AGONIST-INDUCED MYOPATHY AT THE NEUROMUSCULAR JUNCTION IS MEDIATED BY CALCIUM

JOHN P. LEONARD and MIRIAM M. SALPETER

From the Section of Neurobiology and Behavior and the School of Applied and Engineering Physics, Cornell University, Ithaca, New York 14853

ABSTRACT

Inactivation of cholinesterases at mammalian neuromuscular junctions (nmj) produces extensive muscle "necrosis." Fine-structurally, this myopathy begins near the nmj with an increase in large-diameter vesicles in the soleplasm, the dissolution of Z-disks, dilation of mitochondria, destruction of sarcoplasmic reticulum, and often a highly specific contracture of the muscle under the endplate. Since a Ca^{++}-activated protease which specifically removes Z-disks is known to exist in mammalian skeletal muscle, we tested the possibility that the myopathy after esterase inactivation is due to the prolongation of acetylcholine lifetime and thus of Ca^{++} influx. We first produced the myopathy near endplates by inactivating esterases with diisopropylfluorophosphate (DFP) followed by nerve stimulation for 1–2 h in vitro. The myopathy was later mimicked by bath application of carbamylcholine without esterase inhibitors. This myopathy could be prevented by inactivating the acetylcholine receptors (AChR) with α-bungarotoxin (α-BGT) or by removing Ca^{++} from the bath with EGTA. These results favor the hypothesis that esterase inhibition leads to an agonist-induced myopathy, which is mediated by Ca^{++} and requires an intact AChR.

KEY WORDS neuromuscular junction · myopathy · anticholinesterase · Z-bands · Ca^{++} · acetylcholine receptor α-bungarotoxin

Numerous studies have confirmed the early observations (2, 12, 27) that inactivation of cholinesterases at mammalian neuromuscular junctions (nmj) produces extensive muscle "necrosis" (8, 10, 11, 13, 15, 19, 20, 21, 32). Fine-structurally, this myopathy begins at the nmj with an increase in large-diameter vesicles in the cytoplasm under the junctional folds (soleplasm), dilation of mitochondria, breakdown of sarcoplasmic reticulum, and dissolution of Z-disks (8, 20, 21). When mouse muscles are bathed with diisopropylfluorophosphate (DFP) in vivo, breakdown of the sarcoplasmic reticulum, mitochondrial damage, and changes in Z-disks can be seen within 2 h. Various studies have shown that similar changes occur with other esterase inhibitors, and require a critical inactivation time of 2 h for major necrosis to occur (2, 21). By 1–3 d, the necrosis is maximal. After 1 wk, some recovery has occurred, and by 2–3 wk, muscle architecture is normal (30). Since an intact nerve supply is a prerequisite for the necrosis to occur (2), and since nerve stimulation enhances this effect, a neurotrophic etiology has been suggested (11, 20, 21), possibly mediated by Na^+ flux (21). Salpeter et al. (30) have recently suggested an alternative mechanism, involving Ca^{++} as the mediator of the necrosis. In mammalian skeletal muscle, biochemical studies have identified a Ca^{++}-activated protease which specifically removes Z-disks from isolated myofibrils in vitro (3,

6, 7, 28, 29). Since agonist-receptor interaction causes Ca^{++} influx in the region of the endplate (9, 23), the myopathy after esterase inactivation may be due to an increase of soleplasm Ca^{++} resulting from the prolongation of acetylcholine (ACh) lifetime. The present study was designed to test this hypothesis.

MATERIALS AND METHODS

An isolated nerve-muscle preparation was used in vitro. The extensor digitorum longus (EDL) muscle was removed from albino mice under nembutal anesthesia (50 mg/kg body weight) and loosely pinned out in a 10-ml sylgard chamber. The medium, which was a bicarbonate-phosphate buffered Krebs solution (pH 7.4) gassed with 95% O_2 5% CO_2, was changed every 10 min. Two groups, differing in agonist treatment, were considered. The first was pretreated in vivo to inactivate cholinesterases (ChE) by bathing the exposed muscle with DFP (10^{-3} M) for 2 h. The development of myopathy was then monitored in vitro. Preincubation with DFP was undertaken in vivo to shorten the time in vitro. The muscle was then excised, placed in the dish containing a Krebs bath, and stimulated indirectly (at 2 Hz) with a suction electrode on the nerve. The second group was bathed in 0.1 mM carbamylcholine (carbachol) (for different periods ranging from 20 min to 3 h without esterase inactivation or nerve stimulation).

