

## IN VIVO RECOVERY OF MUSCLE CONTRACTION AFTER $\alpha$ -BUNGAROTOXIN BINDING

HELEN C. FERTUCK, WILLIAM WOODWARD, and MIRIAM M. SALPETER. From the Section of Neurobiology and Behavior, School of Applied and Engineering Physics, and Department of Physics, Cornell University, Ithaca, New York 14853

The discovery that  $\alpha$ -bungarotoxin ( $\alpha$ -BTX), a polypeptide isolated from the venom of *Bungarus multicinctus*, binds specifically and irreversibly to the nicotinic acetylcholine receptor (AChR) (3) has provided a tool for the study of the localization and turnover of the receptor in vertebrate muscle. Previous studies by Berg and Hall (1, 2) have suggested that the recovery after  $\alpha$ -BTX binding at adult neuromuscular junctions is relatively slow compared to that of the postdenervation extrajunctional receptor. However, no physiological data nor precise time scale for the recovery of junctional receptors was given.<sup>1</sup> In the present study we approached the question of recovery of junctional AChR after  $\alpha$ -BTX by investigating the in vivo recovery of nerve-stimulated muscle contraction in mouse sternomastoid muscle, after inactivating the response with  $\alpha$ -BTX.

### *In Vivo Neuromuscular Preparation*

The right sternomastoid muscle was exposed in anesthetized mice, and a loop of the innervating nerve (branch of the spinal accessory) was drawn

<sup>1</sup> After this paper was submitted for publication, a study by Chang and Huang (1975. *Nature [Lond.]*, **253**:643-644) did in fact give a time scale for recovery of inactivated junctional receptor. This study is similar to that of Berg and Hall (1) in that it bases its criterion of recovery on the removal of [<sup>125</sup>I] $\alpha$ -BTX radioactivity from a diaphragm preparation and comes to conclusions similar to those of Berg and Hall.

into a suction electrode. The muscle could thus be stimulated indirectly (neurally) without cutting the nerve. It could also be stimulated directly (field stimulation) by a pair of blunted needle electrodes in contact with the muscle. Muscle concentration was monitored by a polished needle inserted into the muscle and attached to an isotonic transducer (Harvard Apparatus Co., Inc., Millis, Mass.). In this preparation, maximum tetanic contraction amplitudes were produced at stimulation frequencies above 70 Hz. We found that normally the maximum tetanic contraction obtainable from the muscle was the same for both the direct and the indirect modes of stimulation, provided the voltage necessary to produce this maximal tetanic contraction was determined each time for each mode of stimulation. Furthermore, the absolute amplitude of the recorded muscle contraction depended on the location of the transducer needle. However, since we recorded the muscle contractions produced by both modes of stimulation by the same transducer needle, we had, at each recording session, an internal standard of muscle contraction, i.e. the field-stimulated response, against which to compare the neurally evoked response.

### *Recovery of Neuromuscular Response after $\alpha$ -BTX*

The maximum tetanic contraction of the exposed muscle was first established by stimulating

at ~80–100 Hz at supramaximal voltage settings. Tetanic contraction was used in order to obtain maximal recruitment of available fibers. (The tetanic contraction amplitude was roughly four times that obtained by single twitch stimulations.) To inactivate the AChR, the muscle was then bathed in  $\alpha$ -BTX ( $10^{-6}$  M in Krebs-Ringer solution) while the nerve was stimulated every 15 min with short (~2 s) trains of pulses (80–100 Hz) until no tetanic muscle contractions could be recorded (Fig. 1). Inactivation took a total of ~1–2 h (about twice as long as to eliminate single twitch responses).<sup>2</sup> The  $\alpha$ -BTX did not affect the muscle response to direct stimulation. The incision was then closed, and the animals were allowed to survive (survival rate was ~50–60%). At varying daily intervals, animals were reanesthetized, and the amplitude of maximal tetanic muscular response to both modes of stimulation was checked. Recovery was tested on a total of 10 animals. No animal was tested on more than two different days to minimize affecting the results by muscle damage.

