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Involvement of Nicotinamide Adenine Dinucleotide in the Action of Cholera Toxin *In Vitro*

(adenylate cyclase/erythrocyte)

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ABSTRACT **NAD is a necessary cofactor for the activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] by cholera toxin. Lysates of certain types of cell that hydrolyze their endogenous store of NAD after cell disruption respond poorly or not at all to cholera toxin. Lysates of pigeon erythrocytes, which lack enzymes that degrade NAD, provide a convenient and reproducible system for assaying the activity of cholera toxin *in vitro* and allow investigation of the mechanism of action of the toxin upon broken cells.**

The protein exotoxin secreted by *Vibrio cholerae* increases the adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] activity of vertebrate cells. Many types of cell are sensitive to the toxin and for all of them a common sequence of events is believed to occur. The toxin appears to interact first with a limited number of specific cell surface receptors. Binding to the receptors is rapid and, after a few minutes exposure to the toxin, cells can no longer be rescued by extracellular antitoxin. After the binding there is a lag, varying from 15 to 60 min at 37° according to the cell type, during which the adenylate cyclase activity remains unchanged. Thereafter, the cyclase activity rises gradually over a period of some hours to reach a level severalfold higher than before. Activation of the cyclase by the toxin appears to be irreversible, since the elevated activity is still apparent in membranes isolated from affected cells (reviewed in ref. 1).

Our knowledge of the mechanism whereby cholera toxin causes this activation is incomplete. The evidence available suggests that the process of intoxication is more complex than a direct interaction of the toxin with adenylate cyclase, and that intracellular factors are somehow involved. Although cholera toxin has little or no effect on many kinds of disrupted cells, Gill and King (2) found that concentrated lysates of pigeon erythrocytes respond well to the toxin. The characteristics of the response *in vitro* differ from those of the response of intact cells in several important respects. Cholera toxin added to a pigeon erythrocyte lysate may elevate adenylate cyclase activity more rapidly, and to a higher level, than is possible with intact erythrocytes. In lysates there is no lag between the addition of toxin and the onset in the activity rise. The addition at any stage of cholera antitoxin immediately prevents further rise in adenylate cyclase activity without reversing any activation that has already occurred. Whereas the entire toxin molecule (six to eight peptides totalling about 84,000 daltons) is necessary for the action on whole cells, only a single peptide (A1: about 24,000 daltons) is needed for adenylate cyclase activation in a lysate. Gill and King concluded that, when cholera toxin acts upon a whole cell, its peptide A1 must penetrate at least as far as the inner surface of the membrane, if not into the cytoplasm itself. They suggested that peptide A1 might be an enzyme that catalyzes some intracellular reaction resulting in the modification of a component of the inner surface of the plasma membrane, where adenylate cyclase is located.

Gill and King noticed that erythrocyte ghosts would not respond to cholera toxin once they had been separated from the soluble erythrocyte cytoplasm. They suggested that the cytoplasm might contain a factor or factors necessary for a toxin-mediated reaction. It is now shown that NAD is one component of the cytoplasm that is required for the action of cholera toxin *in vitro*. NAD partially substitutes for the complete cytoplasm in enabling ghosts to respond to cholera toxin and the hydrolysis of NAD renders lysed erythrocytes refractory to cholera toxin.

METHODS

Medium A consists of 0.13 M NaCl, 5 mM KCl, 2 mM $MgCl_2$, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (Hepes), and NaOH to give pH 7.3 at 37°. Medium B contains 0.08 M NaCl, 12 mM $MgCl_2$, 3 mM EDTA, 0.2 mM dithiothreitol, 0.3 mM papaverine, 2 mM ATP, 13 mM phosphoenolpyruvate, 20 μg/ml of adenylate kinase, 40 μg/ml of pyruvate kinase, 0.25 mg/ml of bovine serum albumin, 40 units of cholera antitoxin per μg of toxin in the tissue sample (a 1.4-fold excess), 0.2 μg/ml of phenol red, 50 mM Tris, and HCl to give pH 8.0 at 37°. These media are the same as used previously (2) except that papaverine has been substituted for theophylline as the inhibitor of cyclic AMP phosphodiesterase in medium B; this allows over double the recovery of cyclic AMP.

