BLOCKING THE ACTION OF CHOLERA ENTEROTOXIN ON ADENLYATE CYCLASE AND THE CYCLIC AMP CONCENTRATION IN RABBIT SMALL INTESTINAL MUCOSAL CELLS WITH SODIUM 2,3-DITHIOPROPANESULFATE

V. A. Yurkiv and V. I. Melikhov

UDC 616.341-008.931-02:615.919:579.843.1]-02:615.279

KEY WORDS: adenylate cyclase; cholera enterotoxin; unithiol.

The connection has now been proved between diarrhea caused by cholera enterotoxin and activation of adenylate cyclase (AC), with a subsequent increase in the intracellular cyclic AMP concentration in the mucosa of the small intestine [6, 11]. A study of the structure of cholera enterotoxin has shown that it is a protein with mol. wt. of 84,000 and consists of five B subunits, linked noncovalently with one A subunit [7, 8]. Each B subunit contains one disulfide bridge [8]. The A subunit consists of two peptides A_1 and A_2, connected by a disulfide bond [7]. Cholera enterotoxin binds with the cell surface through interaction between its B subunits and the oligosaccharide moiety of ganglioside GM_1, after which the A subunit penetrates inside the cell [7]. It is suggested that inside the cell the disulfide bond in the subunit A molecule is ruptured, as the result of which two peptides A_1 and A_2 are formed [7]. Peptide A_2 evidently is required only for the toxin molecule to penetrate inside the cell. Removal of peptide A_2 converts peptide A_1 into the active state, i.e., endows it with catalytic activity [4, 5].

The substrate for ADP-ribosylation, which is catalyzed by protein A_1, is the regulatory GTP-binding subunit of AC, located on the inner side of the cytoplasmic cell membrane [10]. For that reason, in experiments on membrane preparations *in vitro*, activation of AC occurred without the participation of B subunits and peptide A_2 [14]. In that case the cholera toxin was converted into the active state (i.e., liberation of protein A_1 was induced) by incubating the toxin with SH-reducing agents [12, 14].

The complex oligomeric structure of cholera enterotoxin can evidently be explained on the grounds that this protein has several functions: binding with the cell receptors, penetration through the membrane, and the subsequent enzyme reaction. It can be tentatively suggested that rupture of the disulfide bonds of cholera enterotoxin reduces its ability to bind with the receptor (ganglioside GM_1) and to penetrate inside the cell, and consequently to reduce its toxic action. As reducing agent, it was decided to use sodium 2,3-dithiopropanesul-

TABLE 1. Effect of Cholera Enterotoxin on AC and PDE Activity and Cyclic AMP Concentration in Mucosa of Rabbit Jejunum in the Absence of Unithiol and also after Combined Injection of Cholera Toxin and Unithiol into Intestinal Lumen (M ± m)

Experimental conditions	AC activity, pmoles cyclic AMP/mg protein/min	PDE activity, pmoles AMP/mg protein/min	Cyclic AMP concentration in mucosa, pmoles/g tissue
Intact animals (physiological saline injected)	28±3 (n=11)	250±12 (n=11)	365±23 (n=11)
Animals after injection of 200 μg cholera enterotoxin	120±12 (n=12)	248±18 (n=12)	856±33 (n=12)
Animals after injection of unithiol:			
5 mg/kg	28±5 (n=6)	275±6 (n=6)	347±10 (=8)
10 mg/kg	25±4 (n=6)	300±10 (n=6)	321±14 (n=8)
Animals after combined injection of 200 μg cholera enteroxin and unithiol			
5 mg/kg	96±9 (n=6)	280±7 (n=6)	666±21 (n=6)
50 mg/kg	36±5 (n=12)	295±16 (n=12)	358±13 (n=12)

Central Research Institute of Epidemiology, Ministry of Health of the USSR, Moscow.
(Presented by Academician of the Academy of Medical Sciences of the USSR S. E. Severin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 92, No. 10, pp. 432-434, October, 1981. Original article submitted April 22, 1981.

 0007-4888/81/9210-1358$07.50

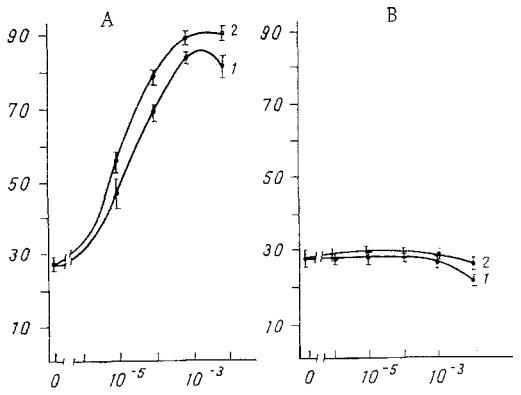

Fig. 1. Dependence of AC activity of membrane preparation of rabbit jejunal mucosa on concentration of thiol compounds. A) In presence, B) in absence of cholera enterotoxin: 1) unithiol, 2) DTT. Abscissa, concentration of thiol compounds (in M); ordinate, AC activity (in pmoles cyclic AMP/mg protein/min).

fate. This Soviet therapeutic preparation, known as unithiol, is less toxic than diothiothreitol (DTT). It contains two SH groups and is evidently capable of rupturing disulfide bridges in proteins [2].