Percent recovery was determined (Fig. 2) by comparing the maximum tetanic contraction obtainable by nerve stimulation with that obtainable by direct muscle stimulation, e.g., normal muscle always gives 100%. Although there was considerable variability from animal to animal, recovery of neuromuscular contraction occurred in 4–8 days (half time of ~3 days), i.e., of 4 animals tested between 4–8 days after inactivation, all recovered to better than 90% within that time.

Our finding of relatively little recovery in 1 day is consistent with the report of Berg and Hall (2). However, if one can equate absence of removal of radioactivity with absence of physiological recovery, the fact that recovery occurs within 4–8 days in our system seems to be at variance with an earlier report by the same authors (1) that 66% of the radioactivity remains at the end plate regions 5 days after [<sup>125</sup>I] $\alpha$ -BTX (see also footnote 1). The different experimental procedures

<sup>2</sup> We have found (see footnote 3) that, after this inactivation endpoint, more than 90% of the available  $\alpha$ -BTX binding sites are occupied, i.e., if after inactivation with nonradioactive  $\alpha$ -BTX the muscle is incubated with [<sup>125</sup>I] $\alpha$ -BTX for an additional 2 h, the amount of radioactivity bound to the end plate (assessed by EM autoradiography) is less than 10% of that which is bound when the initial inactivation is done by radioactive  $\alpha$ -BTX and is localized in essentially the same place.

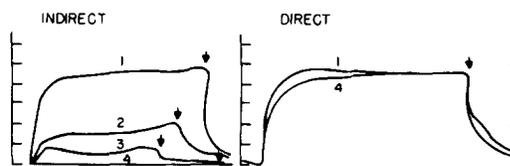


FIGURE 1 Stimulation mode. Tetanus 0 min (curve 1), 60 min (curve 2), 102 min (curve 3), and 145 min (curve 4) after  $\alpha$ -BTX. Amplitude of tetanic muscle response to indirect (nerve) and direct (field) stimulation in the presence of  $\alpha$ -BTX. Normally (curve 1) the maximum muscle contraction is the same for both modes of stimulation. After 145 min in  $\alpha$ -BTX (curve 4) the response to indirect (nerve) stimulation is not above background, whereas the response to the direct stimulation is unchanged (arrows represent end of stimulation period).

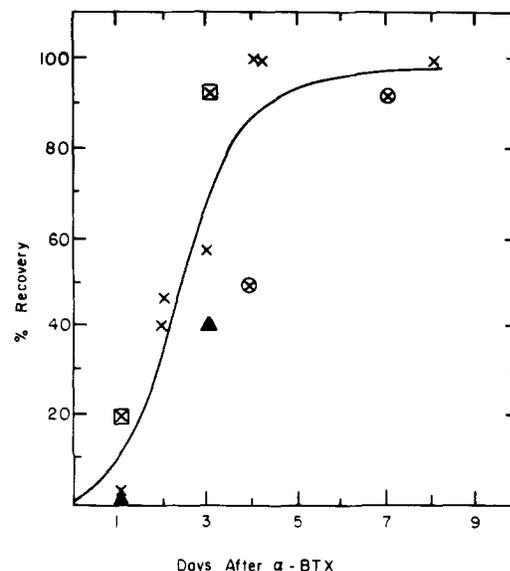


FIGURE 2 Percent recovery of the neuromuscular response. ([Amplitude of neurally evoked contraction]/[amplitude of field stimulation contraction])  $\times$  100%. Three animals ( $\blacktriangle$ ,  $\boxtimes$ , and  $\otimes$ ) were each tested on two different occasions to show individual variations in speed of recovery. All other animals ( $\times$ ) were tested only once. The solid line represents the average recovery curve, but, due to the individual animal variations, is not intended to represent a universal recovery function.

may account for the difference in results. We looked at recovery of a physiological response, while they looked at removal of radioactivity. A possibility remains that some of the radioactivity seen by Berg and Hall is due to migration and internalization of the  $\alpha$ -BTX (or an  $\alpha$ -BTX receptor complex) which then remained in the tissue for prolonged periods.

