Studies on the Time Course and Rate-Limiting Steps in the Activation of Adenylate Cyclase in Rat Liver by Cholera Toxin

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Cholera toxin stimulates adenylate cyclase in rat liver after intravenous injection. The stimulation follows a short latent period of 10 min, and maximum stimulation was attained at 120 min. Half-maximal stimulation was achieved at 35 min. In contrast with this lengthy time course in the intact cell, adenylate cyclase in broken-cell preparations of rat liver *in vitro* were maximally stimulated by cholera toxin (in the presence of NAD^+) in 20 min with half-maximal stimulation in 8 min. Binding of cholera toxin to cell membranes by the B subunits is followed by translocation of the A subunit into the cell or cell membrane, and separation of the A_1 polypeptide chain from the A_2 chain by disulphide-bond reduction, and finally activation of adenylate cyclase by the A_1 chain and NAD^+ . As the binding of cholera toxin is rapid, two possible rate-limiting steps could be the determinants of the long time course of action. These are translocation of the A_1 chain from the outside of the cell membrane to its site of action (this includes the time required for separation from the whole toxin) or the availability of NAD^+ for activation. When NAD^+ concentrations in rat liver were elevated 4-fold, by the administration of nicotinamide, no change in the rate of activation of adenylate cyclase by cholera toxin was observed. Thus the intracellular concentration of NAD^+ is not rate-limiting and the major rate-limiting determinant in intact cells must be between the time of toxin binding to the cell membrane and the appearance of subunit A_1 at the enzyme site.

Cholera toxin stimulates adenylate cyclase in most mammalian cells (Sharp & Hynie, 1971; Kimberg *et al.*, 1971; Sharp, 1973; Finkelstein, 1973). After exposure of the cells to toxin it is known that specific binding of the toxin to ganglioside GM_1 on the cell surface occurs rapidly and irreversibly (King & van Heyningen, 1973; Cuatrecasas, 1973; Staerk *et al.*, 1974; S. van Heyningen, 1974; W. E. van Heyningen, 1974; Moss *et al.*, 1976a). Contact of the cells with toxin for only a few minutes results, after a short latent period, in a gradual and progressive increase in the activity of adenylate cyclase (Guerrant *et al.*, 1972). Direct stimulation of adenylate cyclase by attachment of the toxin to the cell membranes was ruled out by the finding that the brush-border membranes of small intestine, the site of initial toxin–cell interaction in the disease state, do not contain adenylate cyclase (Parkinson *et al.*, 1972). The toxin, a protein of some 84000 mol.wt. (LoSpolluto & Finkelstein, 1972), can be separated into its subunits by treatment with urea or sodium dodecyl sulphate (Lonnroth & Holmgren, 1973;

Finkelstein *et al.*, 1974; Mendez *et al.*, 1975; Gill, 1976; Kurosky *et al.*, 1977; Lai *et al.*, 1976; van Heyningen, 1976; Ohtomo *et al.*, 1976). The ‘light’ subunits (B) with a mol.wt. of 11604 (Lai, 1977; Kurosky *et al.*, 1977) are responsible for binding to membrane gangliosides. The ‘heavy’ subunit (A) with a mol.wt. of approx. 30000 is responsible for the activation of adenylate cyclase. Subunit A consists of two polypeptide chains joined by a single disulphide bond, which are referred to respectively as A_1 (which with NAD^+ can alone activate adenylate cyclase in broken-cell preparations) and A_2 .

It is thought that, after the B subunits bind to membrane ganglioside GM_1 , either subunit A crosses the membrane (separately or with whole toxin) and is reduced to the A_1 and A_2 chains or the reduction of the A_1 and A_2 chains at the membrane allows the A_1 chain to separate from the B components, after which it crosses the membrane and is free to stimulate adenylate cyclase by a reaction involving NAD^+ (Gill, 1975; Flores *et al.*, 1976; Martin *et al.*, 1977). The activation process may be an ADP-ribosylation (Moss *et al.*, 1976b) resulting in inhibition of a guanosine triphosphatase in the membrane (Levinson & Blume, 1977) and consequent dependent stimulation of adenylate cyclase (Levitzki, 1977). To define the

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rate-limiting steps in the action of cholera toxin the following information has been sought: (1) the time course of activation of adenylate cyclase in intact relative to broken cells; (2) the time required to separate subunit A from subunit B in cytosol; (3) the time required for reduction of subunit A to the A_1 and A_2 chains in cytosol; (4) the possible rate limitation of NAD^+ concentrations *in vivo*; and (5) an indirect estimate of the time taken for translocation of subunit A as the A_1 chain across the membrane. For these studies we have used rat liver, a tissue that has a well-characterized adenylate cyclase and that has been used for investigating the action of cholera toxin (Beckman *et al.*, 1974; Flores *et al.*, 1976; Martin *et al.*, 1977).

