THE KINETICS OF CURARE ACTION AND RESTRICTED DIFFUSION WITHIN THE SYNAPTIC CLEFT OF MOTOR NERVE TERMINALS ON FROG SKELETAL MUSCLE

Thesis by

David Lee Armstrong

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- Donc, quand la moralité de l'explorateur paraît bonne, on fait une enquête sur sa découverte.

- On va voir?

- Non. C'est trop compliqué. Mais on exige de l'explorateur qu'il fournisse des preuves. S'il s'agit par exemple de la découverte d'une grosse montagne, on exige qu'il en rapport de grosses pierres.

Antoine de Saint-Exupéry

Le Petit Prince

Acknowledgements

My parents, Dawn and Otis Armstrong, provided this opportunity for me, and I lovingly dedicate this thesis to them. George Streisinger first encouraged me to study at Caltech, and Itzchak Parnas introduced me to experimental electrophysiology with contagious enthusiasm. Henry Lester patiently supported and guided me throughout this study. Both Henry and Brian Seed taught me to seek physical explanations for biological phenomena. Elizabeth Sandoz helped me analyze the data and denervate the frog muscles for this thesis. Seymour Benzer, Jim Hudspeth and Felix Strumwasser gave me productive scientific advice, and I always received kind and reliable assistance from the staff of the Biology Division. I am grateful to Herschel Mitchell for showing me that excellence in both athletics and science can be pursued humanely. David Weisblatt and I discovered that 'specialized biophysical machine' together in 19 Beckman. Barry Rothman and Nancy Shinowara cheerfully befriended me in the lab when I was confused about the 'meaning of it all,' and three crazy guys made this past year especially productive and enjoyable for me: Menasche 'too long' Nass, Simon 'the Rock' Levinson, and Mauri 'too white' Krouse. Outside the lab, I shared the good life with J. A. Prufrock and company; in particular Clyde Okuda, Betsy Sandoz and David Pollard helped me keep my insanity. And thanks to Darlene for the sweet rolls.

ABSTRACT

1. The kinetics of curare inhibition were measured at the postsynaptic membrane of frog sartorius and cutaneous pectoris muscle fibers. Acetylcholine (ACh) and d-tubocurarine (dTC) were iontophoresed from twin-barrel micropipettes, and the muscle fiber's membrane potential was recorded intracellularly. By itself dTC produced no change in membrane potential, but dTC-receptor binding was assayed by observing changes in the response to a constant pulse of ACh.

2. The responses to both ACh and dTC had brief latencies, reached their maxima rapidly, and were highly sensitive to the dose. Under these conditions, the kinetics of drug action are not slowed by access of the drugs to the synaptic cleft.

3. After a pulse of dTC, recovery from inhibition proceeds slowly along an exponential time course with a rate constant, $1/\tau_{off} \approx 0.5\ sec^{-1}$. The recovery rate does not depend on the maximal level of inhibition and varies only slightly with temperature ($Q_{10} = 1.25$).

4. After a sudden maintained increase in dTC release, inhibition develops approximately exponentially until a steady-state level of inhibition is reached. The apparent rate constant for the onset of inhibition, $1/\tau_{on}$, is greater than $1/\tau_{off}$. When the steady-state inhibition reduces the ACh response to 1/n of its control value, $1/\tau_{on} = n(1/\tau_{off})$.

5. When the ACh sensitivity is reduced with cobra toxin, both $1/\tau_{on}$ and $1/\tau_{off}$ increase. Thus, the kinetics of dTC inhibition depend on the density of acetylcholine receptors in the synaptic cleft. If the density of acetylcholinesterase is reduced in the cleft by collagenase, $1/\tau_{off}$ increases only twofold.

6. When the nerve terminal is removed after collagenase action, and the drugs are iontophoresed directly onto the exposed postsynaptic membrane, $1/\tau_{off}$ increases more than tenfold.

7. Bath-applied dTC competitively inhibits the responses to brief iontophoretic ACh pulses with an apparent equilibrium dissociation constant, $K_D = 0.5$ μM. This suggests that dTC molecules equilibrate with the receptors on a millisecond time scale.

8. On denervated frog muscle cells, extrasynaptic receptors have a lower apparent affinity for dTC. After a pulse of dTC, inhibition decays tenfold more rapidly at these extrasynaptic sites than at the nerve-muscle synapse.

9. It is suggested that dTC inhibits synaptic receptors more effectively because the nerve terminal restricts diffusion within the synaptic cleft and each dTC molecule binds repeatedly to several acetylcholine receptors before escaping from the cleft. Consequently, the receptors transiently buffer the concentration of dTC in the cleft, and the macroscopic kinetics of inhibition are much slower than the molecular rates of dTC binding.

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INTRODUCTION

Curare (dTC) is a potent inhibitor of synaptic transmission at motor nerve terminals on vertebrate skeletal muscle (Bernard, 1857; Eccles, Katz and Kuffler, 1941). But dTC does not reduce neurally stimulated acetylcholine (ACh) release (Dale, Feldberg and Vogt, 1936; Fletcher and Forrester, 1975). Langley (1905) suggested that dTC forms a reversible chemical complex with the nicotinic receptive substance of muscle, and thereby prevents ACh from exerting its excitatory action. Later, electrophysiological studies confirmed that at equilibrium dTC competitively inhibits ACh-induced depolarizations and conductance increases (Jenkinson, 1960; Adams, 1975). At the molecular level, fluctuation analysis has revealed that dTC reduces neither the elementary conductance nor the lifetime of individual ACh channels in the membrane (Katz and Miledi, 1972). Instead, dTC apparently competes for an agonist binding site and reduces the frequency of channel opening (Sheridan and Lester, 1975, 1977). Using irreversible elapid α-toxins, the acetylcholine receptor protein from muscle cells has been isolated in membrane fragments and purified; and indeed dTC competitively inhibits radiolabeled toxin binding (Brockes and Hall, 1975; Colquhoun and Rang, 1976). These studies of dTC antagonism all suggest that dTC binds to the nicotinic acetylcholine receptor in a reversible bimolecular reaction.

Nevertheless, little is known about the molecular rate constants for dTC binding. Because dTC does not open channels, its binding cannot be measured directly with electrophysiological techniques, but must be inferred from changes in the response to a constant dose of agonist. Del Castillo and Katz (1957a) introduced a technique for iontophoresing brief pulses of both agonist and antagonist from twin micropipettes onto the same localized population of receptors. They observed

that dTC's inhibitory effect decayed over several seconds, much more slowly than expected from dTC's diffusion in solution. They suggested that this slow recovery represented the molecular rate of dissociation of the dTC-receptor complex.

However, other experiments suggest that dTC leaves the receptors rapidly enough to be displaced significantly during brief ACh responses (Blackman, Gauldie and Milne, 1975). Further information was obtained from voltage-jump relaxation experiments in which the frequency of channel opening is measured by the dependence of the relaxation rate on the agonist concentration. Elapid α-toxins eliminate a fraction of the receptor population, but the opening rate remains unchanged for the remaining receptors. In the presence of dTC, on the other hand, the opening rate decreases uniformly over the entire population. This suggests that individual receptors experience a decreased average availability to agonist molecules and that dTC binds to, and dissociates from, the receptor on a millisecond time scale (Sheridan and Lester, 1977).

This paradox may be resolved by recognizing that a drug's apparent rate of diffusion, and therefore its macroscopic kinetics, will be slowed by rebinding to its own receptors (Crank, 1956; Rang, 1966; Thron and Waud, 1968; Colquhoun, Henderson and Ritchie, 1972). At the nicotinic nerve-muscle synapse, the neurally evoked postsynaptic current (PSC) decays more slowly after inhibiting the acetylcholinesterase (AChE), but the individual ACh channel lifetime remains the same (Katz and Miledi, 1973). In the absence of AChE, rebinding to receptors evidently slows acetylcholine's diffusion out of the cleft since the PSC decay rate increases again after the receptor density is reduced by toxin (Katz and Miledi, 1973; Magleby and Terrar, 1975; Colquhoun, Large and Rang, 1977). This rebinding is not surprising considering the high density of ACh receptors in the synaptic cleft (Matthews-Bellinger and Salpeter, 1978); and given dTC's much higher affinity for the receptor

(Jenkinson, 1960), dTC's diffusion in the cleft should be slowed substantially by rebinding to receptors (Adams, 1975; Sheridan and Lester, 1977).

Therefore, we have reinvestigated the kinetics of dTC inhibition at the frog nerve-muscle synapse. Our experiments indicate that dTC leaves the synaptic cleft slowly because the nerve terminal obstructs its diffusion and consequently each dTC molecule rebinds to several acetylcholine receptors before escaping. Such buffered diffusion can account for the slow kinetics observed by del Castillo and Katz (1957a). Although the actual molecular binding rates are too fast to measure accurately with the iontophoretic technique, these binding steps must be complete within a few tens of milliseconds.

Some of these results have already appeared in abstract form (Armstrong and Lester, 1977).

METHODS

Preparation. Sartorius and cutaneous pectoris muscles were dissected from adult Rana pipiens throughout the year. In a few cases, the muscles were denervated two to six weeks before the experiment. For denervation, animals were anesthetized in a solution of 5% ether in water, and a few millimeters of nerve were cut out where it entered the muscle (Dreyer and Peper, 1974d). The frogs were kept at room temperature and were fed meal worms. The recording chamber contained a layer of Sylgard (Dow Corning), to which the muscles were pinned with fine tungsten wire and mounted on the stage of a compound microscope. Usually the muscle was viewed with bright field optics. When better visualization was required, a monolayer of cutaneous pectoris fibers was prepared (Dreyer and Peper, 1974a) and viewed with Nomarski differential interference contrast optics (McMahan, Spitzer and Peper, 1972).

The standard Ringer had the following composition (mM): NaCl, 110; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 4; Na-Hepes (N-2 hydroxyethylpiperazine-N' ethane-sulphonic acid), 2 mM (pH 7-7.2). For bath-applied drugs, we used a push-pull perfusion system made from 10 ml syringes. Dye measurements showed that three washes (requiring about 90 sec) changed the drug concentration in the 3 ml bath to within a few percent of that in the reservoir, usually without disturbing the electrodes. Except where noted, the experiments were conducted at room temperature (19-22°C). The temperature in the bath was changed by introducing cold (2°C) or hot (30°C) Ringer from the reservoir. The temperature then slowly returned to normal over several minutes.

Pharmacological manipulations. Acetylcholine receptors were irreversibly blocked with cobra toxin T_3 (Lester, 1972) which was kindly provided by D. Eaker from the venom of Naja naja siamensis (Karlsson, Arnberg and Eaker, 1971). The muscle was exposed to cobra toxin (60 nM) until the ACh sensitivity was reduced more than tenfold (15-25 min). The chamber was then flushed with Ringer solution, and the sensitivity remained stable thereafter.

