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Antagonism of Carbamylcholine-Induced Depolarization by Batrachotoxin and Veratridine

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SUMMARY

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Depolarization of muscle end plates by carbamylcholine was reduced by 35-64% in muscles that had been treated with batrachotoxin or veratridine. Tetrodotoxin was used to prevent or reverse the membrane depolarization elicited by batrachotoxin or veratridine in the surface fibers of muscles, prior to the addition of carbamylcholine. But even when tetrodotoxin was added initially to prevent any depolarization of the muscle membrane by batrachotoxin, the latter toxin retained its inhibitory effect on the carbamylcholine response. Treatment with tetrodotoxin alone or tityustoxin followed by tetrodotoxin had no effect on the end plate depolarization induced by carbamylcholine. The inhibitory effect of veratridine on the response to carbamylcholine, but not that of batrachotoxin, was reversible. Prior treatment with batrachotoxin reduced responses to a bath-applied combination of acetylcholine plus neostigmine as well, while having no effect on responses to microiontophoretically applied acetylcholine, on miniature end plate potentials, or on end plate currents. The muscle depolarization elicited by batrachotoxin was unaffected by α-bungarotoxin, an acetylcholine receptor antagonist, or by histrionicotoxin, an antagonist of the ion conductance modulator associated with the acetylcholine receptor. The ability of batrachotoxin to alter the end plate response to carbamylcholine was found to be noncompetitive. The results are consonant with activation of tetrodotoxin-insensitive sodium channels by batrachotoxin and veratridine and the subsequent lack of participation of these activated channels in the depolarization of end plates by carbamylcholine. The depolarization elicited by carbamylcholine in a batrachotoxin-treated preparation thus reflects only the activation of acetylcholine receptor-ion conductance modulator complexes.

INTRODUCTION

Acetylcholine receptor activation-inactivation by nicotinic agonists is generally associated with an initial, intense depolarization of the end plate membrane, followed by blockade of neuromuscular transmission; in the presence of this blockade the membrane slowly repolarizes but becomes insensitive to cholinergic activation (1, 2). During experiments involving receptor activation-inactivation (desensiti-

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zation) by nicotinic agonists in frog skeletal muscle, it was noted that prior membrane depolarization by the steroidal alkaloid batrachotoxin resulted in partial antagonism of depolarization elicited by bath-applied carbamylcholine (3). The apparent ability of batrachotoxin to alter acetylcholine receptor activation-inactivation by nicotinic agonists afforded a unique opportunity to examine agonist-induced alterations in membrane phenomena at the neuromuscular junction. Batrachotoxin has been shown to increase sodium conductances in nerve and muscle fibers (4–8). The plant alkaloid veratridine (9, 10) and the scorpion venom tityustoxin (11) have also been reported to increase resting sodium conductances in electrogenic membranes. The effect of batrachotoxin was therefore compared with the effects of veratridine and tityustoxin on carbamylcholine-induced depolarization of the muscle end plate.

METHODS

Experiments were carried out *in vitro* on the diaphragm, soleus, and extensor digitorum longus (extensor) muscles of female Wistar rats (180–200 g) at 23° and 37° and on sartorius muscles of the frog *Rana pipiens* at 28°. The techniques for preparation of the muscles, for recording from surface fibers, and for recording the responses to microiontophoretically applied acetylcholine have been described (5, 8, 11–14). The microiontophoretic application of acetylcholine was made within 50–150 μm of the focal region of the end plate. Unless otherwise stated, drug-containing solutions were introduced into the muscle bath within 5–10 sec subsequent to removal of the normal physiological solution. Drug effects on the membrane potential were monitored by sampling membrane potentials only at end plate regions of surface fibers.

The physiological solution for mammalian tissue had the following millimolar composition: NaCl, 135.0; KCl, 5.0; $MgCl_2$, 1.0; $CaCl_2$, 2.0; $NaHCO_3$, 15.0; Na_2HPO_4, 1.0; glucose, 11.0. Appropriate alterations in the NaCl concentration were made when the $MgCl_2$ concentration was increased. Chloride-free (9 mM Cl^-) physiological solution was prepared by replacement of NaCl with 67.5 mM Na_2SO_4. The mammalian solution was continuously aerated with 95% O_2–5% CO_2, resulting in a pH of 7.1–7.3.

The physiological solution for amphibian tissue had the following millimolar composition: NaCl, 115.5; KCl, 2.0; $CaCl_2$, 1.8; NaH_2PO_4, 0.7; Na_2HPO_4, 1.3. When $MgCl_2$ was added to the amphibian solution, no adjustment was made in the concentration of NaCl. These solutions were bubbled with 100% O_2, and the pH was between 6.9 and 7.1.

Drugs were dissolved in an appropriate physiological solution at the time of their use. Carbamylcholine (chloride salt), tetrodotoxin, neostigmine (bromide salt), and acetylcholine (bromide salt) were obtained from Sigma Chemical Company, and veratridine, from Aldrich Chemical Company. Purified tityustoxin (mol wt 7000) was generously supplied to us by Dr. Carlos R. Diniz (Department of Biochemistry, Faculty of Medicine of Ribeirão Preto, University of São Paulo).

