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Reversible Neuromuscular Blockade by Modified Neurotoxin from *Naja naja siamensis*[1] (39875)

ALAN J. BLOCK AND JAMES K. BUBIEN

Astra Pharmaceutical Products, Inc., Pleasant Street Connector, P.O. Box 1089, Framingham, Massachusetts 01701

Introduction. The pharmacological actions of the toxic constituents in cobra venom have been clarified and extensively reviewed (1-4). It is now acknowledged that the purified "neurotoxin" component obtained from many snakes within the family Elapidae induces a nondepolarizing neuromuscular paralysis of varying degrees of reversibility. For instance, of the subspecies of *Naja naja, N.n. atra* and *N.n. nigricolis* produce neurotoxins whose effects are readily reversible, whereas neurotoxins from either *N.n. naja* or *N.n. siamensis* induce a paralysis of skeletal muscle which is virtually irreversible (4-6).

Karlsson and Eaker (7) chemically modified two irreversible native neurotoxins obtained from *N.n. naja* and *N.n. siamensis*. By increasing the degree of carbamylation or acetylation of free amino groups within each toxin molecule, they were able to reduce successively but not abolish the *in vivo* lethality. Coincident with the loss of toxicity, modification also effected a more rapid recovery from the toxicity induced by the neurotoxins. Consequently, the authors inferred that the modified neurotoxins were bound to the acetylcholine receptor less firmly than their native counterparts. The present investigation sought to quantify further *in vitro* the degree of potency lost upon modification of *N.n. siamensis* neurotoxin and to establish whether or not these modified neurotoxins fulfill the criteria for truly competitive, reversible, neuromuscular blocking agents.

Methods. All experiments were performed on frog (*Rana* species) rectus abdominus muscle bathed at room temperature (19-22°) in Ringer solution bubbled with compressed air. The tissues were subjected to a resting load of 1-2 g and their contractions were monitored with Harvard (Model 386 or 356) transducers coupled to pen recorders. After a 30-min quiescent period, the experiment proceeded according to one of the following protocols.

(A) Effect of time and toxin concentrations. The Ringer Solution bathing the muscles was drained off and replaced by Ringer solution containing acetylcholine at a concentration of 1×10^{-5} *M*. After the muscle response reached a plateau, the bathing fluid was drained off and the muscle was washed twice with fresh Ringer solution and allowed to relax completely. Such a response-relaxation cycle could be repeated every 20 min throughout the day with no deterioration of responses. Once the contractile responses had stabilized, each tissue was exposed to a single concentration of siamensis toxin. Ringer solution, containing toxin plus acetylcholine (10^{-5} *M*), was introduced for ~2 min at 20-min intervals. Between responses the tissues were rinsed with Ringer-toxin mixture to ensure continuous exposure to toxin at the desired concentration. When the reduced acetylcholine-evoked responses either reached a plateau or disappeared, the tissues were rinsed with fresh toxin-free Ringer solution, and the acetylcholine dose schedule was continued without toxin to assess reversibility of blockade. At least five control responses to acetylcholine were evoked before addition of toxin; all subsequent responses were expressed as a percentage of the last control response.

(B) Effect of toxin on cumulative dose-response curves for acetylcholine. In the control period, each tissue was exposed to cumulative submaximal concentrations of acetylcholine (3×10^{-7} to 3×10^{-5} *M*). Then cumulative concentrations up to 3×10^{-2} *M*

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were similarly administered in the presence of fixed concentrations of toxin which had been in contact with the tissue for a predetermined equilibration time according to the data obtained in protocol A, above. Again, only one dose of toxin was examined on each tissue.

(C) pA_2 determinations, according to Schild (8). Dose ratios were calculated by employing a modified three-point assay system in which tissue responses to acetylcholine evoked after equilibration with toxin (90 minutes) or with *d*-tubocurarine (20 min) were compared with responses (20–80% of maximal) prior to the addition of the antagonists. Responses of tissues not exposed to antagonists did not change during comparable "equilibration" periods. Maximal contractile responses were evoked at the end of each experiment with KCl, 1×10^{-10} *M*; in separate experiments maxima elicited by acetylcholine, 1×10^{-2} *M*, and KCl, 1×10^{-1} *M*, did not differ ($P > 0.05$).

Drugs. Native and fully modified (hexacarbamylated and hexa-acetylated) siamensis neurotoxins (7) were supplied by Dr. E. Karlsson. Solutions of each were prepared daily in 50 m*M* acetate buffer (pH 5), and their concentrations were verified by ultraviolet spectroscopy at 280 nm (7, 9). Final dilutions of toxin, *d*-tubocurarine, and acetylcholine were prepared in Ringer solution and are expressed in the text as molar concentrations in the bath.