The AChE activity was irreversibly blocked in some experiments by incubating the muscle for 30 min in methanesulphonyl fluoride (5 mM) (Kordas, Brzin and Majcen, 1975). In other experiments, AChE was removed from the synaptic cleft by applying 50-100 µl of purified collagenase (Boehringer Mannheim, 5 mg/ml) onto the muscle surface (Hall and Kelly, 1971; Betz and Sakmann, 1973). After 20-30 min, the muscle was washed thoroughly with Ringer.

Electrical arrangements. Acetylcholine and d-tubocurarine were iontophoresed from twin-barrel micropipettes, and the muscle cell's membrane potential was recorded intracellularly with conventional electrophysiological techniques

(del Castillo and Katz, 1957a). The intracellular microelectrodes were filled with 3 M KCl and had resistances between 5-15 MΩ. The bath was grounded through an agar bridge leading to a chlorided Ag wire. A virtual ground circuit (WPI model 180) maintained this electrode at ground potential and measured the currents flowing through the iontophoretic pipettes. Current pulses were passed through the iontophoretic pipettes by a circuit containing a high-voltage operational amplifier connected as a boot-strapped follower (see Dreyer and Peper, 1974b). The current through each barrel was the sum of a maintained braking current and two command pulses with independently variable amplitude and temporal characteristics.

The responses to ACh were amplified and filtered through high-pass and low-pass filters (time constants 1 sec and 1 msec), and several traces were stored on the oscilloscope where they were photographed. Each photograph contains two sets of traces. The lower traces show the iontophoretic current pulses. For clarity, positive current flowing out of the iontophoretic pipette is displayed as a downward deflection. The upper traces show the changes in membrane potential in response to these pulses.

Iontophoresis. We have used the quantitative iontophoretic techniques developed at the frog nerve-muscle synapse (Peper and McMahan, 1972; Dreyer and Peper, 1974b,c; Kuffler and Yoshikami, 1975). Iontophoretic microelectrodes were constructed from fused twin-barrel capillaries containing a glass fiber in each barrel. The shank of one barrel was injected with a solution of acetylcholine chloride (2 M); the shank of the other barrel was usually injected with a solution of d-tubocurarine chloride (25 mM) and KCl (0.1 M). Although the KCl certainly reduced dTC's transport number out of the pipette, the lower electrode resistance

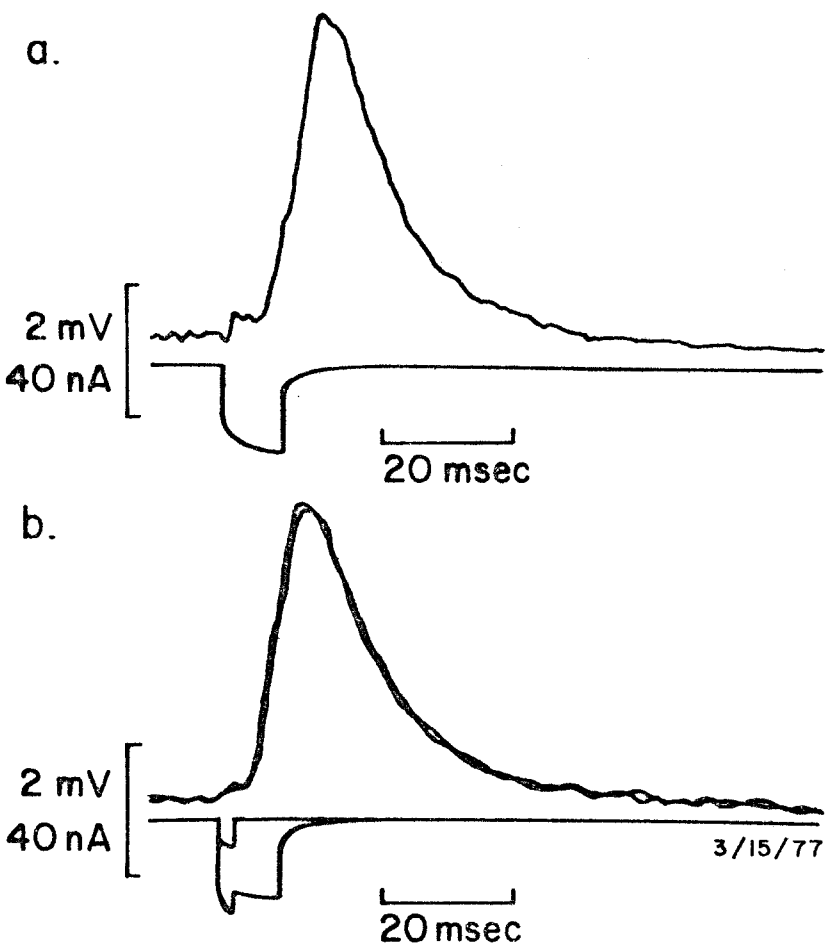
allowed the reliable passage of large currents. Electrodes were accepted for use if the ACh barrel's resistance was 120-250 mΩ, if the dTC barrel's resistance was 300-600 MΩ and if each barrel reliably passed 100 nA for 50 ms.

Often there was capacitative or resistive coupling between the two barrels of the iontophoretic pipette: an outward current pulse through the dTC barrel also released enough ACh to depolarize the cell. Coupling was negligible in the opposite direction because the ACh pulses were small and the dTC solution was dilute. The coupling was studied by replacing the dTC solution with D-glucosamine hydrochloride (25 mM) to eliminate inhibitory effects caused by binding of dTC to the receptors. The coupling artifact (Fig. 1a) is eliminated by delivering an inward pulse of current to the ACh barrel simultaneously with the glucosamine pulse. This balancing current is adjusted to eliminate the coupling exactly (Fig. 1b). Electrical coupling was balanced out in this manner throughout the experiments. Nevertheless, several iontophoretic electrodes showed negligible interbarrel coupling, and such electrodes gave the same results as those requiring balance currents.

Katz and Miledi (1977) observed that a massive iontophoretic dose of dTC produced a small (<0.1 mV) local hyperpolarization at endplates of anti-esterase treated frog muscle. In the present study, the largest dTC pulses were 100-fold smaller than those which produced hyperpolarization in the experiments of Katz and Miledi (1977).

Conduct of the experiments. When an apparent nerve terminal had been located visually and the muscle fiber had been impaled, the iontophoretic pipette was positioned until brief pulses (2-5 ms) of ACh produced responses that began with brief latencies (<5 ms) and rose rapidly to their maxima (≤15 ms) (see Fig. 5a). Initially, the braking currents were set at 3-4 nA on each barrel, but at this point

Fig 1. Electrical coupling between the two barrels of an iontophoretic micropipette filled with ACh and glucosamine. A, a pulse of outward current (30 nA x 10 msec) through the glucosamine barrel also releases enough ACh to depolarize the cell. B, two responses to a much smaller ACh pulse (10 nA x 2 ms). In one sweep, the ACh pulse is delivered alone; in the other trace, it is delivered simultaneously with the glucosamine pulse, but a small inward current (3 nA) is also applied to the ACh barrel during the glucosamine pulse. The two ACh responses superimpose, showing that the coupling has been balanced out. The records were traced from the original photographs.


a.
2 mV
40 nA
20 msec
b.
2 mV
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they were reduced to the point where leakage of either ACh or dTC was just prevented. Acetylcholine pulses were then adjusted to produce small responses (<5 mV) to avoid effects of nonlinear summation (Martin, 1955). Acetylcholine sensitivity was measured as the slope of the linear part of the dose-response curve in mV/nC (Kuffler and Yoshikami, 1975). Control ACh sensitivities ranged from 200 to 1200 mV/nC depending on the quality of visualization for placement of the pipette.

In some experiments, the nerve terminal was displaced so that drugs could be iontophoresed directly onto the postsynaptic membrane (Kuffler and Yoshikami, 1975). In preparation 50-100 µl of partially purified collagenase (Sigma type I, 2500 units/ml) was released onto the muscle surface by pressure ejection from a pipette. Within 10-30 min, the unmyelinated nerve terminals could be located visually. Calcium-free Ringer solution was then flushed through the chamber to prevent interference from neurally released acetylcholine. Even after washing, the enzymes continued to act: spontaneous miniature endplate potentials (mepp) first became large (presumably because of AChE removal) but eventually disappeared altogether. The latter was taken as a signal that synaptic disjunction had occurred (Betz and Sakmann, 1973), and the iontophoretic pipette was then used to displace the nerve terminal mechanically.

RESULTS

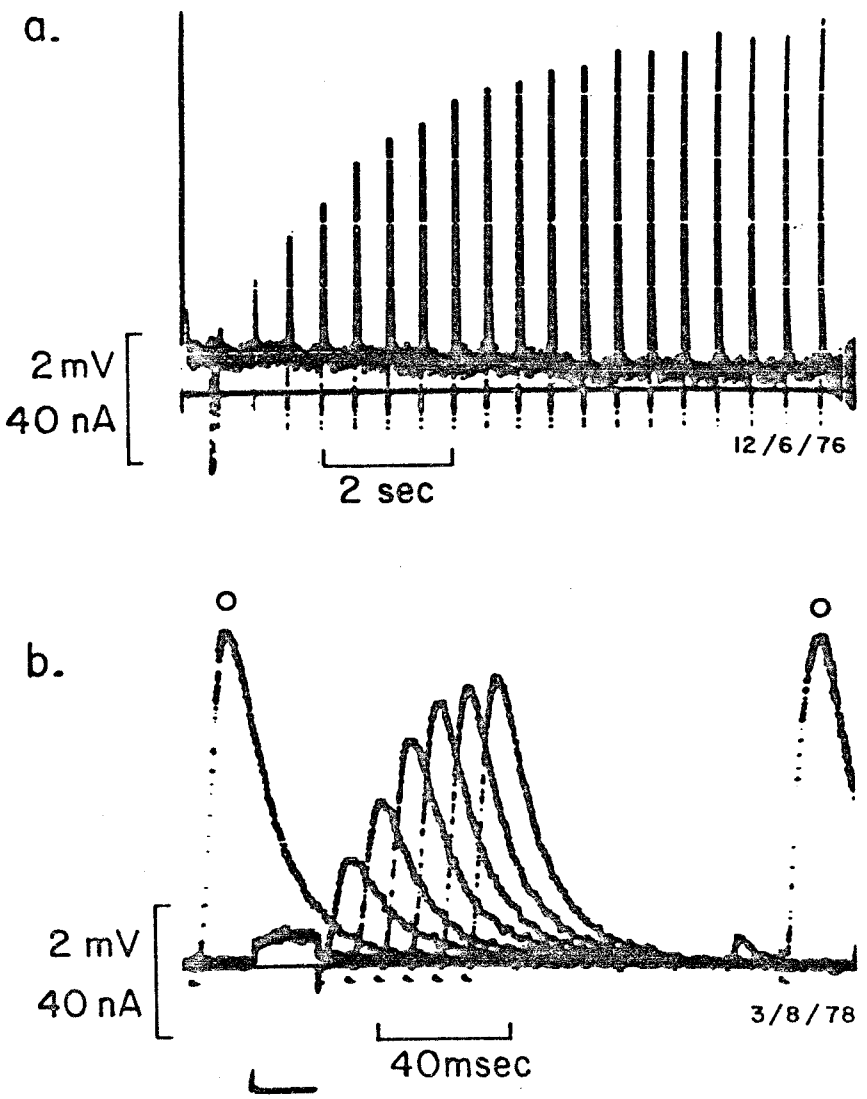
The nerve-muscle synapse recovers slowly from dTC inhibition

