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Molecular Basis for the Pathological Actions of *Clostridium perfringens* Iota Toxin

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***Clostridium perfringens* type E iota toxin is composed of two separate and independent polypeptide chains that act synergistically in mouse lethal assays. The light chain is an enzyme that mono(ADP-ribosyl)ates certain amino acids. The enzyme displays substantial activity when homopoly-L-arginine is used as a substrate, but it shows little activity when polyasparagine, polylysine or polyglutamic acid are used. In keeping with the properties of an ADP-ribosylating enzyme, the toxin possesses the following characteristics. It produces incorporation of radioactivity into polyarginine when adenine-labeled NAD is used, but radioactivity is not incorporated when nicotinamide-labeled NAD is used. Irrespective of labeling, enzymatic activity is accompanied by the release of free nicotinamide. After incorporation of ADP-ribose groups into polyarginine, enzymatic and chemical techniques can be used to release the incorporated material. Snake venom phosphodiesterase releases mainly AMP; hydroxylamine releases AMP and ADP-ribose. The heavy chain of iota toxin has little or no enzyme activity, and it does not substantially affect the enzyme activity of the light chain. The heavy chain may be a binding component that directs the toxin to vulnerable cells. The data suggest that iota toxin is a representative of a novel class of ADP-ribosylating toxins.**

Clostridium perfringens is a gram-positive, anaerobic organism that is relatively ubiquitous in its distribution (7). There are five types of *C. perfringens*, designated types A, B, C, D, and E (15). Each of these organisms produces a semi-unique spectrum of protein toxins. *C. perfringens* type E produces at least six soluble toxins, one of which is iota toxin (7, 15).

Iota toxin was first described more than four decades ago (1), but the substance was not isolated and purified to homogeneity until recently (31; B. G. Stiles and T. D. Wilkins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B128, p. 39). When tested in vivo, the toxin produces death in mouse lethality assays (1, 23); when tested in situ, it produces dermonecrosis in guinea pigs (1). Although the full structure-function relationships of the toxin have not been determined, one aspect of the structure of the substance is important here. Iota toxin is composed of two separate polypeptide chains (31; Stiles and Wilkins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985). The individual chains possess relatively little toxicity, but the combination of chains is very potent. The data suggest that iota toxin is a true binary toxin.

Binary toxins of microbial origin are not common substances. Only a small number have been reported, including the components of anthrax toxin (26), leukocidin (18), and one of the botulinum toxins (10, 21). The mechanism of action of two of the binary toxins has been partially determined. One of the components of anthrax toxin is an enzyme that possesses adenylate cyclase activity (14). One of the components of the botulinum binary toxin is also an enzyme, possessing ADP-ribosylating activity (25; Leppla, personal communication).

Iota toxin appears to share certain properties with the botulinum binary toxin, including the ability to promote the movement of fluids across membranes (5, 11, 19, 20, 24). There are also the obvious similarities that both toxins are clostridial in origin and both can be released into the gut of a host that has succumbed to an opportunistic infection. These similarities suggest that the two toxins should be compared in terms of cellular and molecular actions.

The present study focused on the molecular properties of *C. perfringens* iota toxin. Data were obtained which show that one of the components of the toxin is an enzyme with mono(ADP-ribosyl)ating activity.

MATERIALS AND METHODS

Growth of bacteria. The reference strain *C. perfringens* type E NCIB 10748 (VPI 1589) was used in this study. The organism was grown in brain heart infusion dialysis flasks as previously described (27). Each flask was inoculated with cultured organisms and incubated at 37°C for 72 h. Cells and debris were removed by centrifugation (8,000 × *g*; 15 min) and subsequent filtration (0.45-μm-pore-size membrane; Gelman Sciences, Inc., Ann Arbor, Mich.).

Toxin purification. The full details on isolation and purification of the toxin have been described elsewhere (Stiles and Wilkins, submitted for publication). For the purposes of this study, two polypeptides were used that migrated as single bands in polyacrylamide gel electrophoresis (13). These two components of iota toxin have previously been referred to as i_a and i_b, but they are referred to here as light chain and heavy chain (see Discussion).

