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Activation by Cholera Toxin of Adenylate Cyclase Solubilized from Rat Liver

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Cholera toxin, or peptide A1 from the toxin, activates adenylate cyclase solubilized from rat liver with Lubrol PX, provided that cell sap, NAD^+, ATP and thiol-group-containing compounds are present. The activation is abolished by antisera to whole toxin, but not to subunit B.

Cholera toxin activates adenylate cyclase in the plasma membrane of a wide variety of eukaryotic cells. The toxin has two types of subunit (van Heyningen, 1974; Finkelstein *et al.*, 1974; Lönnroth & Holmgren, 1975; Sattler *et al.*, 1975): subunit B binds irreversibly to a ganglioside (G_{M1}) in the outer plasma membrane (van Heyningen, 1974; van Heyningen *et al.*, 1971; Cuatrecasas, 1973), whereas subunit A (which consists of two polypeptide chains A_1 and A_2 joined by a disulphide bond) activates the enzyme. Purified subunit A has low activity with some intact cells, but is more active in broken cells (van Heyningen & King, 1975; Gill & King, 1975; Bitensky *et al.*, 1975; Sahyoun & Cuatrecasas, 1975): this suggests that it must enter the cell before it can interact with the adenylate cyclase. It has been thought, however, that some interaction with the membrane was essential for the activity of the toxin, and membrane fragments have always been included in experiments. I show in this present paper that membrane fragments are not essential. Toxin (as well as subunit A or peptide A_1) activates a detergent-solubilized adenylate cyclase from rat liver. Unidentified cytoplasmic components are also required.

Some experiments (Mullin *et al.*, 1976; Ledley *et al.*, 1976) suggest that cholera toxin may have structural and mechanistic similarities to some of the glycoprotein hormones such as thyrotropin. Results with cholera toxin may therefore have a wide significance.

Methods

Preparation of the enzyme

The livers of freshly killed rats were perfused *in situ* with cold 0.13M-KCl/0.02M-potassium acetate, pH7.4, removed, washed in 0.01M-Tris/HCl buffer, pH7.4, and then homogenized with ten strokes of a Dounce homogenizer in 5ml of 0.01M-Tris/HCl/5mM-EDTA/0.25% sucrose/20mM-$MgCl_2$ (pH7.4)/2g of liver. The mixture was centrifuged for 10min at 1000*g*. The supernatant from this centrifugation was removed, further centrifuged for 30min at 100000*g*, then made 0.5% with respect to Lubrol PX [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.], and 5mM each with respect to NAD^+, ATP and dithiothreitol (neutralized with NaOH). The low-speed pellet was washed once in the homogenization buffer and then sonicated for 15s in the above high-speed supernatant containing Lubrol and cofactors by using a Rapidis 150 sonicator (Ultrasonics). This mixture was further centrifuged for 30min at 100000*g*. The supernatant (which was visibly clear) was used as the soluble adenylate cyclase preparation after removal of Lubrol by incubation for 30min at 0°C in the presence of Amberlite XAD-2 (Beckman *et al.*, 1974). All these operations were carried out at 2°C.

Incubations and assay

Samples (50μl) of the solubilized enzyme were incubated for 5min at 15°C with 10μl of a solution of toxin [prepared by the method of Finkelstein & LoSpalluto (1970) and kindly donated by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, U.S.A., for whom it is prepared under contract], or of peptide A_1 [prepared by gel filtration in 1M-urea (van Heyningen, 1976)] diluted with 0.1M-Tris/HCl/0.2M-NaCl/serum albumin (1mg/ml), pH7.0. Then 40μl of this solution was incubated usually for 30min at 15°C with 60μl of assay medium containing 0.1M-Tris/HCl, 3mM-EDTA, 10mM-$MgCl_2$, 0.2mM-dithiothreitol, 2mM-ATP, 0.5mM-papaverine, 0.2% albumin and an ATP-regenerating system (12mM-phosphoenolpyruvate, 50μg each of pyruvate kinase and adenylate kinase/ml) adjusted to pH8 (at 15°C). The reaction was stopped by boiling the solution, and triplicate 20μl samples were removed for assay of cyclic AMP. Each enzyme assay was done at least in duplicate. Production of cyclic AMP was linear with time up to 1h. Cyclic AMP was assayed essentially by the method of Brown *et al.* (1971), by using

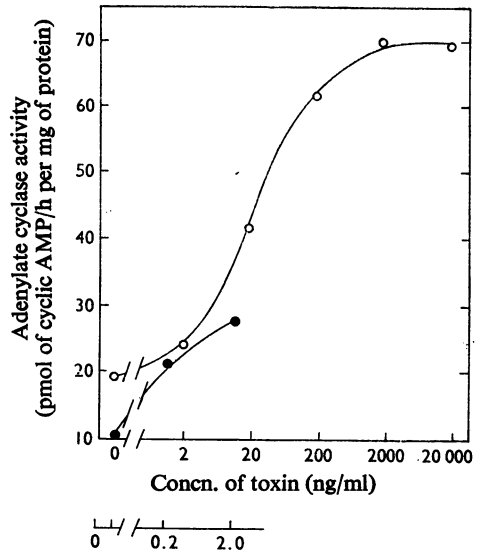

Fig. 1. *Effect of preincubating different concentrations of toxin* (○) *and of peptide* A_1 (●) *with solubilized adenylate cyclase for* 5 *min at* 15°C, *followed by assay for* 30 *min at* 15°C

Experimental details are given in the text.