Washed pigeon erythrocytes were lysed in one packed-cell volume of medium A by rapid freezing and thawing. Fifty microliter portions of the lysate were incubated with cholera toxin for the times specified in the tables. In medium A, cholera toxin activates adenylate cyclase but endogenous cyclic AMP phosphodiesterase hydrolyzes most of the cyclic AMP formed and little of the nucleotide accumulates. Ten microliter aliquots of the activated lysates were diluted to 50 μl in medium B and incubated for 1 hr at 37°. Medium B allows cyclic AMP to accumulate and contains sufficient cholera antitoxin to neutralize the toxin present and prevent further activation of adenylate cyclase. The amount of cyclic AMP formed was determined by a protein kinase binding

Abbreviation: EF, elongation factor.

assay, as described earlier (2). Adenylate cyclase activities are calculated as the pmol of cyclic AMP that accumulated in medium B in 1 hr in the presence of activated lysate or ghosts from 1 μl of packed cells.

NAD was determined by a sensitive isotope dilution assay (3). A sample containing 5–200 pmol of unlabeled NAD was mixed with 50 pmol of [^{32}P]NAD and the specific activity of the mixture was determined by reacting the NAD with 40 pmol of Elongation Factor 2 (EF 2), using diphtheria toxin as the catalyst (EF 2 + $NAD^+ \rightarrow$ ADP-ribose-EF 2 + nicotinamide + H^+). The [^{32}P]ADP-ribose EF 2 was separated from unreacted NAD by filtration, precipitated with trichloroacetic acid, and analyzed for radioactivity.

NADH was determined by its ability to participate in a coupled reaction in which the addition of NADH results in a transient emission of light. The method was suggested by James Becvar and Tom Baldwin and is similar to that described by Stanley (4). NADH reduces FMN in the presence of an NADH-dependent FMN reductase and the $FMNH_2$ stimulates light emission in the presence of luciferase and aldehyde. Both enzymes were from the luminescent bacterium *Beneckea harveyi*. The peak light intensity was measured a few seconds after NADH was added to a mixture of the other reagents.

NAD glycohydrolase (EC 3.2.2.5) from *Neurospora* was purchased from the Aldrich Chemical Co., Inc., streptococcal NAD glycohydrolase was given by Dr. A. W. Bernheimer, the pig brain and horse liver enzymes by Dr. Jonathan Everse.

RESULTS

Requirement for NAD in the response of erythrocyte ghosts and lysates to cholera toxin

When a freshly prepared lysate of pigeon erythrocytes is incubated with cholera toxin, the activity of membrane-bound adenylate cyclase increases 20- to 50-fold (Table 1, first column). The toxin has no effect, however, if incubated solely with nucleated ghosts that have been separated from the soluble phase of the cytoplasm by brief centrifugation. The first indication that the lack of response could be attributed to the removal of NAD was the demonstration that some of the responsiveness to toxin could be restored by the specific addition of NAD (Table 1, second column). Although the restoration was incomplete, it was consistently observed and could not be duplicated by any other purine nucleotide known to be present in erythrocytes, such as ATP, GTP, and their derivatives.

The failure of NAD to restore the toxin sensitivity to its full original value is in part due to the decay of adenylate cyclase that accompanies manipulations or incubations of ghosts, particularly when separated from cell supernate, and in part due to the presence in cell cytoplasm of an additional factor involved in the action of cholera toxin, as described later.

