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Short Communications

Subunit A from Cholera Toxin is an Activator of Adenylate Cyclase in Pigeon Erythrocytes

By SIMON VAN HEYNINGEN*
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

and CAROLYN A. KING
Sir William Dunn School of Pathology, University of Oxford, South Parks Road, Oxford OX1 3RE, U.K.

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Intact cholera toxin and its purified subunit A both activate the adenylate cyclase of pigeon erythrocyte membranes, but subunit B does not. The activation by subunit A is unaffected by treatments that inhibit whole toxin by interfering with the binding of subunit B to cell membranes.

Cholera toxin (mol.wt. 82000) is a powerful activator of adenylate cyclase (for general review, see Finkelstein, 1973), and is composed of two different types of subunit, A (mol.wt. 27000) and B (mol.wt. about 14500) (van Heyningen, 1974; Finkelstein *et al.*, 1974; Lönnroth & Holmgren, 1973; Cuatrecasas *et al.*, 1973). An aggregate of subunit B ('choleragenoid', mol.wt. 54000) can be isolated from culture filtrates of *Vibrio cholerae* at the same time as the toxin. The native toxin and choleragenoid both bind to cell membranes and compete for ganglioside G_{M1}, the probable natural receptor; but only the binding of intact toxin results in the activation of adenylate cyclase. It has therefore been suggested that the role of subunit B is to recognize the binding site on the cell surface (van Heyningen *et al.*, 1971; Pierce, 1973; King & van Heyningen, 1973; Holmgren *et al.*, 1974; Cuatrecasas, 1973*a*,*b*,*d*).

The present paper presents experiments showing that, although it is inactive *in vivo* and does not bind to ganglioside, subunit A is itself an activator of adenylate cyclase in pigeon erythrocytes, which have a low enzyme activity which is increased severalfold by cholera toxin (Gill & King, 1974).

Methods

Preparation of subunit A. Pure subunit A was prepared by the method of van Heyningen (1974) and further purified by diluting the solution tenfold with water, adjusting it to pH 8.5 with NaOH, and passing it through a column (Pasteur pipette) of DEAE-cellulose (Whatman DE 52) equilibrated with 5mM-Tris–HCl buffer, pH 8.5. Subunit A was eluted with 0.1M-Tris–HCl, pH 7.0, and was homogeneous on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate or in 6.25M-urea at pH 3.2, and in the ultracentrifuge. It showed no reaction with rabbit anti-(subunit B) antiserum, but reacted weakly with rabbit antitoxin.

Adenylate cyclase assay. Erythrocytes were isolated from fresh heparinized pigeon blood by centrifugation (5min, 1200*g*) to remove the plasma and buffy coat, followed by suspension in an equal volume of a medium containing 130mM-NaCl, 5mM-KCl, 2mM-$MgCl_2$ and 20mM-2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonate (Hepes) buffer, adjusted to pH 7.3 (37°C) with NaOH. Most of the remaining white cells were removed by passage through cotton-wool. After a second centrifugation, the packed cells were resuspended in medium, and incubated under various conditions at a final erythrocyte dilution of 25–50% (v/v). After incubation, the cells were washed with 20vol. of ice-cold medium, centrifuged and resuspended. Antitoxin (from the Swiss Serum and Vaccine Institute, Berne, Switzerland) was added when necessary [1unit (Craig *et al.*, 1972) per 50ng of toxin] and the cells were lysed by incubation for 3min at 37°C with the θ-toxin of *Clostridium perfringens* (a thiol-activated haemolysin kindly donated by Dr. Smythe of the Karolinska Institute, Stockholm, Sweden). The nucleated 'ghosts' were then washed in ice-cold medium, and sedimented by centrifugation (7min, 1200*g*). For experiments with lysed cells, the cell suspension was lysed before the experimental incubation, after which the 'ghosts' were washed as above. There was no sign of lysis of the cells before addition of θ-toxin even after a 2h incubation.

The adenylate cyclase activity of 10μl samples of washed 'ghosts' was measured by suspending them in a medium containing 10mM-EDTA, 5mg of serum

* Present address: Department of Biochemistry, University of Edinburgh, Teviot Place, Edinburgh EH8 9AG, U.K.

albumin/ml, 1mM-cyclic AMP and an ATP-regenerating system (8mM-phosphoenolpyruvate, 50μg of pyruvate kinase/ml and 50μg of adenylate kinase/ml) in 40mM-Tris–HCl, pH8.0. The solution was made 0.2mM in [8-^{14}C]ATP (0.5μCi; The Radiochemical Centre, Amersham, Bucks., U.K.) and incubated for 15min at 37°C. Then 0.1ml of a solution containing 5mM-cyclic AMP and 5mM-ATP was added, and the reaction stopped by boiling for 2.5min. The cyclic AMP was then purified on Dowex-50 and $BaSO_4$ essentially by the method of Krishna *et al.* (1968), and a 3ml sample was counted for radioactivity in 10ml of Aquasol (New England Nuclear Corp., Dreieichenhain, W. Germany).

Results

Cholera toxin activated adenylate cyclase in the membrane of pigeon erythrocytes, whether it was incubated with intact or previously lysed cells (Fig. 1). A time-course (Fig. 2) of the effect on intact cells shows a lag such as has been observed in all whole-cell systems (e.g. Cuatrecasas, 1973*c*). With lysed cells the activation was immediate.

Isolated subunit A also activated the enzyme in intact or lysed cells, showing a similar lag with intact cells (Fig. 2). As with whole toxin, less protein was needed to produce a given response with lysed cells than with intact cells (Fig. 1), but even with lysed cells the subunit had a lower specific activity than the toxin (perhaps an intrinsic difference, but perhaps due to inactivation during preparation).