Methods and Materials

Activation of adenylate cyclase in vivo

Female Sprague-Dawley rats were injected, under diethyl ether anaesthesia, with 100 μ g of cholera toxin in 0.5 ml of Tyrode-Ringer solution (Beckman *et al.*, 1974) via a polyethylene catheter into the right jugular vein. The rats were left for different times up to 3 h. At the appropriate time they were anaesthetized again and the livers perfused *in situ* through the portal vein with ice-cold 75 mM-Tris/HCl/25 mM-MgCl₂, pH 7.4, to remove blood. Portions (0.5–1 g) of liver were homogenized in 10 vol. of ice-cold Tris/HCl/MgCl₂ buffer by ten strokes of a Dounce homogenizer. The homogenates were centrifuged for 5 min at 1200g at 4°C and the supernatants removed. The pellets were resuspended in 10 vol. of Tris/HCl/MgCl₂ buffer with five strokes of the Dounce homogenizer, and this suspension was diluted 6-fold with the Tris/HCl/MgCl₂ buffer for assay of adenylate cyclase activity. The enzyme was assayed by the method of Krishna *et al.* (1968) as described for studies on rat liver (Flores & Sharp, 1975; Flores *et al.*, 1976). The amount of protein in the incubation mixture varied between 15 and 25 μ g, and the incubation was carried out for 5 min at 37°C.

Activation of adenylate cyclase in rat liver particulate preparation

The particulate preparation was made by homogenizing 1 g of perfused rat liver in 10 ml of the Tris/HCl/MgCl₂ buffer in a Dounce homogenizer and centrifuging at 1200g for 5 min. The pellet was resuspended in 10 ml of fresh buffer, centrifuged again and resuspended in 10 ml of buffer. The cytosol preparation used was prepared by homogenizing 4 g of liver in 10 ml of the buffer, centrifuging at 1200g for 5 min, and then centrifuging the supernatant at 100000g for 30 min. All operations were performed at 4°C.

For activation of adenylate cyclase *in vitro* two types of experiment were performed. In one the cholera toxin was preincubated for 15 min with the cytosol preparation before being mixed with the particulate preparation. In the other no preincubation was performed. Thus an estimate of the rate of conversion of cholera toxin into its 'active' form could be obtained. Three samples (each 450 μ l) of the cytosol preparation were mixed with 50 μ l of concentrated NAD^+ solution so that the final concentration was 4 mM. Cholera toxin (4 μ g) was added to one of these preparations and all three preparations were incubated for 15 min at 22°C. Then 4 μ g of cholera toxin was added to a second cytosol preparation and all three preparations were then added to 500 μ l portions of the particulate preparation and 50 μ l of NAD^+ to maintain a final concentration of 4 mM. Incubation was continued for 20 min at 22°C. At 5, 10 and 20 min 200 μ l samples of the incubations were taken, mixed vigorously with 5 ml of ice-cold buffer to stop the reactions and centrifuged at 1200g for 5 min. The pellets were resuspended in 0.25–0.5 ml of buffer for assay of adenylate cyclase activity and of protein content (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Similar experiments were performed with a maximally effective concentration of subunit A.

Elevation of liver NAD^+ concentration by nicotinamide and the effect of cholera toxin in vivo

Rats were anaesthetized and injected intraperitoneally with either nicotinamide in 0.9% NaCl (1 g/kg body wt.) or an equivalent volume of 0.9% NaCl. NAD^+ was measured at 2, 4 and 5 h after the injections. Both sets of animals were anaesthetized and their livers exposed and freeze-clamped with aluminium blocks precooled in liquid N_2 . The frozen liver was powdered in liquid N_2 in a mortar and 0.3–1.0 g was mixed with 5 ml of 0.6 M-HClO₄. The mixture was homogenized with a Teflon pestle and stored overnight at 4°C. This storage had no effect on the NAD^+ concentration. NAD^+ was determined by the method of Klingenberg (1974).

To determine the effect of raised NAD^+ concentration on adenylate cyclase activity and the effect of cholera toxin, the nicotinamide and 0.9%-NaCl-injected rats were further injected with toxin 4 h after the first injections. Liver adenylate cyclase activity was determined as described for the time course *in vivo* at 20, 60 and 120 min after toxin injection.

Preparation of subunit A

Subunit A of cholera toxin was separated from the B subunits by incubation with 0.1% sodium dodecyl sulphate. Portions (100 μ g) of toxin in 25 μ l of 0.1% sodium dodecyl sulphate were then subjected to

electrophoresis on 10% (w/v) polyacrylamide/0.1% sodium dodecyl sulphate gels by the method of Weber & Osborn (1969). The gels were scanned at 280nm in a Gilford recording spectrophotometer to locate the separated subunits. Appropriate zones were cut from the gels, minced and extracted in Tris/HCl/MgCl₂ buffer by stirring overnight. The mixture was centrifuged, and the supernatant collected, freeze-dried and redissolved; then it was again subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and re-extracted. Protein content was determined by the method of Lowry *et al.* (1951).