Variations in the experimental conditions for the preparations of the second group consisted of: (*a*) inactivating AChR by applying α-BGT (1×10^{-6} M) to the exposed muscle in vivo for 3 h before placing the muscle in the carbachol containing bath, or (*b*) removing Ca^{++} with 5 mM EGTA, or (*c*) replacing NaCl with choline chloride or with Tris HCL, or (*d*) adding D-600 (1×10^{-6} or 1×10^{-4} M) to decrease Ca^{++} fluxes (14, 18). In each case, the EDL preparation was pre-incubated for 30 min in Krebs' solution modified as listed above before adding carbachol (1×10^{-4} M) to the bath. To determine whether the continuous presence of carbachol was necessary or whether the myopathy was a "triggered" event, pulse experiments were performed in which the application of carbachol was discontinuous. In one case, the muscle was exposed to carbachol for 20 min and then to normal Krebs' for 2 h and 40 min; in a second case, the muscle was exposed to three 20-min pulses of carbachol, each being followed by 40 min in normal Krebs' solution.

At the end of each experiment the muscles were washed for 30 min in Krebs solution without Ca^{++} to promote muscle relaxation. The muscle was then pinned out at approximately resting length for 30 min in 4% formaldehyde (pH 7.4 buffered with 0.067 M NaH_2PO_4-Na_2HPO_4) freshly made from paraformaldehyde and then transferred to fresh 4% formaldehyde and kept overnight at 4°C. The muscle fibers near the endplate regions were cut into 1- to 2-mm cubes, fixed in 1% osmium tetroxide for 1 h at 4°C, washed in cold distilled water, stained en block with 2% uranyl acetate at 4°C for 1 h, dehydrated in graded ethanols and propylene oxide, and embedded in Epon 812 (22). Pale-gold sections (~1,000 Å) were cut from random regions of the muscle, stained with uranyl acetate and lead citrate, and examined in the electron microscope.

The extent of myopathy was judged using the two most consistent early fine-structural changes during the development of the necrosis: accumulation of vesicles under the neuromuscular junction, and Z-disk dissolution. The analysis for the extent of vesiculation under the endplate was performed by a random point method. Sections were scanned in a Philips 300 or 201 electron microscope until ~10 endplates from different regions of the muscle were found. The area analyzed consisted of a region between the postsynaptic membrane and the first myofibril (soleplasm). A grid of points was punched through the micrographs. (Such points are random relative to the tissue because the orientation of the grid does not depend on the orientation of the tissue.) The number of points overlying large vesicles was then tabulated (a vesicle diameter >130 nm was chosen for convenience). A vesiculation index for each endplate was the ratio of points falling over vesicles to that falling over the entire tabulated region. The Z-disk damage was measured by counting the number of myofibrils under the endplate in longitudinal sections which have missing or disrupted Z-disks.

RESULTS

When isolated mouse EDL nerve-muscle preparations in which the esterases have been inactivated by DFP are stimulated in vitro via the nerve (group 1), several morphological changes occur at the endplate regions which resemble the early changes described for the in vivo studies. By 2 h in vitro, considerable damage can be seen. These changes can be mimicked in an esterase intact preparation by the application of 0.1 mM carbachol without nerve stimulation (group 2). These results are summarized in Tables I and II. The most striking immediate effect (within 1 h) is the appearance of many large vesicles in the soleplasm (see Fig. 1). This vesiculation is restricted to a small (~10–50 μm) region near the endplate, and almost reaches its maximum amount by 20 min in carbachol (Table II). Mitochondria at the endplate are often swollen and lacking cristae while mitochondria in extra-junctional areas of the muscle fiber and in the axon are normal. The sarcoplasmic reticulum (SR) sacs in the region of the nmj also begin to breakdown. Subsequently (by 2–3 h), the Z-disks of the myofibrils nearest the endplate begin to spread and then disappear. There is very often also a specific contracture of the myofibrils under the endplates which ends abruptly beyond the endplate region (see Fig. 1) and is much more

TABLE I

The Role of Ca^+ in Carbachol-induced Myopathy

Condition	Time in vitro 1 h: Relative Area Occupied by Vesicles‡	Time in vitro 1 h: Index of Z disk damage§	Time in vitro 3 h: Relative Area Occupied by Vesicles‡	Time in vitro 3 h: Index of Z disk damage§
1. Controls without Carb				
(a) in Krebs'	—	—	2	0.00
(b) in Tris HCl without Na^+	—	—	5	0.00
2. 0.1 mM Carb‖	24	1.50	29*	3.33*
3. 0.1 mM Carb, No Ca^{++}, & 5 mM EGTA	5	0.00	6*	0.00*
4. 0.1 mM Carb after 1 μM α-BGT in vivo	5	0.00	4	0.00
5. 0.1 mM Carb, and 0.1 mM D-600	9	0.50	3	1.20
6. 0.1 mM Carb, No Na^+ and Choline Chloride	25	0.11	25	2.33
7. 0.1 mM Carb, No Na^+, and Tris HCl	—	—	23	1.25

* These values were based on 2 experiments with 10–15 endplates evaluated per experiment; all other values were based on one experiment with ~10 endplates evaluated per experiment.

