Cholera Toxin Requires Oxidized Nicotinamide–Adenine Dinucleotide to Activate Adenylate Cyclase in Purified Rat Liver Plasma Membranes

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Activation of adenylate cyclase in isolated rat liver plasma membranes by cholera toxin was demonstrated. The activation requires the presence of NAD⁺ and ATP and is irreversible.

Cholera toxin has been shown to activate adenylate cyclase in a large number of tissues (Finkelstein, 1973; Gill & King, 1975; Gill, 1975; Bennett *et al.*, 1975; Bennett & Cuatrecasas, 1975a). In most of these studies, however, whole cells were preincubated in the presence of cholera toxin, the cells were subsequently broken and the particulate fraction was assayed for adenylate cyclase activity. There are few reports of activation of adenylate cyclase by cholera toxin in broken-cell preparations. Gill (1975) has demonstrated that adenylate cyclase is activated by cholera toxin in a washed particulate fraction from pigeon erythrocytes provided that NAD⁺ is present. Sahyoun & Cuatrecasas (1975) have shown that cholera toxin decreases the rate of loss of adenylate cyclase activity during incubation of a particulate fraction from fat-cells.

The purpose of the present study was to establish whether or not cholera toxin would activate cyclase in purified liver plasma membranes, a system where the properties of the adenylate cyclase are relatively well understood (Rodbell *et al.*, 1975) and to establish if there is any involvement of NAD⁺ in this process.

Experimental

Materials

ATP, cyclic AMP, NAD⁺, NADP⁺, NADPH, GTP, AMP-P(NH)P (adenylyl imidodiphosphate) and GMP-P(NH)P (guanylyl imidodiphosphate) were obtained from Boehringer (London) Corp., Bell Lane, Lewes, East Sussex BN7 1LG, U.K. Phosphocreatine and creatine kinase (EC 2.7.3.2) were from Sigma (London) Chemical Co., London S.W.6, U.K. Dithiothreitol was from BDH (Poole, Dorset, U.K.). [α -³²P]ATP was from The Radiochemical Centre, Amersham, Bucks., U.K. Cholera toxin (lot 1071) was a kind gift from Dr. R. Finkelstein, Dallas, TX, U.S.A. (prepared under contract for NIAID).

Methods

Adenylate cyclase was assayed by the method of Salomon *et al.* (1974) where ATP was the substrate. Where AMP-P(NH)P was the substrate, assays were performed as described by Houslay *et al.* (1976).

Rat liver plasma membranes were isolated and purified by the method of Pilgis *et al.* (1974).

Results

Cholera toxin activated the adenylate cyclase of rat liver plasma membranes with an absolute requirement for NAD⁺. In the presence of 1 mM-NAD⁺ the activation is apparent within 10 min at the lowest cholera toxin concentration used (0.1 μ g/ml), and within 5 min at the highest concentration (20 μ g/ml). The extent of the activation is concentration-dependent and the effect appears to be saturable (Fig. 1a). In the absence of NAD⁺ no effect was observed when the membranes were incubated with cholera toxin under the same conditions for up to 4 h.

The effect of varying the NAD⁺ concentration is shown in Fig. 1(b). Increasing NAD⁺ concentrations increased the extent of activation observed in the presence of cholera toxin (10 μ g/ml) and also decreased the time before any activation was observed. No activation was observed before 12 min at the lowest NAD⁺ concentration used (0.1 mM), whereas at 5 mM-NAD⁺ a marked activation was seen at 5 min.

A series of experiments was carried out to determine whether the effect of cholera toxin was reversible. Membranes were incubated for 20 min under various conditions, centrifuged, washed and then assayed as shown in Table 1. Preincubation in the presence of 1 mM-NAD⁺ and cholera toxin (10 μ g/ml) resulted in an irreversibly activated adenylate cyclase (Expt. D). Preincubation in the presence of

Fig. 1. Effect of various concentrations of cholera toxin and NAD^+ on adenylate cyclase activity