RESULTS

Effect of batrachotoxin on depolarization induced by carbamylcholine. When carbamylcholine (0.035–0.7 mM) was applied to frog sartorius muscles previously exposed to tetrodotoxin (0.6 μM) for 30 min, the membrane potential at the end plate was significantly reduced. A dose-effect relationship and double-reciprocal plot of the data are shown in Fig. 1A and B. In a typical experiment (Fig. 1C) the control membrane potential before application of 0.7 mM carbamylcholine in the presence of tetrodotoxin was -98.6 ± 1.2 mV (mean ± SE; $n = 5$ fibers/muscle) and declined to -15.2 ± 2.1 mV ($n = 11$ fibers/muscle) at the peak effect of the drug, i.e., during the first few minutes of drug application. Subsequently the muscle membrane repolarized in the presence of this and all other concentrations of carbamylcholine, reaching 95% of control values after 30 min (Fig. 1C). During the first

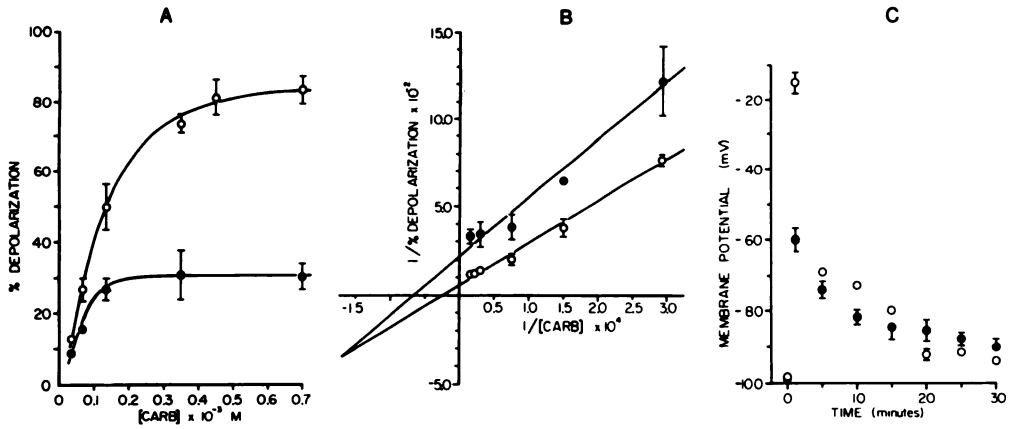

FIG. 1. *Dose-response and double-reciprocal plots and time course of carbamylcholine-induced depolarization at muscle end plates in the presence of tetrodotoxin, and effect of prior exposure of sartorius muscles to batrachotoxin on carbamylcholine response.*

○, carbamylcholine (CARB) in the presence of tetrodotoxin (0.6 μM); ●, carbamylcholine in the presence of tetrodotoxin with prior exposure of the muscle to batrachotoxin (0.15 μM) and tetrodotoxin for 90 min. The data for depolarization were taken at the peak effect of carbamylcholine during the first 3 min of exposure to the drug. The values for carbamylcholine plus tetrodotoxin were obtained from end plates of frog sartorius muscles exposed to tetrodotoxin for 30 min and then to carbamylcholine plus tetrodotoxin for 30 min. The values for carbamylcholine plus tetrodotoxin and batrachotoxin were obtained from end plates of muscles exposed to tetrodotoxin for 30 min, to tetrodotoxin plus batrachotoxin for 90 min, and then to carbamylcholine plus tetrodotoxin and batrachotoxin for 30 min. Each point in A and B is the mean ± standard deviation for three to five muscles (25–40 fibers); where no standard deviation appears, the value was too small to be shown. Each point on the dose-response curve (A) for carbamylcholine plus tetrodotoxin is significantly different from the corresponding points obtained after batrachotoxin treatment at least at the 0.01 level. The lines in the double-reciprocal plot (B) were obtained from linear regression analysis of the data in Fig. 1A, where V_{max}, K_m, slope, and correlation coefficient (r) for ○ are 1.72, 4.00, 2.41, and 0.99 and for ● are 0.47, 1.56, 3.34, and 0.98, respectively. C. Time course of depolarization-repolarization by carbamylcholine (0.7 mM); each point is the mean ± standard error for 5–11 fibers of one muscle. Where no standard error appears, the value was too small to be shown.

few minutes of exposure to carbamylcholine plus tetrodotoxin, the muscle fibers were quiescent throughout their length except for a significant local shortening that could be observed in the area immediately adjacent to the end plate. Membrane potentials could, however, be recorded at the end plate during that time. When the muscle was first exposed to tetrodotoxin (0.6 μM) for 30 min, then to batrachotoxin (0.15 μM) plus tetrodotoxin for 90 min, the initial depolarizing response to carbamylcholine (0.7 mM), applied in the presence of batrachotoxin plus tetrodotoxin, was reduced by 54% (Fig. 1C). After 30 min of exposure to the drug, the surface fibers had repolarized to 91% of control (Fig. 1C). Although batrachotoxin produced no depolarization, subsequent responses to carbamylcholine were reduced by 35–64%, depending upon the concentrations of the nicotinic agonist. A double-reciprocal plot of the values obtained for depolarization by carbamylcholine in the presence of tetrodotoxin alone, when compared with that in muscle previously exposed to batrachotoxin, reveals noncompetitive antagonism (Fig. 1B).

