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SEQUENCE OF EVENTS IN THE ACTIVATION OF ADENYLATE CYCLASE BY CHOLERA TOXIN

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Summary

The toxin of Vibrio Cholera causes fluid secretion from the small intestine by stimulation of adenylate cyclase and elevation of intracellular cyclic AMP concentrations. The toxin is a protein composed of subunits responsible for binding to cell membranes and a subunit responsible for the activation of adenylate cyclase. The binding subunits (B) are non-covalently bonded to the active subunit (A). The latter is composed of two polypeptides A_1 and A_2 linked by a disulphide bridge. Exposure of the intestine to toxin results in rapid binding to the brush border membrane. Thence follows a gradual increase in adenylate cyclase activity, and stimulation of electrolyte and fluid secretion. Enzyme localization studies show that the brush border does not contain adenylate cyclase. Thus the stimulation of adenylate cyclase by toxin which interacts with the brush border must be indirect. From recent studies it seems that an activator of adenylate cyclase can be found in cytosol from toxin-treated cells. Incubation of toxin with cytosol or dithiothreitol results similarly in the formation of an activator. Preincubation of toxin with cytosol results in more rapid activation of adenylate cyclase in liver cell membranes than direct addition of cytosol and toxin. Preincubation of cholera toxin for activation, by cytosol, is presumed to be due to the splitting of the disulphide bond between the A_1 and A_2 components of the active subunit. The stimulatory ability resides in A_1 and both A_1 and NAD are required for the activation of adenylate cyclase. The toxin-stimulated adenylate cyclase has similar characteristics to the enzyme stimulated by non-hydrolysable analogs of GTP such as guanylylimidodiphosphate (GppNHp). Stimulation by either cholera or GppNHp is irreversible, the responses to catecholamines are enhanced and the enzyme can be solubilized in the activated state.

From this and evidence of mutual interference in the actions of toxin and guanylylimidodiphosphate it is suggested that the toxin affects the nucleotide regulatory site of adenylate cyclase.

Zusammenfassung

Das Toxin von Choleravibrionen löst durch Stimulation der Adenylatzyklase und Erhöhung der intrazellulären Konzentration an diesem AMP eine Flüssigkeitssekretion im Dünndarm aus. Das Toxin ist ein Protein, das aufgebaut ist aus Untereinheiten, die verantwortlich sind für die Bindung an Zelleinheiten und einer anderen Untereinheit, die verantwortlich ist für die Aktivierung der Adenylatzyklase. Die Bindungs-Untereinheiten (B) sind nicht-kovalent verbunden mit der aktiven Untereinheit (A). Die Letztere setzt sich zusammen aus zwei Polypeptiden A_1 und A_2, die durch eine Disulfidbrücke verbunden sind. Einwirkung von Toxin auf den Darm resultiert in einer raschen Bindung an die Bürstensaummembran. Darauf folgt ein allmählicher Anstieg der Adenylatzyklaseaktivität und eine Stimulation der Elektrolyt- und Flüssigkeitssekretion. Untersuchungen über die Enzymlokalisation zeigen, dass der Bürstensaum keine Adenylatzyklase enthält. Folglich muss die Stimulation der Adenylatzyklaseaktivität durch das Toxin, welches mit dem Bürstensaum reagiert, indirekt sein. Nach neueren Untersuchungen scheint es, dass ein Aktivator der Adenylatzyklase sich im Cytosol toxinbehandelter Zellen findet. Inkubation von Toxin mit Cytosol oder Dithiothreitol resultiert in ähnlicher Weise in der Bildung eines Aktivators. Präinkubation von Toxin mit Cytosol resultiert in einer schnelleren Aktivierung der Adenylatzyklase in Leberzellmembranen als direkter Zusatz von Cytosol und Toxin. Präinkubation von Choleratoxin zur Aktivierung durch Cytosol wirkt vermutlich durch die Spaltung der Disulfidbrücke zwischen den A_1- und A_2-Komponenten der aktiven Untereinheit. Die Fähigkeit zur Stimulation ist der Komponente A_1 eigen, und sowohl A_1 als NAD sind erforderlich für die Aktivierung der Adenylatzyklase. Die toxinstimulierte Adenylatzyklase hat eine ähnliche Charakteristik wie das durch das nicht hydrolysierbare Analogum von GTP, wie z.B. Guanylimidodiphosphat (GppNHp), stimulierte Enzym. Stimulation sowohl durch Choleratoxin als durch GppNHp ist irreversibel; die Empfindlichkeit gegenüber Katecholaminen ist erhöht und das Enzym kann im aktiven Zustand solubilisiert werden. Aus diesen Befunden und aus solchen über wechselseitiger Interferenz zwischen den Aktionen von Toxin und Guanylylimidodiphosphat wird angenommen, dass das Toxin die nukleotid-regulatorische Stelle der Adenylzyklase beeinflusst.