Radioisotope assay for ADP-ribosylation. The heavy and light chains of iota toxin were assayed for ADP-ribosylating activity by techniques previously reported (4, 16). Substrate (10 mg/ml) was suspended in dimethylglutaric acid buffer (100 mM, pH 7.0) that contained 5 μCi of [^{3}H]NAD plus other ingredients as indicated under Results (total volume, 100 μl). The solution was incubated at 33°C for various times. When the substrate in question was homo-poly-L-arginine, the ADP-ribosylated product was precipitated by adding phosphate buffer (1 ml, 100 mM, pH 7.0). The mixture was centrifuged (4,500 × *g*), and the pellet was washed three times. The final pellet was dissolved in 0.5 ml

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TABLE 1. Gradient formation for HPLC separations[a]

Condition[b]	Time (min)	Flow rate (ml/min)	% Buffer A[c]	% Buffer B[d]
A	0	0.5	100	0
B	9.99	0.5	100	0
C	10.00	1.0	100	0
D	20.00	1.0	90	10
E	22.00	1.0	90	10
F	25.00	1.0	0	100

[a] An extensive presentation of chromatograms can be found in reference 2.
[b] Isocratic conditions were used from A to C and from D to E. A linear gradient was used between C and D and between E and F.
[c] 20 mM Tris hydrochloride (pH 8.0)
[d] 50 mM Tris hydrochloride (pH 8.0)–1 M NaCl.

of 1.0 mM HCl, followed by 1.0 ml of 100 mM dimethylglutaric acid buffer (pH 7.0). A sample of the solution was added to a scintillation cocktail, and radioactivity was quantified by liquid scintillation spectrometry. Experimental values were corrected by subtracting background activity in solutions without enzyme.

When the substrate in question was a homo-poly-L-amino acid other than polyarginine, the reaction mixture and incubation procedures were identical to those described above. At the end of experiments, [^{3}H]ADP-ribosylated homopolymer was separated from unreacted [^{3}H]NAD by fractionation on a Sephadex G-25 column (12 by 270 mm). The column was eluted with unbuffered saline (1 ml/min), and fractions of eluate were quantified for radioactivity as described above.

Irrespective of the substrate used or the separation procedures, each assay was performed in duplicate, and each experiment was done at least twice. Thus, each result represents the mean of at least four observations.

HPLC assay for ADP-ribosylation. The methods used for high-performance liquid chromatography (HPLC) were basically similar to those of Brown et al. (2). The technique allowed for resolution of NAD, nicotinamide, AMP, and ADP-ribose.

The reaction conditions were the same as described above for the radioisotope assay. At the end of incubation, samples were injected into a Waters Associates HPLC system with a gradient controller. The gradient was created with 20 mM Tris hydrochloride (pH 8.0) and 50 mM Tris hydrochloride (pH 8.0) containing 1 M NaCl. The conditions for elution of the compounds are given in Table 1. The elution times were: NAD, ~6 min; nicotinamide, ~10 min; AMP, ~20.5 min; and ADP-ribose, ~21.5 min.

Compounds were separated on an anionic-exchange column (Mono-Q; Pharmacia Fine Chemicals, Piscataway, N.J.). The A_{254} was monitored and plotted on a strip-chart recorder. Fractions were collected at 1-min intervals. Results with unlabeled compounds were quantified by measuring the areas under the curve; results with labeled compounds were quantified by liquid scintillation spectrometry.

Cleavage of ADP-ribosylated homo-poly-L-arginine. Two techniques were used to release incorporated products from homopolymer. In the first, ADP-ribosylated polyarginine was incubated with snake venom phosphodiesterase as described under Results. The products were characterized by HPLC (details are given below). In the second, ADP-ribosylated polyarginine was reacted with hydroxylamine by the technique of Wielckens et al. (30). The procedure was as follows. At the end of incubation, the washed and precipitated polyarginine was dissolved in 150 μl of 5 mM HCl. A sample (50 μl) of 2 M NH_2OH was added, and the solution was adjusted to pH 7.4 with concentrated NaOH. The material was heated at 56°C for 15 min, and it was maintained at 37°C for an additional 60 min. Samples of the reaction mixture were injected into the HPLC.

The hydroxylamine method of Wielckens et al. (30) releases the ADP-ribose moiety incorporated into protein, either as the ADP-ribose molecule or as AMP. The amount of radioactive ADP-ribose and AMP released from polyarginine was determined after the homopolymer was incubated with toxin and tritiated NAD. The results of exposure to hydroxylamine were compared with those of exposure to water. The data are expressed as the percentage of radioactivity lost from polyarginine and as the percentage of radioactivity recovered in ADP-ribose and AMP.