### *Effect of Actinomycin D*

Various studies with actinomycin D or inhibitors of protein synthesis (2, 4, 6) have led to the conclusion that the production and turnover of the extrajunctional AChR involves an active metabolic process.

To see whether this is also true for the junctional receptors, we looked at the effect of actinomycin D on the recovery of function after  $\alpha$ -BTX in the sternomastoid preparation. (We did not use cycloheximide or other inhibitors of protein synthesis, as they are too toxic for long-term *in vivo* studies.) Grampp et al. (6) have shown that, from 12 h to 3½ days after a single injection of actinomycin D, the incorporation of [<sup>3</sup>H]uridine into RNA of mouse muscle homogenates is reduced by 50%.

In our study, two groups of animals (four mice each) were treated as follows: Group 1 had their neuromuscular contractions inactivated with  $\alpha$ -BTX as described above. After these animals had recovered from the anesthesia, they received an intraperitoneal injection of actinomycin D (0.5 mg/kg weight). Group 2 received a similar injection of actinomycin D after a sham operation in which no  $\alpha$ -BTX was administered. The animals were weighed at daily intervals, and 3–4 days later they were anesthetized, and their muscle response was determined as described above.

The dose of actinomycin used was not lethal to any of the animals, but considerable weight loss (~5 g in 3–4 days) was seen in both groups. When neuromuscular response was tested 3–4 days after the actinomycin D injection, we found that, in the animals without  $\alpha$ -BTX, actinomycin D did not affect the response to indirect stimulation (at 80–100 Hz), whereas in the animals pretreated with  $\alpha$ -BTX, actinomycin D prevented the normal recovery of neurally evoked muscle contraction. By 4 days, recovery was less than 10%. These results suggest that the *in vivo* recovery seen after inactivation of the junctional receptor with  $\alpha$ -BTX is an active metabolic process involving RNA synthesis, a finding similar to that previously reported for the extrajunctional receptor.

Fambrough (4) had reported that ACh sensitivity at diaphragm neuromuscular junctions is unaffected by actinomycin D for 3 days *in vitro* and on that basis suggested that in that time scale there is essentially no turnover of junctional AChR. To be consistent with this interpretation given by Fambrough, one could conclude that at the adult neuromuscular junction there is normally little or

no turnover of AChR and that inactivation by  $\alpha$ -BTX triggers *de novo* synthesis of receptor and degradation of the complexed receptor or otherwise triggers an active process necessary to reactivate the receptor. Some caution is required with such an interpretation both of our own and Fambrough's data, however, since there is evidence that actinomycin D may also inhibit normal degradation and release of cellular material and that synthesis and degradation may be linked (7, 9–11). The fact that actinomycin D does not affect neurally-evoked muscle contraction in non- $\alpha$ -BTX-treated animals may then reflect an interference with normally occurring degradation of receptor. The data are thus also compatible with an interpretation that normally there occurs a steady state of junctional AChR turnover which is revealed both by the post- $\alpha$ -BTX recovery and its interference by actinomycin D. At present we cannot distinguish between these alternatives.

### *Fine Structure and EM Autoradiography*

Preparations examined by electron microscopy immediately after  $\alpha$ -BTX inactivation and at various times during recovery showed essentially normal muscle and end plate morphology.

In a previous study (5), we found that [<sup>125</sup>I] $\alpha$ -BTX bound only to a zone at the top of the junctional folds and was not uniformly distributed throughout the folds. Subsequently, we determined that this constitutes a density of roughly 30,000 sites per  $\mu\text{m}^2$  of postsynaptic membrane within this zone.<sup>3</sup> This raises questions regarding the role of the depths of the junctional folds. The possibility that membrane flow may be involved either during the formation of new receptors or during the removal of the BTX-receptor complex was explored by preliminary studies during recovery; EM autoradiography was used by the procedure of Salpeter and Bachmann (8). First, to determine whether during recovery one can detect new sites at positions other than their normal location, we inactivated neurally evoked contraction with non-radioactive  $\alpha$ -BTX in one animal and then, 2 days later, used [<sup>125</sup>I] $\alpha$ -BTX to inactivate the newly recovered response (which was ~40% of normal). We found that the localization of radioactivity was restricted to the tops of the junctional folds, and, thus, was similar to that seen in the normal animals. Secondly, to study possible movement of bound radioactivity during re-