When carbamylcholine (0.35 mM) was allowed to mix slowly with the solution bathing the muscle, end plate potentials (Fig. 2) and miniature end plate potentials recorded in magnesium-treated sartorius muscles were blocked after 5 min. When the physiological solution was changed within 5–10 sec to one containing carbamylcholine (as in the initial experiments with batrachotoxin), end plate potentials and miniature end plate potentials disappeared within seconds. In another set of

experiments, a solution containing carbamylcholine (0.35 mM) in addition to batrachotoxin plus tetrodotoxin was allowed to mix slowly with the bath solution during the microiontophoretic application of acetylcholine near the end plate of batrachotoxin- and tetrodotoxin-treated muscles (about 50–100 μm from the focal region); at 5 min the acetylcholine potentials were blocked although the membrane potential was virtually unchanged (Fig. 3). Thus batrachotoxin had no effect on the ability of carbamylcholine to block synaptic potentials, even though depolarization by carbamylcholine was reduced by the toxin.

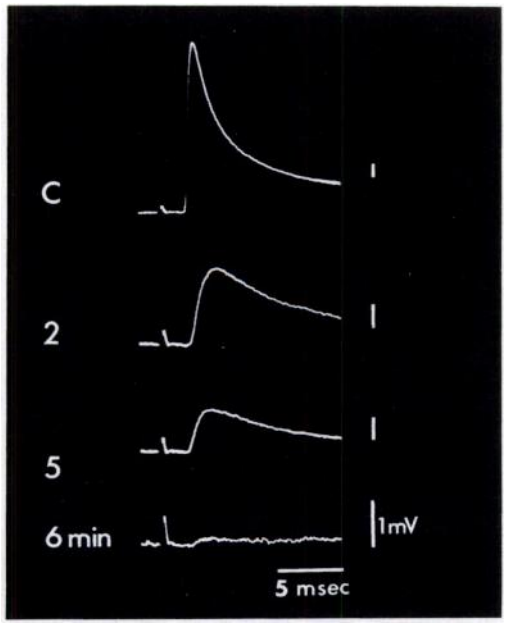

FIG. 2. *Effect of carbamylcholine on end plate potential of frog sartorius muscle*

The preparation was paralyzed with 12 mM Mg^{2+}, and a solution containing carbamylcholine (0.35 mM) was then slowly added to the bath at a rate of approximately 0.3–0.4 ml/min Control (c). The stimulus frequency was 0.5 Hz.

In the soleus muscle of the rat a similar protocol was followed. Batrachotoxin (0.15 μM) was applied first, followed by batrachotoxin plus tetrodotoxin (0.6 μM), and then carbamylcholine (3.5 μM) was applied together with batrachotoxin plus tetrodotoxin. The prior exposure to batrachotoxin resulted in a 30% reduction of the maximal membrane depolarization induced by carbamylcholine alone. It is important to note that tetrodotoxin had no effect on the carbamylcholine-induced depolarization at the end plates of the various muscles.

Effect of batrachotoxin on end plate depolarization induced by acetylcholine. The transient membrane depolarization elicited by acetylcholine applied microiontophoretically 50–150 μm distant from the focal end plate area of sartorius muscles was unaffected by prior exposure to batrachotoxin (0.15 μM) plus tetrodotoxin (0.6 μM) (Fig. 4). The acetylcholine sensitivity recorded 50–150 μm outside the focal end plate area prior to drug treatment was 115 ± 45 mV/nC (mean ± SE; range,

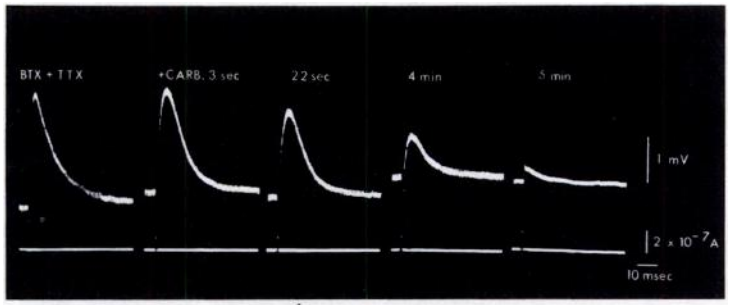

FIG. 3. *Effect of batrachotoxin on desensitization of acetylcholine receptor by carbamylcholine*

The frog sartorius muscle was first depolarized with batrachotoxin (BTX) (0.15 μM) applied for 30 min and then repolarized to normal by simultaneous exposure to batrachotoxin plus tetrodotoxin (TTX) (0.6 μM). The response to microiontophoretically applied acetylcholine near the end plate was then determined every 3 sec. Carbamylcholine (CARB) (0.35 mM) was then slowly perfused through the bath. The acetylcholine potential was blocked in the cell shown in this figure and in all other cells within 5–6 min. The upper trace is the acetylcholine potential, and the lower trace is the current applied through the acetylcholine micropipette.

