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THERAPEUTIC APPROACHES TO THE TREATMENT OF BOTULISM

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In vitro experiments have been done on isolated phrenic nerve-hemidiaphragm preparations. The purpose of the experiments was twofold: firstly, to evaluate a host of drugs as potential botulinum neurotoxin antagonists; and secondly, to evaluate the possibility that botulinum neurotoxin is an ADP-ribosyltransferase. The drugs that were tested included aminopyridines, guanidine, calcium, theophylline, forskolin, isobutylmethylxanthine, and cholera toxin. Various of the drugs had effects on neuromuscular transmission, and some had narrow spectrum utility as clostridial toxin antagonists, but none had the broad spectrum utility that would be needed for a clinically useful drug. In related experiments, a series of studies were conducted to determine whether the toxins have ADP-ribosyltransferase activity. The data did not support the hypothesis, though additional work needs to be done. (H.W.)

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FOREWORD

In conducting the research described in this report, the investigator (s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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1. Statement of Problem

Pharmacological methods are being sought to prevent or reverse the effects of botulinum neurotoxin. During the past year, emphasis has been placed on two problems: i.) finishing studies aimed at evaluating aminopyridines, guanidine and related drugs as pharmacological antagonists, and ii.) initiating studies aimed at assessing the liklihood that the neurotoxin is an ADP-ribosyltransferase.

2. Pharmacological Antagonists

A. Background

Botulinum neurotoxin exists in seven serotypes that are designated A,B,C,D,E,F and G. The various forms are relatively or absolutely distinct immunologically, but they share a similar macrostructure and perhaps a similar mechanism of action. Of the seven serotypes, three account for most cases of human botulism (A,B and E) and one accounts for most cases of animal botulism (C). The present study deals with types A,B,C and E botulinum neurotoxin.

Each of the botulinum neurotoxins is synthesized as a single chain polypeptide with a molecular weight of approximately 150,000 (For a review on structure, see Sakaguchi, 1983; for a review on mechanism of action, see Simpson, 1986a). When this precursor is exposed to proteolytic cleavage, it is converted to a fully active molecule in which a heavy chain ($Mr \sim 100,000$) is linked by a disulfide bond to a light chain ($Mr \sim 50,000$). The heavy

chain is thought to be responsible for binding and
internalization of the toxin. The light chain is assumed to
be an enzyme that acts intracellularly to block
acetylcholine release.

There are many similarities between botulinum
neurotoxin and tetanus toxin (DasGupta and Sugiyama, 1977;
Habermann and Dreyer, 1986; Simpson, 1986a). The latter
substance is synthesized and activated almost identically to
botulinum neurotoxin. Furthermore, tetanus toxin has a two
chain structure that appears to have the same functional
domains as botulinum neurotoxin. The most compelling
evidence for similarity comes from a recent study on
homology. The gene for tetanus toxin has been sequenced,
and from this an amino acid sequence has been deduced (Eisel
et al., 1986; Fairweather and Lyness, 1986). A comparison
of the complete primary structure of tetanus toxin with the
partial primary structure of several botulinum neurotoxins
reveals substantial homology (Eisel et al., 1986). These
various lines of evidence suggest that botulinum neurotoxin
and tetanus toxin may be evolutionary descendants of the
same ancestral parent.

The idea of commonality in origin, structure and
activity is appealing, but there is at least one observation
that may be a challenge to the hypothesis. A number of
authors have reported that aminopyridines such as 4-
aminopyridine and 3,4-diaminopyridine have markedly
different activities in antagonizing clostridial

neurotoxins. The drugs are potent antagonists of type A botulinum neurotoxin, but they are only weak antagonists of the other neurotoxins (Dreyer and Schmitt, 1981; Habermann et al., 1980; Kauffman et al., 1985; Lewis, 1981; Lundh et al., 1977; Lundh and Thesleff, 1977; Sellin et al., 1983; Simpson, 1978; 1986b). The data on aminopyridines have prompted questions about commonalities among the toxins.