<sup>3</sup> Fertuck, H. C., and M. M. Salpeter. Manuscript in preparation.

covery, we partially inactivated neuromuscular response by [ $^{125}$ I] $\alpha$ -BTX in three animals, killed the animals at 2, 4, or 8 days later, and again prepared their end plates for examination by EM autoradiography. We found that some labeling ( $\sim 20$ – $25\%$  of that seen immediately after full inactivation of the muscle response by [ $^{125}$ I] $\alpha$ -BTX) was still evident at the neuromuscular junction even 8 days after incubation with radioactive toxin, at which time muscle contraction was fully recovered. The localization of the residual label was again primarily in a zone at the top of the junctional folds (Fig. 3). (No information is at present available regarding the nature of this residual radioactivity.) We, however, saw no evidence of mass internalization of radioactivity down the folds, into the muscle, or into any extrajunctional region. EM autoradiography is capable of localizing only the radioactivity which is retained during fixation and other tissue processing. We can, therefore, not exclude the possibility that internalization of radioactivity in some soluble form is in fact part of the normal recovery process. There is, however, no indication, in these preliminary studies, of internalization or membrane flow of bound radioactivity.

### CONCLUSION

Our method of measuring recovery involved the overall response of the muscle *in vivo*. Since we wanted to study the response *in vivo* and therefore could not measure isometric contraction in absolute terms, we devised a measure of recovery which was based on a comparison between the contrac-

tion capacity of the muscle when stimulated by the nerve and that when stimulated directly. This provided a valid quantitative measure of recovery if recorded with the same recording needle, since the two responses are normally identical, and since the direct stimulation response was unaffected by  $\alpha$ -BTX. Our measure did not allow us to draw any conclusions, however, regarding the rate of recovery at the individual end plates. The question is left open as to whether the gradual recovery of overall muscle function represents a variable rate of recovery of receptor at the different junctions, or whether the rate of recovery of ACh receptor is identical at all the junctions, yet different end plates have different threshold conditions for response.

We can conclude, however, that full recovery of neurally evoked contraction after  $\alpha$ -BTX inactivation occurs *in vivo* over a 4–8-day period (half-time  $\sim 3$  days) and that it seems to be an active metabolic process involving RNA synthesis. This *in vivo* functional recovery is slower than that reported for the turnover of extrajunctional receptors after denervation, but is faster than that reported for the removal of end plate-bound radioactivity from diaphragm preparations. The quantitative relationship between functional recovery and removal of radioactivity remains to be resolved.

### SUMMARY

Acetylcholine receptors were inactivated *in vivo* at the mouse neuromuscular junction using  $\alpha$ -bungarotoxin ( $\alpha$ -BTX). It was found that neurally



FIGURE 3 EM autoradiogram showing [ $^{125}$ I] $\alpha$ -BTX label 8 days after binding at the neuromuscular junction of mouse sternomastoid muscle. The residual label is not distributed over the full depth of the junctional folds (*jf*) but is primarily concentrated in a zone at the top of the folds adjacent to the axon (*a*), and thus similar to that seen immediately after incubation with [ $^{125}$ I] $\alpha$ -BTX (5).  $\times 21,500$ .

produced muscle contraction recovered within 4–8 days (halftime ~3 days). Actinomycin D interfered with this recovery, but did not affect normal nerve-stimulated muscle contraction. If the response was initially eliminated by [<sup>125</sup>I]α-BTX and the end plates examined by EM autoradiography, no evidence of mass internalization of bound radioactivity during recovery was seen. The fine structure of the end plates and muscle was unaltered during the post-α-BTX recovery period.

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