Rat liver plasma membranes (0.4 mg) were incubated in 1.0 ml of assay medium (25 mM-Tris/HCl, pH 7.4, 1 mM-dithiothreitol, 20 mM-phosphocreatine, 50 units of creatine kinase/ml, 10 mM-MgCl₂, 0.5 mM-cyclic AMP, 0.5 mM-ATP, 5 μ Ci of [α -³²P] ATP/ml) at 30°C. Samples (0.1 ml) were removed at timed intervals and added to 0.1 ml of stopping solution (2% sodium dodecyl sulphate, 40 mM-ATP, 1.4 mM-cyclic AMP). The Figure shows the production of cyclic AMP in the presence of cholera toxin as a percentage of the production of cyclic AMP in the absence of cholera toxin. (a) 1 mM- NAD^+ was present throughout. Cholera toxin was present in the following concentrations: $\circ$, 0; $\bullet$, 0.1 μ g/ml; $\square$, 0.5 μ g/ml; $\blacksquare$, 1.0 μ g/ml; $\triangle$, 2.5 μ g/ml; $\blacktriangle$, 10 μ g/ml; ∇ , 20 μ g/ml. (b) shows the effect of 10 μ g/ml of cholera toxin in the presence of: $\circ$, no NAD^+ ; $\bullet$, 0.1 mM- NAD^+ ; $\square$, 0.3 mM- NAD^+ ; $\blacksquare$, 0.7 mM- NAD^+ ; $\triangle$, 1.0 mM- NAD^+ ; $\blacktriangle$, 2.0 mM- NAD^+ ; ∇ , 5.0 mM- NAD^+ .

Table 1. Effect of preincubation of rat liver plasma membranes with cholera toxin and NAD^+ on the subsequent response of adenylate cyclase to cholera toxin and NAD^+

Rat liver plasma membranes (0.3 mg) were preincubated for 20 min at 30°C in 1 ml of the medium described in the legend to Fig. 1, except that no radioactive ATP was present. Further additions were as shown in the Table. The membrane suspension was centrifuged at 14000g for 4 min and the pellet washed in 1 ml of 50 mM-Tris/HCl, pH 7.4, containing 1 mM-dithiothreitol. The pellet was resuspended in 0.2 ml of the same buffer and 0.03 ml samples were assayed (20 min, 30°C) in a total volume of 0.1 ml of assay medium with the additions shown in the Table. The data presented are means $\pm$ S.E.M. of three parallel incubations.

Expt.	Assay conditions ...	Adenylate cyclase (nmol of cyclic AMP/mg of protein)			
		(i)	(ii)	(iii)	(iv)
	Preincubation conditions	—	Cholera toxin (10 μ g/ml)	NAD^+ (1 mM)	Cholera toxin (10 μ g/ml) + NAD^+ (1 mM)
A	—	0.62 $\pm$ 0.013	0.663 $\pm$ 0.058	0.44 $\pm$ 0.006	0.872 $\pm$ 0.063
B	Cholera toxin (10 μ g/ml)	0.65 $\pm$ 0.08	0.559 $\pm$ 0.015	0.88 $\pm$ 0.03	0.984 $\pm$ 0.05
C	NAD^+ (1 mM)	0.52 $\pm$ 0.011	0.505 $\pm$ 0.007	0.419 $\pm$ 0.077	0.828 $\pm$ 0.011
D	Cholera toxin (10 μ g/ml) + NAD^+ (1 mM)	1.17 $\pm$ 0.04	1.12 $\pm$ 0.007	1.14 $\pm$ 0.04	1.071 $\pm$ 0.021

cholera toxin alone did not activate the adenylate cyclase unless the enzyme was assayed in the presence of NAD^+ (Expt. B). Thus the effect of the exposure of membranes to cholera toxin was irreversible, but NAD^+ was required for the effect to be expressed in terms of activation of adenylate cyclase. This was not

the case with exposure to NAD^+ alone (Expt. C), where subsequent addition of toxin to the washed membranes had no effect. This might be expected, as the membranes had been exposed previously to NAD^+ *in vivo*.

It has been noted that NAD^+ alone inhibits

Table 2. *Effect of ATP on activation of adenylate cyclase by cholera toxin*

Rat liver plasma membranes (0.3 mg) were incubated for 30 min at 30°C in 1 ml of medium containing 25 mM-Tris/HCl, pH 7.4, 1 mM-dithiothreitol, 5 mM-NAD⁺ and further additions as below. At the end of the incubation the membrane suspension was centrifuged at 14000 *g* for 4 min and the pellet was washed in 1 ml of 50 mM-Tris/HCl, pH 7.4, containing 1 mM-dithiothreitol. The pellet was resuspended in 0.1 ml of the same buffer and 0.03 ml samples were assayed (15 min, 30°C) in a total volume of 0.1 ml of assay medium containing 5 mM-NAD⁺. The data presented are means $\pm$ S.E.M. of three parallel incubations.