Materials

Cholera toxin was obtained from Schwarz-Mann, Orangeburg, NY, U.S.A.; cyclic [³H]AMP and [α -³²P]ATP were from New England Nuclear Corp., Boston, MA, U.S.A.; pyruvate kinase, myokinase, alcohol dehydrogenase, cyclic AMP, phosphoenolpyruvate, nicotinamide and NAD⁺ were from Sigma Chemical Co., St. Louis, MO, U.S.A.; ATP was from Calbiochem, La Jolla, CA, U.S.A.

Results

Effect of cholera toxin in vivo

The time course of activation of adenylate cyclase in rat liver, after intravenous injection of cholera toxin, is shown in Fig. 1. At 10min after the injection, no change in adenylate cyclase was detected, but at 20min a significant 38% increase had occurred ($P < 0.01$). Maximum activation was observed after 120min, when a 5.5-fold increase in activity was reached. Half-maximal activation required 35min.

Fig. 1. Time course of activation of adenylate cyclase by cholera toxin in rat liver *in vivo*

Cholera toxin was injected intravenously at zero time. Enzyme activity was significantly elevated at all times after 10min. Vertical bars represent $\pm$ S.E.M. Numbers of experiments are printed below the plotted points.

Effect of cholera toxin in vitro

The time course of activation of adenylate cyclase in rat liver particulate preparation was next tested under two conditions: (1) when the particulate preparation was mixed with cholera toxin, a cytosol preparation and NAD⁺ at zero time, and (2) when the cholera toxin was preincubated with the cytosol preparation for 15 min at 22°C before mixing with the particulate preparation and NAD⁺ at zero time. A control of liver particulate preparation, cytosol and NAD⁺ but without the addition of cholera toxin was studied simultaneously. The results are shown in Fig. 2. Without preincubation of cholera toxin, enzyme activity was elevated at 5 min ($P < 0.01$), half-maximal activation was achieved in 8 min, and near-maximal activation (5-fold stimulation) at 20 min. These times are in contrast with 35 and 120 min for the time course *in vivo* (Fig. 1). When the toxin was preincubated with cytosol to separate subunits A and B, and subunit A into A₁ and A₂ chains, an even more rapid stimulation of adenylate cyclase was observed. In these experiments (Fig. 2) the half-maximal activation required only 4 min and stimulation was maximal at 10 min. At both 5 and 10 min, adenylate cyclase activity was significantly higher when toxin had been preincubated with cytosol than when toxin was added without preincubation ($P < 0.001$ and < 0.02 respectively). By 20 min no significant difference existed ($P < 0.3$). In the control preparation, without toxin, no significant

Fig. 2. Time course of activation of adenylate cyclase by cholera toxin in broken-cell particulate fractions of rat liver

●, Cholera toxin was preincubated with cytosol for 15 min before addition to a particulate preparation and NAD⁺. ○, Cholera toxin, cytosol, NAD⁺ and the particulate preparation were mixed at zero time. □, Control preparation (no toxin). Vertical bars represent $\pm$ S.E.M. for seven experiments.

Fig. 3. NAD^+ content of rat liver at different times under control conditions (○) and after the intraperitoneal injection of 1g of nicotinamide/kg body wt. (●)

Vertical bars represent $\pm$ S.E.M. Numbers of experiments are printed under the bars.

Fig. 4. Time course of activation of adenylate cyclase in rat liver by cholera toxin *in vivo* under control conditions (○) and 4h after the injection of 1g of nicotinamide/kg (▲)

Vertical bars represent $\pm$ S.E.M. for seven experiments.

differences from zero-time controls were detected at 5, 10 or 20min.

Effect of subunit A *in vitro*

Exactly similar experiments were performed with purified subunit A of cholera toxin as were performed with intact toxin *in vitro*, to assess the time required for conversion of subunit A into the active A_1 chain. In these studies (results not shown), no differences in the rate of activation of adenylate

cyclase were detected. Thus the time required for reduction of the disulphide bond by cytosol must be extremely short.