Results. Effect of time and toxin concentration. The effect of siamensis native toxin (A) and its acetylated derivative (B) on the contractures produced by acetylcholine (10^{-5} *M*) is illustrated in Fig. 1. The administration of toxin reduced subsequent contractile responses elicited by acetylcholine. For the native toxin this decrement continued unabated and the rate of decline increased with the toxin concentration. In contrast, as exemplified with acetylated toxin in (B), each modified toxin induced an apparent equilibrium blockade, the maximum effect of which was expressed at approximately 50 min. Subsequent to the times indicated by the arrows, the tissues were rinsed with toxin-free Ringer solution while the acetylcholine dose schedule was continued. Thus, although native toxin produced practically irreversible blockade of responses to acetylcholine, acetylated neurotoxin produced readily reversible blockade. Similar reversibility was seen with the carbamylated derivative.

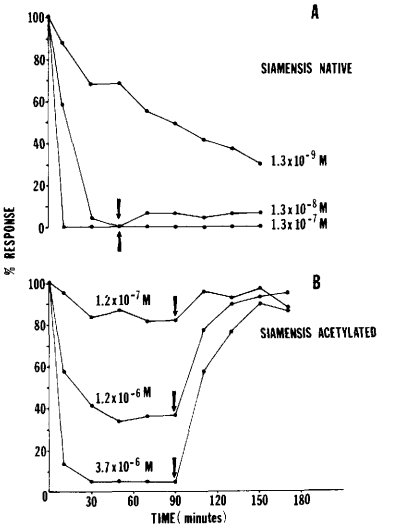

FIG. 1. Time course of inhibition produced by siamensis native (A) and acetylated (B) neurotoxin. Each point represents one response to acetylcholine 1×10^{-5} *M* and each curve is from a single muscle. Toxin, at the concentration indicated, was added at time zero and washed out at the arrow.

Effect of toxin upon dose-response curves for acetylcholine. In the presence of each toxin, the dose-response curves for acetylcholine were shifted to the right. Within the concentration range (1.2×10^{-7} to 3.7×10^{-6} *M*) of acetylated as well as carbamylated siamensis neurotoxins studied, these shifts were parallel, with no depression of the maximal response. Figure 2 illustrates this for siamensis carbamylated neurotoxin. In contrast, although native neurotoxin at 3.8×10^{-9} *M* caused a parallel rightward shift without depressing the maximal response, at higher concentrations it produced rightward shifts of decreasing slopes with a considerable depression of the maxima, characteristic of its known irreversible blockade.

pA_2 Determination. The pA_2 plots for *d*-tubocurarine and the acetylated siamensis neurotoxin are illustrated in Fig. 3. Determined in exactly the same manner, the pA_2 for the carbamylated siamensis toxin was

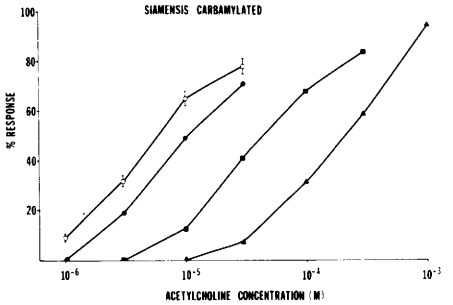

FIG. 2. Effect of different concentrations of siamensis carbamylated neurotoxin upon dose-response curves for acetylcholine. Open circles represent the means of six control responses, one from each of six muscles; the vertical bars indicate the standard errors. The closed symbols are the averages of two responses obtained from two muscles within the control group, but evoked by acetylcholine after the muscles were exposed to a fixed concentration of toxin for 90 min. Toxin concentrations: solid circles (●), 1.2×10^{-7} *M*; solid squares (■), 1.2×10^{-6} *M*; solid triangles (▲), 3.7×10^{-6} *M*.

6.77 and the slope of its regression line was 1.07. None of the slopes of the regression lines for each antagonist differed from unity ($P > 0.05$).

Discussion. The purified polypeptide neurotoxin obtained from the Thailand cobra, *Naja naja siamensis,* is known to produce irreversible, nondepolarizing, neuromuscular blockade (4, 6). The methods for isolation, purification, and modification of the neurotoxin have been published elsewhere (7, 9). The present *in vitro* experiments have shown that masking all free amino groups of native neurotoxin renders its blockade reversible to such an extent that these derivatives fulfilled the criteria for truly competitive neuromuscular antagonists, such as *d*-tubocurarine.

