A Preclinical Evaluation of Aminopyridines as Putative Therapeutic Agents in the Treatment of Botulism

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4-Aminopyridine and 3,4-diaminopyridine were evaluated for their abilities to delay the onset of paralysis due to botulinum neurotoxin types A, B, and E. Experiments were done on phrenic nerve-hemidiaphragm preparations excised from mice. At a concentration that produced an enhancement in muscle twitch amplitude, 4-aminopyridine and 3,4-diaminopyridine delayed the onset of paralysis due to botulinum toxin type A. Under the same conditions, the drugs did little to protect tissues against botulinum toxin types B and E. 3,4-Diaminopyridine was also evaluated for its ability to reverse the paralysis due to botulinum toxin. Experiments were done on rat phrenic nerve-hemidiaphragm preparations that had previously been poisoned in vivo. The drug produced transient increases in neuromuscular transmission, with the effect being greater for botulinum neurotoxin type A than for botulinum neurotoxin types B and E. Equivalent types of experiments were done with tetanus toxin. The results with 3,4-diaminopyridine showed that tetanus toxin resembled botulinum toxin types B and E. The data help to clarify the role of aminopyridines as therapeutic agents in the treatment of botulism. They also provide insights into the mechanism of action of clostridial neurotoxins.

Botulism is a neurological disorder caused by an exotoxin from the organism Clostridium botulinum. The disease is characterized by progressive muscle weakness that can result in complete flaccid paralysis. Unfortunately, there is no known cure for the disorder. Patients who contract the illness are typically provided with supportive therapy (e.g., respiratory support). Recovery appears to be a function of the ability of the nervous system to repair the damage produced by the toxin.

Botulinum toxin acts on peripheral nerve endings that store and release the transmitter acetylcholine (2, 15). It shows greatest affinity for those cholinergic nerve endings that innervate striated muscle, including the muscles of respiration (e.g., diaphragm and intercostal muscle). The precise mechanism of toxin action is blockade of nerve-depolarization-induced release of acetylcholine. This action accounts for the ability of the toxin to produce muscle weakness and flaccid paralysis.

Botulinum toxin is a protein with an Mr of ~150,000 (10, 15). It is composed of two polypeptide chains (Mr’s, ~100,000 and 50,000) that are linked by a disulfide bond. The toxin appears to proceed through a sequence of three steps in producing its neuroparalytic effects (14, 15). There is an initial binding step, a subsequent membrane penetration step, and an eventual poisoning step that occurs inside cholinergic nerve endings. The binding and internalization steps are thought to be mediated by the heavy polypeptide chain (L. L. Simpson, Annu. Rev. Pharmacol. Toxicol., in press). The intracellular poisoning step may be mediated by the light chain.

The incidence of human botulism is relatively low, and thus programs of immunization are not deemed appropriate. When individual patients contract the disease, there is the possibility of administering antitoxin, but this is of limited value. Antitoxin will neutralize circulating titer of botulinum toxin, but it cannot cross the plasma membrane of nerve cells to neutralize internalized toxin. Therefore, alternate pharmacological means have been sought to overcome the effects of botulism.

The fact that the toxin depresses transmitter release has encouraged efforts to find therapeutic drugs that will promote acetylcholine release. The aminopyridines (4-aminopyridine [4-AP] and 3,4-diaminopyridine [3,4-DAP]) are one group of agents that has been tested. These drugs act on nerve membranes to promote influx of calcium, and this in turn greatly promotes efflux of acetylcholine (4, 9, 18). The pharmacological actions of 4-AP and 3,4-DAP, as well as certain preliminary observations (7), suggest that these drugs may be useful in the treatment of botulism. More specifically, they might (i) slow the onset of neuromuscular blockade, (ii) increase the lethal dose, or (iii) provide symptomatic relief.

The present study is an attempt to evaluate two of these possibilities. Data are described that help to evaluate 4-AP and 3,4-DAP as agents that can slow the onset of neuromuscular blockade or provide symptomatic relief. Findings are also described that provide additional insight into the mechanism of botulinum neurotoxin action.