In the present experiments, we have confirmed the observation that several seconds are required for the disappearance of inhibition after iontophoretically-applied pulses of dTC (del Castillo and Katz, 1957a; Waud, 1967). In the experiment of Fig. 2a, for example, a single brief pulse of dTC rapidly produces inhibition; recovery then proceeds along a roughly exponential time course with a time constant, $\tau_{off} \approx 2.5$ sec. Two observations show that this slow recovery is not an artifact of the twin-barrel iontophoretic micropipettes. Firstly, when separate ACh and dTC iontophoretic pipettes are positioned individually at the synapse with Nomarski optics, very similar kinetics are observed. Furthermore, another antagonist, hexamethonium, shows much faster kinetics when tested with twin-barrel micropipettes (Fig. 2b).

The episode method for faster kinetics

Hexamethonium recovery proceeds at rates much faster than 1 sec^{-1}. Such kinetics cannot be measured accurately with trains of identical ACh pulses because at frequencies higher than two pulses per second, desensitization reduces the response to successive pulses. Instead, kinetics are measured in a series of episodes. Episodes occur at intervals of several seconds; during each episode there is one antagonist pulse (usually dTC), at a fixed time, followed with a variable delay by a single ACh pulse. Since each series of episodes lasts a few minutes, control responses to the same ACh pulse are measured with no antagonist present before and after the series. This ensures that the ACh response recovers completely and does not change because of electrode movement during the series. With this

Fig. 2. A, recovery from dTC inhibition. One pulse of dTC (40 nA x 50 ms) was delivered just before the second ACh pulse in a train of identical brief ACh pulses. Note that on this time scale, the ACh responses appear as vertical lines whose height equals the peak response. B, a series of episodes showing recovery from hexamethonium inhibition. Each episode contained the same hexamethonium pulse, at a fixed time, followed with a variable delay by a single ACh pulse. Episodes were repeated every 5 seconds. Open circles (O) indicate control responses to the same ACh pulse before and after the series. Note the much smaller time scale.


a.
2 mV
40 nA
2 sec
12/6/76
b.
2 mV
40 nA
40msec
3/8/78

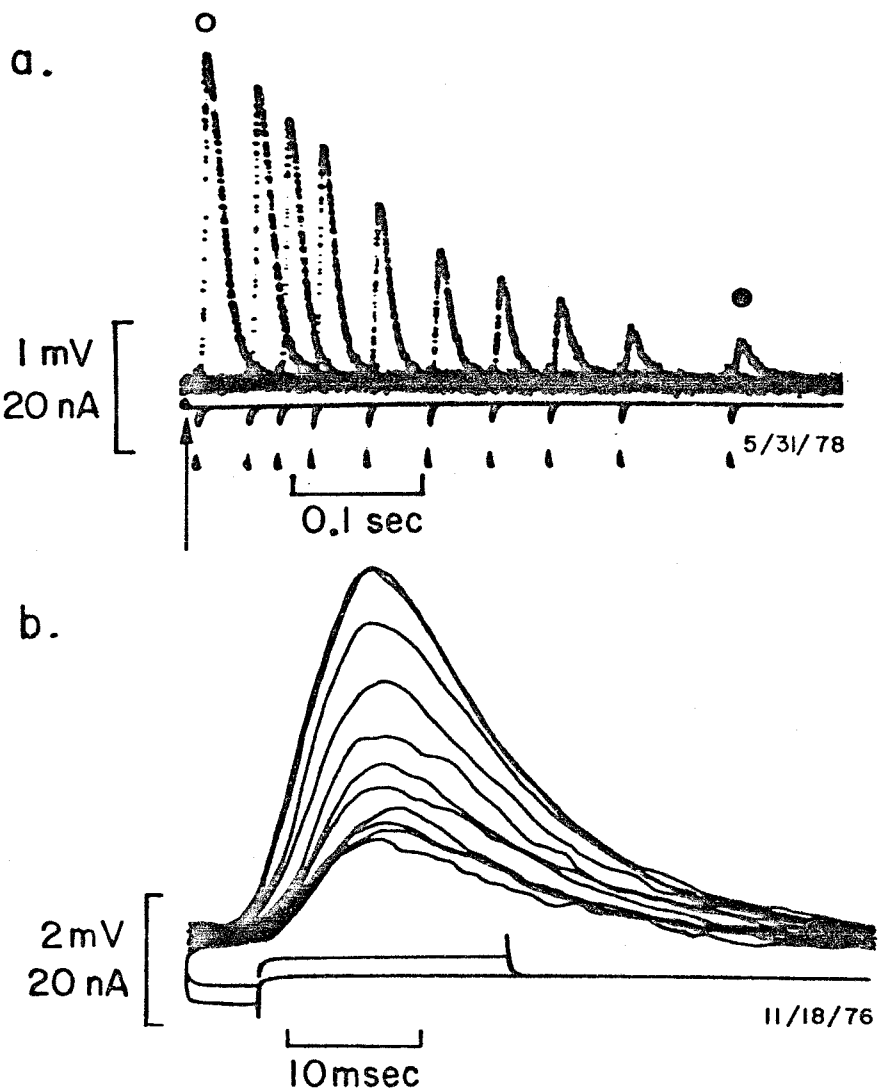
method, we observed that after a hexamethonium pulse, recovery from inhibition proceeds with a time constant, τ_{off} = 25 msec. This is roughly one hundred times faster than recovery after a dTC pulse. This difference may be related to hexamethonium's much lower affinity for the ACh receptor (Blackman, Gauldie and Milne, 1975).

Onset of dTC inhibition

The rapid onset of inhibition is examined in more detail in Fig. 3 with the episode method. In Fig. 3a, inhibition develops roughly exponentially with a time constant, τ_{on} = 120 msec, which is tenfold faster than recovery at this synapse. In Fig. 3b, from another fiber, the waveforms of the ACh responses are shown during the onset of inhibition. Note that all the ACh responses have brief latencies and take the same time to reach their peak response. One must infer that inhibition develops as the concentration of dTC rises near a localized population of receptors rather than as dTC spreads over the synapse. The ACh responses also rise to their peaks rapidly (<15 msec).

In preliminary experiments, we occasionally settled for iontophoretic pipette placements which produced ACh responses that rose more slowly ($\geq$40 ms), presumably because the pipettes were rather far from the receptors. At these sites, the onset of dTC inhibition was actually slower than recovery, as in the experiments of Waud (1967). Waud's iontophoretic pipettes were probably also some distance from the synaptic cleft judging from his low ACh sensitivities ($\sim$4 mV/nC), and he concluded that the kinetics which he observed were slowed by diffusion through the external solution. An alternative explanation for the slow decay of dTC inhibition might be some such barrier to dTC's immediate access

Fig. 3. Onset of dTC inhibition following a sudden maintained increase in dTC release. A, single ACh pulses were delivered during a long dTC pulse (1 sec). Each episode contained the same ACh and dTC pulses, but we varied the delay between the beginning, ↑, of the dTC pulse and the ACh pulse. Episodes were repeated every 20 seconds. O, control response to ACh pulse alone; ●, steady-state inhibition of ACh response after maintaining dTC release for 20 seconds. B, the oscilloscope sweep was triggered at the same time as the ACh pulse. A train of eleven ACh pulses was delivered at four pulses per second. About 30 msec after the second ACh pulse, dTC release began (5 nA) and was maintained until the train was over. Note that the ACh response reached a steady-state level of inhibition.


a.
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10msec

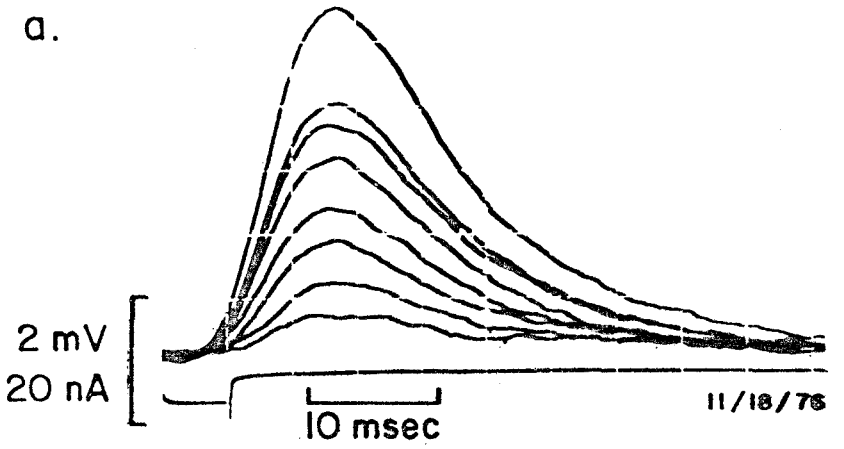
to the synaptic cleft. However, any such barrier would have to be remarkably selective to account for the rapid action of hexamethonium and the still more rapid action of ACh. The receptors themselves show such selectivity, but ACh receptor density is reduced by two orders of magnitude within just a few microns of the synaptic cleft (Kuffler and Yoshikami, 1975; Matthews-Bellinger and Salpeter, 1978). And del Castillo and Katz (1957b) observed that dTC pulses which inhibit iontophoretic ACh responses also inhibit neurally evoked ACh responses equally effectively. Thus, there are virtually no extrasynaptic drug receptors to bind dTC.

Inhibition is not slowed by access of dTC to the synaptic cleft

With the improved visualization under Nomarski optics, the iontophoretic pipette can be accurately positioned next to the nerve terminal so that the time course of iontophoretic ACh potentials closely mimics that of neurally stimulated ACh potentials (Kuffler and Yoshikami, 1975). Under these circumstances, the time course of the iontophoretic ACh potentials is determined not by diffusion but by the iontophoretic pulse duration and the time constant of the muscle fiber's membrane (Dreyer and Peper, 1974c).