Animals and toxicity testing. A limited number of experiments were done on mice (male Swiss-Webster, 20 to 30 g). The sole purpose of the experiments was to determine whether the individual chains from the botulinum toxin and from the *C. perfringens* toxin would combine to form a hybrid binary toxin. The various components were added together and administered intravenously in a volume of 0.1 ml (0.154 M NaCl). Because of the nature of the experiment and the data being obtained, it was deemed inappropriate to allow animals to die. When mice became visibly ill, they were sacrificed painlessly with a volatile anesthetic (ethyl ether).

Reagents. Unlabeled NAD and NADase were purchased from Sigma Chemical Co. (St. Louis, Mo.). Tritiated NAD labeled in the adenine moiety (nicotinamide [4-^{3}H]adenine dinucleotide; 30 Ci/mmol) was obtained from ICN Pharmaceuticals, Inc. (Irvine, Calif.). Tritiated material labeled in the nicotinamide moiety ([2,4-^{3}H]nicotinamide adenine dinucleotide; 3.2 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.). Homo-poly-L-amino acids (arginine, asparagine, glutamic acid, and lysine) and snake venom phosphodiesterase were purchased from Sigma. The heavy and light chains of botulinum toxin were generously provided by I. Ohishi (University of Osaka Prefecture, Osaka, Japan).

RESULTS

Enzyme activity. The light and heavy chains of iota toxin were tested for ADP-ribosylating activity. In the initial experiments, toxin was mixed with homo-poly-L-arginine and adenine-labeled NAD.

The light chain of iota toxin produced time- and concentration-dependent incorporation of radioactivity into the homopolymer fraction (Fig. 1). This result was obtained at toxin concentrations of 10^{-9} to 10^{-7} M and at elapsed times of 1 to 4 h. When the heavy chain was tested at 10^{-9} M and for an elapsed time of 1 h, it possessed only 0.7% of the activity of the light chain.

Various mixtures of heavy and light chains were tested for enzyme activity (ratios tested were 4:1, 2:1, 1:1, 0.5:1, 0.25:1). Irrespective of the ratio tested, there was no substantial effect of the heavy chain on the light chain. Enzyme activity was equal to, or only slightly greater than (15 to 20%), that which could have been accounted for on the basis of additivity. The data indicate that interaction between the two chains in terms of toxicity (see below) cannot be explained in terms of synergistic enzyme activity.

Additional experiments were done to determine whether other poly-amino acids subject to mono(ADP-ribosyl)ation (i.e., asparagine and lysine [29]) or poly(ADP-ribosyl)ation

(i.e., glutamic acid and lysine [29]) were substrates for the light chain of iota toxin. Unlike polyarginine, these other poly-amino acids were not effective as substrates for the toxin (Table 2).

The experiments just described suggest that the light chain of iota toxin is an ADP-ribosylating enzyme. An additional series of experiments was done to confirm the nature of the enzymatic activity. This work involved the use of tritiated NAD labeled in the nicotinamide group, unlabeled NAD, and NADase.

In the presence of nicotinamide-labeled NAD, the light chain of iota toxin (10^{-9} to 10^{-7} M) failed to produce significant incorporation of radioactivity into polyarginine. Furthermore, when a 50-fold molar excess of the nicotinamide-labeled NAD was used in the presence of adenine-labeled NAD, there was a predictatable decrease (>90%) in the amount of radioactivity incorporated into polyarginine. In the latter experiment, the nicotinamide-labeled material presumably acted as a competitive substrate and displaced the adenine-labeled material.

In the next series of experiments, the light chain of iota toxin (10^{-9} M) was incubated with a large molar excess of unlabeled NAD (10^{-4} M) and with polyarginine. At the end of various incubation times, the reaction mixture was injected into the HPLC. The results showed that the toxin produced time-dependent loss of NAD and time-dependent appearance of nicotinamide. For example, when the mixture was incubated for 24 h, approximately 82% of the NAD was lost. Within the limits of resolution of the HPLC, there appeared to be nearly a stoichiometric appearance of nicotinamide. In keeping with the expected outcome, there was virtually no appearance of free ADP-ribose.