Before proceeding with a general discussion, several technical points should be clarified. For example, to minimize error in the determination of pA_2, it is necessary to establish an equilibrium between an antagonist and the receptor (8). In the present investigation, 50 min were required to attain a steady level of inhibition by toxin (e.g., Fig. 1B). However, to be absolutely certain that equilibration had occurred, an exposure to toxin of 90 min was employed for the determination of pA_2. The control dose-response curves for acetylcholine were not carried to their maxima for two reasons. First of all, complete recovery of the tissue from the effects of high concentrations of acetylcholine takes a long time. This, coupled with the chosen 90-min equilibration time for the neurotoxin, would have severely decreased the amount of data obtainable in a given day. Second, desensitized nicotinic cholinergic receptors are protected against the inhibitory effects of siamensis neurotoxin (10). Thus, to avoid the possibility of acetylcholine-induced desensitization, overly high doses of this agonist were avoided. Yet, the possible occurrence of desensitization even at the doses employed cannot be excluded, since the slight (10%) reversibility observed with native siamensis neurotoxin at 1.3×10^{-8} *M* (Fig. 1A) could represent a recovery from desensitization which had previously protected a small population of receptors.

Assuming that the chemical treatment did not induce changes other than acetylation or carbamylation of free amino groups, our data tend to support the conclusion of Karlsson and Eaker (7) that these groups are not essential for toxicity (i.e., neuromuscular paralysis). Granted, a major reduction in potency occurred with modification, yet each derivative was still more potent than *d*-

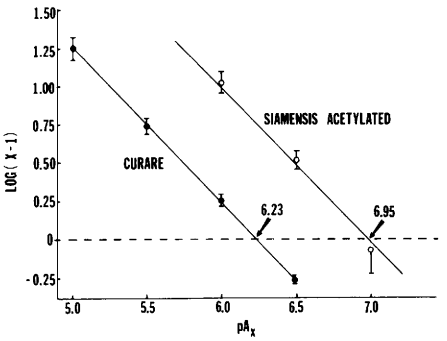

FIG. 3. pA_2 Plots for antagonism of acetylcholine contracture in frog rectus abdominus muscle by *d*-tubocurarine (closed circles) and acetylated siamensis neurotoxin (open circles). Each point represents the mean of between 6 and 10 determinations at each concentration of antagonist, and the vertical bars indicate the standard errors. The arrows and numbers above them indicate pA_2 values; the slopes of the regression lines for *d*-tubocurarine and acetylated siamensis neurotoxin were 1.00 and 1.09, respectively.

tubocurarine. Furthermore, while not essential for receptor recognition, it appears that the free amino groups do regulate the receptor-binding properties of the toxin.

It is not certain how chemical modification of the neurotoxin molecule renders it a less potent, reversible blocker of acetylcholine receptors. The masking of these functional amino groups per se or the attendant alteration in overall ionic charge (derivatives are anionic, whereas native neurotoxin is strongly cationic at pH 7.4) may have produced the observed results. Conformational changes of the peptide, near to but probably not involving the primary active site, might also have occurred. Nevertheless, while effecting a loss of potency and an increase in reversibility, it is unlikely that the process of modification abolished the integrity of the disulfide bonds, since their reduction would render the neurotoxin inactive (4).

Moreover, since modification reduced further the disparity between the characteristics of the neuromuscular blockade induced by *d*-tubocurarine (MW 772) on the one hand and the elapid polypeptide neurotoxins (MW 6000–8000) on the other, we must reappraise the notion put forward by Lee (3) that the lesser reversibility of paralysis induced by cobra neurotoxin was due to its large molecular size.

Summary. The objective of this study was to determine whether modified neurotoxins (7) from *Naja naja siamensis* produced competitive neuromuscular blockade. Each modified toxin as well as native neurotoxin from *N.n. siamensis* was tested at several concentrations for its inhibitory effects upon acetylcholine-induced contractures in frog rectus abdominus muscles *in vitro*. Whereas native neurotoxin induced irreversible blockade, neuromuscular blockade with either the carbamylated or acetylated derivatives was deemed to be truly competitive because: (i) Inhibition of acetylcholine contractures was readily reversible upon washing with toxin-free solution; (ii) dose–response curves for acetylcholine were shifted parallel to the right without depression of maxima; (iii) slopes of regression line for pA_2 determination were not different from unity.

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