Fig. 4a shows the waveform of individual ACh responses during recovery from steady-state inhibition. The largest response is a control response in the absence of dTC; the smallest response is the response to the same ACh pulse after 15 seconds of maintained dTC release. Each of the intermediate ACh responses occurred in a separate episode with a variable delay after the end of the dTC pulse. As the delay increases, more recovery takes place, but recovery is still slow: τ_{off} = 2 sec which is 200-fold slower than the falling phase of the ACh potentials. Note that all the ACh responses have brief latencies and rise rapidly

Fig. 4. Recovery from dTC inhibition. A, the same ACh pulse was delivered with a variable delay (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 15 sec) after the end of a long (15 sec) dTC pulse. Episodes were repeated every 30 seconds. The smallest and largest responses are steady-state responses in the presence and absence of dTC. In this case also, the oscilloscope sweep was triggered at the same time as the ACh pulse so that the waveforms could be compared. B, two independent episodes, d.c.-coupled recording. Both episodes show the response to a 1 nA reduction of the ACh braking current for about 2 sec (↓↑). In one episode a pulse of dTC was delivered while the cell was depolarized. Note the small additional depolarization at the beginning of the dTC pulse; interbarrel coupling was not compensated in this experiment (see Methods).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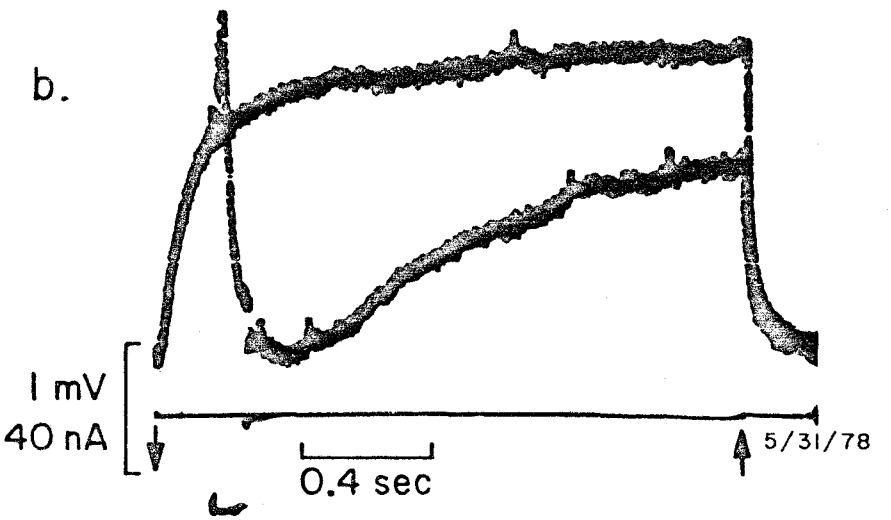
b.
1 mV
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5/31/78

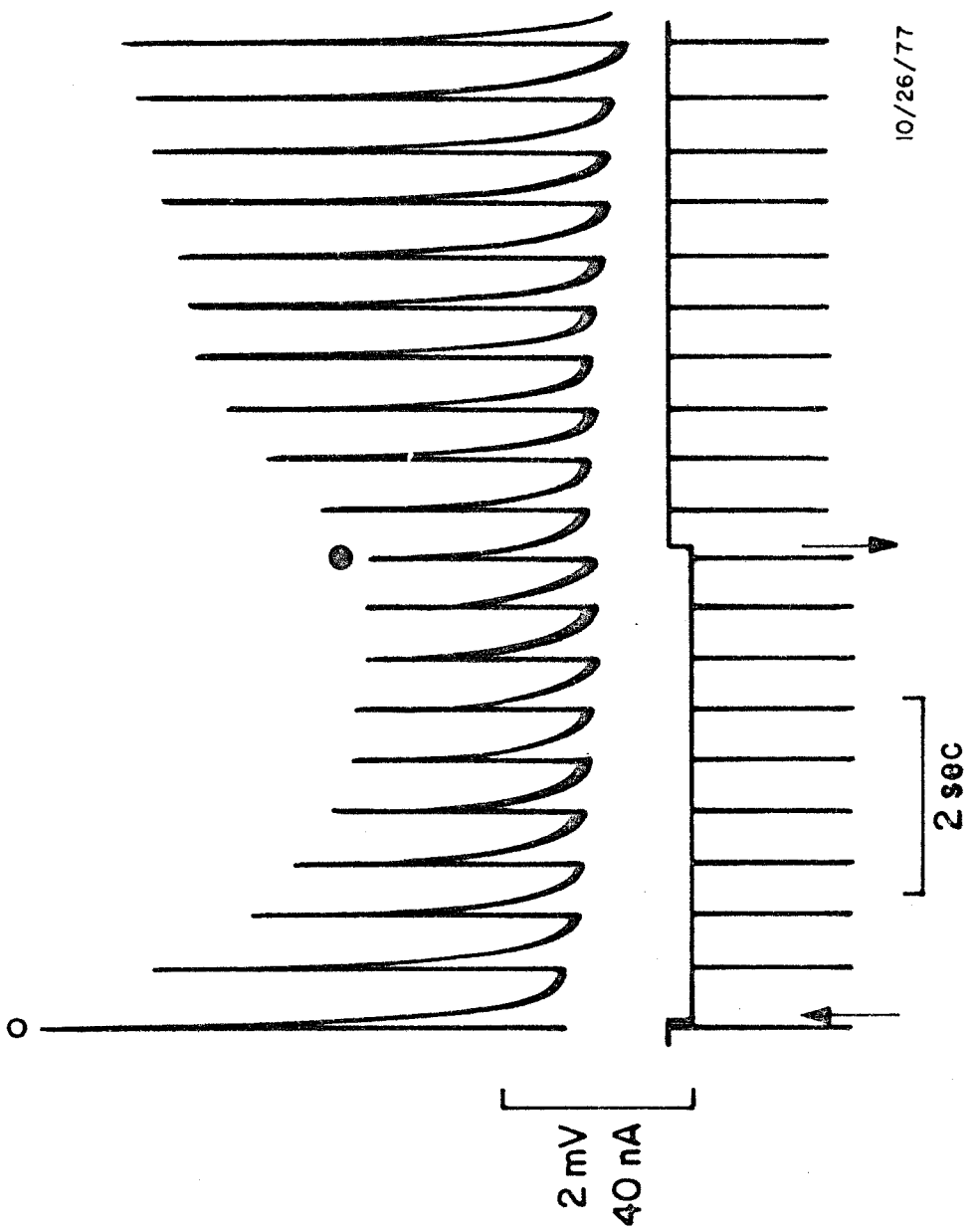
to their peaks. Furthermore, the ACh responses' peak times remain the same during recovery; thus recovery is not caused by ACh diffusing to more distant uninhibited receptors (c.f. Feltz and Mallart, 1971).

The time course of dTC action can also be examined against the background of a steady ACh response (del Castillo and Katz, 1957a). In Fig. 4b, the ACh braking current is reduced just enough to depolarize the fiber by a few mV for a few seconds. The response to dTC has a brief latency and rapidly reaches maximal inhibition. Nevertheless, recovery still proceeds slowly; much more slowly, in fact, than does repolarization after the much longer ACh pulse. Notice also that the dTC pulse completely inhibits the ACh response. This suggests that the spatial concentration profile of dTC envelops that of ACh. Taken together, these results make it very unlikely that diffusion barriers outside the synaptic cleft limit dTC's access to the receptors (c.f. Waud, 1967).

Relation between the rates and the steady-state level of inhibition

If the time course of inhibition were determined by the molecular rates of dTC binding to the receptor, as del Castillo and Katz suggested (1957a), then the macroscopic rates of inhibition should depend on the dTC concentration in a predictable manner: the rate of onset should increase with concentration but the rate of recovery should remain constant (Hill, 1909). Fig. 5 shows the response to a train of identical brief ACh pulses delivered at a frequency of two pulses per second. After a control response (ΔV_0), dTC release suddenly begins and continues at a constant rate for several seconds. Subsequent ACh pulses produce smaller responses as inhibition develops until a steady-state is reached (ΔV_∞). When the dTC release is stopped suddenly, the ACh response recovers gradually

Fig. 5. Time course of inhibition. A train of identical brief ACh pulses was delivered at two pulses per second. A long dTC pulse (↑ ↓) begins just after the first ACh pulse and lasts 5 seconds. O, ΔV_0; ●, ΔV_∞ (see text). This figure was traced from the original photograph for clarity.


10/26/77
2 sec
2 mV
40 nA
0

to its original amplitude. Several such records were taken at the same synapse with varying levels of dTC release. In Fig. 6, the time course of inhibition is analyzed graphically for three different levels of release. Both the rates of onset and recovery are roughly exponential, but the rate of onset is faster. Furthermore, only the rate of onset varies with the level of inhibition.

We have examined the quantitative relationships among the apparent rate constants of onset ($1/\tau_{on}$) and recovery ($1/\tau_{off}$), and the steady-state level of inhibition. When dTC is released at a constant rate for several seconds, the ACh response is eventually inhibited to some steady-state level, presumably because the dTC concentration has reached a steady-state value near the receptors. We define $n = \Delta V_0 / \Delta V_\infty$ as an index of the steady-state level of inhibition and therefore the dTC concentration near the receptors. If we assume that dTC binds to the receptor in a reversible bimolecular reaction, these experiments (such as Fig. 5) are formally similar to concentration-jump studies which can be analyzed with simple relaxation theory (Eigen, 1954). The predicted relationship, $1/\tau_{on} = n\,(1/\tau_{off})$, adequately summarizes the data (Fig. 7). When inhibition is small ($n \rightarrow 1$), $1/\tau_{on}$ should approach $1/\tau_{off}$, and this is observed (Fig. 7a). Furthermore, $1/\tau_{on}$ increases linearly as n increases, but $1/\tau_{off}$ is constant. The slope of the line relating $1/\tau_{on}$ and n should equal the observed recovery rate constant, $1/\tau_{off}$. This prediction is tested in Fig. 7b. For each synapse, the ratio of $(1/\tau_{on})/n$ to $1/\tau_{off}$ should equal one. In fact, the ratio equals 0.97 ± 0.17 (mean $\pm$ S.D. at eight synapses).

The results in Fig. 7 are consistent with the notion that the kinetics of inhibition do measure the molecular rates of dTC binding to the receptor. However, it is unlikely that one can produce a perfect 'concentration-jump' of dTC near the receptors. The following argument explains this statement. The

Fig. 6. Kinetics of inhibition at the synapse from the experiment of Fig. 5. Semilogarithmic plots of inhibition versus time for three different levels of dTC release. A, recovery plotted as the remaining fraction of steady-state inhibition versus the time after the end of the dTC pulse. B, onset of inhibition plotted as the normalized fraction of the original response versus the time after the beginning of the dTC pulse. A and B have identical time scales.