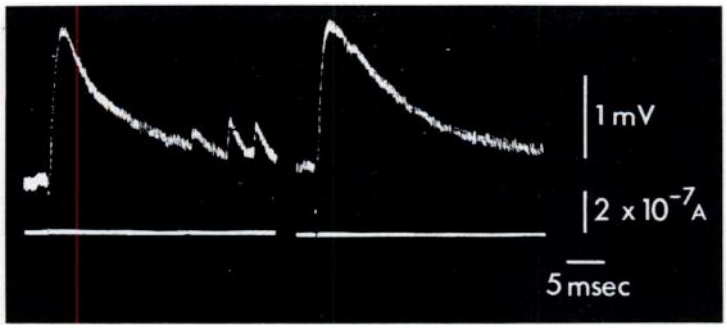

FIG. 4. *Effect of batrachotoxin on acetylcholine sensitivity near the end plate of frog sartorius muscle*
On the left is the control response to microiontophoretically applied acetylcholine in the presence of tetrodotoxin (0.6 μM), and on the right, the response to acetylcholine after exposure to batrachotoxin (0.15 μM) for 30 min and then to batrachotoxin plus tetrodotoxin for an additional 30 min. The pulse duration on the left is 0.1 msec, and on the right, 0.2 msec. The upper trace is the response to microiontophoretically applied acetylcholine, and the lower trace is the current applied through the acetylcholine micropipette.

34–168 mV/nC in eight fibers of three muscles). After exposure to batrachotoxin (0.15 μM) for 30 min and then to tetrodotoxin (0.6 μM) plus batrachotoxin, the acetylcholine sensitivity was 117 ± 15 mV/nC (range, 102–147 mV/nC in three fibers of two muscles). These results confirm earlier observations on the absence of effect of batrachotoxin and tetrodotoxin on acetylcholine sensitivity (4, 5).

Different results were obtained when acetylcholine was bath-applied to paired sartorius muscles (Fig. 5). After treatment with neostigmine (3.3 μM) plus tetrodotoxin (3.0 μM) for 30 min, the muscle was bathed in a solution containing acetylcholine (10 μM) plus neostigmine and tetrodotoxin, and the membrane potentials were sampled at the end plate area as before. During the first few minutes the membrane potential approached −20 mV, and in the continued presence of acetylcholine it repolarized toward control values (Fig. 5). The second of the pair of muscles was first exposed to batrachotoxin (0.15 μM) plus neostigmine (3.3 μM), then to tetrodotoxin (3.0 μM) plus batrachotoxin and neostigmine, and then to acetylcholine (10 μM) plus tetrodotoxin, batrachotoxin, and neostigmine. The maximal acetylcholine-elicited depolarization was reduced by 63% in muscle previously exposed to batrachotoxin (Fig. 5).

Effect of α-bungarotoxin and histrionicotoxin on depolarization induced by either carbamylcholine or batrachotoxin. The action of carbamylcholine (0.35 mM) was completely blocked by prior exposure of the sartorius muscle to α-bungarotoxin (5 μg/ml) applied for 1 hr. In contrast, the depolarizing effect of batrachotoxin (0.15 μM) was unaffected in either rate or magnitude of membrane depolarization by prior exposure to α-bungarotoxin. Even though α-bungarotoxin irreversibly blocked neuromuscular transmission within 1 hr, batrachotoxin (0.15 μM) still depolarized the membrane of surface fibers from −96.2 ± 1.4 mV (n = 20/muscle) to −6.6 ± 1.2 mV (n = 13/muscle) 20 min after exposure to the toxin. Coincidentally, batrachotoxin does not protect against the binding of α-bungarotoxin to the acetylcholine receptor (15).

Prior exposure to histrionicotoxin (35 μM) completely blocked the effect of carbamylcholine (0.35 mM) on frog sartorius muscles. Concentrations of histrionicotoxin as low as 0.9 μM significantly reduced the late phase of membrane depolarization induced by carbamylcholine, although having little effect on the initial depolarization (15).[1] Prior treatment with histrionicotoxin (35 μM) for 1 hr did not, however, antagonize the depolarization elicited by batrachotoxin (0.15 μM). Within 15 min after the addition of batrachotoxin the membrane potential of surface fibers declined from −93.7 ± 0.3 (n = 8) to −21.2 ± 3.0 mV (n = 8).

[1] Unpublished observations.