‡ Only vesicles with diameters >130 nm were counted; 300–1,500 random points for each experiment reported. Relative area = points overlying vesicles/total points in soleplasm (%). By the Mann-Whitney U test, treatments 6 and 7 are not significantly different from treatment 2 ($P \gg 0.05$) while treatments 1, 3, 4, and 5 are significantly different from treatment 2 ($P \gg 0.01$).

§ This index is defined as the total number of myofibrils under the endplates with damaged Z-lines divided by the number of endplates examined. This index is more variable and more difficult to quantify than the vesiculation index; thus, a test for statistical significance was not done.

‖ Similar results were obtained when the muscle was preincubated with DFP and stimulated for 2 h at 2 Hz.

severe if the prefixation relaxation bath (see Materials and Methods) is omitted after the carbachol treatment. However, in some cases an extreme contracture was seen much beyond the endplate region (see also reference 15).

Blockage of AChR with α-BGT (1×10^{-6}M) or removal of Ca^{++} from the medium with 5 mM EGTA completely prevented the appearance of large vesicles in the soleplasm and of the Z-disk damage in all cases (Table I, Figs. 2 and 4). In contrast, removing Na^+ from the bath did not prevent the characteristic vesicular damage (Table I, Fig. 3) but did decrease the extent of Z-band damage somewhat. D-600, a compound that reduces Ca^{++} influx in other systems (14, 18), had a partial effect in protecting the muscle from damage in 1 μM concentration, and was about twice as effective in 0.1 mM concentration. A single 20-min period in carbachol followed by 2 h and 40 min in Krebs' solution showed finestructural changes that were less severe than those after 1 h in carbachol. Three 20-min periods in carbachol interposed by 40 min in Krebs' produced changes which resembled those seen after 1 h in carbachol and were less severe than those after 3 h in carbachol, even though the total time in vitro was

TABLE II

Effect of Time in Carbachol on Extent of Myopathy

Condition	Relative area occupied by vesicles	Index of Z-disk damage
1. 0.1 mM Carb for 20 min followed by 2 h and 40 min in Krebs'	18*	0.42*
2. 0.1 mM Carb for 20 min at the start of each of 3 h	20	1.91
3. 0.1 mM Carb for 3 h	29*	3.33*

* These values were based on two experiments with 10–15 endplates evaluated per experiment; the other values were based on one experiment with 11 endplates evaluated.