Cholera toxin stimulates fluid secretion in the small intestine without obvious damage to the intestinal cells (LEITCH et al., 1967; SACK and CARPENTER, 1969; BANWELL et al., 1970). At high rates of secretion the major structural change in the intestine is closure of intercellular spaces (DI BONA et al., 1974). It appears that cholera toxin is rapidly bound to the intestinal cells so that only brief exposure to toxin is required to elicit a progressive increase in fluid secretion which reaches maximal values at 4 - 6 hours and which, in dogs, persists for more than 24 hours (GUERRANT et al., 1972). It is known that the secretion of fluid is caused by increased intracellular concentrations of cyclic AMP (SCHAFER et al., 1970) generated by stimulation of adenylate cyclase (SHARP and HYNIE, 1971; KIMBERG et al., 1971).

The toxin is a protein of M.W. estimated at 84,000. It can be separated into its constituent subunits by treatment with urea or sodium dodecyl sulfate (LO SPALLUTO and FINKELSTEIN, 1972; LONNROTH and HOMGREN, 1973; van HEYNINGEN, 1973; FINKELSTEIN et al., 1974; MENDEZ et al., 1975; HOLMGREN and LONNROTH, 1975; KUROSKY et al., 1976; OHTOMO et al., 1976). As currently understood, the toxin consists of subunits (B) responsible for binding to cell membranes and an active subunit (A) which contains the adenylate cyclase activating ability. The subunits are non-covalently bonded and the active subunit A consists of two polypeptide chains, designated A_1 and A_2, connected by a single disulphide bond (LAI et al., 1976; KUROSKY et al., 1976). Amino acid sequencing studies are progressing so that knowledge of the complete structure of the toxin may soon be available. This would solve the discrepancies in the literature regarding molecular weights for the different subunits and components of the toxin (see Table 1).

The B subunits are responsible for a highly specific binding of the toxin to ganglioside GM_1 on the cell surface (van HEYNINGEN, 1974; KING and van HEYNINGEN, 1973; HOLMGREN et al., 1973; CAUTRECASAS, 1973; van HEYNINGEN, 1974a). In the intestinal cells, adenylate cyclase is localized in the basal and lateral membranes (PARKINSON et al., 1972). As the toxin is taken up by the brush border membranes, which do not contain adenylate cyclase, then the activation of adenylate cyclase must be an indirect process. Either the active subunit is translocated from the brush border to the basal and lateral membranes or transmission of a signal to the basal and lateral membranes must occur. A considerable amount of information is available about the binding of toxin to cell membranes, the initial phase of its action. Similarly much is known about the activated adenylate cyclase, the final phase of toxin action (CHEM et al., 1971, 1972; SHARP et al., 1973).