MATERIALS AND METHODS

Toxins, reagents, and animals. Type A botulinum toxin was generously supplied by E. J. Schantz (University of Wisconsin, Madison). Botulinum toxin types B and E were kindly provided by B. R. DasGupta (University of Wisconsin, Madison). The three neurotoxins were obtained in various states of purity. For the purposes of this study, each neurotoxin was bioassayed on phrenic nerve-hemidiaphragms (see below). Equiactive amounts of the toxins (i.e., amounts of toxin that produced paralysis in 100 to 120 min) were then used in the various studies. Tetanus toxin was generously provided by R. O. Thomson and A. J. Beale (Wellcome Research Laboratories, Beckenham, England). The aminopyridines (4-AP and 3,4-DAP) were purchased from Sigma Chemical Company (St. Louis, Mo.). Experiments were done on male Swiss Webster mice (body weight, 25 to 30 g) and on male Sprague-Dawley rats (body weight, 200 to 250 g).

Phrenic nerve-hemidiaphragm preparation. Phrenic nerve-
Aminopyridines and Botulinum

RESULTS

Dose-response data with aminopyridines. Within a narrow concentration range, both 4-AP (10⁻³ to 10⁻¹ M) and 3,4-DAP (10⁻¹ to 10⁻³ M) produced an initial enhancement in the muscle twitch response due to nerve stimulation (Fig. 1). At higher concentrations, there was substantial and sustained depression of muscle responses.

4-AP and botulinum toxin. Phrenic nerve-hemidiaphragms from mice were incubated (4°C, 45 min) with botulinum toxin type A, after which they were washed and transferred to tissue baths without added toxin. For control tissues, the phrenic nerve was stimulated, and the elapsed time for onset of paralysis was monitored. Experimental tissues were treated identically, except that the bathing medium contained 4-AP (10⁻⁴ M). The data revealed that the aminopyridine provided protection against the rate of onset of neuromuscular blockade. The paralysis time for control tissues (n = 6) was 64 min; that for experimental tissues (n = 6) was 135 min.

An equivalent effect was obtained when the drug was added repeatedly to neuromuscular preparations. 4-AP (10⁻⁴ M) was present when tissues were placed in the bathing medium, and a similar concentration was added at the following three later times: (i) when the decay in neuromuscular transmission first became apparent, (ii) when transmission was 50% blocked, and (iii) when transmission was 90% blocked. Under these conditions, the drug provided

mean for each datum point is equal to or less than 10% of the respective mean.
protection against the onset of paralysis due to botulinum toxin type A (Fig. 2).

A similar procedure was used to study the interaction between 4-AP and botulinum toxin types B and E (Fig. 2). Interestingly, 4-AP did not provide much protection against toxin types B and E, even when tested under the same conditions that provided protection against toxin type A.

3,4-DAP and botulinum toxin. Tissues from mice were incubated with type A botulinum toxin as described above, and afterward they were transferred to baths without added toxin. 3,4-DAP (10^{-4} M) was present in the bathing medium. The diamino compound was strikingly effective in delaying the onset of paralysis. When a correction was made for the initial effects of 3,4-DAP on neuromuscular transmission (Fig. 3), the drug still displayed a statistically significant ability (P < 0.001) to delay the onset of toxin-induced paralysis.

The same approach was used to study the interaction between 3,4-DAP and neurotoxin types B and E. The results were qualitatively similar to those obtained with 4-AP. When tested under conditions that provided substantial protection against neurotoxin type A, 3,4-DAP provided relatively little protection against neurotoxin types B (P ≈ 0.05) and E (P > 0.05) (Fig. 4).

The experimental procedure described above is one that eliminates the possibility that 3,4-DAP could interfere with the binding of toxin. The drug was not added to tissues until the binding of toxin was complete. The procedure does allow the possibility that 3,4-DAP could interact with either the internalization step or the intracellular poisoning step. Conceivably, the ability of the drug to promote internalization of toxin could offset the ability of the drug to stimulate acetylcholine release (see Discussion). Therefore, an experiment was done to minimize the possible effect of 3,4-DAP on toxin internalization.

Tissues were incubated as described above and transferred to bathing medium. Phrenic nerves were stimulated, and the onset of neuromuscular blockade was monitored. The diamino compound (10^{-4} M) was not added until the tissues were 50% paralyzed, i.e., until there was evidence that the toxin had been internalized. The results showed that 3,4-DAP provided substantial protection against botulinum toxin type A but only minimal protection against botulinum toxin types B and E (Fig. 5).