Effect of intracellular NAD^+ concentrations on the rate of activation of adenylate cyclase

To investigate whether NAD^+ concentration is rate-limiting *in vivo*, an attempt was made to increase it by the administration of nicotinamide (Greengard *et al.*, 1964; Clark & Pinder, 1969) and to reassess the time course of action of cholera toxin. Nicotinamide (1g/kg) was injected intraperitoneally into rats and liver NAD^+ concentration determined at 2, 4 and 5h after injection. Control rats were injected with 0.9% NaCl. At zero time NAD^+ concentration was $3.4 \pm 0.2 \mu\text{mol/g}$ of protein. This value was unchanged by the 0.9% NaCl injection after 2, 4 and 5h. After injection of nicotinamide the expected increase in NAD^+ was observed, with maximum values at 4h, to $13.9 \pm 0.6 \mu\text{mol/g}$ of protein. Thus NAD^+ concentration in the liver was almost 4 times as high after nicotinamide injection as in control rats. The results are shown in Fig. 3. After this, two groups of rats were injected with either 0.9% NaCl or nicotinamide and left for 4h so that in the latter group NAD^+ concentrations were increased maximally. Both groups were then injected intravenously with cholera toxin ($1 \mu\text{g/g}$ body wt.), and adenylate cyclase activity in liver was assayed at 0, 20, 60 and 120min. The results are shown in Fig. 4. Adenylate cyclase was stimulated by cholera toxin at the same rate and to the same extent in both groups of rats. Thus normal NAD^+ concentrations in liver are not rate-limiting for the effect of cholera toxin.

Discussion

Stimulation of adenylate cyclase by cholera toxin is characterized by a latent period, of variable length in different tissues, and a prolonged phase of gradually increasing activity. Maximum activation requires some hours and appears to be not markedly quicker when the dose of toxin is increased. In attempting to define the timing of the toxin action it is clear that there are several steps in the activation process which theoretically could be rate-limiting. Thus the process involves binding to cell membranes, translocation of the A_1 chain across the membrane (either in the whole toxin, in the A subunit or as the A_1 chain after separation) and finally the stimulation of adenylate cyclase by the A_1 chain and NAD^+ . The initial binding of cholera toxin to cell membranes is extremely rapid, as shown by direct binding studies (Cuatrecasas, 1973; Boyle & Gardner, 1974; Walker *et al.*, 1974) and by stimulation of adenylate cyclase in tissues exposed only briefly to the toxin (Guerrant *et al.*, 1972). This step will have only a small effect on

the time course of action. It is also clear from the present study and others (Flores & Sharp, 1975; Gill, 1976; Flores *et al.*, 1976; Martin *et al.*, 1977) that, with the membrane barrier effectively removed by homogenization and with sufficient NAD^+ concentration, cholera toxin can rapidly stimulate adenylate cyclase. The NAD^+ concentrations reported in the present work are $3.4 \pm 0.2 \mu\text{mol/g}$ of protein. As in 15 determinations 1 g of liver wet wt. was shown to be equivalent to $217 \pm 7 \text{ mg}$ of protein, then the NAD^+ concentration was $0.74 \mu\text{mol/g}$ wet wt. This value closely agrees with previous reports (Greengard *et al.*, 1964; Clark & Pinder, 1969). By assuming a dry weight equivalent to 27% of the wet weight (Clark & Pinder, 1969), this value would equate with a concentration of NAD^+ of 1 mM in the tissue water. As 1 mM- NAD^+ has been shown to promote maximum activation of adenylate cyclase by cholera toxin in rat liver homogenates (Flores *et al.*, 1976) and as elevation of NAD^+ concentration by nicotinamide failed to influence the time course of action of cholera toxin *in vivo*, it seems that the NAD concentration in the intact tissue is not rate-limiting. Thus the initial step (binding) and the final step (activation) are rapid processes. Rate limitation must then reside in either the separation of the A_1 chain from the toxin or the translocation of the A_1 chain across the membrane. At the present time, it is not possible to distinguish between these possibilities, because the locations at which dissociation of the A subunit and reduction to the A_1 chain occurs are not known. If the whole toxin crosses the membrane, then the time taken to cross would be the major rate-limiting step. This is because in a dilute cytosol preparation, even at 22°C , the separation of the A_1 chain from whole toxin contributes only 4 min to the activation process. Under similar conditions the conversion of subunit A into the A_1 and A_2 chains was so rapid that no difference in the time course was found between the actions of subunit A and the A_1 chain. If the dissociation of subunit A and reduction to the A_1 chain takes place before passage through the membrane, then the rate-limiting step cannot be determined from the present data. The rate at which subunit A dissociates and is converted into the A_1 chain when it is on the cell surface and separated from the cell cytosol is not known. If this is still rapid, then the rate limitation would be the time required to cross the membrane; if it is slow, then dissociation and disulphide-bond reduction at the membrane could be rate-limiting.

In conclusion, the long time course of activation of adenylate cyclase in intact cells is not due to slow binding or to a slow activation process after the subunit A_1 reaches the enzyme. NAD^+ concentrations are not limiting *in vivo*. The rate limitation is due to the slow access of subunit A_1 to adenylate cyclase. This may be due to either a slow dissociation of the whole toxin into its constituent subunits at the

membrane or a slow translocation of the A_1 chain (as whole toxin, subunit A or the A_1 chain) across the membrane.

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