In the final experiments, NADase (0.01 U per reaction tube) was incubated (1 to 4 h, 35°C) with adenine-labeled NAD before enzyme assay. At the end of the assay, samples of the reaction mixture were injected into the HPLC. The identity of labeled compounds (polyarginine, NAD, nicotinamide) was determined on the basis of coelution with larger amounts of unlabeled compounds (see Materials and Methods) (Table 1).

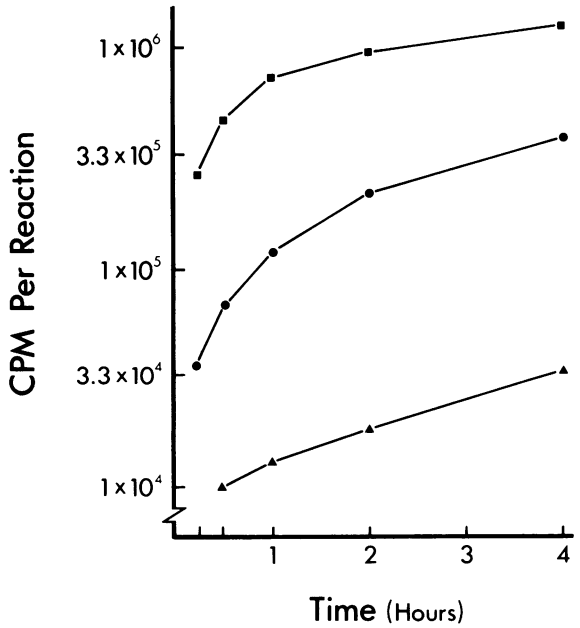

FIG. 1. Counts per minute of radioactivity incorporated into homo-poly-L-arginine. The reaction was carried out as described in Materials and Methods. Three concentrations of the light chain of iota toxin were tested (▲, 10^{-9} M; ●, 10^{-8} M; ■, 10^{-7} M).

TABLE 2. Homo-poly-L-amino acids as substrates for iota toxin[a]

Chain	CPM with:			
	Arginine	Asparagine	Glutamic acid	Lysine
Heavy	<1,000	<1,000	<1,000	<1,000
Light	92,764	<5,000	<5,000	<5,000

[a] The reaction (1 h, 33°C) was carried out in the presence of 10^{-8} M heavy or light chain, 5 μCi of adenine-labeled NAD, and 100 μg of poly-amino acid in a volume of 100 μl. The results represent the ADP-ribosylated protein collected after chromatography on Sephadex G-25.

NADase expressed time-dependent glycohydrolase activity, and this was associated with a decrease in the subsequent incorporation of radioactivity into polyarginine. For example, incubation of NADase with NAD for 4 h produced almost complete loss of NAD and a simultaneous appearance of its products (i.e., nicotinamide). In the subsequent ADP-ribosylation assay, the loss of NAD was associated with a marked reduction (>90%) in the amount of radioactivity found in polyarginine.

Phosphodiesterase experiments. Polyarginine (100 μg) was incubated (4 h, 33°C) with adenine-labeled NAD (5 μCi) and the light chain of iota toxin (10^{-8} M). At the end of incubation, the poly-amino acid was precipitated and washed as described under Materials and Methods. The pellet was suspended and divided into two aliquots. Half of the material was incubated (33°C) for an additional 4 h without modification; the balance of the material was similarly incubated, but in the presence of snake venom phosphodiesterase (100 μg). Both solutions were injected into the HPLC.

The reaction mixture that had not been exposed to phosphodiesterase had one major peak, which was radioactive polyarginine. There was a small peak that represented unreacted [^{3}H]NAD. The reaction mixture that was exposed to phosphodiesterase lost most (~80%) of its radioactive polyarginine. Radioactivity was recovered predominantly, but not exclusively, in AMP. There were other radioactive products that were not characterized (see Discussion).

Hydroxylamine experiments. ADP-ribosylation assays were performed as described above, and the individual reaction tubes were treated with water (control) or hydroxylamine (experimental) as described under Materials and Methods.