Table 1. Estimated molecular weights for subunits of cholera toxin

B	A	A_1	A_2	
10,000	28,000	23,000		Finkelstein et al., 1972
8,000	28,000			Lonnroth, I. and Holmgren, J., 1973
8,000	36,000	27,000	8,000	Cuatrecasas et al., 1973
8,000	28,000			Staerk et al., 1974
15,000	25,000			van Heyningen, 1974 (a)
9,000	30,000			Mendez et al., 1975
10,000		22,000	7,500	Mendez et al., 1975
14,000	27,000	22,000	5,000	van Heyningen, 1976
9,500		20,000	7,500	Lai et al., 1976
11,000	28,000	23,000	2,500	Ohtomo et al., 1976
9,700		24,000	9,700	Kurosky et al., 1976
10,600	29,000	23,500	5,500	Gill, 1976
8,500	27,000	21,000	7,500	Lipson and Sharp, 1976

Little information exists on the intermediate stages of the action of toxin, i.e. the period between initial binding to the brush border membrane and the stimulation of adenylate cyclase. Some experiments designed to investigate this little known area will be described here, experiments which have been made possible by an in vitro broken cell system for the activation of adenylate cyclase by cholera toxin. From 1970, when the action on adenylate cyclase was discovered, until 1975, the action of cholera toxin was studied on intact cells. This was because cholera toxin failed to stimulate adenylate cyclase in homogenates or purified membrane preparations. With the discovery by GILL (1975) that NAD was an essential cofactor it became possible to stimulate adenylate cyclase in broken cell systems. Several broken cell systems have been studied (FLORES et al., 1976; FLORES and SHARP, 1975; GILL and KING, 1975) and rapid progress in unravelling the action of cholera toxin is being made.

In investigations on the stages between binding of cholera toxin to cell membranes and activation of adenylate cyclase we have looked for an intracellular activator of the enzyme. For this purpose rats were injected intravenously with either cholera toxin (test) or saline (controls). Four hours after the injections the rats were anesthetized and their livers perfused to remove blood. The livers were then rapidly removed and homogenized. Cytosol preparations were made by centrifugation at 100,000 x g. These preparations were found to be free from adenylate cyclase activity. From a third control rat, a particulate (adenylate cyclase)

