age, and color, it was impossible to conclude from this series of experiments what changes took place in the state of the lipid layer in cholera diarrhea.

In the experiments of series II *in vivo* two segments of small intestine from the same animal were used as experimental material. The mutual position of these segments varied. Statistical analysis of the results of this series of experiments (n = 5) showed no significant differences with respect to the parameters $2A_{zz}$ and $\Delta H_{1/2}$ for probe I in the experimental and control specimens.

Injection of CT may give rise to a general stress response, and this may be reflected, for example, in differences between the sensitivity of different animals to CT [2]. Stress

may also affect the parameters of the lipid bilayer of the control specimens in the experiments of series II *in vivo*. To abolish this effect, in the experiments of series III the control segment of the intestine was removed at the beginning of the operation, after which CT was introduced into the experimental segment. The effect of the operation itself and of the anesthetic was monitored in a parallel series of experiments in which, instead of toxin, the same value of the original solution was injected.

In the experiments of series III (n = 9) a decrease in the parameter $2A_{zz}$ (on average by 0.5 G) was observed in BB membranes from diarrheal segments of the small intestine compared with control specimens, in agreement with the results of the experiments of series I. This change varied from 0.2 to 1.2 Hz, but in all the experiments its sign was the same and, consequently, the use of Wilcoxon's signed rank sum test [4] is significant ($P < 0.01$). Particularly marked differences between experiment and control were observed in relation to the kinetics of reduction of the radical fragment of probe I by ascorbate (Fig. 2). In preparations of BB membranes isolated from diarrheal segments of intestine the rate of recovery was 15-20% greater, and together with the decrease in parameter $2A_{zz}$, this is evidence of the "liquefaction" of the lipid bilayer of the BB membranes through the action of cholera toxin.

The significance of this effect was also confirmed by the fact that on replacement of the CT solution in the medium by original medium without toxin (experiments of series IV) the opposite tendency was observed: If the anesthetic and operation had any effect, they led to a change of opposite sign in the parameter $2A_{zz}$ (n = 5).

The results of the experiments *in vivo* as a whole thus indicate that CT reduces the microviscosity and the degree of orderliness of the BB membranes, in agreement with data in the literature [5] on changes in the lipid composition of the membranes. However, as the results of the experiments of series II and III show, these structural changes were evidently due to the action of cholera toxin on the animal and were not a truly diarrheal effect.

It can be tentatively suggested that structural changes in enterocyte membranes in diarrhea are at least to some extent reversible and disappear when the membranes are isolated from the cells. To detect any changes of this type in the lipid and protein regions of the BB membrane, experiments were carried out *in vitro* using lipid probes I and II and protein label IV.

To begin with direct interaction between CT and BB membranes was studied. Although the concentration of GM_1 gangliosides, which are the primary receptors of CT, in the BB membrane is very low [7], there could be a cooperative or indirect (through enzyme systems in the BB membrane) influence of CT binding on the structure of the membrane. Data in the literature show that extremely low concentrations of prostaglandins (10^{-12}-10^{-9} M) may act on the degree of orderliness of the erythrocyte membrane [9].

Interaction of CT with BB membranes was studied within the concentration range of toxin from 10^{-9} to 10^{-6} M. The EPR spectra of the two probes I and III and of label IV were unchanged both immediately after addition of CT and during incubation for 1 h at 37°C, on the basis of which it can be concluded that binding of CT does not significantly change the state of the BB membrane.

The subsequent stages of the action of CT are linked with activation of AC and an increase in the intracellular concentration of cyclic 3',5'-AMP. However, all the reactions induced by cyclic AMP and leading to a change in transport of Na^+ and Cl^- ions and of water through epithelium are not yet known.

Schemes of the action of cyclic AMP in cells given in the literature assume either direct activation of enzymes or activation of protein kinases, which can subsequently phosphorylate proteins in the presence of ATP [8]. Starting from the schemes of action of cyclic AMP mentioned above, its action *in vitro* was simulated on isolated vesicles of BB membranes.

In view of the absence of data on localization of protein kinases in enterocytes, interaction of BB membranes with cyclic AMP was studied in the presence both of ATP and of cytosol of cells which may contain water-soluble protein kinases. Since cyclic AMP and protein kinases act inside the cell, in order to make the inner surface of the membrane accessible, the experiments were carried out on vesicles treated with small quantities of the detergent Triton X-100.

The cyclic AMP concentration was changed to 50 μM, which is more than an order of magnitude higher than in enterocytes during diarrhea [5]. The cytosol obtained on isolation of BB membranes was concentrated three to fourfold and was added in the presence of 5 mM $K_3Fe(CN)_6$, which prevents reduction of the radical fragments of the probes.

Addition of ATP or Mg-ATP complex in a concentration of 1-5 mM leads to structural changes in the protein and lipid regions of the membrane, manifested as inhibition of movement of the protein label (the parameter $2A_{zz}$ was increased from 64.3 ± 0.1 to 64.8 ± 0.1 G) and of probe II, localized on the surface of the membrane (the correlation time of the probe was increased from 1.44 ± 0.1 to 1.55 ± 0.1 nsec). Meanwhile the mobility of probe I, located in the hydrophobic region of the bilayer, increased ($2A_{zz}$ was reduced from 46.8 ± 0.1 to 43.6 ± 0.1 G). BB membranes are known to possess ATPase activity. Changes in the EPR spectra may be due to conformational changes in these proteins during binding and hydrolysis of ATP.

Addition of cytosol also changed the local surroundings of probe I and of the protein-bound label IV to some extent. However, all the changes described above were observed in the absence of cyclic AMP also. Meanwhile, no extra effects induced by addition of cyclic AMP could be observed, within the limits of error of measurement.

Under the conditions used to simulate the initial stages of cholera diarrhea *in vitro*, no structural changes thus were found in the BB membranes, whether during binding CT or in response to an increase in the cyclic AMP concentration. The negative results of these experiments do not rule out the possibility of such changes in the cell completely, for not all the essential conditions of *in vivo* experiments could be reproduced in the present investigation. Moreover, these changes themselves may be local in character, and further research is required to reveal them.

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