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Biochemistry

ADP-ribosylation of membrane proteins catalyzed by cholera toxin: Basis of the activation of adenylate cyclase

[GTPase/NAD/pigeon erythrocyte/poly(ADP-ribose)]

D. MICHAEL GILL AND ROBERTA MEREN

Harvard University, Department of Biology, 16 Divinity Avenue, Cambridge, Massachusetts 02138

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ABSTRACT **In the presence of ATP and a cytosolic factor, cholera toxin fragment A_1 catalyzes the transfer of ADP-ribose from NAD to a number of soluble and membrane-bound proteins of the pigeon erythrocyte. Evidence is presented that suggests that the most readily modified membrane protein (M_r 42,000) is the adenylate cyclase-associated GTP-binding protein. Its modification by toxin is stimulated by guanine nucleotides. Adenylate cyclase activity increases in parallel with the addition of ADP-ribose to this protein and decreases in parallel with the subsequent reversal of ADP-ribosylation by toxin and nicotinamide. The protein is only accessible to toxin A subunits if the erythrocytes are lysed. When adenylate cyclase activity reaches a maximum, the number of ADP-ribose residues bound to this protein (about 1500 per cell) is similar to the reported number of β-adrenergic receptors.**

The activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1.] by cholera toxin involves the interaction of some part of the cyclase system exposed on the inner surface of the plasma membrane with NAD, a nucleoside triphosphate, and a cytoplasmic protein (1–4) and is conveniently studied in lysed pigeon erythrocytes. The likelihood that the NAD might function as an adenosine diphosphoribose donor in a toxin-catalyzed reaction of the type NAD^+ + protein $\rightleftarrows$ ADP-ribosyl protein + nicotinamide + H^+ was increased by the demonstrations that the active A_1 fragment of cholera toxin catalyzes the slow hydrolysis of NAD to ADP-ribose and nicotinamide (5) and the transfer of ADP-ribose to arginine and related compounds (6) and to itself (C. King, personal communication; ref. 7).

If the postulated acceptor were adenylate cyclase or an associated membrane protein, it should be possible to demonstrate a toxin-dependent transfer of ADP-ribose from radioactive NAD to a small number of sites on pigeon erythrocyte ghosts. There is, however, a large toxin-independent incorporation of ADP-ribose which was difficult to decrease without losing the toxin response. We now report the resolution of this problem and the demonstration of several toxin-specific ADP-ribose acceptors, principally a 42,000 M_r membrane protein.

METHODS

The medium used throughout consisted of 0.13 M NaCl, 0.01% sodium azide, Trasylol (aprotinin: FBA Pharmaceuticals) at 2 kallikrein inactivator units/ml, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), and NaOH to give pH 7.3 at 37°. Washed and purified pigeon erythrocytes suspended in an equal volume of this medium were lysed by rapid freezing and then thawing. The lysate was centrifuged at 10,000 × *g* for 5 min to separate ghosts and cytosol. The cytosol was mixed with well-washed pig brain NAD glycohydrolase (Sigma) at a ratio of 1 ml/10 mg, agitated at 37° for 30 min, and then centrifuged to remove the enzyme. The ghosts were washed three times in 20 vol of medium. There are about 8×10^9 ghosts per ml (packed).

Portions of lysate reconstituted from 1 part washed ghosts and 2 parts NAD-depleted cytosol fraction were incubated for 30 min at 25° or 37° with 10 mM thymidine, 5 mM ATP, 0.02 vol of activated cholera toxin (or activation solution, for controls) and [^{32}P]NAD: either 10 μM [*both phosphates*-^{32}P]NAD (100–200 mCi/mmol) prepared by Robert Benjamin (8) or 1–10 μM [*phosphate in AMP*-^{32}P]NAD (5–17 Ci/mmol) (9). Toxin was activated by preincubation (10 min, 37°) in 1.25% dodecyl sodium sulfate/2 mM dithiothreitol/serum albumin (1 mg/ml).

For estimation of incorporation and of cyclase activity, the volume was 100 μl and the incubation was ended by adding 1 ml of cold medium. The ghosts were recovered by centrifugation and resuspended. A portion of the ghost suspension equivalent to 10 μl of packed ghosts (50 μg of membrane protein) was removed for cyclase assay; the remaining ghosts were washed once more, precipitated with 5% trichloroacetic acid, filtered, and assayed for radioactivity. Adenylate cyclase was assayed in a medium including 2 mM ATP and a regenerating system but with no deliberate addition of GTP (3).