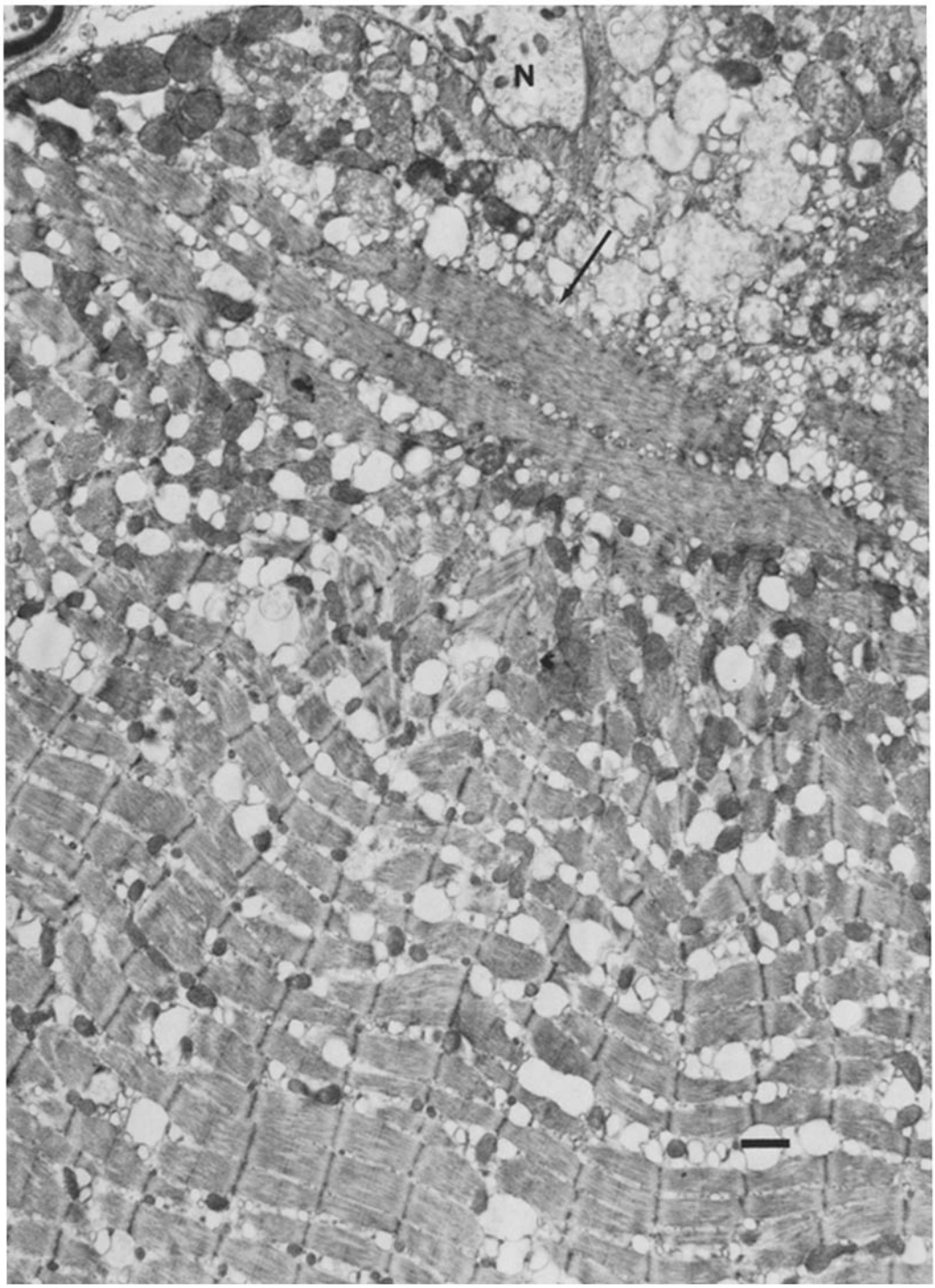

FIGURE 1 Endplate region of isolated mouse EDL muscle after 1 h in carbachol (nerve terminal = *N*). Note swollen mitochondria, dilated sarcoplasmic reticulum, specific contracture, vesiculation of soleplasm, and absence of Z-bands in myofibrils nearest the endplate (arrow). × 7,350.

