CHOLERA TOXIN MEDIATED ACTIVATION OF ADENYLATE CYCLASE IN INTACT RAT HEPATOCYTES

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1. Introduction

Cholera toxin exerts its action upon target tissues by causing the irreversible activation of the enzyme, adenylate cyclase [1,2]. This activation is apparently dependent upon the A-subunit of the toxin, NAD^+ and ATP [1,2]. The mechanism by which this effect is achieved is probably the ADP-ribosylation of a guanine nucleotide regulatory protein associated with the catalytic unit of adenylate cyclase [3].

In intact cells, cholera toxin first binds rapidly and irreversibly to cell surface receptors, which are believed to be G_{M1} gangliosides [2]. This is followed by a characteristic lag phase, the duration of which appears to vary widely from about 20 min to about 4 h depending upon cell type [1,2,4,5] before the onset of the irreversible activation of adenylate cyclase on the inner surface of the membrane.

We have shown in isolated plasma membranes from rat liver that both the extent of activation of adenylate cyclase and the lag time of onset could be influenced by manipulating either the NAD^+ or cholera toxin concentrations [6]. These lag times however were very short compared with those found using various other types of intact cells [1,2,4,5].

This study reports on our attempts to influence the lag time for onset, and the degree of cholera toxin activation of adenylate cyclase in isolated rat hepatocytes to compare with our observations using isolated rat liver plasma membranes [6].

2. Materials and methods

Adenylate cyclase was assayed as described previously [7] with modifications as in [8].

Isolated intact hepatocytes were prepared from 24 h starved 200–300 g male Sprague–Dawley rats [9] and incubated as described previously in detail [10].

Cells (3–5 mg dry wt./ml) were pre-incubated for 15 min at 37°C prior to the start of the experiment with their respective added substrates, and then regularly gassed for 20 s every 10 min with an O_2 : CO_2 gas mixture (19:1). This was essential to maintain a stable adenylate cyclase activity. At selected intervals, 1 ml samples were taken and immediately placed on ice to quench the reaction [11], prior to centrifugation at 14 000 × g_{av} for 6 min at 4°C in a Jobling Model 320 microfuge. The pellets were then resuspended in 150 μl of 1 mM $KHCO_3$ (pH 7.2), and disrupted by repeatedly (× 12) syringing them using a 1 ml plastic syringe and 25G needle. A washed membrane fraction was obtained by diluting such a disrupted pellet with 0.1 ml of $KHCO_3$ (pH 7.2) and centrifuging as before prior to resuspending in 150 μl of 1 mM $KHCO_3$, pH 7.2.

ATP, lactate and pyruvate were assayed by standard procedures [12–14].

Glucagon was a gift from Dr W. W. Bromer of Eli Lilly Co., Indiana, U.S.A.

Cholera toxin and lactate were from Sigma. Enzymes and other Biochemicals were from Boehringer. All other chemicals were of A.R. grade from B.D.H.

3. Results and discussion

In rat liver plasma membranes both ATP and NAD^+ are essential co-factors for cholera toxin mediated activation of adenylate cyclase [6]. The degree of activation of adenylate cyclase in isolated plasma membranes by cholera toxin, is markedly dependent upon NAD^+ concentration, which can also affect the lag time. At 10 μg/ml cholera toxin and 0.5 mM NAD^+ (approximately that found in the liver cell) we might expect to see nearly a 2-fold increase in adenylate cyclase activity, with a lag time of onset of about 5 min [6]. This contrasts with the situation in intact hepatocytes where under similar conditions we obtained a 7.8-fold (± 0.5, S.E.M., n = 10) increase in activity with a 10 min lag (fig.1).

No difference in lag time or degree of activation was achieved when using either pyruvate (10 mM) or lactate (10 mM) as substrates (fig.1) which achieved a 50-fold difference in cytoplasmic NAD^+/NADH ratio (as judged by intracellular pyruvate/lactate ratio). Similarly if the other essential factor, ATP, was depleted in the cells using 10 mM fructose (from 10.1 nmol/mg to 3.6 nmol/mg, equivalent to a concentration change from about 4 mM to 1.5 mM ATP), then again this had no effect on either the time of the lag or extent of activation achieved by 10 μg/ml cholera toxin (fig.1). It appears then that these parameters of cholera toxin mediated adenylate cyclase activation are relatively insensitive to such changes in the NAD^+/NADH ratio or the ATP concentration in intact hepatocytes but not in isolated membranes [6] or solubilised preparations [15]. This may well be because the mechanism of activation by cholera toxin differs from that in whole cells.

The extent of activation of adenylate cyclase achieved was identical over the range of cholera toxin studied (0.5–20 μg/ml in a 90 min period (fig.2). This contrasts markedly with the situation in plasma membranes where the degree of activation was highly dependent upon the cholera toxin concentration, being little if any at 0.1 μg/ml toxin and 4-fold at 20 μg/ml toxin [6].