For polyacrylamide gel analysis, the ghosts of 10–300 μl of incubation mixture were washed twice in 1–2 ml of medium, deep-frozen to reopen those that had sealed during incubation, and washed once more. The pellet was agitated gently for 15 min at 37° in 0.2 ml of medium with additional 0.3 M NaCl, 0.5% Lubrol PX, and 0.15% cetyltetramethylammonium bromide [a mixture that extracts >95% of the radioactive proteins].* Proteins were precipitated with 1 ml of acetone (−20°, 1 hr), redissolved in 30 μl of gel sample buffer with heating, and subjected to electrophoresis on slab gels for 4 hr at 300 V (main gel, 7.5–15%; stacking gel, 5% acrylamide 0.1% dodecyl sodium sulfate). Gels were stained in Coomassie blue and destained in the presence of Bio-Rex mixed bed resin RG 501-X8, 20–50 mesh, which adsorbs any radioactive NAD and ADP-ribose that elutes from the gel.

RESULTS

Table 1 lists the "background" and toxin specific products formed from NAD in pigeon erythrocyte lysates. All represent the transfer of the entire ADP-ribose moiety of NAD, but not of nicotinamide, and in all cases incorporation was decreased by unlabeled NAD but not by unlabeled ADP-ribose.

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* Gill, D. M. (1977) *Proceedings of the 13th Joint Conference on Cholera. The U.S.-Japan Cooperative Medical Science Program*, pp. 195–274.

Table 1. Acid-insoluble ADP-ribosyl derivatives formed from NAD in pigeon erythrocyte lysates

Derivatives	ADP-ribose/cell*
Toxin-independent:	
Poly(ADP-ribose); nuclear; very large and heterogenous; inhibited by thymidine, nicotinamide, and ATP; stimulated by ADP-ribose; stimulated and then inhibited by DNase	Up to 100,000 (25°)
Unknown soluble product; migrated rapidly on polyacrylamide gels but did not form bands; formation was inhibited by nicotinamide, stimulated by ADP-ribose, and not affected by ATP	20,000 (37°)
M_r 115,000 ± 5000 ghost protein; detergent-soluble (see Fig 1)	100 (25°)
Toxin-dependent†:	
Major ghost product (M_r 42,000)	1,500 (25°)
Minor ghost products: M_r 98,000, 18,000, 20,000, 35,000, and 200,000 and others	1,000 (25°)
Cytosolic products; overall V_{max} = 10^6/cell equivalent/hr at 50 μg of toxin per ml; major‡, M_r 13,000, *15,000, 24,000,* and *29,000*; minor, M_r, *22,000, 58,000,* 67,000, 71,000, 92,000, 105,000, and 200,000 and others	10,000 (25°)

* Approximate number of residues incorporated per cell equivalent during a 30-min incubation at the indicated temperature with 10 μM NAD alone or with 10 μg of toxin per ml and 5 mM ATP.

† All are detergent-soluble and form discrete bands on polyacrylamide gels (see Fig 1). ADP-ribosylation required cytosol and a nucleoside triphosphate and occurred to a greater extent at 25° than at 37°.

‡ Those in italics also were found in ghost fractions.

Nuclear synthesis of poly(ADP-ribose) initially obscured the expected toxin-specific incorporation and had to be decreased to about 1% without impairing the ability of the lysate to respond to cholera toxin. Several approaches were adopted.

(*i*) Selective inhibitors of poly(ADP-ribose) synthesis were added: thymidine and ATP [the latter is also a cofactor for the toxin (3)]; 10 mM nicotinamide was added when studying incorporation into soluble proteins because it inhibits interfering reactions more than the toxin reaction.

(*ii*) The lysate was separated into ghost and cytosol fractions and the ghosts were washed several times. During washing they lost much of their ability to synthesize poly(ADP-ribose).

