EFFECT OF CHOLERA FILTRATE ON THE ADENYLATE CYCLASE SYSTEM IN VITRO

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UDC 616-008.939.633.2-02:576.851.315.097.29

An in vitro model using homogenates of rat intestine and liver is suggested for the study of the effect of a filtrate of a Vibrio cholerae culture on the adenylate cyclase system. Optimal conditions of adenylate cyclase function were determined for this model. The cholera filtrate was shown to induce stable activation of adenylate cyclase and not to change cyclic AMP phosphodiesterase activity. Phosphodiesterase activity in the intestinal homogenate was also shown to be about 2.5 times higher than in the liver homogenate. The model seems to be promising for study of the mechanism of action of cholera toxin.

KEY WORDS: cholera filtrate; adenylate cyclase system.

The principal link in the pathogenesis of cholera is considered to be activation of adenylate cyclase (AC) of the epithelium of the small intestine by cholera toxin [1, 5, 9]. A change in the level of cyclic AMP in the endothelial sites leads to disturbance of transport of materials in them [1, 4, 5, 8]. Since cholera toxin does not change cyclic AMP phosphodiesterase (PDE) activity [1, 5, 14], another mechanism controlling the level of intracellular cyclic AMP must exist in the cells of the mucous membrane, for which fluctuations in cyclic AMP content are not typical. Such a role may probably be played by the prostaglandin system [12, 15].

The highly contradictory nature of data in the literature on the action of cholera toxin on prostaglandin metabolism and their interaction with the AC system can be partly explained by the fact that workers who have used model systems have worked with widely different preparations of cholera toxin, starting with Vibrio cholerae and ending with a highly purified preparation of the toxin [6, 7, 11, 15].

The object of this investigation was to create an in vitro model with which to study the action of a filtrate of a culture of V. cholerae (cholera filtrate) on AC and PDE, and also to study the character of action of cholera filtrate of the intestine and liver, in which the AC system performs a different function [2]. Since the above-mentioned enzyme systems differ in their spatial localization in the cell, tissue homogenates were chosen as the test object.

EXPERIMENTAL METHOD

To obtain homogenates of the small intestine and liver, male Wistar rats weighing 200-250 g were used. Before the experiment the rats were deprived of food for 24 h. The animals were decapitated, the intestine was quickly removed and washed with Hepes-buffer (30 mM Hepes, from Sigma; 7.5 mM $MgSO_4$, pH 7.4), and the liver was perfused with the same buffer and removed. The tissue was homogenized in 10 volumes of Hepes-buffer in a Potter-Elvehjem homogenizer (30 frictions). The homogenates were filtered through four layers of gauze. All operations were carried out at 0-4°C. Protein in the homogenates was determined by Lowry's method. Aliquots of homogenates were preincubated, mixed with cholera filtrate (40 μg, from Sigma) and NAD (4 mM, from Reanal; here and subsequently the final concentrations are shown) at 30°C. The volume of medium was 500 μl. The concentration of cyclic AMP was determined by a radioimmunological method using standard kits from the Radiochemical Centre, Amersham (England) [13]. Radioactivity was determined in tolule-Triton scintillator from Beckman (USA) on a Mark-3 counter (from Searl, Holland). AC activity was determined in 1 ml of incubation medium containing protein of the test homogenate, 30 mM Hepes, 7.5 mM $MgSO_4$, 7 mM theophylline (from Sigma), and 3 mM ATP (from Sigma). The reaction was triggered by the

Institute of Human Morphology, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR A. P. Avtsyn.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 88, No. 10, pp. 414-416, October, 1979. Original article submitted December 28, 1978.

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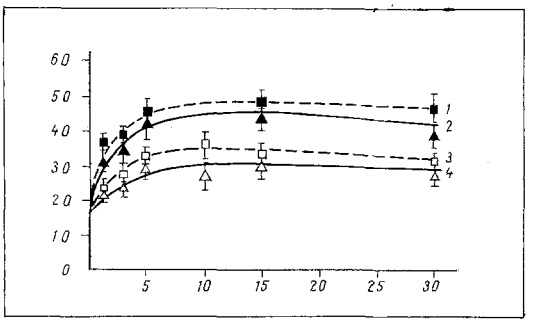

Fig. 1. Adenylate cyclase reaction velocity in homogenates of intestine (2, 4) and liver (1, 3) as a function of time. Points are mean values of five experiments. 1, 2) Cholera filtrate; 3, 4) control. Abscissa, reaction time (in min); ordinate, quantity of cyclic AMP formed (in pmole/mg protein).

TABLE 1. Dependence of Cyclic AMP Synthesis on Protein Content in Homogenates Treated with Cholera Filtrate ($M \pm m$; $n = 5$)

Protein content, μg	AC activity, picomoles/mg protein/5 min	
	intestine	liver
50	30,0±3,50	30,0±3,30
100	34,9±4,05	37,0±7,46
200	43,0±5,60	45,3±4,69
500	48,0±6,74	51,7±6,17
1000	28,0±7,46	34,3±3,78

TABLE 2. Patients with Cholera Filtrate ($M \pm m$; $n = 5$)