An accepted approach for determining site and mechanism of action of pharmacological agents is to compare the magnitude of evoked responses in the presence of antagonists. The present study compares the neuromuscular blocking actions of botulinum neurotoxins types A,B,C and E and tetanus toxin. Comparisons have been carried out in the presence or absence of antagonists that inhibit internalization of toxins or inhibit intracellular toxicity. The results have been compared with previously published data on antagonism of binding. The collective findings are used to deduce the extent of relatedness among the clostridial neurotoxins.

B. Materials and Methods

Tissue preparations. Phrenic nerve-hemidiaphragms were excised from mice (20-30 g; female, Swiss-Webster, Ace Animal, Inc.) and suspended in a 20 ml tissue bath containing a physiological solution that was bubbled with 95% O_2 and 5% CO_2. Unless otherwise indicated, the physiological solution had the following composition (millimolar): NaCl, 137; KCl, 5; $CaCl_2$, 1.8; $MgSO_4$, 1.0;

$NaHCO_3$, 24; NaH_2PO_4, 1.0; and glucose, 11. The solution was supplemented with gelatin (0.02%) to diminish nonspecific inactivation of toxins.

Phrenic nerves were stimulated supramaximally with bipolar electrodes. Parameters of nerve stimulation were 0.2 Hz square waves of 0.1 to 0.3 msec duration. Muscle twitch was recorded by a force-displacement transducer connected to a physiological recorder. Toxin-induced paralysis of neuromuscular transmission was measured as a 90% reduction in muscle twitch amplitude evoked by nerve stimulation.

Toxins and drugs. Types A,B,C and E botulinum neurotoxin were purchased from Wako Chemicals (Dallas, TX). Tetanus toxin was purchased from Calbiochem (San Diego, CA) and cholera toxin was purchased from Sigma Chemical Co. (St. Louis, MO). Ammonium chloride was obtained from Fisher Scientific Co. (Fair Lawn, NJ), methylamine hydrochloride was obtained from Aldrich Chemical Co. (Milwaukee, WI), and all other drugs were purchased from Sigma Chemical Co.

Assay for neurotoxins. The various neurotoxins were not purified to homogeneity. The removal of auxiliary proteins and in some cases nucleic acid renders the neurotoxins unstable. Therefore, experiments were done with the stable neurotoxin-auxiliary protein complexes.

Each of the four botulinum neurotoxins and tetanus toxin was bioassayed on the phrenic nerve-hemidiaphragm. For each toxin, a concentration was chosen that produced

paralysis within 100 to 120 minutes and this was the concentration used in all experiments. Equiactive concentrations of toxin were re-bioassayed on tissues in the presence of drugs thought to be antagonists. Drugs that actually behaved as antagonists produced an apparent decrease in toxicity, i.e., produced an increase in the amount of time necessary for onset of toxin-induced paralysis. Results are presented in terms of absolute changes in paralysis times.

C.   Results

Ammonium chloride and methylamine hydrochloride. The paradigm used with ammonium chloride and methylamine hydrochloride was the same as that used with all potential antagonists. It consisted of a sequence of three experiments, as follows: i.) a determination of the concentration range within which a suspected antagonist can be applied to the isolated neuromuscular junction, ii.) a determination of whether and at what concentration(s) the antagonist alters the activity of botulinum neurotoxin and tetanus toxin, and iii.) a determination of the time(s) during toxin-induced paralysis when the antagonist must be added to exert its protective effect.

Previous studies have shown (Simpson, 1983), and preliminary experiments here have confirmed, that high concentrations of ammonium chloride and methylamine hydrochloride depress neuromuscular transmission. The highest concentration of ammonium chloride that can be used

is about 8 mM; that of methylamine hydrochloride, about 20 mM.