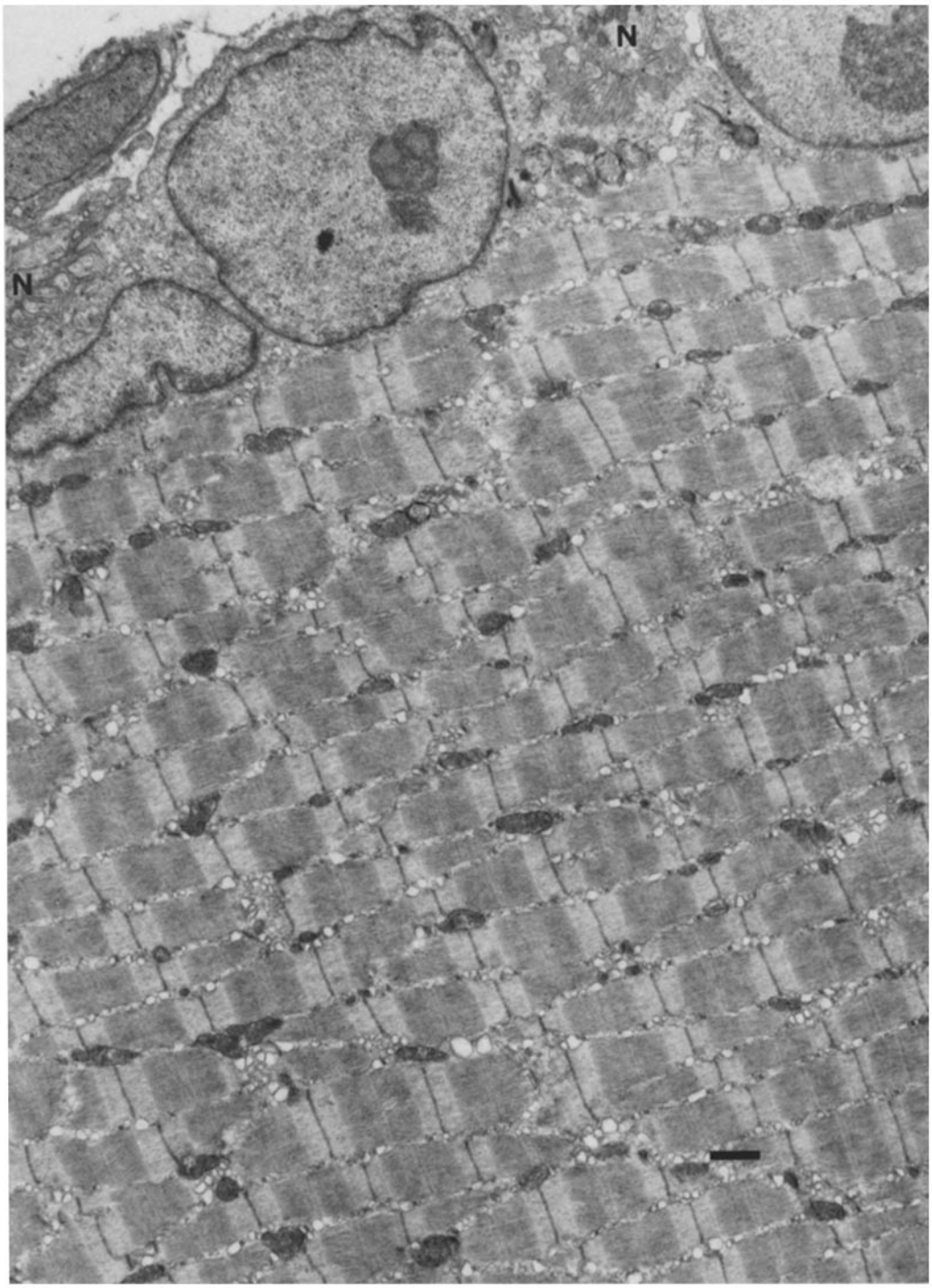

FIGURE 2 EDL muscle treated for 1 h with carbachol (as in Fig. 1) after inactivating ACh receptor in vivo with α-BGT. Note normal appearance of muscle, especially absence of dilated sarcoplasmic reticulum, vesiculation or damage to myofibrils under endplate. (nerve terminal = *N*). × 7,350.

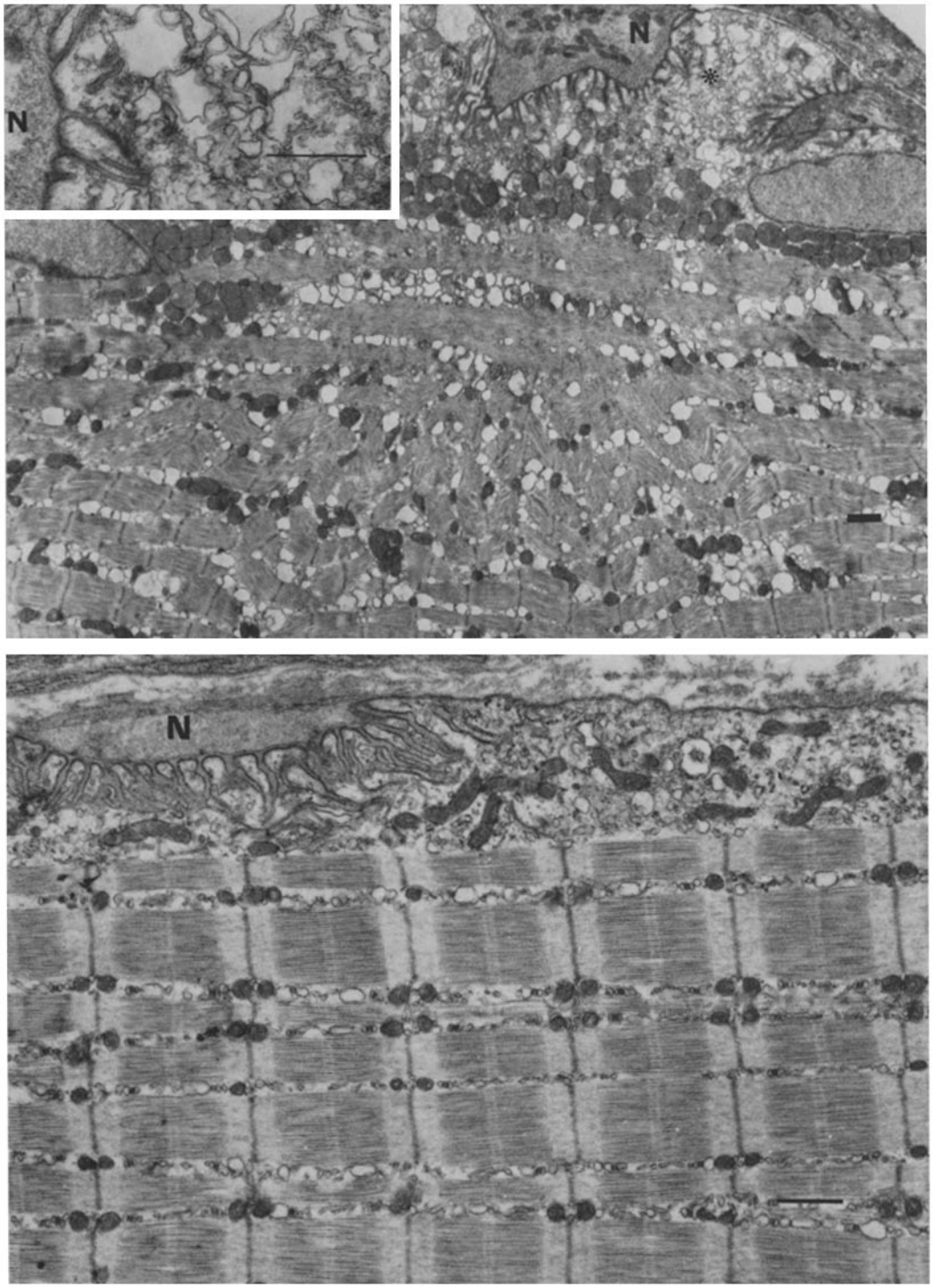

FIGURE 3 EDL muscle treated for 3 h with Na^+-free Krebs' containing carbachol. Note similarities with respect to Fig. 1. (nerve terminal = *N*) × 5,000. *Inset* is enlarged view of area in upper right (*). × 15,000.