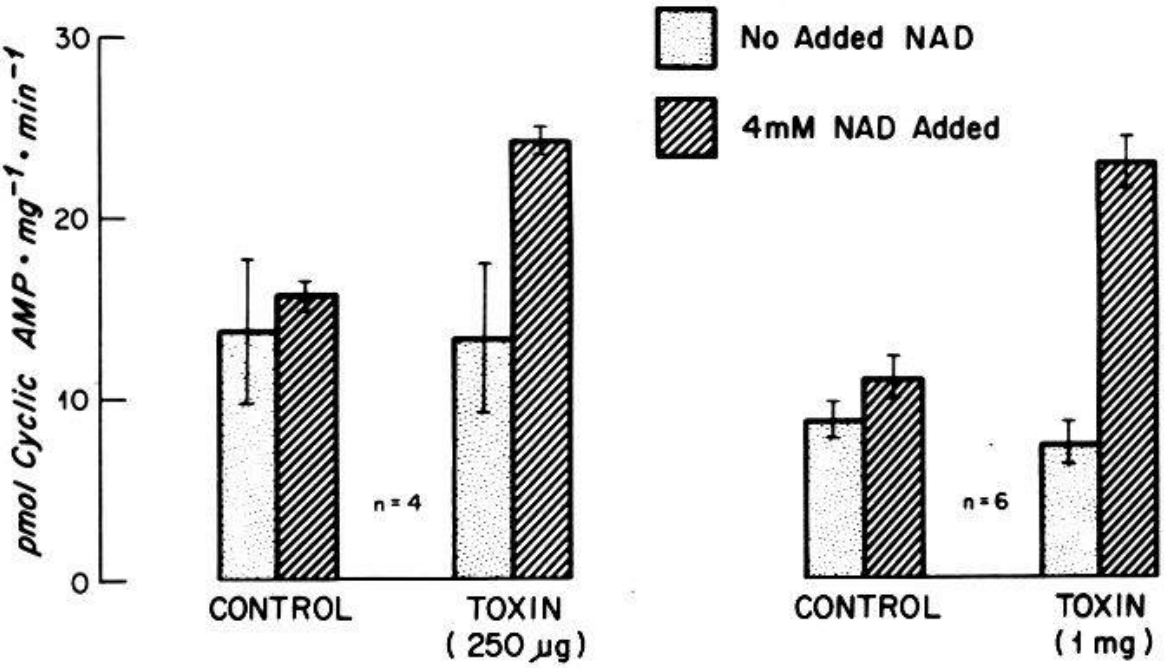

Figure 1. Activation of adenylate cyclase by cytosol from liver cells of rats treated with cholera toxin in vivo.
Rats were treated with either 250 µg or 1 mg of cholera toxin injected intravenously. Control rats were injected with saline. Four hours after the injections cytosol preparations were made from the livers of the toxin treated and control rats. These cytosols were then added to a particulate preparation from a third rat liver in the presence and absence of NAD to test for the ability to stimulate adenylate cyclase.

preparation was made by homogenization, centrifugation at 1200 x g, and resuspension of the pellet in Tris-$MgCl_2$ buffer. To test whether the cytosol from toxin-treated rat liver contained an activating factor, the two cytosol preparations (test and control) were mixed with the particulate preparation from the third rat, in the presence and absence of NAD. The mixtures were incubated for 10 minutes at 22°C. To stop the reaction an excess of cold buffer was added and then the whole was centrifuged at 1200 x g. The pellets were resuspended in fresh buffer and assayed for adenylate cyclase activity. It was found that cytosol from toxin-treated rat liver stimulated adenylate cyclase in the presence, but not in the absence of NAD. Cytosol from the control rat had no effect on adenylate cyclase activity in the presence or absence of NAD. Thus the cytosol from toxin-treated rat liver contains an activating factor (see fig. 1). Further studies will be required to determine whether this is subunit A, whether it was inserted into the cytosol as a consequence of toxin binding to the cell membranes or whether it is an artefact of the preparation of cytosol.
Following this demonstration, tests were performed to see if cytosol could generate an activating factor from cholera toxin. It was already known that cholera toxin required both NAD and a cytosol component for stimulation of adenylate cyclase, but the precise sequence

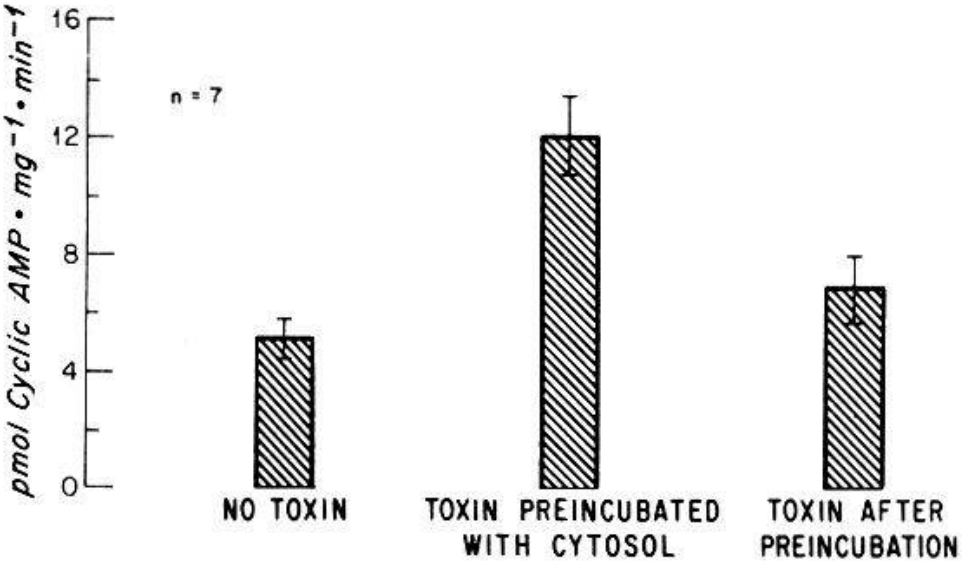

Figure 2. Effect of preincubations of cholera toxin with cytosol on the rate of activation of adenylate cyclase.
Three cytosol preparations were preincubated for 15 minutes with 4mM NAD. To one, no further additions were made; to the second, cholera toxin was added at the beginning of the preincubation period and to the third, cholera toxin was added at the end of the preincubation period. At 15 minutes, all the mixtures were added to a particulate preparation of rat liver. Adenylate cyclase activity in this preparation was assayed after 5 minutes with the cytosol mixtures.