Additions	Adenylate cyclase (nmol of cyclic AMP/mg of protein)
(1) —	0.58 $\pm$ 0.013
(2) Cholera toxin (10 μ g/ml)	0.64 $\pm$ 0.003
(3) ATP (0.5 mM), phosphocreatine (10 mM), creatine kinase (50 units/ml)	0.60 $\pm$ 0.02
(4) ATP (0.5 mM), phosphocreatine (10 mM), creatine kinase (50 units/ml), cholera toxin (10 μ g/ ml)	1.63 $\pm$ 0.08

Table 3. *Effect of GMP-P(NH)P, GTP, glucagon and F⁻ on the response of adenylate cyclase to cholera toxin*

Rat liver plasma membranes (0.04 mg) were incubated for 20 min at 30°C in 0.1 ml of assay medium (see legend to Fig. 1). NAD⁺ (1 mM) was present in all incubations and other additions were as shown in the Table. The results are means $\pm$ S.E.M. of three parallel incubations.

Addition	Adenylate cyclase (nmol of cyclic AMP/mg of protein)	
	No cholera toxin	Cholera toxin (10 μ g/ml)
—	0.684 $\pm$ 0.036	1.378 $\pm$ 0.061
NaF (10 mM)	3.874 $\pm$ 0.03	2.951 $\pm$ 0.096
GMP-P(NH)P (10 μ M)	3.783 $\pm$ 0.036	3.252 $\pm$ 0.048
GMP-P(NH)P (10 μ M) +glucagon (1 μ M)	5.331 $\pm$ 0.093	4.277 $\pm$ 0.043
Glucagon (1 μ M)	2.409 $\pm$ 0.063	2.019 $\pm$ 0.029
Glucagon (1 μ M)+ GTP (10 μ M)	3.325 $\pm$ 0.325	3.493 $\pm$ 0.077
GTP (10 μ M)	1.518 $\pm$ 0.045	2.787 $\pm$ 0.072

adenylate cyclase (see Table 1). The highest concentration of NAD⁺ used in this study was 5 mM (Fig. 1b). This halved the activity of adenylate cyclase. Addition of cholera toxin increased this activity sixfold, i.e. threefold over the control in the absence of NAD⁺.

The specificity for NAD⁺ is apparently unique among the cofactors to which the membrane is likely to be exposed *in vivo*. Neither NADH, NADP⁺ or NADPH, at concentrations up to 5 mM, had any effect in allowing activation of adenylate cyclase by cholera toxin (10 μ g/ml) over a period of 30 min at 30°C, whereas in the presence of NAD⁺ a threefold activation was observed.

Gill (1975) has suggested that ATP is required for the action of cholera toxin on adenylate cyclase in pigeon erythrocyte 'ghosts'. The experiment shown in Table 2 shows that this is also the case with purified liver membranes. Membranes incubated with cholera toxin and NAD⁺ in the absence of ATP and then washed free of toxin show no effect of cholera toxin when assayed for adenylate cyclase activity. If ATP was present a large stimulation was observed. It is noteworthy in this connexion that when AMP-P(NH)P was used in place of ATP as substrate for adenylate cyclase, cholera toxin did not activate the enzyme.

The effect of cholera toxin (10 μ g/ml) was examined in the presence of 1 mM-NAD⁺ and maximally activating concentrations of a number of ligands known to affect adenylate cyclase (Table 3). Cholera toxin markedly inhibited the response of F⁻ (10 mM) and caused a slight inhibition of the response to GMP-P(NH)P (10 μ M), glucagon (1 μ M) and a combination of glucagon and GMP-P(NH)P. Where glucagon (1 μ M) was added together with GTP (10 μ M) there was no effect on the activation achieved by cholera toxin and NAD⁺, but in the presence of GTP (10 μ M), cholera toxin caused a marked activation, about double that seen normally.

Discussion

We have demonstrated that both NAD⁺ and ATP must be present for the activation of adenylate cyclase in purified rat liver plasma membranes by cholera toxin. The activation of adenylate cyclase in the presence of cholera toxin and NAD⁺ is irreversible. The binding of cholera toxin to membranes is also irreversible, resulting in activation of adenylate cyclase on subsequent addition of NAD⁺ in the absence of toxin. Both the length of the lag before onset of activation and the extent of activation appear to be dependent on the NAD⁺ concentration. This is in contrast with the observations of Sahyoun & Cuatrecasas (1975), who found no requirement for NAD⁺ in the effect of cholera toxin on adenylate cyclase in a fat-cell particulate fraction. However, no attempt appears to have been made to establish that the preparation was free of NAD⁺.