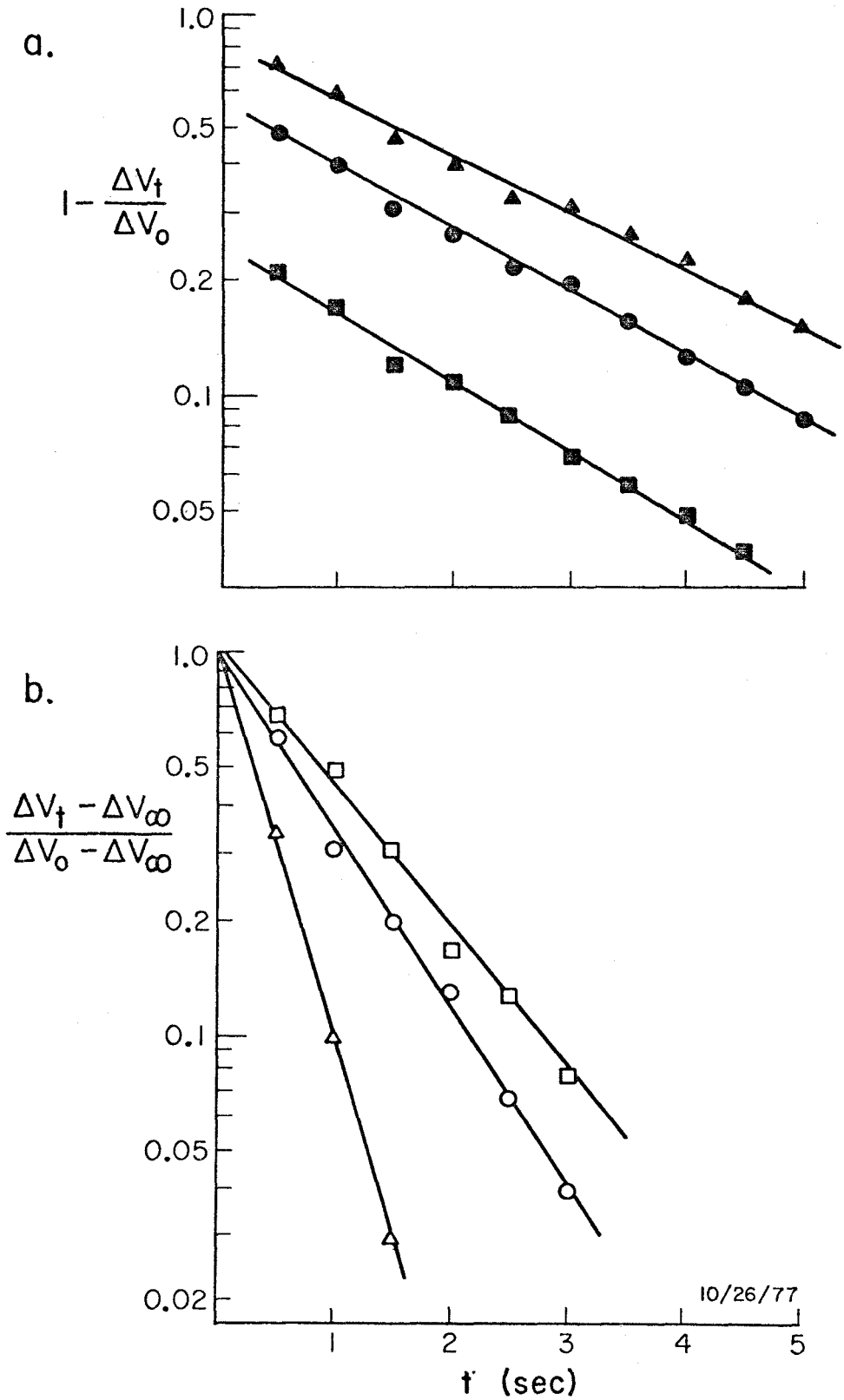
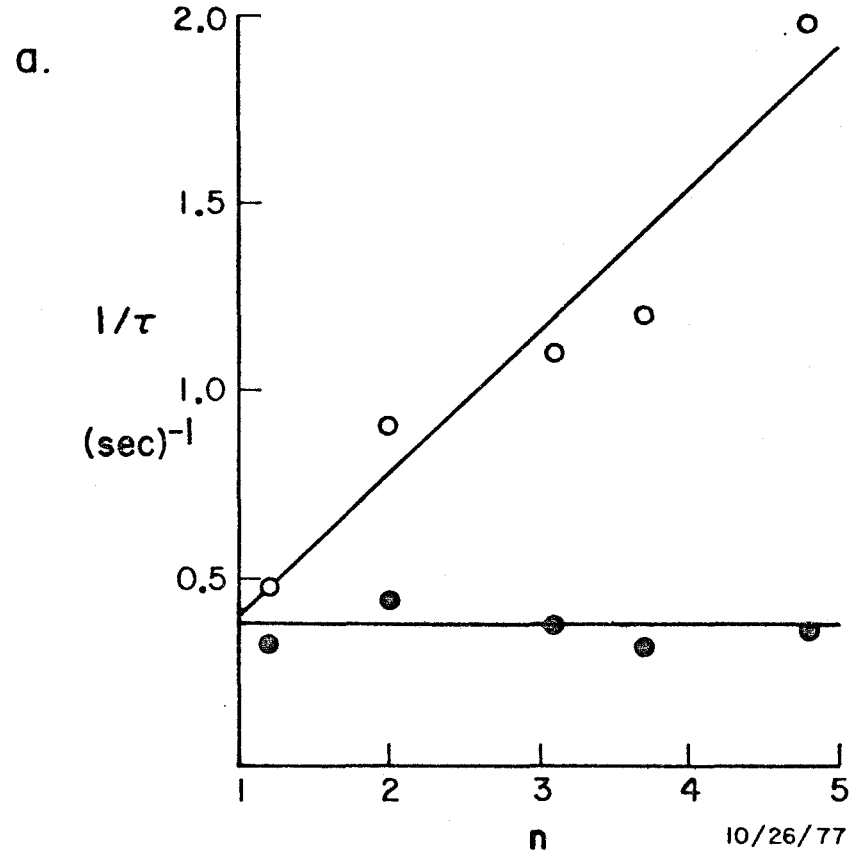
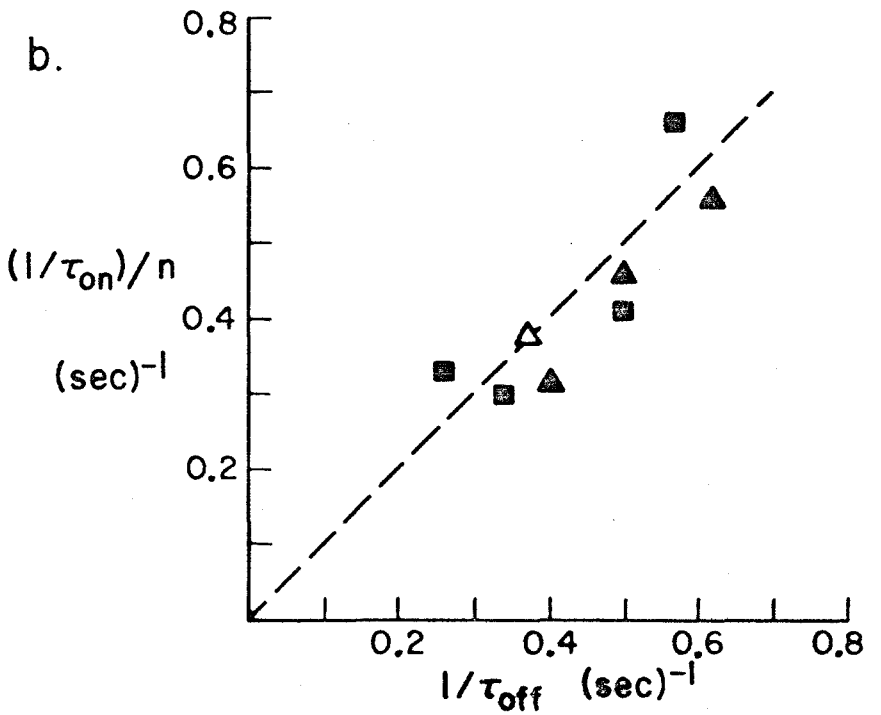

a.
1.0
0.5
$1-\frac{\Delta V_t}{\Delta V_o}$
0.2
0.1
0.05
b.
$\frac{\Delta V_t - \Delta V_\infty}{\Delta V_o - \Delta V_\infty}$
1.0
0.5
0.2
0.1
0.05
0.02
10/26/77
1
2
3
4
5
t (sec)

Fig. 7. Relation between the rates and steady-state level of inhibition at the synapse from the experiment in Fig. 5. A, rate constants of onset, $1/\tau_{on}$, and recovery, $1/\tau_{off}$ (slopes of the curves in Fig. 6) versus n, an index of the steady-state level of inhibition ($n = \Delta V_0 / \Delta V_\infty$; see text). O, $1/\tau_{on}$; ●, $1/\tau_{off}$. Straight lines were fit by the method of least squares. B, predicted $1/\tau_{off} = (1/\tau_{on})/n$ versus experimentally observed $1/\tau_{off}$. The dashed line indicates the theoretical relationship. ■, sartorious fibers; ▲, cutaneous pectoris fibers. Δ, fiber from the experiment in Fig. 7a. For all eight fibers, $1/\tau_{off} = 0.45 \pm 0.12$ sec^{-1} (mean ± S.D.).


a.
2.0
1.5
1.0
0.5
1/τ
(sec)⁻¹
1
2
3
4
5
n
10/26/77

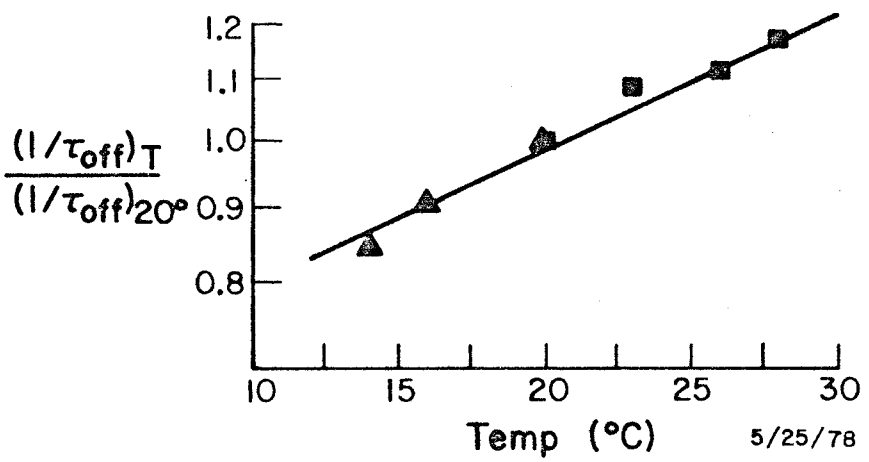

b.
0.8
0.6
0.4
0.2
(1/τon)/n
(sec)⁻¹
0.2
0.4
0.6
0.8
1/τoff (sec)⁻¹

ACh receptor concentration in the cleft is about 300 μM (Matthews-Bellinger and Salpeter, 1978). Suppose the dTC concentration in the bath were 0.5 μM, its equilibrium binding constant. Then half the receptors would have dTC bound. Therefore, the total amount of dTC in the cleft would be 150 μM (mostly bound to receptors) even though the concentration of free dTC in the cleft was 0.5 μM. The cleft contains 300 times as much dTC as it would if it had no receptors. Thus, the receptors buffer the dTC concentration in the cleft; and following a rapid jump upward or downward of the dTC concentration in the bulk solution, the cleft concentration is expected to lag. One concludes that for a high affinity drug like dTC, the kinetics of action could be limited by the drug's buffered diffusion in the cleft. In fact, the results in Fig. 7 are predicted for both a true concentration-jump relaxation and for kinetics dominated by buffered diffusion (Rang, 1966; Colquhoun et al., 1972). Which is the case for dTC?