Duration of incubation, min	AC activity, picomoles/mg protein/5 min	
	intestine	liver
1	49,8±3,68	52,0±7,92
3	51,0±6,45	57,0±10,63
5	53,3±6,74	62,1±11,76
10	50,8±9,39	51,3±5,11
15	45,0±4,93	49,9±7,88
30	43,0±5,39	45,0±7,93

addition of ATP to the sample as it was being incubated at 30°C and stopped by the addition of 3 ml absolute ethanol. For better extraction of cyclic AMP and precipitation of proteins, the sample was boiled for 2 min. Protein was sedimented by centrifugation (4000g, 15 min) and the extract kept. The residue was subjected to repeated alcoholic extraction. The pooled extracts were evaporated in vacuo at 30°C. The residue was dissolved in 0.5 ml Tris-EDTA-buffer (0.05 M Tris, 4 mM EDTA, from the Radiochemical Centre, Amersham) and the cyclic AMP content was determined. PED activity was estimated from the degree of destruction of added cyclic AMP. The composition of the incubation medium was: 30 mM Hepes, 7.5 mM $MgSO_4$, 200 μg protein of the test homogenate, 80 pmole cyclic AMP (Radiochemical Centre). The sample was incubated for 1-30 min at 30°C with regular mixing. To inhibit PED activity, 7 mM theophylline was added. The reaction was stopped by 3 ml absolute ethanol. Cyclic AMP was extracted by the method described above.

EXPERIMENTAL RESULTS

As a result of these experiments, optimal conditions were determined for the study of the AC system in model experiments in vitro. The rate of formation of cyclic AMP in homogenates of intestine and liver during the first 5 min was approximately a linear function (Fig. 1). This time was chosen for the reaction. AC activity in the homogenate (preincubated for 10 min at 30°C with cholera filtrate) increased significantly – by 1.41 times compared with the basal level both in the intestine and the liver.

Investigation of the rate of synthesis of cyclic AMP during the action of cholera filtrate as a function of protein content showed that in the presence of 0-200 μg protein the function was approximately linear for both homogenates (Table 1). Protein in a concentration of 200 μg per sample was used subsequently. The marked decrease in cyclic AMP synthesis in the presence of 1000 μg protein (Table 1) can be attributed to a deficiency of substrate for AC, for the ATP concentration remained the same despite differences in the protein content.

For cholera toxin to be able to act, a definite time interval is necessary for binding with the receptor and for activation of AC by the A_1-polypeptide formed [10]. Preincubation of the homogenates with cholera filtrate was therefore carried out. Maximal activation of AC was observed after 5 min (Table 2). The very brief increase in AC activity under these circumstances can be explained by the possible synergistic effect of the other components of the cholera filtrate and, in particular, of neuraminidase [2]. With an increase in

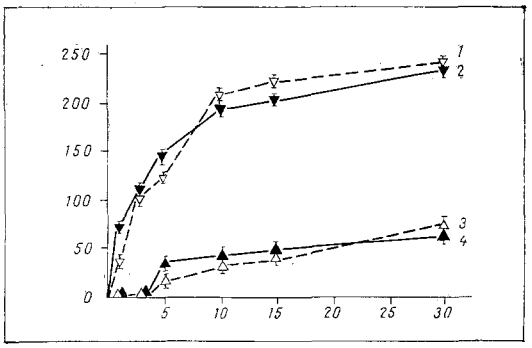

Fig. 2

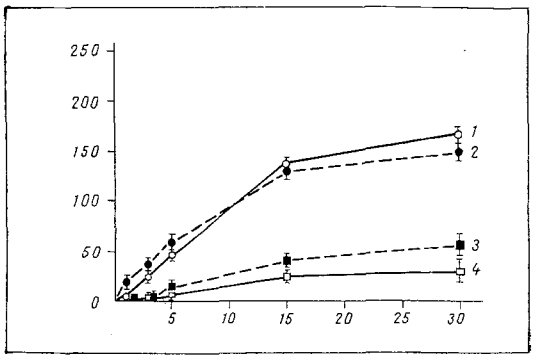

Fig. 3

Fig. 2. Phosphodiesterase reaction in intestinal homogenate as a function of time. Points show mean values for five experiments. 1) Control without theophylline; 2) experiments with cholera filtrate and without theophylline; 3) control with theophylline; 4) experiment with cholera filtrate and theophylline. Abscissa, the action time (in min); ordinate quantity of cyclic AMP destroyed (in pmole/mg protein).

Fig. 3. Phosphodiesterase reaction in liver homogenate as a function of time. 1) Control without theophylline; 2) experiment with cholera filtrate but without theophylline; 3) experiment with cholera filtrate and theophylline; 4) control with theophylline. Remainder of legend as in Fig. 2.

the duration of preincubation the activity of the enzyme fell somewhat, became stabilized, and thereafter remained approximately constant. An interval of 10 min was chosen as the optimal duration of preincubation.

AC function is closely connected with that of cyclic AMP PDE [1, 2]. PDE activity in the intestinal homogenate was found to be almost 2.5 times higher ($P \leq 0.05$) than in liver homogenate (Figs. 2 and 3). Meanwhile, the cholera filtrate did not change the activity of this enzyme in the homogenates, for the PDE activity remained the same as before, whether after the action of cholera filtrate and theophylline or without them (Figs. 2 and 3).

It can thus be concluded that the cholera filtrate, in the in vitro model systems suggested, reproduces the principal effects of pure cholera toxin [6, 14], i.e., it activates AC but does not change PDE activity. This model is evidently suitable for investigation not only of the adenylate cyclase, but also of the prostaglandin system of the cell and also for studying relations between them during the action of cholera toxin.

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