![FIG. 3](http://iai.asm.org) Comparison of the paralysis times of untreated tissues (○) and 3,4-DAP-treated tissues (●). In the case of the latter, the muscle twitch amplitude (percentage of control) was normalized by subtracting the enhanced response due to the aminopyridine (e.g., part B of Fig. 2). The data illustrate the marked effect of 3,4-DAP in delaying the onset of paralysis.

![FIG. 4](http://iai.asm.org) Comparison of the paralysis times of untreated (○) and 3,4-DAP-treated (●) tissues. In the case of the latter, the muscle twitch amplitude (percentage of control) was normalized by subtracting the enhanced response caused by the drug (e.g., part B of Fig. 2). The data show that the drug had a modest effect on type B toxin and virtually no effect on type E toxin.

Reversal of neuromuscular blockade. Phrenic nerve-hemidiaphragms were removed from rats that had been pretreated 2 to 6 days previously with botulinum toxin. To be included in the study, tissues had to satisfy two criteria. (i) They had to be seriously poisoned with botulinum toxin (i.e., minimal response to presynaptic nerve stimulation but full response to postsynaptic, K^+-induced muscle depolarization). (ii) The tissue had to maintain a stable, albeit reduced, response (i.e., the toxin was not still in the nerve endings and causing additional paralysis).

3,4-DAP (10^{-4} M) was added to poisoned tissues that were suspended in tissue baths, and the drug did produce recovery in neuromuscular transmission. However, there were two factors that tended to limit the importance of this response. First, the enhanced response in poisoned tissues was similar to that in control tissues. There was an initial increase in muscle twitch, but this waned with time. Second, there was a differential response for the several toxins. 3,4-DAP produced the greatest increase in twitch amplitude for tissues that had previously been poisoned with toxin type A (n = 7, increase = 327 ± 26%). The increases for toxin types B (n = 5, increase = 159 ± 19%) and E (n = 5, increase = 141 ± 16%) were of a lesser magnitude.

3,4-DAP and tetanus toxin. Recent findings suggest that botulinum neurotoxin and tetanus toxin share many similarities in terms of their abilities to block neuromuscular transmission (3, 11). Therefore, 3,4-DAP was examined to determine whether it could delay the onset of paralysis due to tetanus toxin. Tissues from mice were incubated in toxin as described above. They were then transferred to baths without added toxin but with 3,4-DAP (10^{-4} M). Phrenic nerves were stimulated, and paralysis times were monitored.

The data revealed that the interaction between tetanus toxin and 3,4-DAP was similar to that between botulinum toxin types B and E and 3,4-DAP. Although the drug was used at a concentration that enhanced twitch response (Fig. 1) and prolonged the onset of paralysis due to botulinum toxin type A (Fig. 3), it provided only slight protection against tetanus toxin. The paralysis times for control (n = 3) and experimental (n = 3) tissues were 90 ± 6 and 127 ± 12 min, respectively.

**DISCUSSION**

Botulinum toxin appears to proceed through a series of three steps in producing its neuroparalytic effects. There is a membrane-binding step, an internalization step, and an
intracellular poisoning step. This sequence suggests several possible points of attack in antagonizing the onset of toxin-induced paralysis. Most obviously, antibody could be used to neutralize circulating titers of toxin. In theory, this could be endogenous antibody, i.e., antibody elicited in response to previous administration of toxoid, but this is of little practical importance. There currently exist no immunization programs for the various types of human botulism. Conversely, exogenous antibody could be administered to patients diagnosed as having the illness. This would serve the useful purpose of neutralizing some toxin, but once again the procedure is of limited value. Administered antibody does not reach the nerve cell interior, and therefore it cannot antagonize the effects of internalized toxin.

In the recent past, alternate strategies have been considered for providing relief from the neuroparalytic effects of botulimum toxin. One of these is to administer drugs that prevent internalization of the toxin (17) or prevent the internalized toxin from reaching a vulnerable intracellular compartment (16). Another is to administer a drug that greatly promotes acetylcholine release. The aminopyridines fall into the latter category (9).

4-AP and 3,4-DAP act on ion channels in excitable membranes. The end result of this action is a greatly promoted influx of calcium ions, which in turn triggers the release of acetylcholine (4, 18). This mechanism of action suggests that aminopyridines might exert one or more effects that would be beneficial in botulism. Indeed, there is already preliminary evidence to support this idea.