Cuatrecasas [4] has suggested that the lag phase for onset of adenylate cyclase activation is independent of cholera toxin concentration in intact fat cells [16] and intact toad erythrocytes [11]. We found that the lag time varied from 20 min at

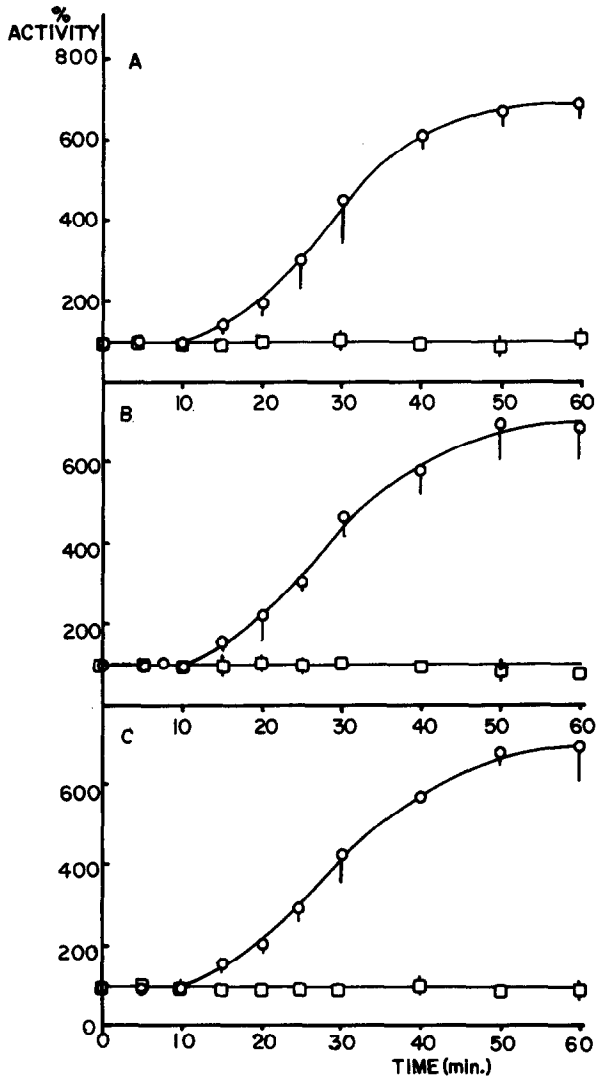

Fig.1. The effect of cholera toxin on the adenylate cyclase activity of intact hepatocytes incubated with different substrates. An hepatocyte suspension was incubated at 37°C in the manner described in section 2 with A. 10 mM pyruvate, B. 10 mM lactate and C. 10 mM fructose as substrates. After 15 min preincubation (T = −15 min), cholera toxin was added to the test incubations (T = zero) to a final concentration of 10 μg/ml. (□) control incubations and (○) 10 μg/ml cholera toxin incubations. The errors given are S.D. on two typical sets of incubations with duplicate adenylate cyclase assays. In the absence of cholera toxin then no change in the basal activity of adenylate cyclase could be detected under any of these conditions. Also if the cells were incubated on ice at 0–4°C, then no change in activity of the cholera toxin treated cells occurred.

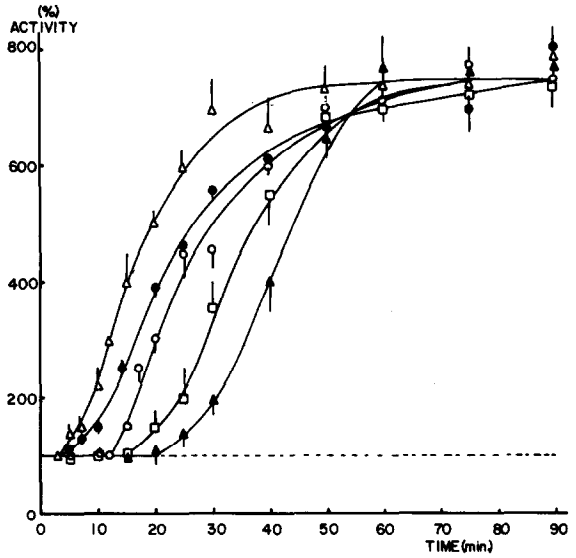

Fig.2. The effect of cholera toxin concentration and the local anaesthetic benzyl alcohol on the lag of onset of adenylate cyclase activation by cholera toxin in intact hepatocytes. Pre-incubations were carried out with 10 mM lactate as substrate. This was added 15 min prior to additions of cholera toxin and benzyl alcohol. Conditions were, (△) 20 μg/ml cholera toxin, (○) 10 μg/ml cholera toxin, (□) 1.0 μg/ml cholera toxin, (▲) 0.5 μg/ml cholera toxin and (●) 10 μg/ml cholera toxin together with 10 mM benzyl alcohol. The errors given are S.D. on two typical sets of incubations with duplicate adenylate cyclase assays. Benzyl alcohol (10 mM) had no significant effect on the level of the control adenylate cyclase activity over the time course of the experiment.

0.5 μg/ml cholera toxin to <5 min at 20 μg/ml cholera toxin (fig.2). However differences of this magnitude may well have been masked by their estimated experimental error of 10–20 min [11].