Several experiments showed that this activity of subunit A was not due to contaminating traces of intact toxin. For example, a sufficient excess of choleragenoid (or of anti-choleragenoid serum) to inhibit the activity of intact toxin on whole cells completely, decreased the activity of preparations of subunit A by amounts varying with the preparation from nil to 35%. Furthermore, preincubation of these subunit A preparations with enough ganglioside to prevent entirely any activity due to the binding of intact toxin to cells, occasionally decreased the activity towards whole cells to an extent comparable with that observed with choleragenoid and anti-choleragenoid, but usually enhanced the activity by up to about 50%. A similar enhancement of its activity by ganglioside was sometimes seen when the subunit was assayed in rabbit skin by the method of Craig (1965). Ganglioside never had any effect on the activity of toxin or subunit on lysed cells.

Conclusion

These experiments show that all preparations of subunit A have an activity in whole cells that is unaffected by treatments that inhibit the activity of the toxin, and so must be due to the subunit itself. That a part of the activity of some preparations was inhibited by choleragenoid, ganglioside etc. is presumably due to contamination of those preparations with not more than 1% (by weight) of intact toxin, which has a much greater specific activity towards whole cells, but which could not be detected by the chemical means used.

This activity in whole and lysed cells (which is inhibited by antitoxin) is the first demonstrable

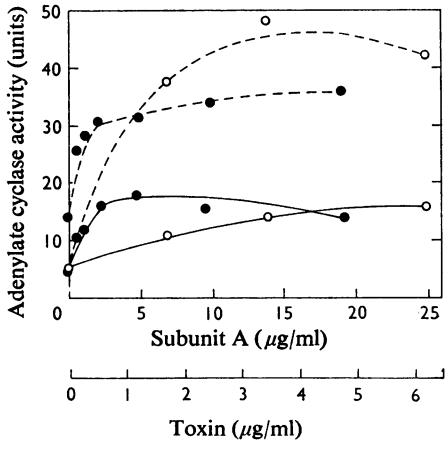

Fig. 1. *Effect of concentration of toxin and subunit A on adenylate cyclase activity in whole cells* (——) *and lysed cells* (----)

(○) Whole toxin (1.5h incubation); (●) isolated subunit A (2h incubation). Adenylate cyclase units are pmol of cyclic AMP/15min per 10μl of 'ghosts'; 9units = 1nkatal per litre of 'ghosts'.

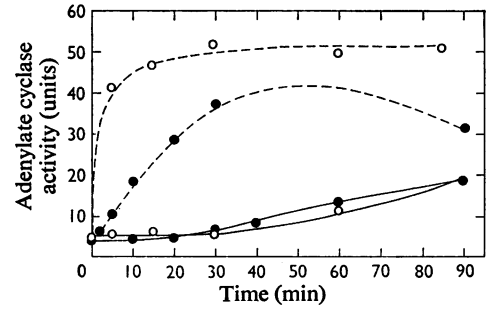

Fig. 2. *Time-course showing the effect of whole toxin* (○, *concentration* 4.6μg/ml) *and of isolated subunit A* (●, *concentration* 25μg/ml) *on adenylate activity in whole cells* (——) *and lysed cells* (----)

Units are as in Fig. 1.

significant activity of isolated subunit A. Isolated subunit is inactive *in vivo* (choleragenesis in infant rabbits; Finkelstein *et al.*, 1974) and only very weakly active in rabbit skin (van Heyningen, 1974).

Our results support the suggestion (Finkelstein, 1973; van Heyningen, 1974; Holmgren *et al.*, 1974) that the two types of subunit have different roles in the activity of the toxin in a manner reminiscent of the two fragments of diphtheria toxin (Gill *et al.*, 1973) and of abrin and ricin (Olsnes *et al.*, 1974). The toxin is perhaps initially bound to the cell through an interaction between subunit B and a ganglioside, ganglioside-like or ganglioside-containing receptor. This in some way permits (or facilitates) the entry of subunit A into the cell where it can activate adenylate cyclase, or perhaps bring about a translocation of the whole toxin molecule, thereby bringing subunit A closer to its target. Treatments that prevent the toxin from binding to the receptor also prevent subsequent activation of enzyme in intact cells; but if the toxin is presented with a lysed cell, the receptor mechanism is bypassed.

However, subunit A has some activity even towards whole cells (albeit little compared with toxin) which cannot result from any binding to ganglioside. This activity might simply be due to slow entry into the cell independent of the usual route taken by the intact toxin. On the other hand, the target molecule might be orientated in the membrane in such a way that the toxin activator site is near enough for direct attack by subunit A to be possible from the outside (although slower than from the inside). If this were so, the role of subunit B might be merely to concentrate the molecules of subunit A near the surface by binding tightly to it, without interacting in any direct way with the target system [cf. certain drugs that attack cells from the outside at a rate which is significant only because of interaction between the membrane surface and a hydrophobic part of the drug molecule (Martin, 1969)]. This would fit in with the observation (Cuatrecasas, 1973*b*; Gill & King, 1974) that incubation of cells with very low concentrations of ganglioside at the same time as with toxin can actually enhance the activity of the toxin. If the ganglioside in the cell membrane is an integral part of the adenylate cyclase-activating system, these results are surprising; but, if the ganglioside-to-subunit B binding serves only to increase the local concentration of subunit A at the membrane surface or to facilitate its entry, such enhancement by ganglioside would be more understandable.

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