Various concentrations of drug were added to tissues simultaneously with toxin, and paralysis times were monitored. It was determined that 8 mM ammonium chloride and 10 mM methylamine hydrochloride produced maximal effects. When higher concentrations of ammonium chloride were tested, the deleterious effects of the drug added to those of the clostridial toxins. When higher concentrations of methylamine hydrochloride were tested, the drug continued to exert a strongly protective effect, but no greater than that seen at 10 mM. As already reported, both antagonists must be added to tissues simultaneously with, or only shortly after, a clostridial toxin (Simpson, 1983). If they are added after toxin-induced blockade has begun to emerge, they no longer exert a protective effect.

Antagonism by calcium. Paralysis times of tissues were measured in the presence of equiactive concentrations of neurotoxin and varying concentrations of calcium (1, 2, 4, 8, 16 and 32 mM). The highest concentration of calcium (32 mM), either with or without osmotic and/or ionic compensation, depressed transmission, but the effect was not total and it was reversible.

The toxins appeared to segregate into two groups, based on their interaction with calcium. Botulinum neurotoxin type A was significantly antagonized by increasing concentrations of calcium, from 1 mM to 16 mM.

When the concentration of calcium was increased to 32 mM, the antagonistic effect was reduced. A qualitatively similar profile was obtained with botulinum neurotoxin types B, C and E, as well as tetanus toxin, but the magnitude of effect was less.

Antagonism by 3,4-diaminopyridine and by guanidine. The dose-response characteristics of 3,4-diaminopyridine action on the mouse phrenic nerve-hemidiaphragm have been reported (Simpson, 1986b). The highest concentration that is practical for use is approximately 0.2 mM. On the other hand, guanidine can be used at concentrations as high as 8 mM.

Several investigators have demonstrated that botulinum neurotoxin is antagonized by aminopyridines and by guanidine (see Introduction). Therefore, varying concentrations of 3,4-diaminopyridine and guanidine were tested for their abilities to antagonize serotype A. The results indicated that 0.1 mM 3,4-diaminopyridine and 3.0 mM guanidine exerted a maximal effect. These concentrations were then tested against other serotypes of botulinum neurotoxin and against tetanus toxin.

3,4-Diaminopyridine and guanidine exerted a somewhat selective effect. They dramatically antagonized botulinum neurotoxin type A; paralysis times were increased more than 2-fold, giving an apparent decrease in toxicity of more than 90%. By contrast, the drugs had little or no effect when tested against botulinum neurotoxin types B, C and E or

against tetanus toxin. Paralysis times increased modestly or not at all.

It has previously been reported that the ability of aminopyridines to exert a protective effect against botulinum neurotoxin type A is time-dependent (Simpson, 1986b). Experiments were done to determine whether there was a similar time-dependency with guanidine. The data showed that when the drug was added to tissues prior to, simultaneously with, or 30 minutes after toxin, its protective effect was maximal. When the drug was added at later times, its protective effect was diminished. The drug had no observable effect on tissues that were fully paralyzed.

The interaction between calcium and 3,4-diaminopyridine or guanidine. Two types of experiments were done. In the first, low levels of calcium (1.0 mM) were used with botulinum neurotoxin type A. The purpose of the experiment was to determine whether a decrease in the calcium concentration would alter the antagonistic activity of 3,4-diaminopyridine or guanidine. In the second experiment, elevated levels of calcium (8.0 mM) were used with botulinum neurotoxin types B, C and E and tetanus toxin. The purpose was to determine whether increased amounts of calcium would allow 3,4-diaminopyridine and guanidine to express an antagonistic effect.

In the initial experiment, the results were striking. Reducing the levels of ambient calcium from 1.8

mM to 1.0 mM greatly diminished the antagonism due to 3,4-
diaminopyridine (0.1 mM) and guanidine (3 mM;). Increasing
the concentration of the antagonists twofold did not restore
the protective effect. In the second experiment, the results
were less striking. Increasing the concentration of calcium
from 1.8 mM to 8.0 mM did little to enhance the activity of
3,4-diaminopyridine and guanidine. Indeed, drug-induced
antagonism of botulinum neurotoxin type A at a calcium level
of 1.8 mM was greater than drug-induced antagonism of any of
the other neurotoxins at a calcium level of 8.0 mM.