(*iii*) The NAD in the cytosol was hydrolyzed by incubation with an insoluble NAD glycohydrolase that was then removed by centrifugation. This technique allowed us to replace endogenous NAD with a low amount of radioactive NAD without diluting the cytosol or decreasing its ability to support the activation of adenylate cyclase. By contrast, removal of endogenous NAD by dialysis, adsorption on charcoal, or column chromatography has generally resulted in major inactivation. Endogenous ATP is also hydrolyzed during the preincubation. Thus, a lysate reconstituted from washed ghosts and NAD glycohydrolase-treated cytosol is almost totally refractory to cholera toxin but is restored to its original sensitivity upon the readdition of both NAD and ATP. With each nucleotide at 5 mM and an incubation of 30 min at 37°, adenylate cyclase activation is detectable with 1 ng of cholera toxin per ml and complete with 100 ng/ml. Under such conditions, one toxin molecule can catalyze the incorporation of more than 50 ADP-ribose residues per hour. For the present experiments we have used NAD at 10 μM or less and correspondingly more toxin (1–10 μg/ml); the same toxin-specific radioactive products were formed as with high NAD and low toxin. The toxin-specific incorporation is 2–3 times the total background incorporation of ADP-ribose.

(*iv*) Much of the residual poly(ADP-ribose) is lost upon extracting the ghosts with detergent and fractionating the extract on polyacrylamide gels. The small amount of poly(ADP-ribose) removed by detergent fails to enter the main gel and the only toxin-independent radioactive band found represents a protein of about 115,000 M_r. The toxin-dependent products are easily recognized (Fig. 1).

Analysis of the Toxin-Specific ADP-Ribosyl Products. Unexpectedly, cholera toxin was found to catalyze the transfer of ADP-ribose to *many* intracellular acceptor molecules both in the membranes and in the soluble fraction (Table 1; Fig 1). The patterns observed were reproducible and did not vary substantially with time or with the addition of protease inhibitors. The same bands were seen when the total (deoxyribonuclease-treated) ghost pellet was fractionated, rather than a detergent extract, but the resolution was inferior.

The radioactive products formed only and always under conditions that resulted in activation of adenylate cyclase (Fig. 1). Thus, ADP-ribosylation of all or any required the presence of thiol-reduced subunit A or of cholera toxin from which A has released by preincubation with dodecyl sodium sulfate (4) and was prevented by antitoxin or anti-subunit A but not by anti-subunit B. ADP-ribosyl transfer was stimulated by a nucleoside triphosphate (e.g., ATP, UTP, 5′-adenylylimidodiphosphate), was inhibited by certain methylxanthine analogs of ATP, and was prevented if endogenous ATP was hydrolyzed by prior treatment with an ATPase. Labeling of the ghost-bound products depended on the presence of cytosol in high concentration. The amount of product was roughly proportional to the

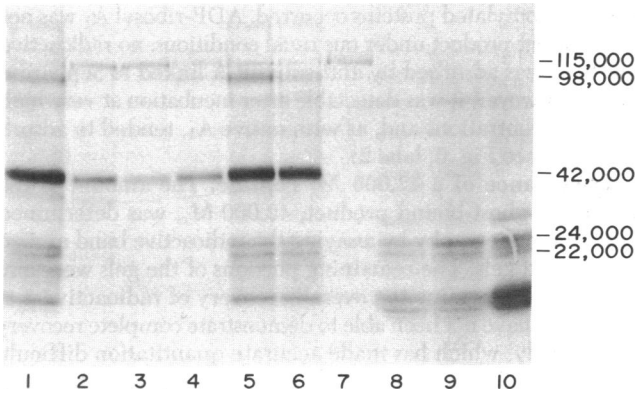


FIG. 1. Gel analysis (autoradiogram) of radioactive products. Incubation: 5 mM ATP, 10 mM thymidine, 10 mM nicotinamide, 5 μM NAD, 10 μg of activated toxin per ml, in 13 μl; 30 min, 37°. Lanes: 1–7, ghosts; 1, no nicotinamide; 2, no added ATP (endogenous ATP only); 3, no cytosol; 4, toxin not preincubated with sodium dodecyl sulfate and dithiothreitol; 5, no thymidine; 6, control; 7, toxin neutralized by anti-subunit A. Lanes 8–10: 3 μl of the soluble fraction; 8, control; 9, incubation at 25°; 10, incubation without ghosts.