A number of investigators have shown that 4-AP and 3,4-DAP can act as antagonists of botulimum neurotoxin (5, 7-9, 12, 13) and tetanus toxin (1, 3). With only one exception (7), these studies were not aimed at evaluating the therapeutic potential of aminopyridines. The goal of the studies was to determine the mechanism of action of the neurotoxins. However, in each case, data were presented that showed that aminopyridines did possess some antagonistic activity. And in the report by Lewis, Jr. (7), preliminary results indicated that aminopyridines could delay the onset of symptoms in animals poisoned with botulimum toxin.

Despite these favorable suggestions, there are two potential and serious drawbacks. First, studies done on isolated tissues that were excised from animals previously poisoned with botulimum toxin have shown a heterogeneity of effect. The aminopyridines were not equivalently effective in relieving the neuroparalytic symptoms produced by different types of botulimum toxin (5, 12). Second, there is a theoretical concern that has diminished enthusiasm for pursuing studies on the aminopyridines. It has been repeatedly demonstrated that the rate of onset of toxin-induced neuromuscular blockade is nerve activity dependent; i.e., the more rapid the rate of nerve stimulation, the more rapid the onset of paralysis. This may be due to the link between exocytosis (viz., the release of acetylcholine) and endocytosis (viz., the internalization of toxin). This allows the possibility that aminopyridines could exert opposing actions. On one hand, they would antagonize poisoning by promoting acetylcholine release; on the other hand, they would enhance toxicity by promoting uptake of toxin molecules.

The present study provides an evaluation of aminopyridines as therapeutic agents in botulism. Experiments were performed that bear directly on two issues, the ability of the drugs to slow the onset of paralysis and the ability of the drugs to provide symptomatic relief. Of the seven serotypes of botulimum neurotoxin, three were examined (A, B, and E). It is these three that account for most cases of human botulism.

When tested at the maximum concentration practical for study (10^{-4} M), both 4-AP and 3,4-DAP slowed the rate of onset of neuromuscular blockade produced by botulimum toxin type A. Of the two, 3,4-DAP produced a more striking effect. Interestingly, neither drug was equally effective in delaying the onset of paralysis due to botulimum toxin types B and E or tetanus toxin.

Failure of the drugs to provide substantial protection against botulimum toxin types B and E cannot be due to drug-promoted uptake of the toxins. When the paralysis times of control tissues and toxin-treated tissues were compared (Fig. 3 and 4), it was obvious that the aminopyridines did not cause tissues to begin to be paralyzed more quickly. Furthermore, the aminopyridines did not provide substantial protection against botulimum toxin types B and E when added to tissues that had already internalized the toxin (Fig. 5).

When tested for its ability to relieve the effects of poisoning in tissues that had previously been exposed to botulimum toxin, 3,4-DAP caused responses to increase in magnitude. The effect was greater for botulimum toxin type A than for botulimum toxin types B and E. These results confirm and extend earlier work in which electrophysiological studies showed that aminopyridines did not affect the rate-limiting promoting acetylcholine release from tissues poisoned with toxin type A than from tissues previously poisoned with toxin type B (12) or type F (5).

The data suggest that 3,4-DAP or a drug similar to it might be useful in the treatment of botulism caused by type A toxin. As indicated by the discussion above, the drug might slow the onset and diminish the ultimate severity of the
disease. Although additional experiments are needed, the available findings suggest that 3,4-DAP may increase the lethal dose of botulinum toxin type A (7).

Aside from therapeutic implications, the work on aminopyridines raises interesting questions about the similarities and dissimilarities of the various botulinum neurotoxins. It is generally assumed that the seven toxins are related and that they are derived from the same ancestral parent. Although the seven toxins are immunologically distinct, there is the presumption that they proceed through the same three-step sequence in exerting their effects and that they possess basically the same intracellular poisoning action.

Although this prevailing belief may be true, there is evidence that the toxins do have important differences. For example, the seven botulinum neurotoxins do not share the same receptor (6). As another example, the various toxins do not interact in the same way with aminopyridines (see above). These findings suggest that a concerted effort should be made to find immunological, pharmacological, and other means to discriminate among the toxins. Until primary structure (e.g., sequence homology) data are available, it will be mainly immunological and pharmacological data that will indicate the extent of relatedness among the various botulinum neurotoxins.

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LITERATURE CITED