Theophylline, forskolin, isobutylmethylxanthine, and
cholera toxin. Four drugs known to elevate tissue levels of
C-AMP were tested for their abilities to antagonize
clostridial neurotoxins. One of these drugs (theophylline)
has previously been examined as an antagonist of one
botulinum neurotoxin serotype (A; Howard et al., 1976).

The addition of theophylline to isolated
neuromuscular preparations produced concentration-dependent
increases in nerve-evoked muscle twitch (0.5 mM to 4.0 mM).
The effect was maximal at 2.0 mM (n = 48; 31 ± 4%; p <
0.01). However, this enhanced response waned with time.
Even in the absence of toxins, the increase in twitch
amplitude returned to control levels within 60 to 100
minutes.

When tissues were pretreated with theophylline (2
mM; 15 minutes) prior to the addition of neurotoxins, the
drug produced an effect that was not universal.

.neophylline significantly antagonized botulinum neur.+oxin type A (n = 12; control = 118 $\pm$ 9 minutes; experimental = 137 $\pm$ 12; $p < 0.05$), but it did not antagonize the other botulinum neurotoxins or tetanus toxin to an extent that attained statistical significance.

Equivalent experiments were done with forskolin (1.0 to 100 M), isobutylmethylxanthine (0.1 to 5 mM) and cholera toxin (10^{-10} to 10^{-7} M). Forskolin did not enhance muscle twitch, nor did it antagonize any of the clostridial neurotoxins. Isobutylmethylxanthine enhanced muscle twitch (e.g., 1 mM; n = 27; 43 $\pm$ 2%; $p < 0.01$), but the enhanced twitch waned with time. When tissues were pretreated with the drug (1 mM; 15 minutes), there was no significant antagonism of toxin-induced neuromuscular blockade.

At concentrations of 10^{-10} to 10^{-7} M, cholera toxin produces characteristic morphological changes and simultaneous increases in tissue levels of C-AMP in a cell line that has been used to study various toxins (Zepeda et al., submitted for publication). These concentrations did not enhance neuromuscular transmission nor did they antagonize clostridial neurotoxins. This result was obtained when cholera toxin was added 1, 2, 3 or 4 hours prior to a clostridial toxin.

D. Implications

Botulinum neurotoxin and tetanus toxin share a number of pharmacological properties. Botulinum neurotoxin acts at low concentrations to block acetylcholine release from

cholinergic nerve endings (Burgen et al., 1949). To produce this effect, the toxin proceeds through a sequence of three steps: an extracellular binding step, an internalization step, and an intracellular poisoning step (Simpson, 1980). Tetanus toxin can also block neuromuscular transmission, although it is less potent than botulinum toxin (Habermann et al., 1980). In addition, tetanus toxin appears to proceed through the same sequence of three steps in producing its effects (Schmitt et al., 1981).

These similarities encourage one to believe that the mechanisms of action of the clostridial toxins can be seen as variations on a common molecular theme. The salient question is this: How great are the variations? Data presented here and elsewhere (Dreyer et al., 1987; Gansel et al., 1987) support the following hypothesis. Botulinum neurotoxin and tetanus toxin proceed through the same sequence of events in producing their neuromuscular blocking effects, but the individual toxins do not necessarily interact with the same components of the nerve ending to produce these effects.

Functional domains and drug antagonism. There are at least three functional domains in the clostridial neurotoxin molecules: a binding domain, an internalization domain, and a poisoning domain. In the present report, experiments have been done with antagonists of the latter two. The published literature already contains numerous reports that deal with binding.