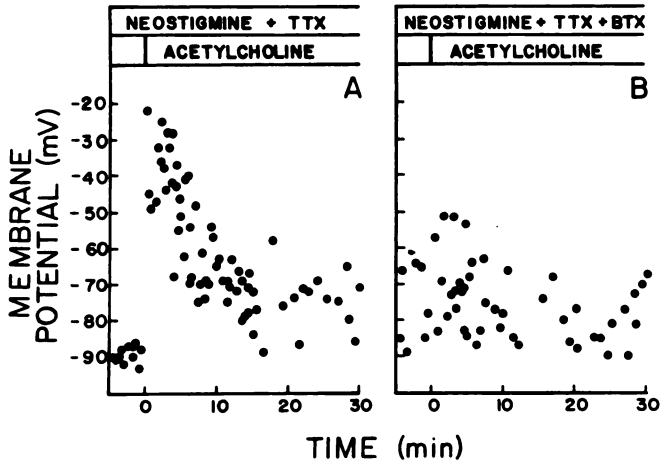

FIG. 5. *Effect of batrachotoxin on end plate depolarization induced by bath-applied acetylcholine in frog sartorius muscles*

A. The control muscle was exposed to neostigmine (3.3 μM) plus tetrodotoxin (TTX) (3 μM) for 30 min prior to the addition of acetylcholine (10 μM). B. The other muscle of the pair was first exposed to neostigmine, depolarized with batrachotoxin (BTX) (0.15 μM) in the presence of neostigmine, repolarized by simultaneous exposure to tetrodotoxin, and then exposed to acetylcholine (plus batrachotoxin, tetrodotoxin, and neostigmine). Each point is a single membrane potential recorded at the end plate of one fiber in a pair of sartorius muscles.

Effect of veratridine on depolarization induced by carbamylcholine. Veratridine, like batrachotoxin, increases sodium ion permeability, probably at a site identical with that at which batrachotoxin acts (16). Rat extensor and soleus muscles were depolarized by 40 mV in the presence of veratridine (10 μM) and were subsequently repolarized to normal values after 30 min of exposure to tetrodotoxin (3 μM) in the presence of veratridine (Fig. 6). When carbamylcholine (7 μM) was applied together with tetrodotoxin and veratridine, there was no significant effect on the membrane potential. After simultaneous exposure to carbamylcholine, tetrodotoxin, and veratridine for 20 min, the muscles were washed for 35 min with normal physiological solution and then carbamylcholine (7 μM) was reapplied. The resulting membrane depolarization was similar to that observed in untreated muscles. Subsequent washing of the muscles with normal physiological solution was followed by slow membrane repolarization.

Effect of tityustoxin on depolarization induced by carbamylcholine. While batrachotoxin and veratridine appear to act at similar sites in the sodium channel (11, 16), tityustoxin, which also increases sodium permeability, appears to act at another site (11). In the presence of 0.1 mM carbamylcholine, the muscle fibers of rat diaphragm were maximally depolarized by about 29 mV in 2 min (Fig. 7), with repolarization occurring at a slow rate. When diaphragm muscles were exposed to tityustoxin (1.43 μM), the membranes of the muscle fibers were depolarized by about 23% in 30 min and then repolarized to normal by application of tetrodotoxin (0.6 μM) plus tityustoxin for 15 min. When carbamylcholine was then applied to this muscle for the first time, there was no significant reduction in depolarization from control values. It should be noted, however, that the membrane depolarization produced by tityustoxin is less than with batrachotoxin or veratridine.

Effect of batrachotoxin on end plate current. Batrachotoxin (0.15 μM) was also applied to frog sartorius muscles during nerve stimulation and concurrent recording of the end plate current. Neither the null potential of the end plate current nor the relationship between peak amplitude of the end plate current and membrane

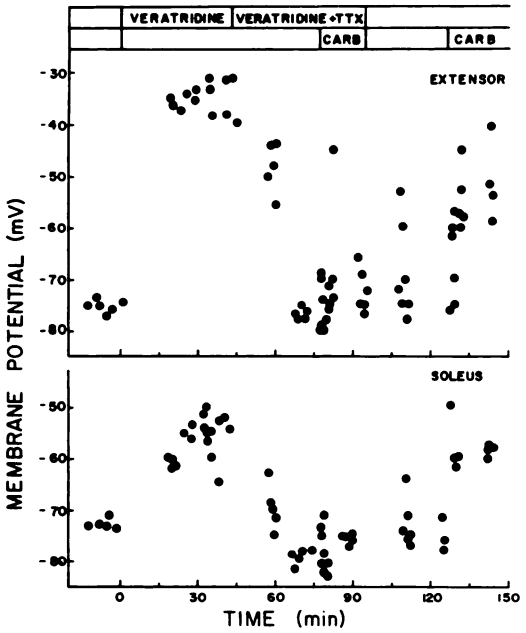

FIG. 6. *Effect of veratridine on depolarization induced by carbamylcholine in rat extensor and soleus muscles*

