MECHANISM OF ACTION OF CHOLERA TOXIN. SPECIFIC INHIBITION OF TOXIN-INDUCED ACTIVATION OF ADENYLATE CYCLASE

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1. Introduction

Cholera toxin, the diarrheogenic protein of *Vibrio cholerae,* has a mol. wt of 84 000 and is composed of subunits of two types, L and H [1–3]. Both subunits are required for biologic activity but only the L type is involved in the high-affinity binding of toxin to the cell surface [1–4]. Recent chemical modification analyses have further elucidated the different functional sites of the toxin [5].

A specific ganglioside, G_{M1}, has been identified as the binding membrane receptor for cholera toxin in intestinal as well as non-intestinal mammalian cells [6–9]. Cell-binding of the toxin is followed by activation of adenylate cyclase which causes various effects in different cell types [10–12]. The activating 'signal' for adenylate cyclase is unknown. Unlike e.g. the stimulation of this enzyme by epinephrine there is a characteristic 'lag' period in the toxin action [10–12]

In the present study we describe for the first time inhibition of cell adenylate cyclase stimulation by cholera toxin which is associated with unchanged cell-binding of the toxin and unimpaired cyclase response to epinephrine and prostaglandin. We propose to have interfered with a cell component required to translate the initial toxin – G_{M1} receptor binding into an adenylate cyclase activating signal.

2. Materials and methods

2.1. *Materials*

Isolated cholera toxin was prepared by R. A. Finkelstein, Dallas, Texas [13]. Radiolabelling of the toxin with ^{125}I was done as described [4]. Prostaglandin E_1 (PGE_1) was generously donated by Dr John Pike, The Upjohn Co. Epinephrine, 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide (EDC), glycine-*O*-methyl ester, *N*-ethylmaleimide, picryl sulfonic acid and succinic anhydride was from Sigma, *N*-cyclohexyl-3 (2-morpholinoethyl) carbodiimide meto-*p*-toluene-sulfonate (CMC) from EGA-Chemie KG, (Steinheim/ Albuch, W. Germany), *N,N'*-dicyclohexyl carbodiimide (DCC) from Merck, and Medium RPMI 1640 and foetal calf serum (FCS) from Flow Laboratories.

2.2. *Incubation of cells and determination of cyclic 3'5'-adenosine monophosphate*

Thymus lymphocytes from CBA mice were prepared as previously described [4] and the cells suspended in the medium used. 10^7 cells were incubated with 10^{-9} M cholera toxin, 10^{-6} M epinephrine or 10^{-4} M PGE_1 at 37°C in 1 ml RPMI 1640 containing 10% FCS (RPMI-FCS). The cells were harvested by centrifugation and cyclic 3'5'-adenosine monophosphate (cAMP) extracted and assayed essentially as described by Bourne et al. [14]. The values reported are those after correction for losses during isolation and varied by less than 10 per cent in duplicate determinations.

2.3. *Chemical modification of cells*

Treatment of thymocytes with group modifying chemicals or other substances was performed for 30 minutes, usually at 37°C, using 10^7 cells per ml RPMI-FCS medium or phosphate-buffered saline. Thereafter the modified cells were washed twice by centrifugation (700 *g*, 1 min) in phosphate-buffered saline and subjected to toxin or hormone stimulation as described above.

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2.4. *Binding of radioiodinated cholera toxin to cells*

Binding of cholera toxin to cells was tested using radioiodinated toxin in mixture with various concentrations of unlabelled toxin according to a previously described procedure [4].

3. Results

3.1. *Modified cells stimulated by cholera toxin or hormones*

Thymocytes suspended in RPMI-FCS medium were pretreated with eight modifying reagents (table 1). After washing, the cells were resuspended in fresh RPMI-FCS medium and incubated with cholera toxin, epinephrine or PGE_1.

Amino group specific cell modification with picryl sulfonic acid or succinic anhydride had no pronounced influence on the cAMP response to cholera toxin.

Carboxyl group specific cell modification was performed with three carbodiimides, EDC and CMC which both are water-soluble and DCC which is only sparsely water-soluble. EDC had a differential effect on cellular cAMP response to cholera toxin on one hand and epinephrine and PGE_1 on the other (table 1). The toxin response was strongly inhibited (88–95 per cent in six different experiments). The cAMP response to the hormones was instead slightly enhanced. When the nucleophilic reagent glycine-*O*-methyl ester was added to EDC the inhibitory effect on the response to cholera toxin was diminished and the enhancement of the epinephrine stimulation was amplified. By itself glycine-*O*-methyl ester was slightly inhibitory for either system. Of the other carbodiimides CMC weakly and DCC strongly inhibited cellular responsiveness to both toxin and epinephrine.

A nondiscriminate inhibitory effect on the response to cholera toxin and epinephrine was observed by treating the cells with the alkylating reagent ethylmaleimide.