There is compelling evidence that the several
serotypes of botulinum neurotoxin and tetanus toxin do not
utilize the same receptor. Both ligand binding studies and
morphological studies show that individual receptors have
unique or semiunique properties. Nevertheless, these
receptors - and the portion of the toxin molecules that bind
to the receptors - share certain properties. For example,
all the toxins have some affinity for gangliosides, and
ganglioside-toxin complexes impede the binding of toxins to
nerve tissue (Kitamura et al., 1980; Rodgers and Snyder,
1981; Kozaki et al., 1984). In addition, all the toxins
appear to have their binding moieties, at least in part, in
the heavy chain (Morris et al., 1980; Bandyopadhyay et al.,
1987). And finally, there is suggestive evidence for
partial competition among heterologous toxins for binding
sites. Botulinum neurotoxin type A can antagonize the
binding of type B (Black and Dolly, 1986; Bandyopadhyay et
al., 1987); tetanus toxin can antagonize the binding of
certain botulinum neurotoxins (Simpson, 1984). In sum, the
data suggest that the toxins bind to the plasma membrane,
but they do not all bind to the same component of the nerve
ending.

The next step in the sequence is thought to be
internalization, and this may be achieved by receptor
mediated-endocytosis. An entire class of drugs has been
identified that antagonizes the action of endogenous (i.e.
peptide hormones) and exogenous (i.e., protein toxins)

substances that enter cells by endocytosis. Among the most thoroughly studied of these substances are ammonium chloride, methylamine hydrochloride and chloroquine (DeDuve et al., 1974). Ammonium chloride and methylamine hydrochloride have been shown to antagonize some of the clostridial neurotoxins (Simpson, 1983), and the range of their effects has been further explored here. The data indicate that the two drugs are very effective antagonists of clostridial toxins. This is in contrast to the findings with chloroquine, which antagonizes botulinum neurotoxin, but not tetanus toxin (Simpson, 1982).

The final step in the process is blockade of acetylcholine release. Calcium (Silinsky, 1985), aminopyridines (Molgo et al., 1975; Yeh et al., 1976) and guanidine (Otsuka and Endo, 1960; Kamenskaya et al., 1975) promote acetylcholine release, and they have been implicated as clostridial neurotoxin antagonists (Thesleff, in press). It has been a general finding that aminopyridines antagonize type A neurotoxin, but they have little or no activity against the other neurotoxins (see Introduction). That general finding has been extended here to include calcium and guanidine. It appears that botulinum neurotoxin type A is most sensitive to changes in the ambient levels of calcium, to the presence of 3,4-diaminopyridine or guanidine, and to the interaction between calcium and 3,4-diaminopyridine or guanidine. An additional finding is that the antagonistic effects of 3,4-diaminopyridine and

guanidine are time-dependent. This finding may have mechanistic implications, and it is discussed below.

Although the intracellular action of the clostridial toxins has not been determined, at least one report provides possible clues. Theophylline has been reported to be an effective *in vitro* antagonist of botulinum neurotoxin type A (Howard et al., 1976). This could mean that the toxin acts through a cyclic nucleotide mechanism, an NAD-dependent process, or both. Therefore, the effect of theophylline has been re-examined, and several additional cyclic nucleotide-modifying drugs were included in the experiments.

Theophylline was found to have a relatively modest effect, and its actions were restricted to botulinum neurotoxin type A. Other procedures that enhance intracellular levels of cyclic-AMP (pretreatment with forskolin, isobutylmethylxanthine, or cholera toxin) did not have a notable effect on any of the clostridial neurotoxins. The data indicate that the toxins do not act primarily through a cyclic-AMP mediated process.

Taken collectively, the data appear to support the hypothesis proposed above. Both botulinum neurotoxin and tetanus toxin proceed through a sequence of three steps in exerting their effects. Insofar as the initial binding step is concerned, several laboratories have shown that the various toxins do not rely upon the same receptor. In relation to the internalization step, conclusions about commonality may be premature. Ammonium chloride and

methylamine hydrochloride are universal antagonists, but
chloroquine is not. It is unclear whether the observations
on chloroquine indicate that there are different mechanisms
for internalization or different mechanisms for proteolytic
processing of toxin. Finally, data here and elsewhere
(Gansel et al., 1987) strongly suggest that the various
clostridial toxins do not have an identical action within
the nerve ending. A host of drugs have now been shown to
have differential effects in antagonizing the toxins.
Again, it appears that the toxins proceed through the same
sequence of steps, but they do not necessarily do this by
interacting with the same components of the nerve ending.