Membrane potentials were first recorded in physiological solution, and then the muscles were depolarized by exposure to veratridine (10 μM); when repolarization was complete, carbamylcholine (CARB) (7 μM) was added to the bathing solution in the presence of veratridine and tetrodotoxin (TTX) (3 μM). After all drugs had been washed out, carbamylcholine was reapplied in the absence of other drugs and membrane potentials were sampled. Each point is a single membrane potential recorded at the end plate of one fiber.

potential was affected by batrachotoxin. Both the rise time (0.90 ± 0.05 msec for control vs. 0.92 ± 0.09 msec for batrachotoxin) and the half-decay time of the end plate current were likewise unaffected by batrachotoxin. In addition, there was no effect on the relationship between the half-decay time of the end plate current and the membrane potential before and after batrachotoxin, or on the slope of this relationship (Fig. 8). This suggests that the falling phase of the end plate current remained voltage-dependent.

DISCUSSION

Batrachotoxin and veratridine significantly reduced the end plate depolarization induced by bath-applied carbamylcholine and acetylcholine (Figs. 1, 5, and 6). In contrast, batrachotoxin never affected the transient depolarization elicited by microiontophoretic application of acetylcholine. After treatment with batrachotoxin, the small amplitude (5–10-mV) potentials produced by microiontophoretic application of acetylcholine at the end plate region were unaltered (Figs. 3 and 4; see also refs. 4 and 5). This action of bactrachotoxin was exerted even when tetrodotoxin was present throughout the experiment to prevent depolarization by the former toxin. Thus batrachotoxin and veratridine depress the membrane depolarization elicited by bath-applied nicotinic agonists at

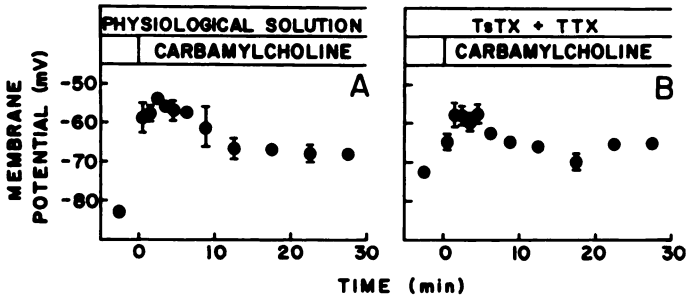

FIG. 7. *Effect of tityustoxin on depolarization induced by carbamylcholine in rat diaphragm muscle*

A. Resting membrane potentials were first recorded in physiological solution; the solution was then abruptly changed to one containing carbamylcholine (0.1 mM) and measurements were continued. B. After control membrane potentials were recorded in physiological solution, the muscle was depolarized to about −60 mV by tityustoxin (TsTX) (1.43 μM); tetrodotoxin (TTX) was then applied simultaneously with tityustoxin, and after 15 min the muscle was repolarized. Carbamylcholine was then added to the bathing solution along with tityustoxin and tetrodotoxin, and the membrane potentials were sampled at the end plate region. Each point is the mean of at least three to five fibers in three muscles. Where no standard error appears, it was too small to be shown.

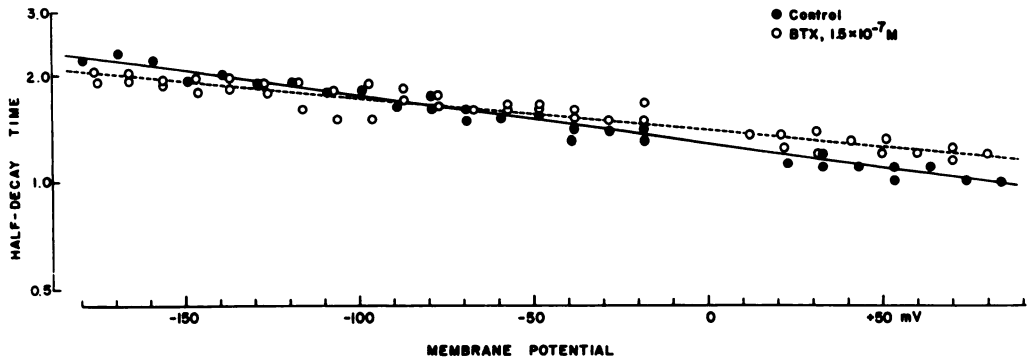

FIG. 8. *Effect of batrachotoxin on relationship between half-decay time of end plate current and membrane potential in frog sartorius muscle*

The experiment was carried out in a sartorius muscle previously exposed to 600 mM glycerol for 1 hr and then "shocked" by washing in a normal physiological solution for an additional hour during stimulation at 0.3 Hz. Subsequently end plate currents were generated to obtain the control recording (●), and the muscle was then exposed to batrachotoxin (BTX) (0.15 μM) for 2 hr, after which further recordings were made (○). Each point is the mean of three or four end plate currents in three to six fibers. Control slope = 2.08 V^{-1}; batrachotoxin slope = 1.49 V^{-1}.