FIGURE 4 EDL muscle after 3 h with carbachol in Ca^{++}-free Krebs' containing 5 mM EGTA. Intermediate magnification to show both soleplasm and myofibrils. Note normal appearance of myofibrils and sarcoplasmic reticulum. (nerve terminal = *N*). × 10,000.

identical (compare condition 2 of Table I with that of Table II).

Our results favor the hypothesis that agonist-induced myopathy is mediated by Ca^{++} involving the ACh receptor and, at least for the first 3 h, is dependent on the length of time in carbachol.

DISCUSSION

The original observations (2, 12, 27) of a "reversible necrosis" at the endplate region of striated muscles after esterase inactivation have been confirmed many times (8, 10, 11, 13, 15, 19, 20, 21, 32). Repeating these experiments on an isolated nerve muscle preparation of mouse EDL that had been pretreated with DFP in vivo and is subjected to nerve stimulation (2 Hz) in vitro yields results similar to those seen in the early stages of in vivo induced myopathy. Application of carbachol, the nonmetabolizable ACh agonist, to the bath of an esterase intact preparation mimics the morphological changes induced by DFP plus nerve stimulation. It is not clear why Fenichel et al. (10) failed to induce myopathy by injection of carbachol. Their in vivo experimental conditions may not have been appropriate. Since α-BGT protects from the myopathy induced by in vivo application of DFP (30) as well as from the in vitro carbachol-induced damage (present study), we believe that in both experimental conditions myopathy is generated by the same mechanism involving agonist-receptor interaction and is not due to the mere presence of excess carbachol or esterase inactivation per se.

Several studies by Dettbarn and colleagues (10, 11, 20, 21, 32) have emphasized the neurotrophic aspect of the necrosis induced by esterase inhibition. The need for an intact nerve supply and the role of ACh and its analogues provides the basis of their conclusions. Laskowski and Dettbarn (21) suggest the possibility that Na^+ influx may mediate the damage. The present study suggests that Ca^{++} rather than Na^+ is primarily involved. This conclusion is based on our finding that low external Ca^{++} essentially prevents the myopathy whereas low external Na^+ has only a small protective effect. Possibly the Na^+ acts indirectly by increasing the free Ca^{++} level in the sarcoplasm.

Studies have shown that Ca^{++} flows into the endplate region of vertebrate muscle in the presence of agonist (9, 23, 31). The presence of a Ca^{++}-activated protease which specifically removes Z-disks from isolated myofibrils in vitro could explain the disappearance of Z-disks from myofibrils near the endplate, if prolonged agonist-receptor interaction elevates intracellular Ca^{++} levels sufficiently. The extreme vesiculation and disrupted SR under the endplate, as well as the swollen mitochondria, may reflect an overloading of the muscle's Ca^{++}-binding capacity which could result in high soleplasm Ca^{++} levels. This Ca^{++} need not have come from the extracellular fluid (ECF), but it is difficult to see how EGTA could have prevented the carbachol-induced myopathy if it did not. Another observation that argues for elevation of Ca^{++} due primarily to an influx from the ECF is the frequent occurence of contractures which are restricted to the endplate region. If these contractures were caused by depolarization-induced release of Ca^{++} from the SR the contracture should extend over a much larger region of the muscle. On the other hand, localized contractures can be produced by focal injections of Ca^{++} and are often used as an indication of such local Ca^{++} influx (16, 26). A similar contracture under the endplate was reported after esterase inactivation (15).

One may wonder whether the characteristic contracture seen under the endplate can itself cause local damage and thus produce the myopathy. We do not believe this to be the case since when we stimulated a muscle (at 2 Hz via suction electrode on the nerve) for 5 h, producing extensive repeated contractures, no typical myopathy or damage of any kind was seen.

Finally, the major protection from necrosis brought about by 0.1 mM D-600 also points to Ca^{++} influx from the ECF as a mediator of the myopathy. D-600 is a very specific inhibitor of Ca^{++} influx into mammalian cardiac muscle (14, 18). However, much higher concentrations of D-600 are required to block depolarization-induced $^{45}Ca^{++}$ uptake into rat brain synaptosomes (24). In preliminary studies using $^{45}Ca^{++}$ and scintillation counting, we have also seen that 0.1 mM D-600 causes a significant but not total decrease in carbachol-induced Ca^{++} influx into the muscle. That 0.1 mM D-600 provides only a partial protection from carbachol-induced myopathy in our preparation is consistent with these findings.

Our results clearly indicate that the early stages of myopathy required agonist-receptor interaction and are mediated by Ca^{++}, since they are prevented by both α-BGT and EGTA. We suggest that Ca^{++} influx in response to prolonged agonist-receptor interaction may overload the Ca^{++} binding mechanisms or otherwise elevate intracellular

Ca^{++} sufficiently to activate the destructive proteases.