Mechanism of toxin action. Three hypotheses have
been advanced to explain the observation that calcium can
antagonize botulinum neurotoxin. These are: i.) the toxin
blocks calcium channels, ii.) the toxin blocks a mechanism
that governs transmitter release, or iii.) the toxin
enhances a mechanism that metabolizes intracellular calcium.
It is instructive to examine these ideas in the context of
the findings on aminopyridines and guanidine.

The idea that the toxin blocks calcium channels has
been repeatedly negated by experimental evidence. The
results with aminopyridine and guanidine are also not
compatible with the idea of channel blockade. If the toxin
were to convert the channel from a high conductance to a low
conductance state, the time-dependency of aminopyridine and
guanidine action should be the opposite of that observed.

The drugs should be more active when added after onset of paralysis rather than prior to paralysis.

The idea that the toxin and calcium interact with a mechanism that governs transmitter release or one that governs intracellular calcium can be envisioned three ways: i.) the toxin and calcium act on one another, ii.) they interact with a common substrate, or iii.) they act on different substrates. The first of these does not seem plausible, but the second two are worthy of consideration.

Botulinum neurotoxin and calcium could act on a common substrate, such as a molecule that promotes transmitter release or one that governs metabolism of calcium. However, the time-dependency of aminopyridine and guanidine action poses a problem. If the toxin were to act on a substrate that governs transmitter release, and if that action were to be expressed as diminished sensitivity to calcium, then aminopyridines, guanidine and other drugs that enhance calcium influx should act as antagonists, and they should do so when added after onset of toxin action. Similarly, if the toxin were to act on a molecule that governs intracellular levels of calcium, and if the effect were to produce enhanced rates of calcium sequestration, then drugs that promote calcium flux should be maximally effective after the onset of paralysis. The only obvious way to reconcile the idea that toxin and calcium act at a common site and that drugs promoting calcium flux are maximally effective when added before onset of paralysis is

to propose that calcium has more than one action. For example, if calcium induced a conformational change in the substrate that made the molecule resistant to the toxin, and at the same time acted to promote transmitter release, that could explain the findings with aminopyridines and guanidine.

The thrust of these findings is that calcium and toxin do not interact in a simple and straightforward manner. One possibility is that they act at the same site, but this would require that calcium exert more than one effect. Alternatively, the toxin and calcium may not act at a common site. They may act remotely from one another, in a molecular sense. For example, they could act at different sites in an intricate cascade that governs transmitter release. In either case, the interaction between the two is more complex than has previously been assumed.

The argument is strongly supported by a recent study by Lupa and Tabti (1986). They examined the effects of botulinum neurotoxin type A on three types of calcium-dependent release phenomena: facilitation, augmentation and potentiation. They found that the toxin enhanced facilitation, had no effect on augmentation, and abolished potentiation. These findings support the notion that there is a complex interaction between botulinum neurotoxin and calcium.

3. ADP-Ribosylation

    A. Background

Three groups of ADP-ribosylating toxins have been described in the literature. The first group is composed of diphtheria toxin and Pseudomonas aeruginosa exotoxin, which catalytically modify elongation factor 2. When the translocase is inhibited, protein synthesis is blocked and cell death ensues. The second group is composed of cholera toxin, E. coli enterotoxin and pertussis toxin. These substances modify regulatory proteins that govern adenylate cyclase, leading to pathological increases or decreases in intracellular levels of nucleotides. However, this group of toxins does not ordinarily cause cell death. The final group is composed of binary toxins obtained from Clostridium botulinum, Clostridium perfringens and Clostridium spiroforme. These agents enzymatically modify actin, and cell death is a typical but not invariant outcome.