concentrations that normally produce depolarization of 12 mV or more, but not the small depolarizations elicited by microiontophoretically applied acetylcholine. Batrachotoxin was without effect either on the acetylcholine sensitivity of junctional or extrajunctional regions of innervated (see Figs. 3 and 4) and denervated muscles (4, 5), on the binding of α-bungarotoxin to acetylcholine receptors, or on the ability of histrionicotoxin to react directly with the ion conductance modulator[2] (15, 17). Batrachotoxin also had no effect on either null potential for the end plate current or the current-voltage relationship of the end plate current (Fig. 8). Conversely, neither α-bungarotoxin nor histrionicotoxin prevented the depolarization of muscle membranes by batrachotoxin. Thus the effect of batrachotoxin and veratridine on the response to nicotinic activation is most likely to involve interaction with a site distinct from either the acetylcholine receptor-ion conductance modulator complex or tetrodotoxin-sensitive sodium channels involved in generation of muscle action potentials. It appears that the interaction between batrachotoxin and carbamylcholine is of a noncompetitive nature (see Fig. 1B).

Under normal conditions an increasing current flow is induced by the interaction of a nicotinic agonist with acetylcholine receptors until the membrane potential approaches the null potential for acetylcholine. A simultaneous increase in sodium conductance for spike generation takes place at the end plate region. In fact, even before the null potential for acetylcholine is reached, a marked increase in resting and active sodium conductances has occurred and generation of action potentials has ensued (18). When a frog sartorius muscle was bathed in a solution containing carbamylcholine (0.35 mM) or acetylcholine (10 μM) with neostigmine in the presence and absence of tetrodotoxin, the membrane potential approached values between −20 and −15 mV, close to the null potential for acetylcholine in this muscle (6, 18). It is quite apparent that the effect of carbamylcholine and acetylcholine prior to treatment with batrachotoxin or veratridine is the result of a direct effect at the end plate region, with subsequent activation of the acetylcholine receptor-ion conductance modulator complex at the end plate region, for the action of both agonists can be

[2] The term ion conductance modulator is a descriptive designation for the molecular entity involved in regulating ion conductance in membranes (17).

blocked by *d*-tubocurarine or α-bungarotoxin.

In order to investigate the direct effect of batrachotoxin and veratridine on the nicotinic responses it was necessary for tetrodotoxin to be present in the bathing solution prior to and during cholinergic activation; first, to prevent or reverse the depolarization elicited by batrachotoxin, veratridine, and tityustoxin, and, second, to block muscle twitches and facilitate the recording of membrane potentials within the first 2 min of application of agonist at the end plate. Under these conditions only a localized activation of the contractile apparatus in the end plate area could be observed in control preparations, as indicated by the shortening of the sarcomeres. Thus the large depolarization elicited by carbamylcholine and acetylcholine in the presence of tetrodotoxin is a reflection of the activation of acetylcholine receptors, their ion conductance modulators, and a tetrodotoxin-resistant channel in or near the end plate region. If the latter had not taken place, no movement of the contractile apparatus directly adjacent to the end plate area would have been observed. Such contractions must result from a spread of agonist-induced depolarization at the junctional region, where a specific increase in sodium conductance complements the nonspecific junctional depolarization. With regard to the relatively small (5–7-mV), transient depolarization produced by microiontophoretic application of acetylcholine, the conductance changes approximate those induced by end plate potentials evoked in the presence of *d*-tubocurarine or high concentrations of magnesium; *i.e.*, only subthreshold end plate potentials were generated. Little, if any, activation of the sodium conductances involved in regenerative activity occurs at this time.

After treating the muscles with batrachotoxin, which causes depolarization of the muscle fiber membranes by increasing sodium conductance (4–8), we again examined the effect of carbamylcholine and acetylcholine. Since a nicotinic agonist would have little, if any, effect on muscle depolarized near the null potential for acetylcholine, although reacting with the receptor, tetrodotoxin was added to the bathing solution pior to the agonist. Under these conditions it was expected that carbamylcholine and acetylcholine would be as effective as under control conditions. The response, however, was reduced by up to 64%. Even when tetrodotoxin was present throughout the experiments and prevented depolarization by batrachotoxin, the response to carbamylcholine was similarly reduced.

The simplest explanation for the effect of batrachotoxin and veratridine would therefore appear to be related to the presence of sodium-dependent action potentials within the end plate region of skeletal muscle. These potentials in the end plate region are characterized by their greater rates of rise, amplitude, and overshoot compared with extrajunctional action potentials and by relative resistance to blockade by tetrodotoxin (19). One possible explanation for the observed reduced response to carbamylcholine in the presence of batrachotoxin or veratridine is that these toxins have altered the function of the end plate tetrodotoxin-insensitive sodium channels, which presumably are located in close proximity to the acetylcholine receptor–ion conductance modulator complex and are coupled to this complex in a membrane potential-dependent manner.