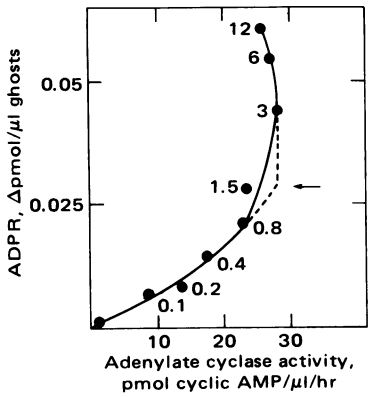


FIG. 2. ADP-ribose incorporation and adenylate cyclase activities at different toxin concentrations. Conditions as in Fig. 1 except 10 μM NAD and variable toxin concentrations, as marked (μg/ml). Radioactivity in the total ghost pellet was corrected for the incorporation without toxin. The intersection of the two portions of the curve (arrow) here represents about 2000 ADP-ribose residues per ghost.

adenylate cyclase activity, provided that the activation was not complete. Extrapolation generally revealed that an average of 1500–3000 ADP-ribosyl residues would be associated with each ghost if every copy of the cyclase was activated (Fig. 2). Continued incubation after this point (or a higher toxin or NAD concentration) results in further ADP-ribose incorporation without further increase (usually with a slight decrease) in cyclase activity.

It was obviously important to determine which, if any, radioactive product was responsible for the activation of adenylate cyclase. We established first that none of the cytosolic acceptors was relevant although some of them (Table 1) tended to associate with the ghost fraction and may represent a significant proportion of the total ghost-associated product, especially at high toxin and NAD concentrations or with extended incubations. The association seems to be incidental because the amount in the ghost fraction varied considerably with experimental conditions and, upon post-incubation in fresh medium, some of the material was lost from the ghost fraction without a corresponding decrease in cyclase activity. In any case, adenylate cyclase was not affected when untreated ghosts were incubated with toxin-treated cytosol in the presence of antitoxin or of NAD glycohydrolase, even though some transfer of ADP-ribosylated proteins occurred. ADP-ribosyl A_1 was not a significant product under our usual conditions: no radioactive material was adsorbed by anti-subunit A linked to Sepharose beads. However, it was detectable after incubation at very high toxin concentrations and, as with native A_1, tended to adsorb to ghosts (see Fig. 3, lane 2).

Importance of a 42,000 M_r Peptide. The amount of the dominant ghost-bound product, 42,000 M_r, was determined after autoradiography by assaying the radioactive band excised from dried gels. The remaining portions of the gels were also assayed to determine the overall recovery of radioactive material. We have not been able to demonstrate complete recovery consistently, which has made accurate quantitation difficult, but we could draw the following conclusions from those gels in which we could account for 90% or more of the radioactivity of the original sample. (*i*) Under limiting conditions, the ADP-ribosyl 42,000 M_r protein represents 50–60% of the total toxin-specific incorporation into ghosts and parallels the activation of adenylate cyclase. (*ii*) The amount present at maximum cyclase activity, determined by an extrapolation similar to that in Fig. 2, is about 1500 per cell, less than 0.01% of the membrane protein; this amount is similar to the estimated number of β-adrenergic receptors and thus possibly to the number of adenylate cyclase molecules in a pigeon erythrocyte (10). (*iii*) Fewer than 200 copies of any other ghost protein are labeled under limiting conditions. (*iv*) Further incubation results in the continued addition of ADP-ribose residues to the 42,000 M_r band (see *Discussion*). (*v*) The 42,000 M_r protein remains the dominant acceptor to high toxin concentrations but is eventually saturated. It is evidently a particularly efficient acceptor. Thus, for example, the 98,000 M_r membrane protein is evidently much more abundant but is only labeled to a greater extent at extraordinarily high toxin concentrations (compare Fig. 1 with lane 4 in Fig. 3).

The 42,000 M_r protein was entirely soluble in the nonionic detergents Lubrol PX and Triton X-100, suggesting that it is membrane-bound. This was confirmed by fractionation. After ghosts had been disrupted with a Yeda press, the radioactive protein was associated with a 100,000 × *g* (membrane) pellet, in contrast to poly(ADP-ribose) which was found only in a low-speed (nuclear) pellet. The protein must be located on the inner face of the cell membrane because it cannot be labeled from outside the erythrocyte. To establish this, intact erythrocytes were suspended in cytosol obtained from other cells and incubated with ATP, [^{32}P]NAD, and toxin A subunits. In order to avoid cell lysis, the A subunits were pretreated only with 5 mM dithiothreitol but not with dodecyl sodium sulfate. As shown in Fig. 3, there was no labeling of the 42,000 M_r protein and little labeling of *any* protein, unless the cells were lysed, despite the relatively enormous concentration of subunit A used. [Although there is a report to the contrary (11), we do not find that isolated subunit A significantly increases the adenylate cyclase of intact erythrocytes.]