of events was unknown. Thus initially it was reasoned that if cytosol alone could generate an activating factor from cholera toxin, then preincubation of toxin with cytosol should produce in the mixture a factor which with NAD would more rapidly activate adenylate cyclase than the simultaneous mixing of cytosol, NAD, cholera toxin and an adenylate cyclase containing membrane preparation. Thus, cytosol was prepared from a rat liver and divided into three portions. To one of these portions, cholera toxin was added and incubated for 15 minutes at room temperature. To the second cytosol was added cholera toxin at 15 minutes and to the third no addition was made. At 15 minutes all three preparations were added to a rat liver particulate preparation in the presence of NAD. The mixture was assayed for adenylate cyclase activity at 5 minutes. From the results shown in figure 2 it can be seen that the toxin preincubated with cytosol produced a more rapid activation than the toxin not preincubated with cholera toxin. Thus either cytosol "prepared" the cholera toxin for activation or an activating factor was generated during the interaction of cholera toxin and cytosol.

Further studies have shown that dithiothreitol can replace cytosol in the generation of the activating factor and that the active subunit A_1 with NAD can stimulate adenylate cyclase.

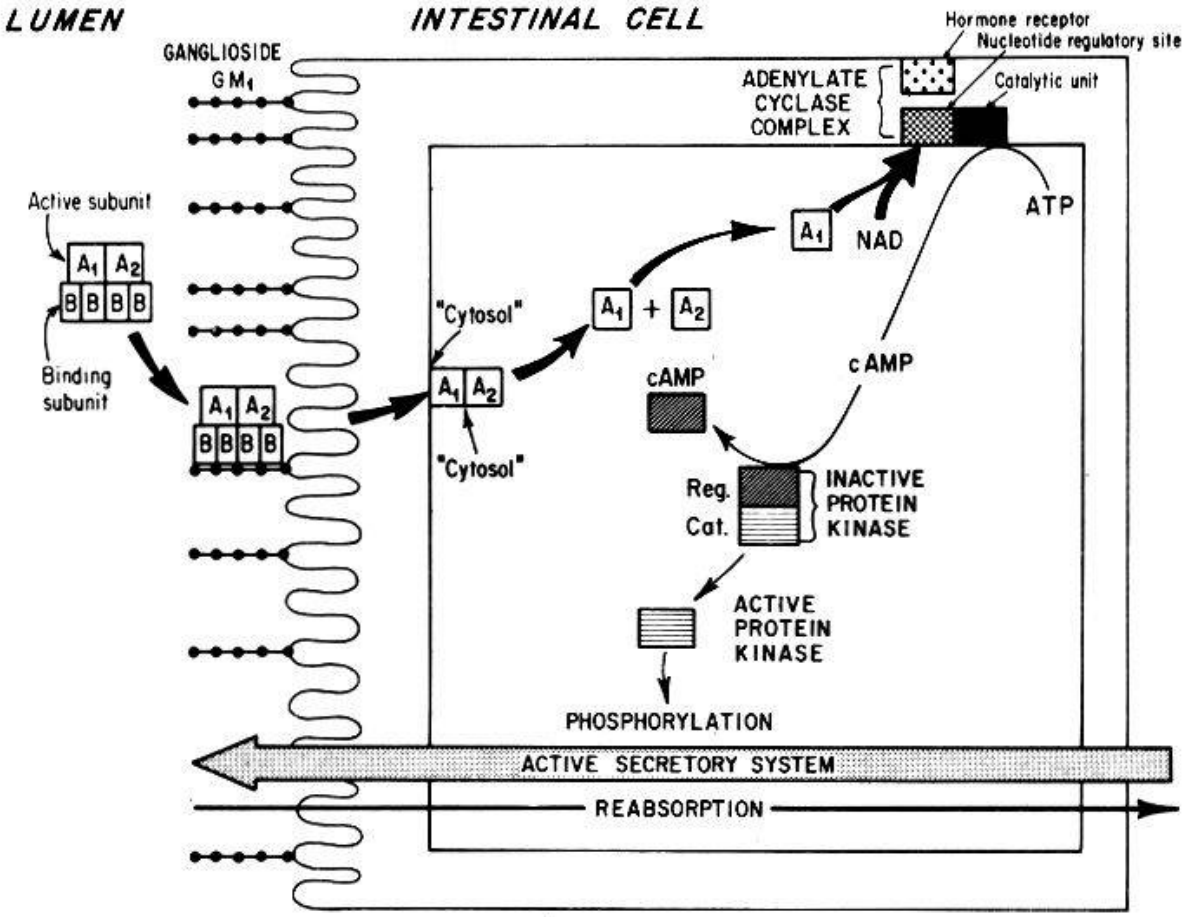

Figure 3. Outline of the possible mechanism by which cholera toxin stimulates adenylate cyclase.

Thus it appears that the activation of toxin is a splitting of disulphide bonds, i.e. the separation of A_1 from A_2.

From these and other studies it is possible to put forward an outline of the action of cholera toxin. The initial interaction of the toxin with the cell is via the B subunits and gangliosides GM_1 at the cell surface. This must result in the A subunit gaining access to the cell interior. Here the A subunit is separated from the B subunits and A_2 is cleaved from A_1. Following its liberation, A_1 in conjunction with NAD activates adenylate cyclase. The mechanism of this activation is still not understood. However there are indications that its site of action on the adenylate cyclase complex involves the so called nucleotide regulatory site.