EXPERIMENTAL METHOD

Cholera enterotoxin (Schwarz/Mann, 200 μg) or unithiol (5 or 50 mg/kg) was injected into the lumen of an isolated loop (about 15 cm) of jejunum of rabbits weighing 800 g. Rabbits of another group received an injection of cholera enterotoxin together with unithiol. Control animals received physiological saline. The animals were killed after the operation [3], 4 h after injection of the reagents.

The jejunal mucosa (about 1 g) was homogenized in a glass homogenizer with Teflon pestle (30 grindings) in 10 ml of solution containing 20 mM Tris-HCl, 1 mM EDTA, pH 7.5 (4°C). The homogenate was filtered through three layers of gauze and centrifuged twice, for 15 min each time, at 4000g. The final residue was resuspended and used as membrane preparation of mucosal AC.

To determine the cyclic AMP concentration, 150-200 mg of mucosa was fixed in 2 ml of 96% ethanol, homogenized, and centrifuged twice for 30 min each time at 1000g. The supernatants were pooled and evaporated to dryness. The residue, after evaporation of the ethanol, was resuspended in 800 μl of solutions containing 50 mM Tris-HCl and 4 mM EDTA, pH 7.5 (4°C). The cyclic AMP concentration was determined with the aid of radioimmunologic kits obtained from the Radiochemical Centre, Amersham, England.

Phosphodiesterase (PDE) activity was determined by the method in [1], AC by the method in [13], and protein by the method in [9].

In the experiments with activation of AC of the membrane preparation by cholera enterotoxin (10 μg/ml) *in vitro* the incubation medium additionally contained 10^{-3} M NADH.

EXPERIMENTAL RESULTS

The dependence of the action of cholera enterotoxin on AC of the mucosal membrane preparation on the concentration of DTT and unithiol is shown in Fig. 1. The sulfhydryl agents clearly had virtually no action on the basal AC activity, but led to the development of threefold activation of the enzyme by cholera enterotoxin. As regards the effectiveness of its action, unithiol was comparable with DTT. It can accordingly be postulated that unithiol, like DTT, ruptures S—S bonds in the cholera enterotoxin molecule and so converts the A_1 subunit into the active state.

The combined action *in situ* of cholera enterotoxin and unithiol in the lumen of a ligated loop of rabbit jejunum reduced AC activation in the mucosa by cholera enterotoxin (Ta-

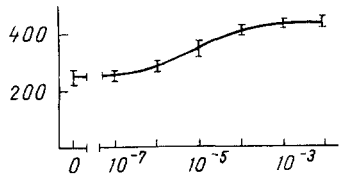

Fig. 2. Dependence of PDE activity of mucosal homogenate on unithiol concentration. Abscissa, unithiol concentration (in M); ordinate, PDE activity (in pmoles AMP/mg protein/min).

ble 1). The cyclic AMP concentration in the mucosa also was reduced at the same time. The small difference between the degree of depression of AC activity and the cyclic AMP concentration in the mucosa of the rabbit jejunum, into which unithiol was injected together with cholera enterotoxin, can evidently be explained by the activating effect of unithiol on PDE (Fig. 2).

These investigations thus showed that the pharmacological agent unithiol, used in the treatment of poisoning by heavy metals [2], can block the action of cholera enterotoxin on the intestinal adenylate cyclase system. The results suggest that this effect develops through the rupture of S—S bonds and a reduction in the ability of the toxin to penetrate inside the cell.

On the basis of these experimental data clinical trials of unithiol are recommended with a view to the use of this substance as a prophylactic agent in cholera. The possibility of using unithiol to reduce penetration of cholera enterotoxin into mucosal cells of the small intestine likewise cannot be ruled out in the treatment of cholera in man.

LITERATURE CITED

1. V. A. Tkachuk, V. G. Lazarevich, M. Yu. Men'shikov, et al., Biokhimiya, No. 9, 1622 (1978).
2. M. D. Mashkovskii, Theraupeutic Substances, 8th Edition [in Russian], Moscow (1978), Part 2.
3. S. N. De and D. N. Chatterje, J. Path. Bact., 66, 559 (1953).
4. D. M. Gill, Proc. Natl. Acad. Sci. USA, 72, 2046 (1975).
5. D. M. Gill and C. A. King, J. Biol. Chem., 250, 6424 (1975).
6. D. V. Kimberg, M. Field, J. Johnson, et al., J. Clin. Invest., 50, 1218 (1971).
7. T. Kohler, L. G. Lipson, J. Flores, et al., Bull. Schweiz. Akad. Med. Wiss., 32, 223 (1976).
8. C. Y. Lai, E. Mendez, and D. Chang, Biochem. Biophys. Res. Commun., 74, 215 (1977).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr, et al., J. Biol. Chem., 193, 265 (1951).
10. J. Moss, J. C. Osborne, P. H. Fishman, et al., Proc. Natl. Acad. Sci. USA, 74, 74 (1977).
11. D. E. Schafer, W. D. Lust, B. Sircar, et al., Proc. Natl. Acad. Sci. USA, 67, 851 (1970).
12. S. Van Heyningen, Biochem. J., 157, 785 (1976).
13. W. A. White, in: Methods in Enzymology (J. G. Hardmann and V. W. O'Malley, eds.), New York (1974), pp. 41-46.
14. A. Wodnar-Filipowicz and C. Y. Lai, Arch. Biochem., 176, 465 (1976).