Batrachotoxin and veratridine are known to increase sodium permeability in a variety of nerve and muscle preparations via specific interactions with sodium channels (4–10, 20). Indeed, even the tetrodotoxin-insensitive sodium channels of denervated muscle are activated by batrachotoxin (4). The "activation" of sodium channels elicited by batrachotoxin and veratridine appears to be due to prevention of the normal time-dependent inactivation of the channels; i.e., the increased sodium permeability induced by these toxins alone or under voltage clamp conditions following step depolarizations is not followed by inactivation (7, 10, 11, 21). The channels remain open and the membrane depolarizes, but the channels are still sensitive to blockade by tetrodotoxin. The threshold for activation of sodium channels following

depolarization appears, in certain preparations, to be lowered by batrachotoxin (22), and in certain other preparations batrachotoxin has a strongly voltage-dependent interaction with sodium channels (23). In view of the specificity of batrachotoxin and veratridine toward sodium channels in a variety of electrogenic membranes, the simplest explanation of the reduction of the carbamylcholine response is that batrachotoxin and veratridine have prevented the normal activation of sodium channels by carbamylcholine-induced threshold depolarization of end plates. A reduction in the external chloride concentration had no effect on the depression of carbamylcholine-elicited depolarization by batrachotoxin.[1] It is therefore unlikely that chloride ions play a role in the reduction of the carbamylcholine response (24–26).

The mechanism whereby batrachotoxin and veratridine prevent the usual activation of end plate sodium channels by carbamylcholine-induced depolarization is unclear. A number of possibilities pertain. The most attractive hypothesis is that batrachotoxin and veratridine prevent, as in other preparations (20), the inactivation of the end plate sodium channels, and may therefore be exerting a conditioning response at the end plate sodium channels somewhat analogous to a conditioning pulse (27). Such conditioning would greatly reduce the effect of nicotinic receptor activation. As a consequence the large membrane depolarization normally induced by high concentrations of carbamylcholine and acetylcholine would now occur only to the extent afforded by activating the acetylcholine receptor–ion conductance modulator complex. A concomitant increase in resting and active sodium conductance for spike generation after activation of the acetylcholine receptor–ion conductance modulator complex would not occur, because these channels have already been activated by batrachotoxin or veratridine. The result is a decrease in the maximal depolarization observed under control conditions with carbamylcholine and acetylcholine alone. Tityustoxin caused only partial depolarization of muscle membranes (Fig. 7). Thus the lack of effect of tityustoxin on the response to carbamylcholine is presumably related to its low effectiveness in interacting with sodium channels. Alternatively, the tetrodotoxin-resistant channels within the end plate may not have a tityustoxin-sensitive site.

If batrachotoxin and veratridine did activate or prevent inactivation of the sodium-specific channels at the end plate, the increase in sodium permeability afforded by these tetrodotoxin-resistant channels would be expected to cause a significant membrane depolarization. The finding that batrachotoxin does not cause an observable membrane depolarization, even though it reduces the effect of bath-applied nicotinic agonists, is therefore surprising. However, activation of sodium channels at the end plate by batrachotoxin or veratridine may take place slowly rather than rapidly as with carbamylcholine, allowing for compensatory activation of $(Na^+ + K^+)$-ATPase in the end plate. The activity of the $(Na^+ + K^+)$-ATPase is unaffected by batrachotoxin or veratridine (28). Furthermore, a marginal, steady-state depolarization of the small end plate region by batrachotoxin or veratridine might be difficult to detect in the presence of tetrodotoxin, even though more transient depolarization by carbamylcholine could be detected.

Other mechanisms ought to be considered. Batrachotoxin or veratridine might, by interaction with the end plate sodium channel, have "uncoupled" this channel from control by the acetylcholine receptor–ion conductance modulator complex. A number of possible mechanisms for such "uncoupling" can be envisaged and must be investigated. The toxins might increase the threshold of the membrane potential change necessary for activation of the sodium channels or might even, by preventing the normal inactivation of the channels, lead to an inactivated channel-toxin complex that could no longer be activated in response to carbamylcholine. Interactions of the toxins with the end plate channels might even confer tetrodotoxin sensitivity, although this possibility seems

unlikely (cf. ref. 4).

In conclusion, batrachotoxin and veratridine are proposed to interact with tetrodotoxin-insensitive, sodium-specific channels at the end plate region that are functionally coupled to the activation of the acetylcholine receptor-ion conductance modulator complex. These sodium channels are thought to subserve the all-or-none properties of the end plate membrane, since their activation is intimately coupled to the postjunctional action of the transmitter. Batrachotoxin and veratridine do not appear to affect the electrical or chemosensitive properties of the acetylcholine receptor-ion conductance modulator complex. It is proposed that the reduction in the depolarizing response to bath-applied nicotinic agonists caused by batrachotoxin and veratridine is of a non-competitive nature and is due to blockade of the nicotinic agonist-elicited activation of sodium channels at the end plate membrane. It now appears possible to investigate activation-inactivation of the acetylcholine receptor-ion conductance modulator complex in the absence of contributions from associated sodium-specific conductances at the end plate.

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