The lag before onset of activation appears to be dependent, to some degree, on the concentration of cholera toxin, ranging from less than 5 min at the higher concentrations used to about 10 min at the

lowest concentrations. This is in marked contrast with the situation in whole cells, where lags of between 30 min and 4 h have been reported (Finkelstein, 1973; Cuatrecasas *et al.*, 1976). Cuatrecasas and co-workers have suggested that the length of the lag is independent of the cholera-toxin concentration. They conclude that the rate-limiting step is the release of the active subunit of the toxin from the inactive cholera-genoid and its subsequent passage through the membrane to interact with adenylate cyclase (Bennett & Cuatrecasas, 1975a,b; Sahyoun & Cuatrecasas, 1975; Cuatrecasas *et al.*, 1976). However, the lag may be explained, at least in part, by the relatively low concentrations of NAD^+ that may be available *in vivo*, in combination with the relatively low concentrations of cholera toxin that were used. The free NAD^+ concentration in liver cells is approx. 0.5 mM and one might expect considerable variation from tissue to tissue. This suggestion is in accord with the observation that cell lysis prevents further activation, presumably owing to dilution of endogenous NAD^+ or its destruction by NAD^+ glycohydrolase. Further, NaCN and NaN_3 both inhibit the activation of adenylate cyclase in intact cells (Bennett & Cuatrecasas, 1975a,b), perhaps as a result of increased cytoplasmic NADH and decreased NAD^+ owing to the inhibition of mitochondrial respiration.

The observed effects of cholera toxin in decreasing the responses to both F^- and glucagon have been noted by other workers (Bennett & Cuatrecasas, 1975a,b; Cuatrecasas *et al.*, 1976). Our novel observations that cholera toxin is inhibitory in the presence of GMP-P(NH)P and does not activate when AMP-P(NH)P is used as substrate lends support to the view that AMP-P(NH)P and GMP-P(NH)P may not only act as analogues of ATP and GTP but have other effects on the enzyme. In the presence of the natural effector GTP the activation by cholera toxin is increased dramatically. Indeed GTP protects the glucagon response from inhibition by cholera toxin. The potentiation of the response to cholera toxin by

GTP suggests that the action of the cholera toxin may be to some extent analogous to the action of stimulating hormones, whose responses are also potentiated by GTP (Rodbell *et al.*, 1975).

The mechanism of action of cholera toxin on adenylate cyclase is poorly understood. The nature of the involvement of NAD^+ and ATP is unknown and there is little information about the interaction of cholera toxin with the other effectors of the enzyme, guanine nucleotides and hormones. The use of cholera toxin with this well-characterized purified membrane system should be helpful in increasing the understanding of the mechanism of action, not only of the toxin but of other effectors of the enzyme.

References

- Bennett, V. & Cuatrecasas, P. (1975a) *J. Membr. Biol.* **22**, 1–28
- Bennett, V. & Cuatrecasas, P. (1975b) *J. Membr. Biol.* **22**, 29–52
- Bennett, V., Mong, L. & Cuatrecasas, P. (1975) *J. Membr. Biol.* **24**, 107–129
- Cuatrecasas, P., Bennett, V., Craig, S., O'Keffe, E. & Sahyoun, N. (1976) in *The Structural Basis of Membrane Function* (Hatefi, Y. & Djavadi-Ohanian, L., eds.), pp. 275–292, Academic Press, New York
- Finkelstein, R. A. (1973) *Crit. Rev. Microbiol.* **2**, 553–623
- Gill, D. M. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2064–2068
- Gill, D. M. & King, C. A. (1975) *J. Biol. Chem.* **250**, 6424–6432
- Houslay, M. D., Metcalfe, J. C., Warren, G. B., Hesketh, T. R. & Smith, G. A. (1976) *Biochim. Biophys. Acta* **436**, 489–494
- Pilkis, S. J., Exton, J. H., Johnson, R. A. & Park, C. R. (1974) *Biochim. Biophys. Acta* **343**, 250–267
- Rodbell, M., Lin, M. C., Salomon, Y., Londo, C., Harwood, J. P., Martin, B. R., Rendell, M. & Berman, M. (1975) *Adv. Cyclic Nucleotide Res.* **5**, 3–30
- Sahyoun, N. & Cuatrecasas, P. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3438–3442
- Salomon, Y., Londos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548