One final question was whether the myopathy was triggered by a short initial influx of Ca^{++} or whether it was produced in response to the prolonged presence of carbachol. This question was relevant since the ACh receptor is known to desensitize within seconds in the presence of agonist (17). The pulse experiments (Table II) were performed to answer this question. We argued that if desensitization prevented Ca^{++} influx, then the damage after 20 min in carbachol followed by 2 h and 40 min in Krebs' should be equal to that after 3 h in carbachol, whereas the damage after three 20-min carbachol periods separated by 40 min in Krebs' should be more extensive. However, if the actual time in carbachol was the important factor, then 3 h in carbachol should show the most damage and 20 min in carbachol should show the least. This is what we obtained.

Our results (Table II) clearly indicate that over a 3-h period the extent of myopathy does depend on the time in carbamylcholine. We do not know how external Ca^{++} mediates the necrosis. If Ca^{++} mediates the necrosis by entering the muscle in the presence of carbachol, then our present result would suggest a continued Ca^{++} influx even under desensitizing conditions. This is not completely surprising. The process of desensitization is not well understood, but it is known that even under conditions of desensitization the endplate does not completely return to the resting state. Nastuk (25) reports residual depolarization even after overnight in high concentrations of carbachol; Adams (1) reports a plateau current in voltage clamped muscle; Creese (4, 5) shows continued Na^+ influx and Evans (9) shows continued Ca^{++} influx in muscle exposed to prolonged carbachol. It is not within the scope of this paper to speculate on the nature of desensitization or to determine the extent of Ca^{++} or Na^+ flux in the desensitized endplates. Our results are compatible with the idea that some Ca^{++} flux may continue in the presence of carbachol over a 3-h period in vitro.

Further study is required to determine the actual Ca^{++} concentration obtained, the meaning of the numerous large vesicles and dilated mitochondria in the muscle, and any possible link between Na^+ and Ca^{++} fluxes. The present study confirmed the suggestion (30) that Ca^{++} which enters in response to prolonged agonist action can cause muscle damage and may explain the necrosis seen after esterase inactivation. Whether influx of Ca^{++} at the endplate is of major importance only to the pathological condition of esterase failure, or is also important to maintenance and growth of muscle structure and in the response of normal muscle to activity, remains to be determined.

The authors thank Knoll AG Chemische Fabriken for the gift of D-600, Joyce Davis, Christie Kroll, Lucy Yin, Marie Read and Mary Johnson for technical assistance, and Ralph Loring and Hedwig Kasprzak for helpful advice and discussion.

This work was supported by National Institutes of Health grant NS-09315.

Received for publication 12 December 1978, and in revised form 20 April 1979.

REFERENCES

1. ADAMS, P. R. 1975. A study of desensitization using voltage clamp. *Pflügers Arch.* **360**:135–144.
2. ARIENS, A.TH., E. MEETER, O. L. WOLTHIUS, and R. M. J. VAN BENTHEM. 1969. Reversible necrosis at the end-plate region in striated muscles of the rat poisoned with cholinesterase inhibitors. *Experientia.* **25**:57–59.
3. BUSCH, W. A., M. H. STROMER, D. E. GOLL, and A. SUZUKI. 1972. Ca^{++} specific removal of Z lines from rabbit skeletal muscle. *J. Cell Biol.* **52**:367–381.
4. CREESE, R., G. I. FRANKLIN, and L. D. MITCHELL. 1976. Two mechanisms for spontaneous recovery from depolarizing drugs in rat muscle. *Nature (Lond.).* **261**:416–417.
5. CREESE, R., G. I. FRANKLIN, and L. D. MITCHELL. 1977. Sodium entry in rat diaphragm induced by depolarizing drugs. *J. Physiol. (Lond.).* **272**:295–316.
6. DAYTON, W. R., D. E. GOLL, M. G. ZEECE, R. M. ROBSON, and W. J. REVILLE. 1976. A Ca^{++} activated protease possibly involved in myofibrillar protein turnover. Purification from porcine muscle. *Biochemistry.* **15**:2150–2158.
7. DAYTON, W. R., W. J. REVILLE, E. E. GOLL, and M. H. STROMER. 1976. A Ca^{++} activated protease possibly involved in myofibrillar protein turnover. Partial characterization of the purified enzyme. *Biochemistry.* **15**:2159–2166.
8. ENGEL, A. G., E. H. LAMBERT, and T. SANTA. 1973. Study of long-term anticholinesterase therapy. Effects on neuromuscular transmission and motor endplate fine-structure. *Neurology.* **23**:1273–1281.
9. EVANS, R. H. 1974. The entry of labeled Ca^{++} into the innervated region of the mouse diaphragm. *J. Physiol. (Lond.).* **240**:517–533.
10. FENICHEL, G. M., W. D. DETTBARN, and T. M. NEWMAN. 1974. An experimental myopathy secondary to excessive acetylcholine release. *Neurology.* **24**:41–45.
11. FENICHEL, G. M., W. B. KIBLER, W. H. OLSON, and W. D. DETTBARN. 1972. Chronic inhibition of cholinesterase as a cause of myopathy. *Neurology.* **22**:1026–1033.
12. FISCHER, G. 1968. Inhibierung und restitution der Acetylcholinesterase an der motorischen Endplatte im Zwerchfell der Ratte nach Intoxikation mit Soman. *Histochemie.* **16**:144–149.
13. FISCHER, G. 1970. Die Azetylcholinesterase an der motorischen Endplatte des Rattenzwerchfells nach Intoxikation mit Paraoxon und Soman bei Applikation von Oximen. *Experientia.* **26**:402–403.
14. FLECKENSTEIN, A. 1971. Specific inhibitors and promoters of calcium action in the excitation-contraction coupling and heart muscle and their role in the prevention of production of myocardial lesions. *In* Calcium and the Heart. Academic Press, Inc., N.Y. 135–188.
15. HUDSON, C. S., J. E. RASH, T. N. TIEDT, and E. X. ALBUQUERQUE. 1978. Neostigmine-induced alterations at the mammalian neuromuscular junction. II. Ultrastructure. *J. Pharmacol. Exp. Ther.* **205**:340–356.
16. JENKINSON, D. H., and J. G. NICHOLLS. 1961. Contractures and permeability changes produced by ACh in depolarized denervated muscle. *J. Physiol (Lond.).* **159**:111–127.
17. KATZ, B., and S. THESLEFF. A study of "desensitization" produced by acetylcholine at the motor end-plate. *J. Physiol (Lond.).* **138**:63–80.
18. KOHLHARDT, M., B. BAUER, H. KRAUSE, and A. FLECKENSTEIN. 1972. Differentiation of the transmembrane Na^+ and Ca^{++} channels in