On incubation of labeled ghosts with trypsin at 50 μg/ml for 10 min, the 42,000 M_r protein was completely converted to a 40,000 M_r peptide with minor amounts of radioactive peptides of 28,000, 19,000, and 17,000 M_r. The failure to digest the

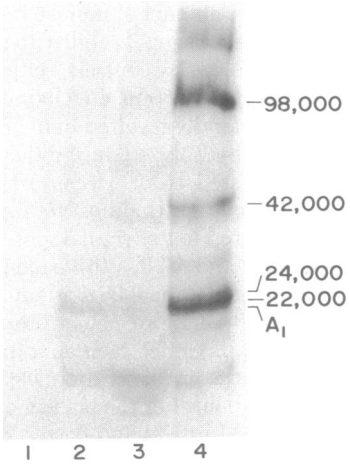


FIG. 3. Intact erythrocytes were suspended in cytosol and incubated with 8 μM [^{32}P]NAD, 5 mM ATP, and isolated toxin subunit A preincubated with 5 mM dithiothreitol, to a final concentration of 100 μg/ml, equivalent to about 300 μg of toxin per ml. After 30 min at 37° the external cytosol was removed by washing, and the erythrocytes were lysed and separated into internal cytosol (lane 1, no bands visible) and ghost (lane 2) fractions. Lanes 3 and 4 represent cytosol and ghost fractions from a parallel incubation of the same volume of lysed erythrocytes incubated with ATP and subunit A. In this case the radioactive NAD was diluted about 1:14 by the erythrocyte NAD. The detergent-soluble product represented about 1200 ADP-ribose residues per ghost (intact) and 200,000 per ghost (lysed). The band in lane 2 marked A_1 appears to be some ADP-ribosyl-A_1.

protein more thoroughly may result from its association with the membrane.

The 42,000 M_r protein migrated slightly more rapidly on polyacrylamide gels when it has not been pretreated with a thiol reducing agent. Such behavior is usually taken to indicate that one or more intrachain disulfide bonds are present and are responsible for some conformational constraint. There are evidently no interchain disulfides.

Reversal of Incorporation of Cyclase Activation. The ADP-ribosylation of ghost proteins could be reversed by incubating the washed modified ghosts with activated cholera toxin (or A) and nicotinamide. Both are essential. Reversal was aided by washing the ghosts first and by adding NAD glycohydrolase to decrease the NAD level. Even then, a high concentration of nicotinamide (100 mM > 10 mM) was required. Presumably the equilibrium is considerably biased toward the forward reaction as is the case with other ADP-ribosyl transfers. Reversal also was aided by decreasing the pH. This suggests that a proton