When reacted polyarginine was exposed to hydroxylamine, the presence or absence of cleavage products varied with treatment conditions. When incubation was at 37°C, there was almost no difference between control and experimental preparations. However, when the reaction tubes were warmed to 56°C for 15 min and then incubated at 37°C, the incorporated radioactivity was significantly hydroxylamine sensitive. For example, after 1 h of incubation the radioactivity in experimental tubes was approximately 40% less than that in the control tubes, and after 4 h the difference was approximately 60%. Virtually all the hydroxylamine-sensitive material was recovered as ADP-ribose and AMP.

Nicotinamide experiments. Nicotinamide produced concentration-dependent inhibition of the ADP-ribosylation reaction (Fig. 2). When the enzyme concentration was 10^{-9} M and when incubation was for 1 h, nicotinamide (0.1 M) diminished by approximately 90% the amount of radioactiv-

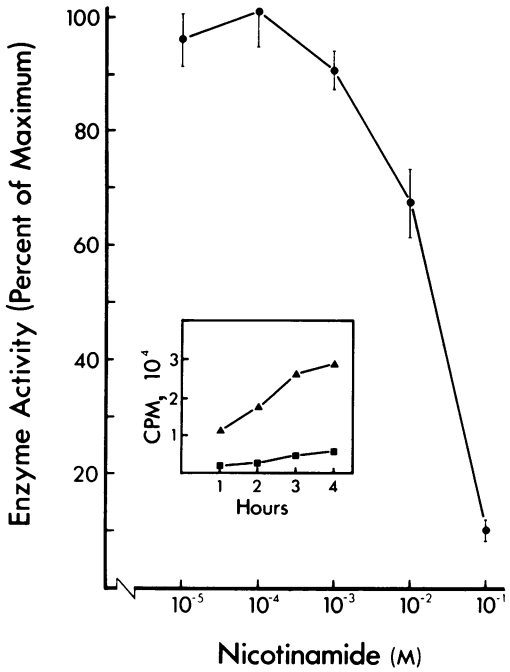

FIG. 2. Effect of various concentrations of nicotinamide on enzyme activity. The reaction (1 h, 33°C) was carried out in the presence of 10^{-9} M light chain, 5 μCi of adenine-labeled NAD, 100 μg of polyarginine and the indicated concentrations of nicotinamide in 100 μl. The inset represents the data from experiments in which incubation time was varied. Nicotinamide was not added to control tubes (▲), but it was added to experimental tubes (●, 10^{-1} M).

ity incorporated into protein. The inhibition was sustained over time.

Binary toxin experiments. Mice (n = 5) were injected with 2.0 μg of the light chain of iota toxin, either alone or in combination with the heavy chain of iota toxin or the heavy chain of the botulinum binary toxin (each at 2.0 μg). The light chain by itself did not produce symptoms of illness within 250 min. When the light chain was combined with the heavy chain of iota toxin, onset of symptoms (respiratory distress) occurred within 100 min. By contrast, when the light chain of iota toxin was combined with the heavy chain of the botulinum binary toxin, no symptoms developed within 250 min. The data indicate that the components of iota toxin act like a binary toxin but that heterologous components of the *C. perfringens* and botulinum toxins do not interact to form a hybrid binary toxin.

DISCUSSION

Two major classes of ADP-ribosylating toxins have been described in the literature. Diphtheria toxin (8) and *Pseudomonas aeruginosa* exotoxin (9) act on elongation factor 2 in eucaryotic cells. By virtue of catalytically modifying the translocase, they inhibit protein synthesis and ultimately cause cell death. Cholera toxin (3, 6), *Escherichia coli* enterotoxin (17), and pertussis toxin (12) act on regulatory proteins that govern adenylate cyclase activity. By modifying nucleotide-binding proteins, they disrupt the ability of eucaryotic cells to control cytoplasmic levels of cyclic AMP.

More recently, one of the botulinum toxins (type C_2) has been found to possess ADP-ribosylating activity (25; S. Leppla, personal communication). This toxin was at one time thought to be part of a homologous series of neurotoxins, but it is now known to be a unique substance. One of its distinctive characteristics is that it is a binary toxin. Although the botulinum binary toxin possesses ADP-ribosylating activity, it appears to be different from other ADP-ribosylating toxins. Unlike diphtheria toxin and *P. aeruginosa* exotoxin, it does not typically cause cell death. The botulinum binary toxin superficially mimics cholera toxin and related substances, because it does promote the movement of fluids across membranes (11, 19, 20, 25), but an action of this toxin on nucleotide-binding proteins has not been reported.