Since our report that cholera toxin caused an enhancement of the stimulatory effect of catecholamines on adenylate cyclase and that the activated enzyme could be solubilized with detergents in the activated state (BECKMAN et al., 1974) we have been impressed by the

similarities of the effects of cholera toxin and certain guanyl nucleotides on the enzyme. After the early report of the effect of GTP on adenylate cyclase (RODBELL et al., 1971) a number of laboratories have found that guanosine triphosphate (GTP) enhances the effect of hormones on the enzyme and can stimulate basal activity. Non-hydrolysable analogues of GTP such as guanylylimidodiphosphate (GppNHp) and guanylylmethylenediphosphate ($GppCH_2p$) have a similar but greater effect than GTP on both basal and hormonal-stimulated adenylate cyclase activity, presumably because of the stability of the terminal phosphate group. These stimulatory effects are irreversible, resisting dilution, washing or solubilization (PFEUFFER and HELMREICH, 1975). Interference of the stimulatory effect of sodium fluoride by GTP or GppNHp has also been reported. Thus the effects of cholera toxin and the analogues of GTP are similar in that they both stimulate adenylate cyclase; they both enhance the effects of catecholamines; they both produce an essentially irreversible stimulation of the enzyme which can then be solubilized in the activated state. Because of these similarities we examined the interrelationship of the effects of cholera toxin and GppNHp (FLORES and SHARP, 1975). It was found that stimulation of adenylate cyclase by the non-hydrolysable analogue of GTP, GppNHp, blocks the stimulatory effect of cholera toxin. Furthermore, the stimulatory effect of cholera toxin could be inhibited by subsequent addition of GppNHp. This mutual interference in the stimulatory effects of the two agents suggests a common site of action at least at some stage of their mechanisms of activation of adenylate cyclase. This site may well be the nucleotide regulatory site. Thus this hypothesis is included in the final summary (figure 3) of the action of cholera toxin.

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