More dramatic evidence of the importance of NAD was obtained when the endogenous NAD, amounting to 60–80 μM, was removed from a complete lysate, leaving other soluble components unchanged. Hydrolysis of the endogenous NAD by incubation with an NAD glycohydrolase reduces and eventually eliminates the response to cholera toxin. The responsiveness can be restored by adding back NAD. Table 2 shows results obtained using the NAD glycohydrolase from *Neurospora;* similar results were obtained using the NAD glycohydrolases from *Streptococcus*, rat liver, and horse brain.

Since these glycohydrolases were able to degrade the added NAD, maximum toxin sensitivity was obtained only when much more NAD was added than was present in the original lysate. It was not possible to use nicotinamide to inhibit any of the glycohydrolases, for nicotinamide is also an inhibitor of the action of cholera toxin.

TABLE 1. *Activation of adenylate cyclase by cholera toxin and NAD in fractions derived from erythrocytes*

	Adenylate cyclase activity, pmol/μl per hr			
			Reconstituted lysate	
Incubation	Complete lysate	Ghosts	Pigeon ghosts + pigeon supernate	Pigeon ghosts + rabbit supernate
No addition	1.3	0.6	1.2	1.2
+ Toxin	34	0.6	12.0	1.3
+ NAD	1.2	0.5	1.2	1.2
+ Toxin + NAD	40	2.4	14.8	10.7
+ Toxin + NADH	38	0.8	—	—

The complete lysate consisted of washed pigeon erythrocytes, free of white cells, suspended in one packed-cell volume of medium A and lysed by rapid freezing and thawing. A portion of this lysate was centrifuged for 60 min at 100,000 × *g* and the particle-free supernate was withdrawn. Another portion of the lysate was centrifuged for 5 min at 2000 × *g* to sediment ghosts. Rabbit erythrocyte supernate was prepared in a similar way as the pigeon supernate except that the erythrocyte lysate was incubated for 2 min at 37° to ensure that all endogenous NAD had been hydrolyzed by the NAD glycohydrolase present in the ghosts. Pigeon ghosts were resuspended in medium A (second column), pigeon erythrocyte supernate (third column), or rabbit erythrocyte supernate (fourth column) to give the same ghost concentration as in the original lysate. All samples were incubated with cholera toxin, 10 μg/ml, and NAD, 1 mM, as described, for 1 hr at 37°. Adenylate cyclase activities were then determined as described in *Methods*.

TABLE 2. *Loss of sensitivity to cholera toxin on hydrolysis of NAD and restoration of sensitivity upon addition of NAD*

	Adenylate cyclase activity, pmol/μl per hr	
	No toxin	+ Toxin
Lysate not preincubated	0.5	20.3
Lysate preincubated alone	0.3	7.2
Lysate preincubated with NAD glycohydrolase	0.3	0.6
Lysate preincubated with NAD glycohydrolase, NAD then added	0.3	7.6

Portions of pigeon erythrocyte lysate were preincubated for 30 min at 37° with or without NAD glycohydrolase from *Neurospora* (0.025 units/ml). Cholera toxin, 20 μg/ml, and in certain cases NAD, 2 mM, were then added, and the incubation was continued for a further 60 min. Adenylate cyclase activities were determined as described in *Methods*. Note that there was a considerable decay of adenylate cyclase activity during the preincubations.

TABLE 3. *Effect of derivatives and analogs of NAD on the activation of adenylate cyclase by cholera toxin; comparison with ADP-ribosyl transfer and dehydrogenation reactions*

Supplement (2 mM)	Adenylate cyclase activity pmol/μl per hr	ADP-ribose donor in diphtheria toxin reaction[a]	Coenzyme activity in dehydrogenase reactions[b]
None	0.6		
NAD	7.6		
NADH	(8.8)[c]	−	−
NADP	2.1	+	− or +
α-NAD	0.6	−	−
Breakdown products of NAD			
NMN	2.0	Uncertain	−
Deamino NAD	2.0	+	++
Nicotinamide, ribose-5-phosphate, ADP-ribose, ADP, AMP, adenosine, or adenine	0.6	−	−
Nicotinamide modifications			
Thionicotinamide AD	9.7	++	+ or ++
Nicotinyl hydroxamic acid AD	2.1	+	+ or ++
3-Acetyl pyridine AD or 3-carbonyl pyridine AD	0.6	+	+ or ++
Ethyl nicotinate AD	1.8	−	−
4-Methyl pyridine AD	0.6	−	−
4-Amino-5-imidazole AD	0.3	−	−
FAD	0.4	−	−
Other compounds			
ATP, GTP, GDP, guanosine, or guanine	0.6	−	−