The relative effects of a number of activating ligands on the adenylate cyclase activity of washed membrane fractions derived from isolated hepatocytes incubated for 60 min with 10 mM lactate, either with or without 10 μg/ml cholera toxin are shown in table 1. The adenylate cyclase activity of the cholera toxin treated cells exhibit a dramatically reduced sensitivity to stimulation by fluoride and glucagon (table 1) in a similar fashion to cholera toxin treated plasma membranes [6]. Indeed the sensitivity of adenylate cyclase in membranes from cholera toxin treated hepatocytes, to stimulating ligands is very similar to that of cholera toxin treated plasma membranes (table 1).

It has been demonstrated [19] that the onset of adenylate cyclase activation by cholera toxin in intact lymphocytes corresponds to the formation of a cap of cross-linked toxin forming at the pole of the cell. Such patching and capping of fluorescent cholera toxin [19,20] also patches and caps the G_{M1} gangliosides [21] indicating that it is this entire complex which is redistributed in the plane of the membrane. The change in the lag period with cholera toxin concentration in intact hepatocytes will thus reflect the amount of toxin bound and undoubtedly the effi-

Table 1
Activation of hepatocyte adenylate cyclase by ligands

Ligand	Fold activation of adenylate cyclase from		
	Control hepatocytes	Cholera toxin treated hepatocytes	Cholera toxin treated plasma membranes (adapted from 6)
NaF	10.4 ± 0.29 (4)	1.96 ± 0.23 (4)	2.14 ± 0.12 (3)
Guanylyl imidodiphosphate	3.3 ± 0.42 (4)	2.69 ± 0.47 (4)	2.36 ± 0.05 (3)
GTP	2.6 ± 0.37 (4)	2.62 ± 0.43 (4)	2.02 ± 0.09 (3)
Glucagon	24.5 ± 0.78 (4)	2.39 ± 0.4 (4)	1.47 ± 0.03 (3)
Glucagon + GTP	37.9 ± 2.48 (4)	3.60 ± 0.37 (4)	2.53 ± 0.10 (3)
Glucagon + guanylyl imidodiphosphate	30.7 ± 3.9 (4)	3.10 ± 0.40 (4)	3.10 ± 0.05 (3)

Values are means ± S.D. for number of separate experiments in parentheses

ciency of redistribution of multivalent toxin at the cell surface.

Patching and capping phenomena are known to be temperature dependent [22], and are presumably dependent upon bilayer fluidity. Indeed a fluid bilayer is a pre-requisite for patch formation due to the interaction of a multivalent ganglioside complex [4] with mutlivalent receptors which aggregate by free lateral diffusion in the plane of the bilayer, to be subsequently removed to a pole of the cell to form a cap. We have shown that the local anaesthetic benzyl alcohol can increase the fluidity of rat liver plasma membranes, indicated by its ability to decrease the lipid phase separation temperature occuring at 28°C [23] and to increase the mobility of an incorporated fatty acid spin label [24]. Such an increase in bilayer fluidity achieved by benzyl alcohol (10 mM) is sufficient to decrease the lag time for onset of activation by 10 μg/ml cholera toxin (fig.2), but does not affect the extent of activation achieved. This would be consistent with the lag period reflecting a cell surface event. This process appears to depend upon membrane fluidity and on metabolic energy to power the capping event [25]. Observations in intact cells, that metabolic inhibitors (KCN, Na Azide and NaF) inhibit both the activation process and capping of fluorescent labelled toxin [11,19], and that cytochalasin B inhibits both processes would support this [19,25].

In isolated plasma membranes this would mean that activation of adenylate cyclase by cholera toxin could not occur via ganglioside binding, but must solely result through direct action of the toxin. Support for such a contention comes from observations that exogenous G_{M1} ganglioside does not inhibit the action of cholera toxin on adenylate cyclase in broken cell preparations, yet it potently inhibits the action in whole cells [2]. Also the activity of the A (activating) subunit is negligible on whole cells, but fully active in broken cell preparations, there being no requirement for the multivalent B (binding) subunit [1,2]. In this instance the degree of activation of adenylate cyclase would be dependent upon both cholera toxin and adenylate cyclase concentrations, with the interactants not being confined to diffusion in the two dimensions of the bilayer.

The lag phase in intact cells does not appear to involve a contribution due to the release of the A subunit from the ganglioside–B-subunit complex, as toxin where the A and B subunits have been cross-linked is still active towards intact cells, and exhibits an identical lag phase to the native toxin [26]. One presumes that in both cases the A-subunit is translocated to the cytosol surface where it might be expected to allow activation of all adenylate cyclase units by free lateral diffusion in the plane of the bilayer. The degree of activation of adenylate cyclase will thus be essentially independent of cholera toxin concentration above a certain critical level required to achieve the formation of an active ganglioside–cholera toxin complex at the cytosol side of the cell surface. This achieves a high local concentration of reactants colliding in the two dimensions of the bilayer leading to the rapid activation of all adenylate cyclase units.

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