There is evidence for the existence of at least one other toxin that may be similar to the botulinum binary toxin. *C. perfringens* produces an iota toxin that is composed of two independent polypeptide chains. The individual chains have relatively little activity, but the combination of chains is highly active. In addition, some of the systemic actions of iota toxin are the same as those of the botulinum binary toxin. These findings suggest that the underlying structure-function relationships of the two toxins are the same.

In the case of the botulinum binary toxin, certain structure-function relationships have already been determined. The heavy chain mediates cell surface binding (22, 24), and the light chain is an enzyme with ADP-ribosylating activity (25). The present study was done to determine whether the structure-function relationships of the *C. perfringens* binary toxin mimic those of the botulinum binary toxin.

The light chain of iota toxin was found to possess ADP-ribosylating activity. When tested in a simple assay system that contained only adenine-labeled NAD and a substrate, the light chain caused radioactivity to accumulate in the homopolymer fraction. As would be predicted with an ADP-ribosylation reaction, nicotinamide was an effective antagonist.

A number of experiments were done to confirm that ADP-ribosylation was involved. The results can be summarized as follows. In the presence of unlabeled NAD and polyarginine, iota toxin produced disappearance of the nucleotide and a concomitant appearance of free nicotinamide. The toxin did not produce free ADP-ribose. This is the expected outcome, because ADP-ribose moieties should be incorporated into polyarginine. The results with labeled NAD supported those with unlabeled material. The toxin produced incorporation of radioactivity into polyarginine when adenine-labeled NAD was present but not when nicotinamide-labeled material was used.

When adenine-labeled NAD was used as a source for the radioactivity incorporated into polyarginine, both enzymatic and chemical means could be used to release the incorporated substance. Phosphodiesterase releases AMP from mono(ADP-ribosyl)ated protein (28). When the enzyme was added to polyarginine that had been exposed to iota toxin and adenine-labeled NAD, it released labeled AMP. There was labeled material in addition to AMP, but this was almost certainly due to the fact that the snake venom enzyme was not homogeneous. It probably contained trace enzymes other than phosphodiesterase. Therefore, a companion experiment was done with hydroxylamine, which releases ADP-ribose and AMP from mono(ADP-ribosyl)ated protein (30). In this case, virtually all the radioactivity that was cleaved from polyarginine was recovered as ADP-ribose and AMP.

The cleavage experiments suggest that mono- rather than poly(ADP-ribosyl)ation was involved. The products of the cleavage experiments indicate this, as do two other observations. First, microbial toxins with enzyme activity (e.g.,

diphtheria toxin, cholera toxin) usually transfer only one ADP-ribose group per substrate group (29). Second, arginine is typically a substrate for mono- rather than poly(ADP-ribosyl)ation reactions (29). These several clues suggest that the toxin is responsible for mono incorporation of ADP-ribose groups.

The discovery that the light chain of iota toxin has ADP-ribosylating activity is interesting, but nevertheless there are substantive questions that remain to be answered. In keeping with the structure-function relationships of other ADP-ribosylating toxins and of the botulinum binary toxin in particular, one would expect that the heavy chain of iota toxin plays a role in cell surface binding. The data reported in this study make it clear that the heavy chain possesses little if any enzyme activity and that this chain does not substantially enhance the enzyme activity of the light chain. The role of the heavy chain is more probably that of tissue targeting the binary toxin, but further work is needed to explore this possibility.

It would also be desirable to determine the intracellular substrate for iota toxin. There is the possibility that the botulinum binary toxin and the iota toxin act on the same intracellular proteins. If this were true, it would suggest that the toxins are the first known representatives of a novel class of ADP-ribosylating enzymes.

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ADDENDUM IN PROOF

During the preparation of this manuscript, two studies were published showing that the *C. botulinum* C_2 binary toxin acts on eucaryotic actin as substrate (K. Aktories, M. Barmann, I. Ohishi, S. Tsuyama, K. H. Jacobs, and E. Habermann, Nature [London] **322**:390–392, 1986; I. Ohishi and S. Tsuyama, Biochem. Biophys. Res. Commun. **136**:802–806, 1986). We (L.L.S. and H.H.Z.) have confirmed their findings and have found that the *C. perfringens* iota toxin also ADP-ribosylates actin.

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