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Absence of binding by botulinum toxin to isolated synaptosomal membranes

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IN A RECENT communication, Drachman and Fandburg[1] showed that type A botulinum toxin, even at high concentrations, was unable to prevent synaptosome uptake of calcium. Calcium is necessary for acetylcholine (ACh) release and, because type A botulinum toxin acts to prevent ACh release, Drachman and Fandburg had reasoned that inhibition of calcium uptake might be the mechanism of toxin action. Their data did not support such a mechanism.

Unfortunately, the aforementioned study did not determine whether type A botulinum toxin binds to synaptosomes. Both electrophysiological[2,3] and histological[4] studies have been ambiguous with regard to whether the toxin affects the central nervous system (CNS). In view of this ambiguity, it seems inappropriate to use synaptosomes for the study of the mechanism of type A botulinum toxin activity until there is evidence that the toxin actually binds either to synaptosomes or to synaptosomal membranes. The studies which follow were designed to seek this evidence. Such work is now particularly timely, because several types of botulinum toxin have recently been separated into their neurotoxic and non-neurotoxic components.[5–7]

To test for possible binding of type A botulinum toxin to central nervous tissue, synaptosomal membranes were prepared according to the technique of Rodriguez de Lores Arnaiz *et al.*[8] Membrane samples were obtained from the cortices of adult female rabbits. Both protein and acetylcholinesterase determinations were comparable with those reported by Rodriguez de Lores Arnaiz *et al.*[8] One-ml samples of synaptosome membrane suspensions ($\simeq 3{\cdot}6$ mg protein/ml, 0·9% NaCl as diluent) were incubated with 5×10^3 mouse LD_{50} (1 $LD_{50} \simeq 29$ pg of toxin protein) of freshly crystallized type A botulinum toxin (kindly provided by Dr. E. J. Schantz). Incubation was conducted at 25° for 30 min. At the end of this period, samples were centrifuged for 20 min at 20,000 *g*. Control samples were similarly incubated and centrifuged, but no tissue fraction was present. Residual toxicity of the supernatant was assayed (Wassermann–Takaki test) by the method of Boroff and Fleck[9] on male albino mice (20–25 g). Injection volume was 0·1 ml. Quite unexpectedly, the supernatants from all tissue fraction incubates were found to be more, rather than less, potent than control samples (see Fig. 1).

In an attempt to characterize the factor potentiating type A botulinum toxin, tissue fraction supernatants were prepared as above, but in the absence of toxin. Intravenous injection of this fluid into

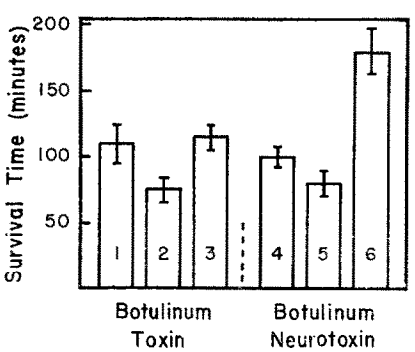

FIG. 1. Survival times of mice injected intravenously with various solutions of botulinum toxin or botulinum neurotoxin. Solutions were 1·0 ml in volume, 0·9% NaCl, to which 5×10^3 LD_{50} of either botulinum toxin or botulinum neurotoxin was added. Incubation was conducted at 25° for 30 min, after which 0·1 ml was injected per mouse (group N = 5). Groups 1 and 4 are the respective controls, groups 2 and 5 are toxin incubations in supernatant from an initial membrane wash, and groups 3 and 6 are toxin incubations in membranes washed 5 times. An increase in mouse survival time denotes loss of toxin potency and a decrease denotes enhancement in toxin potency. Thus, both botulinum toxin and botulinum neurotoxin are enhanced (groups 2 and 5) by the first tissue wash, but only botulinum neurotoxin (group 6) loses potency after incubation with synaptosomal membranes.

mice caused no visible untoward effects. In another test, supernatant was prepared and type A botulinum toxin was added to this, thus never exposing the toxin to synaptosome membranes. Toxicity tests showed that the supernatant before or after dialysis potentiated type A botulinum toxin. Apparently the potentiating factor was eluted from suspended membranes. In a companion sequence of studies,* a variety of phosphatides have been found to potentiate type A botulinum toxin. The potentiating factor may therefore be one or more membrane phosphatides which are lost to the supernatant during incubation.

Sequential washing of membranes markedly diminishes the potentiating capacity of resulting supernatants. Because of this, the original experiment of exposing toxin to synaptosomal membranes was rerun with membrane samples washed five times with 0·9% NaCl. The results are shown in Fig. 1. Type A botulinum toxin, as indicated above, is potentiated by incubation in the first wash. Although potentiation by synaptosome membranes was reduced by repeated washing, botulinum toxin incubated with washed membranes did not lose its potency when the membranes were centrifuged away. This result was obtained regardless of incubation time (up to 1 hr) or temperature of incubation (up to 40°). Apparently, type A botulinum toxin does not bind to rabbit cortex synaptosomal membranes.