Lysed pigeon erythrocytes were preincubated with NAD glycohydrolase and then incubated with cholera toxin and NAD or a derivative as in Table 2.

[a] Ability to replace NAD in the diphtheria-toxin-catalyzed transfer of ADP-ribose to elongation factor 2. ++: good substrate; +: poor substrate; −: very poor or cannot substitute. Data from ref. 5 and unpublished results of John Philbrick, Harvard University.

[b] Ability to act as the oxidant in reactions catalyzed by various NAD-linked dehydrogenases. From citations in ref. 6.

[c] NADH is rapidly oxidized to NAD, as explained in the *text*.

Once adenylate cyclase has been activated by cholera toxin, NAD glycohydrolase does not reduce its activity and NAD is no longer required. Nor does the removal or addition of NAD affect the basal adenylate cyclase activity or the extent of stimulation by epinephrine or by fluoride ions. NAD seems to be required only for the activation in the presence of cholera toxin.

Specificity of the NAD requirement

The ability of certain nucleotides and nucleotide derivatives to replace NAD for the activation of adenylate cyclase was tested in pigeon erythrocyte lysates after treatment with NAD glycohydrolase. The results are listed in Table 3, which also compares the effectiveness of the compounds as cofactors in the action of cholera toxin with their ability to replace NAD as a coenzyme in dehydrogenase reactions and in the ADP-ribose transfer to EF 2 catalyzed by diphtheria toxin. Apart from a few minor differences, those NAD analogs that can partially or completely replace NAD are the same in all three cases. Only one compound, the thionicotinamide analog of NAD, is as effective as NAD itself in the action of cholera toxin. A few derivatives of NAD, including NMN, deamino NAD, and NADP can replace NAD to a limited extent. Adenine and guanine nucleotides, nucleosides and bases, FAD, the alpha isomer of NAD, NAD substituted in the 4 position of nicotinamide, and certain more radically modified NAD derivatives cannot replace NAD at all.

By direct assays of the pyridine nucleotides it was found that NADH added to a lysate is oxidized quantitatively to NAD in less than 1 min at 37°. Thus the addition of NADH to an NAD-depleted lysate is equivalent to the addition of NAD and results in the restoration of toxin sensitivity (Table 3). However, when NADH is added to ghosts, little NAD is formed and there is only a slight restoration of toxin-sensitivity (Table 1). Conversely, the toxin sensitivity of a complete lysate is decreased by ethanol and alcohol dehydrogenase which, at the pH used, convert most of the endogenous NAD to NADH. It is clear, therefore, that NAD itself, and not NADH, is the cofactor responsible for the sensitivity of pigeon erythrocyte lysates to cholera toxin. It is reasonable to suppose that NAD must be similarly involved in the response to cholera toxin of intact erythrocytes and other cells.

Relation between toxin sensitivity and NAD glycohydrolase activity of lysed erythrocytes

Pigeon erythrocytes were originally chosen for a study of the action of cholera toxin *in vitro* because they are metabolically simple, easily lysed cells which have an appreciable adenylate cyclase activity. The choice proved to be fortunate for an additional reason, namely that they lack the membrane-bound NAD glycohydrolase which is present in the erythrocytes of other mammalian and avian species examined (7). (Some examples are given in Table 4.) When present, this enzyme is activated upon cell lysis, rapidly degrades NAD, and renders lysates insensitive to cholera toxin. For mammalian erythrocytes the hydrolysis of NAD is so rapid that a definite activation of adenylate cyclase can only be observed if cholera toxin is added before lysis and is, therefore, present

during the few seconds after lysis that some undegraded NAD remains. (For rabbit erythrocytes we obtained 0.20 ± 0.18 pmol/μl per hr without toxin and 0.60 ± 0.32 pmol/μl per hr with 15 μg/ml of toxin, five samples in each group.)