Temperature dependence of recovery rate

A high temperature dependence characterizes the binding kinetics of most ligand-protein interactions. At the acetylcholine receptor, for example, channel opening and closing rates have a $Q_{10} \sim 3$ (Anderson and Stevens, 1973; Sheridan and Lester, 1975) although these transition rates could be governed by conformational changes rather than binding steps. Nevertheless, if molecular binding rates determine the kinetics of inhibition, one expects a high temperature coefficient for dTC action. However, the recovery rate constant, $1/\tau_{off}$, varies only slightly with temperature: $Q_{10} = 1.25$ between 14° and 28°C (Fig. 8). On the other hand, buffered diffusion does not depend on the molecular rate constants, but only on the equilibrium binding constant, which has a Q_{10} near unity (Jenkinson,

Fig. 8. The relation between the rate of recovery from inhibition and temperature. The recovery rate, $1/\tau_{off}$, is normalized to $1/\tau_{off}$ at 20°C and plotted on a logarithmic scale. The recovery rate changes e-fold every 45°C; this corresponds to a $Q_{10} = 1.25$. Data from two fibers: ▲, ■ .


1.2
1.1
1.0
0.9
0.8
$\frac{(1/\tau_{off})_T}{(1/\tau_{off})_{20°}}$
10
15
20
25
30
Temp (°C)
5/25/78

1960). This suggests that diffusion, rather than the molecular dissociation rate determines the rate of recovery from inhibition. However, we have shown that any delays in diffusion must occur within the synaptic cleft, where the most probable fate for a dTC molecule appears to be binding to an ACh receptor.

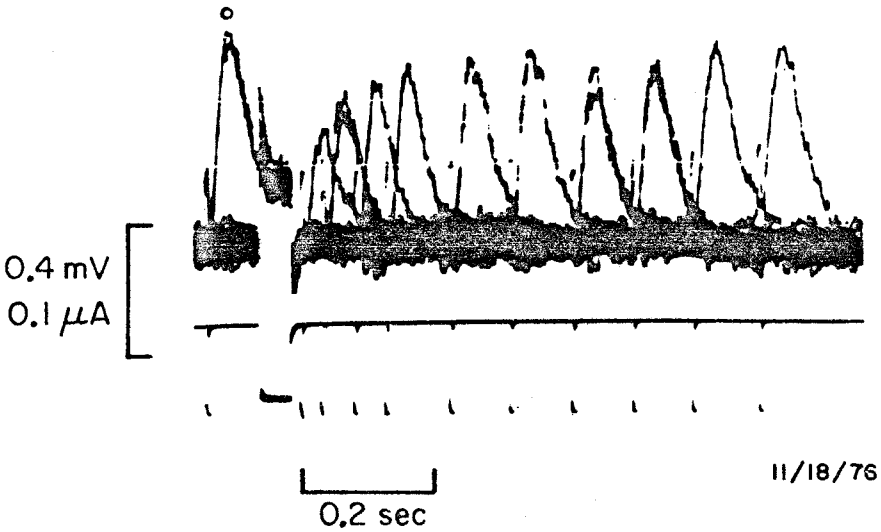
Cobra toxin and the time course of dTC inhibition

If the time course of inhibition were determined by dissociation of dTC from the receptor without rebinding, then the recovery rate should not depend on the density of receptors in the synaptic cleft. Elapid α-toxins bind irreversibly to ACh receptors and block both their activation by ACh and their binding to dTC (Adams, 1975; Moody, Schmidt and Raftery, 1973). We have used the ACh sensitivity of the postsynaptic membrane as a measure of the density of unblocked receptors (Hartzell and Fambrough, 1972; Land, Podleski, Salpeter and Salpeter, 1977). As more receptors are blocked by toxin, the ACh sensitivity decreases. When we reduce the ACh sensitivity with cobra toxin, both the rate of onset and the rate of recovery from inhibition after a dTC pulse are faster (Fig. 9). Thus, the kinetics of dTC inhibition depend on the receptor density in the synaptic cleft. The buffering capacity of the cleft will also depend on receptor density. This suggests that the time course of inhibition reflects repeated binding of each dTC molecule to several ACh receptors as it diffuses within the synaptic cleft.

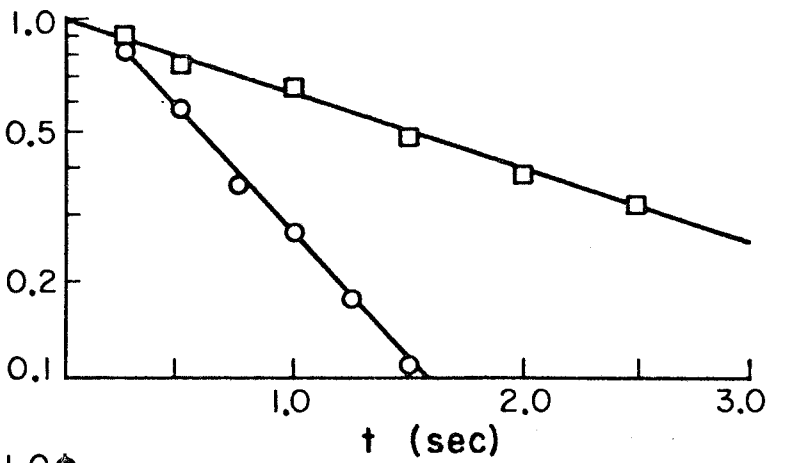
The results with toxin were quite variable, but on the average the recovery rate increased fourfold after a tenfold reduction in ACh sensitivity. One wonders whether this variability arose because other molecules in the synaptic cleft also bind dTC and slow its diffusion. An obvious candidate is the acetylcholinesterase (AChE) which binds dTC with high affinity, $K_D \sim 30$ μM (Mooser and Sigman, 1974),

Fig. 9. Cobra toxin increases the rates of dTC inhibition. A, recovery from inhibition: each ACh pulse was delivered in a separate episode with a variable delay after the same dTC pulse. Episodes were repeated every 30 seconds. Open circle, (O) two superimposed responses to control ACh pulses before and after the series of episodes. Before the series, cobra toxin had reduced the ACh sensitivity at this synapse from 330 to 3.8 mV/nC. B and C, semilogarithmic plots of the rate of onset (O, ●) and the rate of recovery (□, ■) from inhibition. B, rates before toxin; C, rates after toxin. Note the different time scales. Rate constants (sec^{-1}): □ , 0.46; O, 1.5; ■ , 6.7; ●, 23.

a.

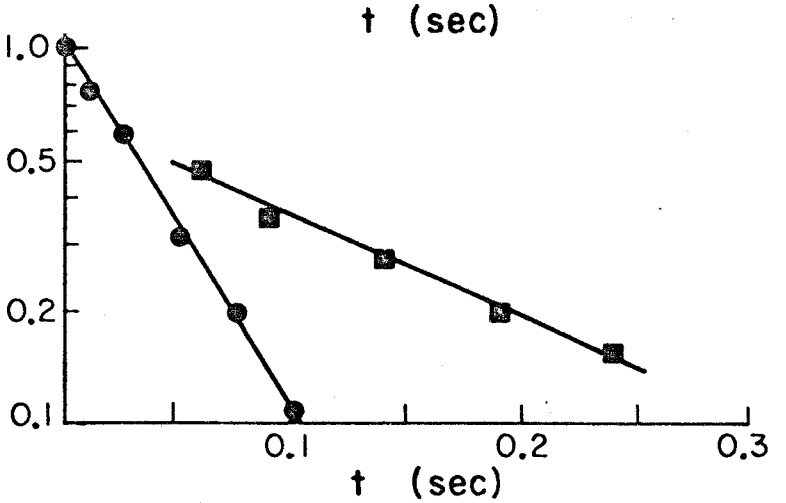


b.



$$\frac{\Delta V_t - \Delta V_\infty}{\Delta V_0 - \Delta V_\infty}$$

c.