mammalian cardiac fibers by the use of specific inhibitors. *Pfluegers Arch. Eur. J. Physiol.* **335**:309–322.
19. LASKOWSKI, M. B., and W. D. DETTBARN. 1977. The pharmacology of experimental myopathies. *Ann. Rev. Pharmacol. Toxicol.* **17**:387–409.
20. LASKOWSKI, M. B., W. H. OLSON, and W. D. DETTBARN. 1975. Ultrastructural changes at the motor endplate produced by an irreversible cholinesterase inhibitor. *Exp. Neurol.* **47**:290–306.
21. LASKOWSKI, M. B., W. H. OLSON, and W. D. DETTBARN. 1977. Initial ultrastructural abnormalities at the motor endplate produced by a cholinesterase inhibitor. *Exp. Neurol.* **57**:13–33.
22. LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409–414.
23. MILEDI, R., I. PARKER, and G. SCHALOW. 1977. Calcium entry across the post-junctional membrane during transmitter action. *J. Physiol (Lond.).* **268**:32–33P.
24. NACHSHEN, D. A., and M. P. BLAUSTEIN. 1978. The "calcium blockers," verapimil and D-600, both block sodium and calcium channels in vertebrate neurons. *Neuroscience Abstr.* **4**:582.
25. NASTUK, R. 1971. Mechanisms of neuromuscular blockade. *N. Y. Ann. Acad. Sci.* **183**:171–183.
26. PARSONS, R. L., and W. L. NASTUK. 1969. Activation of contractile system in depolarized skeletal muscle fibers. *Am. J. Physiol.* **217**:364–369.
27. PREUSSER, H. J. 1967. Die Ultrastruktur der motorischen Endplatte in Zwerchfell der Ratte and Veranderungen nach Inhibierung der Acetylcholinesterase. *Z. Zellforsch. Mikrosk. Anat.* **80**:436–457.
28. REDDY, M. K., J. D. ETLINGER, D. A. FISCHMAN, M. RABINOWITZ, and R. ZAK. 1975. Removal of Z-lines and α-actinin from isolated myofibrils by a calcium activated neutral protease. *J. Biol. Chem.* **250**:4278–4284.
29. REVILLE, W. J., D. E. GOLL, M. H. STROMER, R. M. ROBSON, and W. R. DAYTON. 1976. A Ca^{++}-activated protease possibly involved in myofibrillar protein turnover: subcellular localization of the protease in porcine muscle. *J. Cell Biol.* **70**:1–8.
30. SALPETER, M. M., H. KASPRZAK, H. FENG, and H. FERTUCK. 1979. Endplates after esterase *in vivo*: correlation between esterase concentration, functional response and fine structure. *J. Neurocytol.* **8**:95–115.
31. TAKEUCHI, N. 1963. Effects of Ca^{++} on the conductance change of the endplate membrane during the action of transmitter. *J. Physiol (Lond.).* **167**:141–155.
32. WECKER, L., and W. D. DETTBARN. 1976. Paraoxon induced myopathy: muscle specificity and acetylcholine involvement. *Exp. Neurol.* **51**:281–291.