As expected, the NAD glycohydrolase in rabbit erythrocyte ghosts prevents lysed pigeon erythrocytes from responding to cholera toxin but the membrane-free supernatant from rabbit erythrocytes does not (Table 4).

NAD glycohydrolase is widely distributed in nature (8) and its activity may well account for the general insensitivity to cholera toxin of disrupted cells.

Involvement of a second soluble factor in the action of cholera toxin

As mentioned earlier, the restoration of toxin sensitivity to ghosts by NAD alone is relatively poor. However, the ghosts respond to toxin to a much greater extent if erythrocyte supernate is also present (compare the second and third columns of Table 1). The supernatant factor that enhances the response is also present in rabbit and turkey erythrocytes. Indeed, rabbit erythrocyte supernate that contains no NAD is a convenient source of the second factor. As can be seen from the fourth column of Table 1, rabbit supernate has no effect on toxin sensitivity unless NAD is added. Dialyzed erythrocyte supernate supports adenylate cyclase activation by cholera toxin and NAD almost as well as does undialyzed supernate. This suggests that the second factor may be a macromolecule, and further indicates that NAD is the only small molecule involved in the action of cholera toxin.

Experiments with radioactive NAD

Pigeon erythrocyte lysates were incubated with cholera toxin together with preparations of NAD labeled in the phosphates, the nicotinamide, or the adenine. In each case the soluble products were fractionated by paper chromatography and high-voltage paper electrophoresis. There was no appreciable reduction in the total amount of NAD nor any evidence for formation of degradation products. It is doubtful, however, that soluble products of a reaction between NAD and adenylate cyclase itself would have been detected, since there may be several orders of magnitude fewer adenylate cyclase molecules than NAD molecules in a cell.

The ghosts present in lysates that had been incubated with radioactive NAD were collected by filtration and their radioactivity was measured. There was no appreciable formation of an insoluble radioactive product in the presence of cholera toxin but, as in the case of the soluble products, it is possible that too little product was formed to detect. Detection of a reaction between NAD and any membrane component is further complicated by the fact that each pigeon erythrocyte ghost binds about 10^5 NAD molecules, representing about 1% of the total cellular NAD. *In vitro*, this NAD exchanges with labeled NAD in a time- and concentration-dependent manner. Much of the bound NAD remains after washing of the ghosts, even with trichloroacetic acid.

TABLE 4. *Toxin sensitivity and NAD hydrolysis rate of erythrocyte lysates*

Origin of erythrocytes	Increase in adenylate cyclase activity, pmol/μl per hr, during 30 min incubation of lysate with cholera toxin at: 0.5 μg/ml	5 μg/ml	50 μg/ml	Half-life of NAD in lysate
Pigeon	16.4	36	51	Stable (>4 hr)
Turkey	0	0.6	5	about 3 min
Rabbit	0	0	0	about 4 sec
Pigeon + turkey (1:1)	0.4	4	17.6	
Pigeon + rabbit (1:1)	0	0	0	
Pigeon (with rabbit erythrocyte supernate)	10	27	40	

Erythrocytes suspended in one packed-cell volume of medium A were lysed by rapid freezing and thawing. Immediately thereafter, cholera toxin was added and the temperature was raised to 37°. For the bottom row, pigeon erythrocytes were suspended in rabbit erythrocyte 100,000 × *g* supernate for lysis. NAD hydrolysis rates were determined by adding a small amount of radioactive NAD to portions of the lysates and periodically measuring the amount of NAD remaining that could participate in the diphtheria toxin reaction.