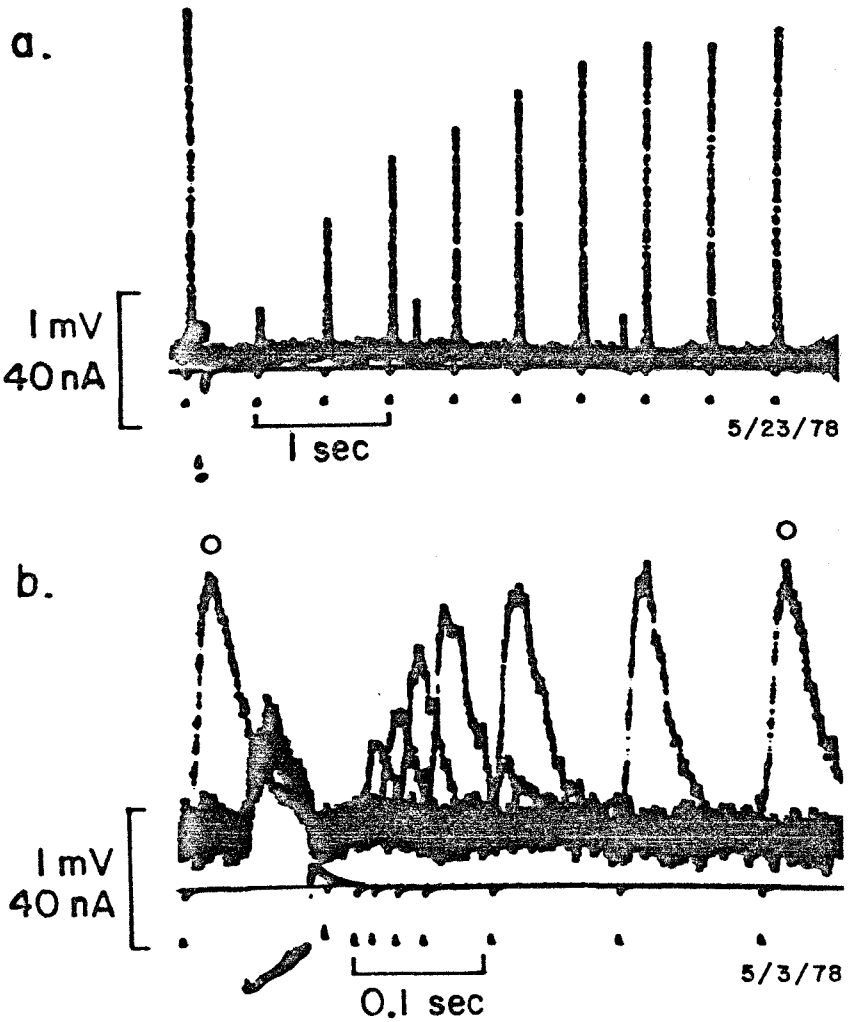
and is present at high concentration in the cleft, ∿ 70 μM (Matthews-Bellinger and Salpeter, 1978). However, irreversibly blocking AChE activity with MSF has no effect on the rate of recovery from inhibition. This might be expected if MSF blocks the enzyme's catalytic site but not the peripheral site of dTC binding (Mooser and Sigman, 1974). Therefore, we used another method to eliminate dTC binding to AChE in the cleft.

The action of collagenase on the recovery rate

Exposing the synapse to collagenase action removes AChE from the synaptic cleft (Hall and Kelly, 1971). Eventually the nerve terminal pulls loose from the postsynaptic membrane, but this 'synaptic disjunction' can be delayed by using purified collagenase (Betz and Sakmann, 1973). The rate of recovery from dTC inhibition is approximately twofold faster after exposure to collagenase: $1/\tau_{off} = 1.0 \pm 0.3$ sec^{-1} (mean ± S.D. at five synapses). An example is shown in Fig. 10a. Both MSF and collagenase effectively eliminate AChE activity in the cleft: the iontophoretic ACh sensitivity increased and spontaneous mepps were also larger. In addition, the iontophoretic ACh responses reached their maxima and decayed more slowly. Histochemical staining (Karnovsky, 1964) was substantially or totally reduced at the light microscope level. Nevertheless, there was no synaptic disjunction as evidenced by large spontaneous mepps.

After prolonged exposure to the action of partially purified collagenase, synaptic disjunction occurs, and the mepps disappear. When the nerve terminal is displaced and the drugs are iontophoresed directly onto the exposed postsynaptic membrane, the recovery rate increases dramatically (Fig. 10b): $1/\tau_{off} = 9.4$ sec^{-1} at four synapses (4.6, 5.4, 11.0, 16.7 sec^{-1}). This is nearly twenty times faster

Fig. 10. The action of collagenase on recovery from dTC inhibition. A, a single dTC pulse (50 nA x 50 msec) was delivered just after the first ACh pulse in a train of identical pulses at two pulses per second (see Fig. 2a). Acetylcholinesterase activity had been blocked previously with collagenase. Note mepps. B, after the nerve terminal was displaced, recovery from dTC inhibition was observed with the episode method (c.f., Fig. 2b). Note the smaller time scale.


a.
1 mV
40 nA
1 sec
5/23/78
b.
1 mV
40 nA
0.1 sec
5/3/78

than recovery at unperturbed synapses. Neither the protease action nor the removal of the nerve terminal reduced the ACh sensitivity of the postsynaptic membrane; in fact, the sensitivity always increased to values greater than 10^3 mV/nC after removing the terminal. Evidently the nerve terminal obstructs diffusion out of the synaptic cleft, and consequently each dTC molecule binds repeatedly to several ACh receptors before escaping. With the terminal intact, the synaptic cleft is only 0.05 μm deep (Matthews-Bellinger and Salpeter, 1978) so even with diffusion coefficients lower than 10^{-6} cm^2/sec, molecules would rattle around in the cleft on a time scale of tens of microseconds. Thus, the probability of rebinding to another ACh receptor is greatly enhanced (Berg and Purcell, 1977). These experiments show that when the enveloping nerve terminal is removed and the postsynaptic membrane is exposed to the bulk solution, or when the number of receptors available to rebind dTC is reduced with toxin, the kinetics of inhibition are much faster. However, under these circumstances, the macroscopic rates of inhibition are still much slower than the millisecond molecular reaction rates inferred by Sheridan and Lester (1977).

Equilibrium studies of dTC antagonism

Whenever dTC and ACh are given sufficient time together to equilibrate with nicotinic receptors (for example, by applying both drugs in the bath), dTC blocks ACh binding in a competitive fashion. We use very brief iontophoretic ACh pulses to assay what fraction of receptors are blocked by dTC at any one moment. The question arises whether dTC molecules bind and dissociate from the receptor rapidly enough to be displaced by the ACh before the response reaches its maximum (∿10 ms). We observe that dTC competitively inhibits these brief

ACh responses and conclude, therefore, that dTC molecules bind and dissociate from the ACh receptor on a millisecond time scale.

As expected for a competitive antagonist, dTC shifts the curve relating peak response and log dose ACh to higher doses without changing the slope of the curves (Gaddum, 1937). Fig. 11a shows representative data from one fiber. The noticeable flattening of the curve in 4 μM dTC may reflect noncompetitive, voltage-dependent block of open channels by dTC (Manalis, 1977, 1978; Colquhoun, Dreyer and Sheridan, 1978). Double-reciprocal plots of the same data (Fig. 11b) also show the expected rotation of the curves around a common intercept on the ordinate (Linweaver and Burk, 1934). The data in Fig. 11a and similar data on synaptic receptors from three other cells are analyzed further by the dose ratio method (Gaddum, 1937): the dose ratio (the ratio of the ACh dose required to produce a given response in the presence of dTC to that required in its absence) is plotted as a function of the dTC concentration (Fig. 12, open symbols). Thus, when the dTC concentration is zero, the dose ratio should be one and this is observed. The relationship is linear as required by competitive antagonism and indicates an apparent equilibrium dissociation constant, K_D = 0.5 μM, which is very close to values obtained at other frog nerve-muscle synapses with bath-applied ACh (Jenkinson, 1960; Adams, 1975). However, Adams' (1975) results with iontophoretic ACh pulses also satisfy the predictions of noncompetitive antagonism. This discrepancy brings up a serious objection to physiological studies of drug antagonism: the dose-response curve saturates before ACh-receptor binding. Unfortunately, the differences between competitive and noncompetitive binding are clearest when the receptors are saturated with ligand.

Fig. 11. Dose-response curves for iontophoretically-applied ACh in the presence of bath-applied dTC: ●, no dTC; O, 0.5 μM dTC; ■, 1.0 μM; □, 2.0 μM; ▲, 4.0 μM. A, peak response versus log dose; curves are drawn by eye. B, double-reciprocal plot of the same data on linear coordinates. The square root of the peak response is used in order to linearize the dose-response curve (Lester, Koblin and Sheridan, 1978). Straight lines were fit by the method of least squares.

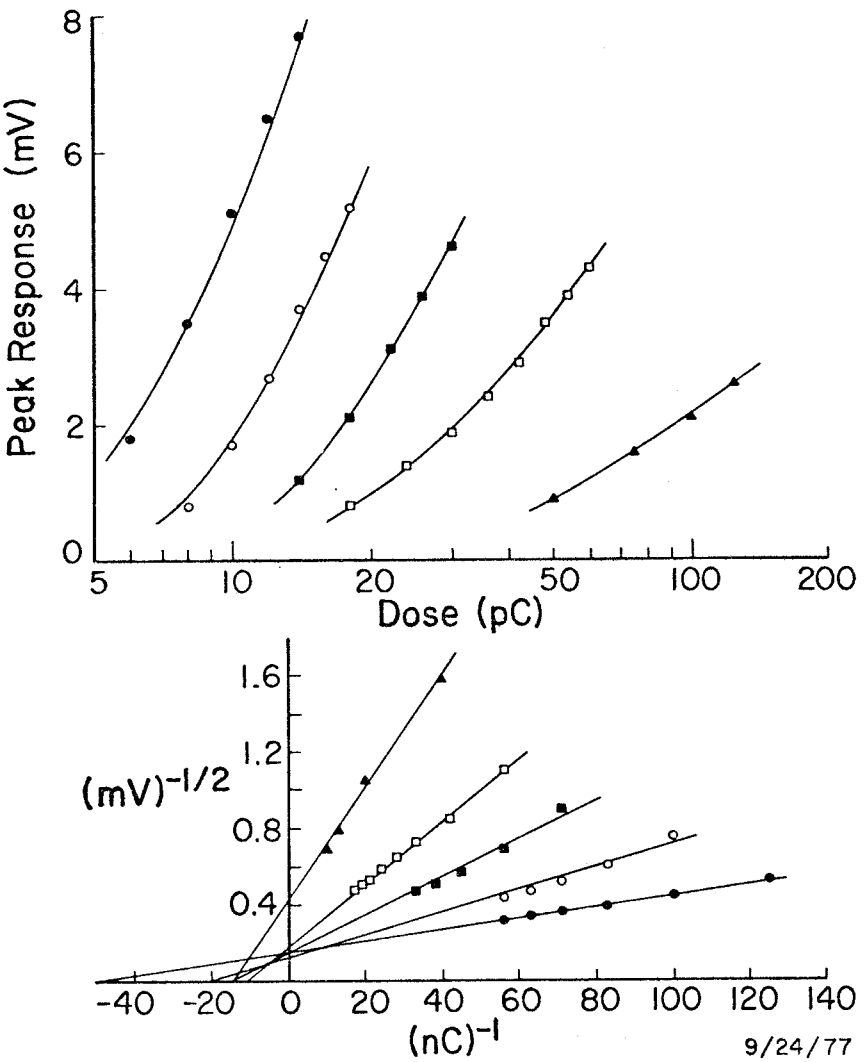
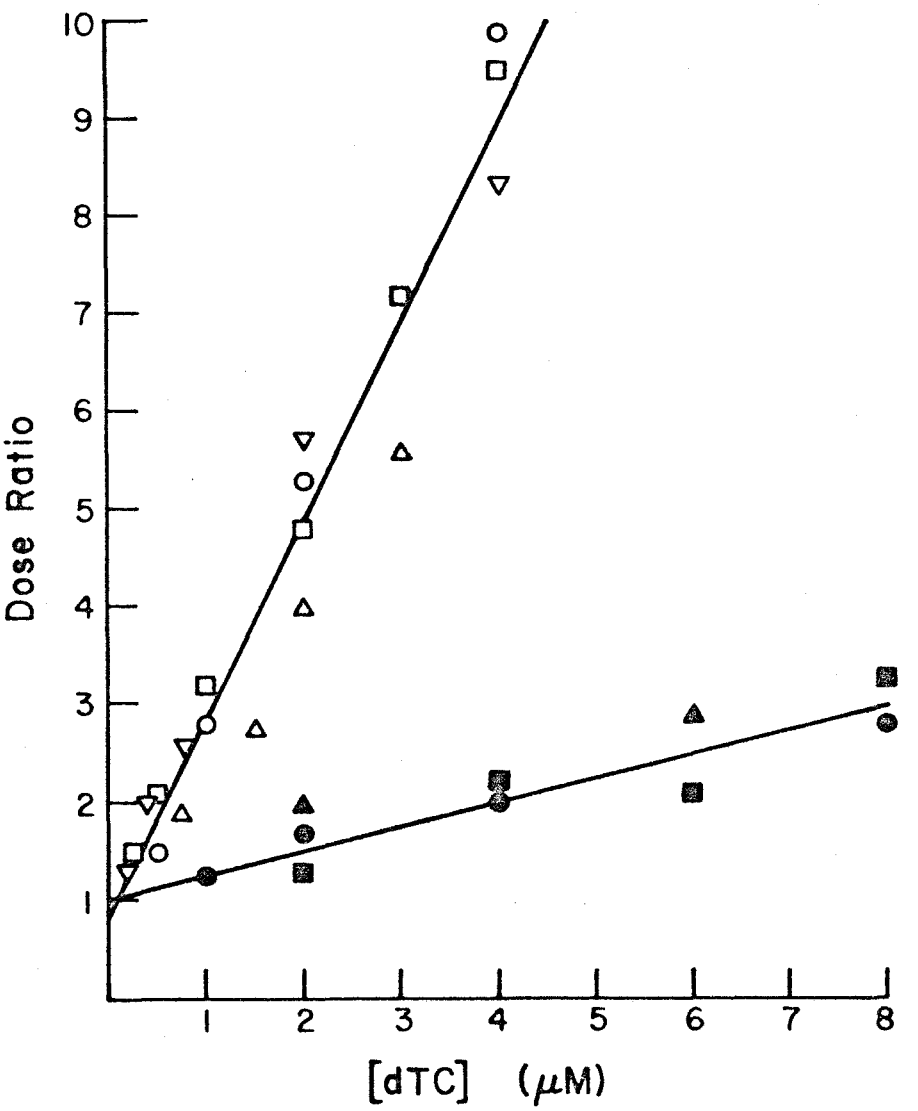