As indicated above, several types of botulinum toxin have been fractionated and their neurotoxic component has been isolated. A sample of type A botulinum neurotoxin, an isolated component of type A botulinum toxin, was obtained through the courtesy of Mr. William Beers. A Wassermann–Takaki test, identical to that run with type A botulinum toxin, was run with type A botulinum neurotoxin. As shown in Fig. 1, neurotoxin activity is also potentiated. But more importantly, the neurotoxic component appears to lose a portion of its toxicity after incubation with synaptosomal membranes.

To account for the disparity in results arising from tests with intact botulinum toxin and its neurotoxic component, we proceeded to demonstrate that indeed two distinguishable compounds were involved. A Sephadex G-200 column was set up [column dimensions, 1·5 cm (i.d.) by 28 cm (height); bed volume, 46·2 cm^3; void volume, 15·2 ml]. Type A botulinum toxin was dissolved in 0·9% NaCl, pH 6·2, the same pH as the tissue fraction samples, and eluted through the column with saline. As can be seen in Fig. 2, the toxin emerged as a single peak near the void volume. This is

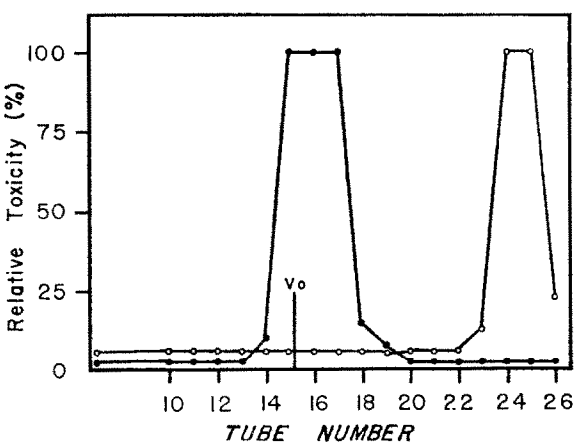

FIG. 2. Elution of botulinum toxin (● — ●) and botulinum neurotoxin (○ — ○) from a G-200 Sephadex column. Both molecules were dissolved in 0·9% NaCl of pH 6·2, and eluted from the column with an identical solution. Samples (1·0 ml) were collected and 0·1-ml aliquots were subsequently injected into mice (group N = 3). Each compound emerged from the column with a single peak, that of botulinum toxin being 16·2 ml and that of botulinum neurotoxin being 24·4 ml. Void volume (V_0) was 15·2 ml. The horizontal baseline is included for graphic, not quantitative, reasons.

the expected result if the components of type A botulinum toxin remain undissociated at the ionic strength of the 0·9% NaCl. On the other hand, type A botulinum neurotoxin similarly treated emerged at *ca.* 1·6 times void volume. This volume is closely in keeping with expectations based on

* L. L. Simpson and M. M. Rapport, to be published.

previous reports of neurotoxin molecular weights.[5–7] Clearly, botulinum toxin and botulinum neurotoxin, both kept for short times in pH 6·2, 0·9% NaCl, are two distinct molecular species.

The data presented here show that type A botulinum toxin does not bind to synaptosomal membranes. This finding is in accord with previous studies which found that the toxin does not bind to homogenates of whole brain,*,† but stands in contrast to studies which found that the toxin does bind to peripheral nerves.[4, 10] In light of our data, it may be questioned whether the observation that type A botulinum toxin does not interfere with calcium uptake by synaptosomes is germane to the pathophysiology of botulism. Unless data are presented to show CNS binding of the toxin, the fact that it does not impede a process can be given no interpretation. In any event, previous data have militated against a competitive antagonism of calcium as a mechanism of type A botulinum toxin action.[11]

The finding that the neurotoxic component of botulinum toxin does lose potency after incubation with synaptosomal membranes does not necessarily indicate that specific binding has occurred. Either nonspecific binding or inactivation due to biochemical reactions may be responsible. Additional studies are needed to resolve this matter.

Division of Neuroscience,
New York State Psychiatric Institute,
New York, N.Y. 10032, *U.S.A.*

M. RAITERI‡
H. KAUFMAN
L. L. SIMPSON

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‡ Department of Pharmacology, Universita Cattolica, Rome, Italy.

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Stimulatory effect of charcoal-broiled ground beef on the hydroxylation of 3,4-benzpyrene by enzymes in rat liver and placenta*†

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YAMAGIWA and Ichikawa[1] demonstrated that coal tar possesses carcinogenic activity in laboratory animals. In 1933, a pure carcinogenic hydrocarbon was isolated from coal tar pitch and identified as 3,4-benzpyrene (BP).[2] This polycyclic, aromatic hydrocarbon is found in certain smoked and cooked

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