DISCUSSION

The activation of adenylate cyclase by cholera toxin is clearly a complex process and much remains to be learned of its mechanism. The activation process involves peptide A1 of cholera toxin, which must be able to reach the inner surface of the cell membrane where adenylate cyclase is embedded. When the toxin acts upon intact cells, the remainder of the toxin molecule is necessary for the insertion of peptide A1 through the cell membrane, but when the membrane is disrupted experimentally peptide A1 is the only part of the toxin molecule that is required. In the present paper it has been shown that NAD is essential for the activation process *in vitro*, and it is reasonable to suppose that it is similarly involved *in vivo*. NAD must be present at the same time as cholera toxin and cell membranes for activation of adenylate cyclase; once the cyclase has been activated, NAD has no effect. Furthermore, its involvement with adenylate cyclase is restricted to the action of cholera toxin and it does not affect the basal adenylate cyclase activity nor the extent to which the cyclase activity is increased by fluoride ions or by catecholamines, which are the only hormones known to stimulate erythrocyte cyclase.

A second component of the erythrocyte cytoplasm is also required for the maximum response of ghosts to cholera toxin. This has not yet been characterized and we know only that it behaves as a macromolecule. Fortunately, the complication that this introduces does not affect the discussion that follows, since washed ghosts can be made to respond to cholera toxin to an appreciable extent simply by the addition of NAD (Table 1). This fact greatly reduces the number of possible models we can construct for the action of cholera toxin.

It is reasonable to assume that cholera toxin may catalyze a reaction between NAD and a target molecule of the red cell membrane, leading to a permanent change in the structure or conformation of adenylate cyclase. This target might be a catalytic component of adenylate cyclase, a regulatory component of the cyclase system, or perhaps some other membrane component which is not normally involved in the regulation of adenylate cyclase activity but which, once modified,

interacts with the cyclase in some new way to cause its activation.

In considering the kind of chemical reaction that might occur between NAD and a membrane target, it has been possible to exclude several of the ways in which NAD participates in known biochemical reactions. It has been shown that NAD is not first converted to NADP, nor first hydrolyzed to NMN and AMP or to nicotinamide and ADP-ribose, since none of these products supports the activation of adenylate cyclase *in vitro* as well as does NAD itself (Table 3). NAD is not first converted to NADH which then reduces a cellular target. NAD cannot act by donating its AMP moiety to a membrane target, in the manner of the reaction catalyzed by *Escherichia coli* DNA ligase, since NMN has a greater cofactor activity in NAD-depleted lysates than would be expected if it acted only after it reacted with ATP to form NAD (Table 3).

In fact, of all the ways in which NAD is involved as a substrate or product in known enzymic reactions, only two remain as possibilities for the cholera toxin reaction. NAD might transfer its ADP-ribosyl moiety to a target in a manner similar to that by which diphtheria toxin catalyzes transfer of ADP-ribose to EF 2. Alternatively, NAD could be reduced to NADH while the target molecule was dehydrogenated. The specificity of the requirement for NAD is equally compatible with either model (Table 3). It does not, unfortunately, allow us to distinguish between the two hypotheses.

If the membrane target were a part of the adenylate cyclase system, or another molecule that interacted with adenylate cyclase on a one-to-one basis, it would be represented relatively few times in each cell. Estimates of the number are necessarily imprecise but it seems unlikely to exceed a few thousand copies per cell and in any case must be insignificant in relation to the NAD content of a cell. Thus it was not altogether surprising to find that during incubation with cholera toxin there was no significant change in the NAD content of a lysate nor any measurable formation of a radioactive product derived from radioactively labeled NAD. If such changes do occur their demonstration may necessitate use of detection methods much more sensitive than those that have been used so far. In any event, it is clearly of importance to determine the precise role of NAD, not only for a full understanding of cholera but also, perhaps more importantly, for an understanding of the functioning of adenylate cyclase.

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