In the recent past, a Japanese laboratory has reported that botulinum neurotoxin types C1 and D are also ADP-ribosyltransferases (Ohashi and Narumiya, 1987; Ohashi et al., 1987). These investigators have suggested that the substrate is a 21,000 dalton protein located in the membranes of nerve and related tissues. The data suggest that the substrate may be a nucleotide binding protein. The work by Narumiya and his associates has been substantially reproduced by Matsuoka et al (1987), and a supporting study will be published shortly by the laboratory of Professor D. Knight.

Research supported by another contract (DAMD17-86-C-6161) has examined this problem in a biochemical manner. The principal finding to date has been that the presence or absence of ADP-ribosyltransferase activity hinges upon the source of toxin being studied. There is at least one plausible explanation for this difference (see below). Therefore, the past quarter has been used to conduct pharmacological experiments that would help to clarify whether clostridial toxins possess ADP-ribosyltransferase activity.

B. Methods

The techniques used for these studies were described above.

C. Results

Experiment 1. Rationale. Narumiya and his associates have reported one interesting difference between the ADP-ribosylating activity of botulinum neurotoxins and that of other ADP-ribosylating toxins. They claim that the neurotoxins do not ADP-ribosylate arginine, but that arginine is an antagonist of the modification of the 21k substrate. The reported IC50 value for L-arginine methyl ester was 14.5 mM. These findings suggest that arginine should be examined to determine whether it modifies neuromuscular transmission and whether it antagonizes botulinum neurotoxin.

Experiment i. Results. Phrenic nerve-hemidiaphragm preparations were incubated in physiological solution at

37°C. L-arginine was added to tissues, and the effect of the drug on directly (muscle) and indirectly (nerve) elicited twitch was monitored .

At concentrations of approximately 20 mM and lower, arginine had no observable effect on neuromuscular transmission. However, when the concentration was incremented beyond this, effects became observable. With incrementing concentrations of 10 mM, there were reproducible depressions in transmission. The effect was a complex one, because both nerve and muscle were affected. However, the decay in neuromuscular transmission was reversible. Even when tissues were not washed, twitch fully recovered.

Tissues were incubated with 90 mM arginine, which is reported to cause approximately 90% inhibition of ADP-ribosylation by types C1 and D toxin (Ohashi et al., 1987). Equiactive concentrations of types A, C1 and D eurotoxin were then tested in the presence and absence of arginine. The data (paralysis times; means, SEM is equal to or less than 10% of the mean) are as follows:

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type A:  Control  121  Experimental 136
type C1: Control  117  Experimental 135
type D:  Control  139  Experimental 124
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The data reveal that arginine, under the conditions tested, is not an effective neurotoxin antagonist.

Experiment 2. Rationale. For all toxin-mediated ADP-ribosylating reactions that have been studied, the

results have shown that the reaction is reversible. Thus,
nicotinamide can be used to antagonize the forward reaction
or to drive the reverse reaction. This observation has been
used to advantage by investigators studying toxin-mediated
ADP-ribosylation of substrate in tissue culture. At
concentrations of 10 mM and higher, nicotinamide and
isonicotinamide are antagonists of the forward reaction.
The possibility that these drugs would also antagonize C1
and D toxins was tested.

Experiment 2. Results. The results with
isonicotinamide were rather similar to those with arginine.
Increasing concentrations of the drug had a depressant
effect on neuromuscular transmission, but the effect waned
with time. The two drugs were also similar in terms of
their ability to alter toxin-induced paralysis. the results
were as follows:

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type A:   Control  113 · Experimental 129
type C1:  Control  131   Experimental 127
type D:   Control  125   Experimental 111
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Experiment 3. Rationale. As discussed above, the
observation that botulinum neurotoxin has ADP-ribosylating
activity appears to hinge on the source of toxin. The toxin
used by Narumiya and his colleagues originated in the
laboratory of Dr. Sakaguchi; the work of Matsuoka et al was
done with material that came from their own laboratory.
Conversely, toxin isolated in other laboratories, including

those at USAMRIID, does not express enzymatic activity.
What could account for this disparity?