3.2. *Binding of cholera toxin to modified cells*

The possibility that EDC affected the GM_1 ganglioside cell receptor for cholera toxin, was investigated. As shown in fig.1 there was no significant difference between untreated and EDC-modified cells in their binding of ^{125}I-labelled toxin.

3.3. *Bicarbonate requirement of EDC*

Since the cAMP stimulating ability of cholera toxin was selectively affected by EDC, the cell modifying ability of this reagent was investigated more closely. When pretreatment of cells with EDC was performed in phosphate-buffered saline instead of the RPMI-FCS tissue culture medium, the inhibitory action disappeared (table 2). Different components of the RPMI-FCS

Table 1
Specific inhibition of cAMP response to cholera toxin by cell modification with EDC[a]

Cell modifying chemical	Picomoles cAMP/10^7 cells in response to,			
	Cholera toxin	Epinephrine	PGE_1	No additive
None	300	122	340	6
Picryl sulfonic acid (1 mM)	400			6
Succinic anhydride (1 mM)	400			6
EDC (1 mM)	34	196	389	6
EDC (1 mM) + Glycine-*O*-methyl ester (5 mM)	170	332		6
Glycine-*O*-methyl ester (5 mM)	146	90		6
CMC (1 mM)	156	80		5
DCC (0.1 mM)	3	2		3
Sodium cyanide (1 mM)	6	6		6
Ethyl maleimide (1 mM)	3	3		3

[a] 10^7 thymocytes in 1 ml RPMI-FCS medium were treated with modifying chemicals and then washed. Thereafter the modified cells were suspended in fresh medium and incubated at 37°C with cholera toxin (10^{-9} M, 50 min), epinephrine (10^{-6} M, 25 min), prostaglandin E_1 (PGE_1; 10^{-4} M, 15 min) or with no stimulant (50 min). Intracellular cAMP was then determined.

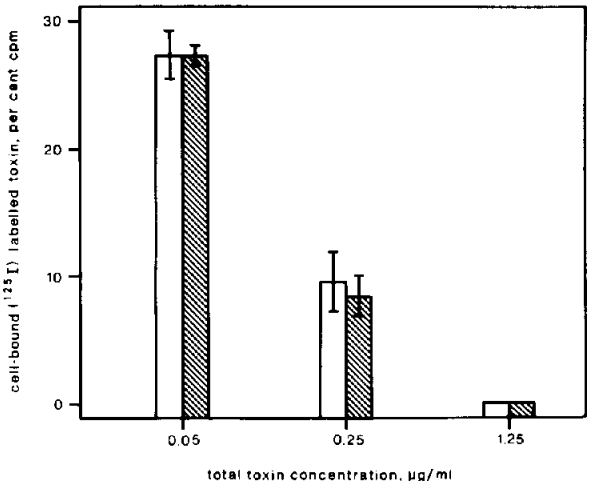

Fig.1. Binding of cholera toxin to untreated cells (open columns) and cells treated with 1 mM EDC (hatched columns) as tested using radiolabelled toxin in mixtures with various concentrations of unlabelled toxin. The means ± 2 SD are indicated.

Table 2
Requirement of bicarbonate for the inhibitory activity of EDC[a]

Cell modifying chemical	Picomoles cAMP/10^7 cells in response to cholera toxin
None	280
EDC alone	250
EDC together with:	
bicarbonate	26
magnesium	192
bicarbonate + magnesium	26
calcium	272
potassium	372
sulfate	280
formate	286
acetate	300
oxalate	274
benzoate	300
glycine	250

[a] 10^7 thymocytes in 1 ml phosphate-buffered saline were pretreated with 1 mM EDC together with 10 mM concentrations of various acids (added as sodium salts) or divalent ions (added as chloride salts). The modified cells were then suspended in fresh RPMI-FCS medium and incubated with cholera toxin (10^{-9} M, 37°C, 50 min) and intracellular cAMP was assayed.

medium like amino acids, vitamins, glucose and salts were investigated for a presumptive synergism with EDC in the inhibition of the cell response to cholera toxin. It was found that the only component which was able to restore the inhibitory action of EDC was sodium bicarbonate. Other salts of carbonic acids tried were ineffective as seen in table 2.

3.4. *EDC inhibition in relation to time and temperature*

When pretreatment of cells with EDC in RPMI-FCS medium was performed at different temperatures, the inhibitory effect on cholera toxin induction of cAMP was found to be highly temperature dependant (fig.2a). At 37°C the inhibition was almost complete, at 23°C slight and at 0°C nil.