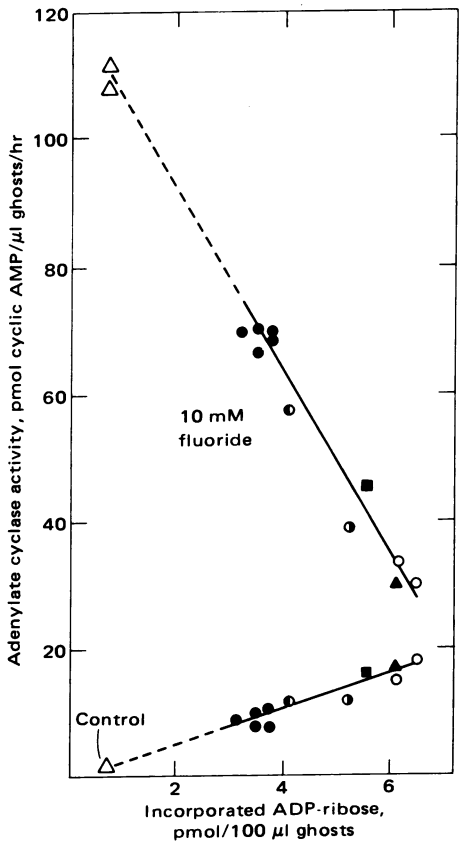


FIG. 4. Decrease of basal adenylate cyclase activity and increase in fluoride-stimulated activity upon reversal of ADP-ribosylation. A reconstituted lysate was partially intoxicated in the presence of 10 μM [^{32}P]NAD (1 μg of toxin per ml, 25°, 30 min, no nicotinamide). Portions were then diluted in 2 ml of buffer and centrifuged. The pellets, containing 85 μl of ghosts, were resuspended in 100 μl of cytosol to (final concentrations) 5 mM ATP, 100 mM nicotinamide, 0.5 unit of NAD glycohydrolase per ml, morpholinopropanesulfonic acid to pH 6.4, and 1 μg of toxin per ml. Incubation under these reversal conditions was for 30 min at 25°. The ghosts were recovered and washed well. Small portions were assayed for adenylate cyclase activity in the absence and presence (upper line) of 10 mM sodium fluoride (ordinate). Acetone precipitates of detergent extracts of the remaining ghosts were assayed for radioactivity (abscissa). △, Control ghosts incubated with NAD but without toxin; ○, toxin-treated ghosts before reversal. ADP-ribosylation reversed by incubation in complete medium (●), without nicotinamide (■), with no additional toxin (◑), with no additional ATP (◐), or at 0° (▲).

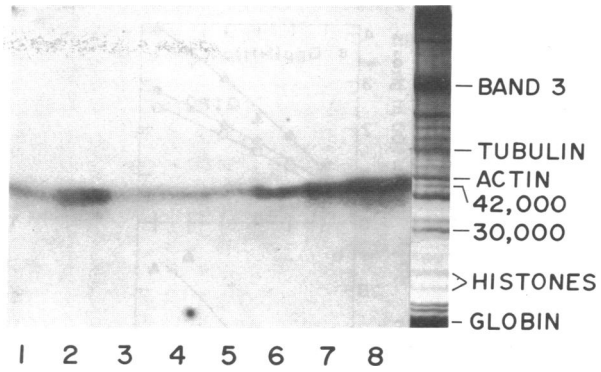


FIG. 5. Specific removal of [^{32}P]ADP-ribose residues from the 42,000 M_r protein. Conditions of labeling and reversal were as in Fig. 4. Extracts of 80 μl of ghosts were fractionated; the appearance after staining is shown on the right. Autoradiogram lanes: 1, full; 2, no NAD glycohydrolase or morpholinopropanesulfonic acid; 3, no morpholinopropanesulfonic acid; 4, only 10 mM nicotinamide; 5, no NAD glycohydrolase; 6, no ATP; 7, no toxin; 8, 0°.

is involved; thus: nicotinamide + H^+ + ADP-ribosyl proteins $\xrightarrow{\text{toxin } A_1}$ NAD^+ + proteins.

As the ADP-ribose is removed, the adenylate cyclase activity decreases from its elevated level. That this decrease is not due to nonspecific inactivation was shown by the accompanying increase in fluoride-stimulated cyclase activity, a property that was decreased by cholera toxin (Fig. 4). Polyacrylamide gel analysis of the ghosts confirmed that much ADP-ribose was removed from the 42,000 M_r protein under any condition that resulted in ADP-ribose removal in general (Fig. 5).

Involvement of GTP. Enomoto and Gill (12; cf. 13) recently found that adenylate cyclase activation by cholera toxin *in vitro* is somewhat dependent upon GTP (distinct from any effect of GTP during the subsequent cyclase assay) and that the dependence is most apparent when whole cytosol is replaced by the macromolecular fraction obtained on a Sephadex G-25 column. Fig 6*b* shows such data, and Fig 6*a* shows that 1 mM GTP and, even better, 5′-guanylylimidodiphosphate also stimulate ADP-ribosylation even in the presence of an excess of ATP.

The rates of toxin-catalyzed ADP-ribosylation and of adenylate cyclase activation were not affected by 10 mM sodium fluoride or 0.1 mM epinephrine.

DISCUSSION

We have shown that cholera toxin catalyzes the transfer of ADP-ribose from NAD to a number of membrane-bound proteins under circumstances that result in the activation of adenylate cyclase. It is reasonable to suppose that ADP-ribosylation of one of these proteins accounts for the cyclase activation and that the event that has been detected here *in vitro* occurs also in intact cells after the passage of fragment A_1 from receptor-bound holotoxin to the cell interior.