One possibility is that there exists
microheterogeneity among type C1 and D toxins. This would
be important, if the following hypothesis were true.
Perhaps the botulinum neurotoxins must undergo lysosomal
processing before they can express enzyme activity. Prior
to activation, the toxins may exist in a conformation that
does not permit enzyme activity; but after activation (viz.,
lysosomal processing), the toxin molecule may be modified
and enzyme activity may become possible.

There may be one pharmacological approach to
addressing this issue. In a number of tissues, there is
temperature-dependence in the endosome-lysosome fusion
process. Most investigators suggest that in the range of 10
to 15° C, fusion does not occur. Therefore, one could
compare the temperature dependence of various preparations
of type C toxin. An absence of toxin-induced paralysis, or
an unusually long time for onset of paralysis, at low
temperatures could implicate a lysosomal processing step.

Experiment 3. Results. Interestingly, mouse phrenic
nerve-hemidiaphragm preparations are very resilient at low
temperatures. Tissues were exposed to temperatures of 2° C
and higher, with increments of 2° C, to find a temperature
at which 90% of the tissues would respond with a stable and
sustained twitch. At 2° and 4°, there were significant

numbers of failures (> 10%). However, at 6° C approximately 90% of the tissues responded.

Tissues were bathed in medium at 36° C, and sufficient type C toxin of Japanese origin was added to produce onset of paralysis in approximately 40 minutes. This same concentration was then added to tissues bathed at 26° C, 16° C, and 6° C. From the data on paralysis, two observations were made. Firstly, there was no sharp discontinuity in the temperature-effect curve. To the contrary, there was a reasonably linear relationship between temperature and paralysis time. The Q10 was about 2.8. Secondly, tissues certainly did paralyze at 6° C. There was no evidence that the fall in temperature had blocked onset of paralysis.

An identical sequence of experiments was done with type C toxin provided by investigators at USAMRIID. This preparation did not possess obvious ADP-ribosyltransferase activity, and thus it is the one that speculatively should require lysosomal processing. The data did not, however, support the speculation. Like the Japanese toxin, the USAMRIID toxin produced paralysis even at 6° C. In addition, the Q10 (about 3.1) was not statistically significantly different (p > 0.05) from that of the Japanese toxin.

Experiment 4. Rationale. Irrespective of whether the toxins do or do not require lysosomal processing, there may still be microheterogeneity in structure. This could

best be gauged by primary sequence comparisons, but the structures have not yet been determined. Another but less ideal method would be to compare the neutralizing activity of type C antitoxin against the various preparations of type C toxin.

Experiment 4. Results. This series of studies has just begun. Ultimately it will include both polyclonal and monoclonal antibodies, but thus far only polyclonal antibodies have been tested.

Two different preparations of type C antibody were titrated to produce a specific lengthening in the paralysis times of tissues, i.e., a doubling in paralysis time. This was done initially with the Japanese toxin, which is available in large quantities. When the antibodies were titrated against this material, they were then tested at the concentration that produced the desired effect, but with USAMRIID toxin as antigen. The results indicated that any given concentration of antibody is equiactive against both toxins. The data were not suggestive of heterogeneity in immune responses.

D. Implications

The work in the P.I.'s laboratory as well as that published in Aktories laboratory, call into question the claims by Narumiya and others that botulinum neurotoxin is an ADP-ribosyltransferase. However, Narumiua has agreed to provide the P.I. with a preparation of neurotoxin that is homogeneous and that possesses ADP-ribosylatransferase

activity.   Resolution of the problem must await arrival of
this material.

4.   Future Plans

During the coming year there are three areas that will
be explored: i.) the testing of monoclonal antibodies
against botulinum neurotoxin and tetanus toxin, ii.) the
testing of drugs that alter the phosphoinositidase - Protein
kinase C cascade, and iii.) the evaluation of techniques
that enhance or inhibit the binding of clostridial
neurotoxins to nerve membranes.

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