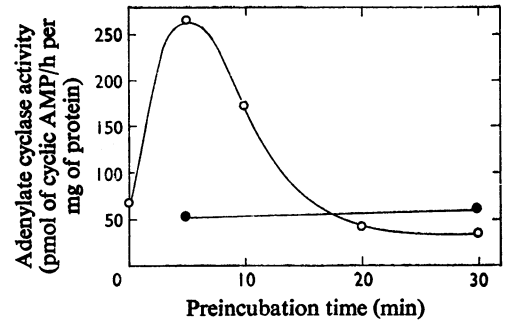

Fig. 2. *Time-course showing the effect of preincubation at* 37°C *in the presence* (○) *and absence* (●) *of* 0.1 *mg of toxin/ml on the activity of solubilized adenylate cyclase assayed for* 15 *min also at* 37°C

Experimental details are given in the text.

a binding-protein preparation from bovine adrenals. The assay was calibrated with standard cyclic AMP and showed a linear response in the range 1–15 pmol; no determinations were made outside this range. Protein concentrations were determined with the Folin reagent (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Results

Adenylate cyclase was solubilized from rat liver with Lubrol PX by a modification of the method of Beckman *et al.* (1974). The crucial point of this modification was that the solubilization of membrane fragments was carried out, not in buffer, but in cell sap that had been freed from lysosomes and other organelles, but to which had been added certain extra cofactors (NAD^+, ATP and dithiothreitol) that seem to be required for toxin activity (Gill, 1975; Flores *et al.*, 1976; Bitensky *et al.*, 1975). The solubilized enzyme (a clear 100000*g* supernatant) was active, and this activity was increased by toxin or purified peptide A_1, as shown in Fig. 1 (as well as by free subunit A), at concentrations down to 20 ng of toxin/ml (about 0.2 nM). Enzyme solubilized in buffer alone (and in the presence or absence of the low-molecular-weight cofactors) had about the same specific activity as that solubilized in the cell sap, but was not affected by toxin, subunit or peptide.

The activation of cyclase was rapid, and had reached a maximum within 5 min at 15°C. At higher temperature (37°C) the activation was more pronounced, but the enzyme was unstable, so that results were sometimes more difficult to reproduce. Further, as shown in Fig. 2, longer incubation sometimes led to decreased activation and even deactivation compared with controls, suggesting that the activated enzyme was rapidly destroyed at 37°C.

The activation by toxin and peptide was not affected by preincubation with ganglioside G_{M1} (which is presumably concerned only with the binding of subunit B to membranes) or with antisera specific for subunit B. But it was decreased by incubation with an antiserum to whole toxin [which reacted weakly with subunit A on immunodiffusion (van Heyningen, 1976)]. Activation was abolished by preincubation with sufficient excess of antiserum to subunit A [prepared by treating the antitoxin with excess of subunit B (van Heyningen, 1976)]. Subunit B ('choleragenoid'; see Finkelstein *et al.*, 1974) had no effect. The solubilized enzyme was activated by 10 mM-NaF to about the same extent as by toxin, and the fluoride-activated enzyme was not further activated by toxin.

Discussion

The experiments described in the present paper show that cholera toxin is active in solution free of membrane fragments. The adenylate cyclase preparation used was 'solubilized' in the conventional

sense (see, for example, Beckman *et al.*, 1974; Sahyoun & Cuatrecasas, 1975) in that it was a clear 100000*g* supernatant from which excess of detergent had been removed. This does not necessarily indicate that the preparation was entirely free of some bound lipid or detergent. Indeed the latter may well be required to keep the enzyme in solution. However, the preparation is undoubtedly free of any sort of membrane fragments that retain their original integrity.

An effect of toxin on broken cells (with intact membrane fragments remaining) has been reported with several tissues, including rat liver [where the concentration of toxin used was comparable with that used in this present work, and some soluble components were also required (Flores *et al.*, 1976)]. But the fact that there is activity in the absence of membrane fragments is added evidence for the idea (van Heyningen & King, 1975; Sahyoun & Cuatrecasas, 1975; Gill, 1976) that the main action of the toxin is by attack of subunit A on adenylate cyclase inside the cell. Subunit A is active by itself, and binding of subunit B to the membrane, although apparently a prerequisite for significant activity towards whole cells, is not an essential part of the activation of the enzyme. Since integral pieces of membrane are not required, the lateral movement of the toxin–ganglioside complex in the membrane (see Revesz & Greaves, 1975) is not directly involved with the activation.

Although membrane fragments are inessential for activation, these experiments confirm the need for soluble factors in the cytoplasm either to activate the toxin or to prevent its inactivation, since the toxin was inactive in buffer alone. There is not necessarily a direct reaction between toxin and adenylate cyclase: there might be a preliminary reaction with some other component which then reacts with the enzyme. The solubilized enzyme may well consist of some intact multi-enzyme complex having regulatory as well as catalytically active components. Under the conditions of these experiments, there will be some dissociation of toxin into free subunit A and an aggregate of subunits B (van Heyningen, 1976; Gill, 1976), so that the observed activation may be actually due directly to free subunit A (or peptide A_1 liberated by thiol-group-containing compounds) rather than to intact toxin.

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