The following observations make it attractive to suppose that the 42,000 M_r protein is the physiologically relevant target in terms of adenylate cyclase activation and, furthermore, that it may be the GTP binding component of the adenylate cyclase system. (*i*) a correlation between the increase in adenylate cyclase activity and the extent of ADP-ribosylation of this protein; (*ii*) a correlation between the decrease in adenylate cyclase activity and the extent of removal of ADP-ribose residues on incubation of the modified membranes with toxin and nicotinamide; (*iii*) an approximate correspondence between the number of copies of this protein per cell that need to be ADP-

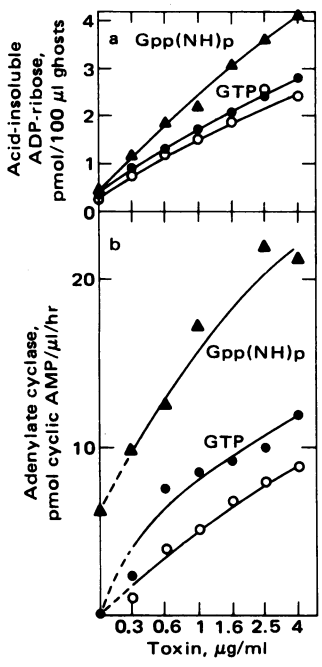
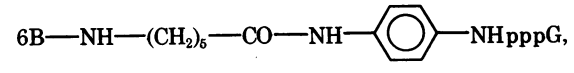


FIG. 6. Stimulation of cholera toxin's action *in vitro* by guanine nucleotides. Incubation: lysate reconstituted from washed ghosts and the macromolecular fraction of cytosol prepared on Sephadex G-25-150, 5 mM ATP, 10 mM thymidine, 1.2 μM NAD, toxin as shown, with no addition (○), 1 mM GTP (●) or 1 mM 5'guanylylimidodiphosphate (Gpp(NH)p) (▲) at 25° for 30 min. Adenylate cyclase activation was incomplete. Separate portions of washed ghosts were extracted with detergent, precipitated with acetone and assayed for radioactivity (*a*) or washed twice to remove guanine nucleotides and assayed for adenylate cyclase activity (*b*). Basal cyclase activity (2 pmol/μl per hr) has been subtracted. The upward displacement of the Gpp(NH)p curve represents cyclase activation by the nucleotide itself.

ribosylated to achieve maximal cyclase activity and the reported number of β-adrenergic receptors per cell (10) and the lack of such a correspondence for any other protein; (*iv*) a size identical to that reported for the cyclase-associated GTP binding protein (10); (*v*) location on the inner surface of the plasma membrane; (*vi*) prior evidence that the GTP binding subunit [or possibly two subunits (14)] modulates adenylate cyclase activity and is required for a cyclase-associated GTPase function (15) [it has been proposed that the active form of adenylate cyclase, with GTP bound, reverts to inactivity when the GTP is hydrolyzed and the split products are released; cholera toxin, by inhibiting the GTP hydrolysis is thought to trap adenylate cyclase in its active state (15–18)]; (*vii*) stimulation by guanine nucleotides of ADP-ribosylation and of adenylate cyclase activation (this could mean that the target protein is a more efficient substrate for the toxin when GTP is bound to it; indeed, because a trace of ghost-bound GTP may have been present in all our experiments, it is possible that the target protein may be a toxin substrate only when bound to GTP). Persistence of the bound GTP might explain the failure of the modified protein to bind to GTP affinity matrices because we were unable to detect specific binding of radioactive product to Sepharose



although a similar matrix adsorbs the unmodified cyclase-associated GTP-binding protein (10).

After about 1500 ADP-ribose residues per cell have been incorporated into the 42,000 M_r protein, it may continue to accept ADP-ribose without a further increase in adenylate cyclase activity. Thus, if it is indeed responsible for the cyclase activation, it is simplest to regard it as a dissociable component of the cyclase system, present in excess over the catalytic and catecholamine receptor components.

The physiological effects of the other ADP ribosylations observed are not clear. The possibility is not excluded that modification of one or more secondary targets may have cellular consequences, and even additional effects on adenylate cyclase. However, because these modifications are relatively slow, they may be inconsequential unless they share the peculiar elegance of the toxin's effect on adenylate cyclase, in which the inhibition of one property (GTPase) disproportionately increases another activity (cyclic AMP synthesis). As is the case for the major target, GTP stimulates the ADP-ribosylation of several (but apparently not all) of these secondary targets as if cholera toxin recognizes some feature common to the nucleotide binding sites of various GTP-binding proteins. This possibility is particularly interesting in view of the remarkable similarities between the actions of cholera and diphtheria toxins. Fragment A_1 of diphtheria toxin catalyzes the transfer of ADP-ribose from NAD to EF2, the eukaryotic polypeptidyl-tRNA translocating factor, which is a protein that binds GTP. After EF2·GTP binds to ribosomes, the GTP is split.

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