Cholera toxin has in all cell types a characteristic lag period between binding and cAMP accumulation [10–12], which in thymus cells is 15–30 min [15]. In order to relate the kinetics of the cellular effect of cholera toxin with the inhibition of this effect by EDC, cells were incubated with the toxin for 50 min. EDC (1 mM) was supplied at 0, 5, 15, 30 and 50 min of this

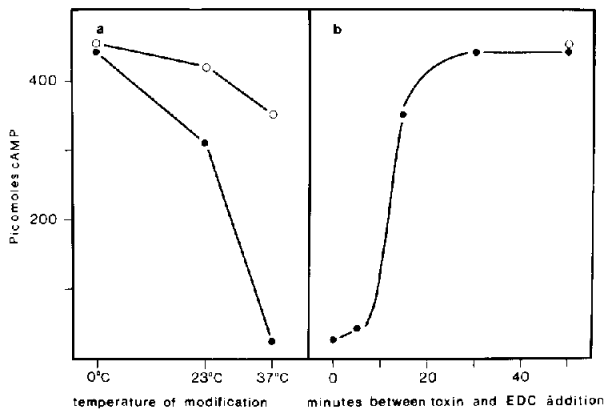

Fig.2. Effect of EDC on cAMP response to cholera toxin in relation to temperature of cell modification (a) or time interval between cell exposure to toxin and reagent (b). (a) 10^7 thymocytes in 1 ml RPMI-FCS medium were incubated at 0, 23 or 37°C for 30 min with (●——●) or without (○——○) 1 mM EDC. Then the treated cells were washed twice, supplied with fresh medium and incubated with 10^{-9} M cholera toxin at 37°C for 50 min. Thereafter intracellular cAMP was determined. (b) Samples with 10^7 thymocytes were incubated at 37°C for 50 min in 1 ml RPMI-FCS medium containing 10^{-9} M cholera toxin. At various times during this incubation 1 mM EDC was added. Intracellular cAMP was determined after completion of the incubation.

incubation. Fig.2b shows that the inhibitor was still effective when supplied 5 minutes after the toxin but not after 30 or 50 min. Thus, EDC inhibits the cellular cAMP response to cholera toxin provided that it is applied early in the toxin's own lag period.

4. Discussion

Cholera toxin and several hormones including epinephrine stimulate cells in a somewhat similar manner. After an initial binding of the agents to specific cell membrane receptors adenylate cyclase is activated, in the case of toxin stimulation with a typical 'lag' period. The ensuing cAMP formation thereafter via specific protein kinases influences various cellular processes.

The binding of cholera toxin to cell membrane G_{M1} ganglioside receptors takes place within a few minutes [4,16]. The significant delay between binding and activation of adenylate cyclase has suggested that intermediary processes are required to translate the initial binding event into an activating signal. The present study was undertaken in an approach to recognize by interference with its function, a possible membrane component of importance for such an intermediary signal.

The fat-soluble carbodiimide DCC has been described to inhibit several cell metabolic processes [17–19]. In our systems DCC inhibited adenylate cyclase stimulation by cholera toxin as well as epinephrine, i.e. it did not interfere with a signal unique for the toxin. A specific inhibition of toxin-induced cAMP was, however, obtained by treatment of cells with EDC, another carbodiimide, in the presence of bicarbonate. The basic levels of cAMP were unchanged and the cAMP response to epinephrine as well as to prostaglandin E_1 slightly potentiated by the treatment indicating that the cyclase enzyme was not inhibited. Further, the binding of cholera toxin to the cell surface was unchanged by the treatment indicating intact binding activity of the G_{M1} ganglioside receptors. The requirement of bicarbonate for the inhibitory action of EDC is as yet unexplained. Possibly bicarbonate reacts with the carbodiimide to form an unstable derivative with enhanced membrane permeability or altered specificity.

The pronounced temperature dependence of the inhibitory activity of EDC-bicarbonate might suggest that a 'fluid-penetrable' cell membrane is required to enable contact between the reagent and the site which is modified. Since the inhibition was evident only during the lag period of the toxin action the EDC-bicarbonate treatment probably affects a membrane structure involved in translating the toxin binding into an adenylate cyclase activating signal. The interpretation of the presented data is compatible with a proposed scheme of interactions of cholera toxin subunits with cell membrane structures [4]. According to this model the initial binding of the toxin by L subunit to G_{M1} receptors opens up a previously hidden membrane structure with which the toxin H subunit can interact. The resulting modulation of this membrane component then leads to activation of adenylate cyclase, either directly via a mechanical configurational change or indirectly via intermediary messengers.

To summarize, we claim to have blocked a cholera toxin specific 'signal' required for activation of adenylate cyclase in thymus cells and propose that this inhibition was due to a chemical modification of a membrane component functionally bridging the G_{M1} ganglioside receptors with adenylate cyclase. In addition to the implications for understanding the mechanism of action of cholera toxin and of adenylate cyclase activation, the study suggests a possible level for specific prophylactic-therapeutic interference with cholera disease.

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