Peak Response (mV)
8
6
4
2
0
5
10
20
50
100
200
Dose (pC)
(mV)-1/2
1.6
1.2
0.8
0.4
-40
-20
0
20
40
60
80
100
120
140
(nC)-1
9/24/77

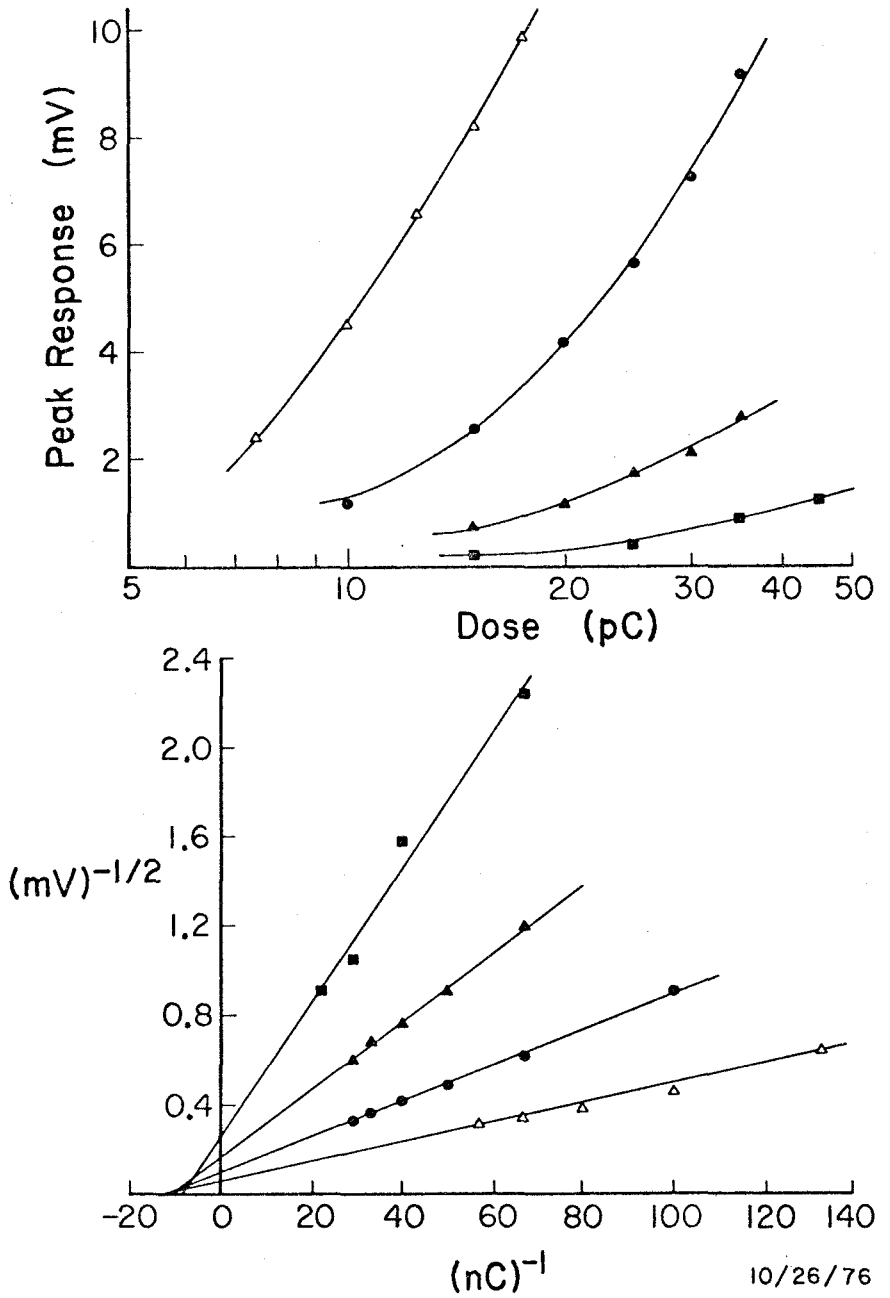
Fig. 12. Dose ratio test of dTC antagonism. Dose ratio = ([dTC]/K_D) + 1. Open symbols, data for synaptic ACh receptors from four fibers: K_D = 0.5 μM. Open circles represent data from the experiment of Fig. 11a. Closed symbols, data for extrasynaptic receptors from three denervated fibers: K_D = 4.0 μM. Straight lines were fit by the methods of least squares.


Dose Ratio
10
9
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
[dTC] (μM)

In order to test whether our techniques can discriminate between competitive and noncompetitive binding, we have investigated the antagonism of iontophoretic ACh responses by cobra toxin. Elapid α-toxins in general are known to be irreversible noncompetitive antagonists of nicotinic acetylcholine receptors on the time scale of physiological experiments (Lester, 1972; Adams, 1975). Dose-response curves for iontophoretically-applied ACh in the presence of toxin (Fig. 13) show none of the features characteristic of competitive inhibition which are illustrated by dTC in Fig. 11 (ignoring the data for 4.0 μM dTC). The differences are clearest in the respective double-reciprocal plots (Figs. 11b and 13b). Toxin reduces the apparent maximum response fivefold but changes the apparent equilibrium binding constant only 30%. This is just the behavior expected of a noncompetitive antagonist. In contrast, dTC decreases the apparent binding constant fivefold without changing the apparent maximum response (±20%), just as expected for a competitive antagonist.

There is another serious limitation on using the iontophoretic technique to study dose-response relations. In such studies, one presumes that the response increases because the drug concentration increases near the receptors. On the other hand, with iontophoretic pulses the response might increase because larger pulses cover a larger area of the receptive membrane. This latter phenomenon clearly occurs on denervated fibers where the extrasynaptic membrane is uniformly sensitive to ACh (Feltz and Mallart, 1971; Dreyer and Peper, 1974d). To avoid this problem in the present study, we routinely use doses whose responses reach thier maxima at the same time (see Figs. 3b, 4a). Then we can be sure that the iontophoretic pipettes always sample the same population of receptors and really vary the drug concentration near them.

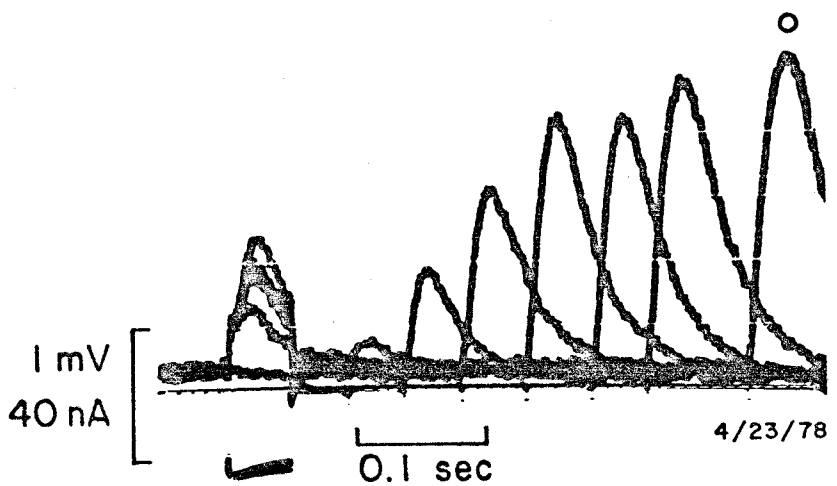
Fig. 13. Dose-response curves (as in Fig. 11) for iontophoretically-applied ACh in the presence of bath-applied cobra toxin (60 nM): Δ, no toxin; ●, 10 minutes after adding toxin; ▲, 20 min; ■, 25 min.


Peak Response (mV)
10
8
6
4
2
5
10
20
30
40
50
Dose (pC)
2.4
2.0
1.6
1.2
0.8
0.4
(mV)^-1/2
-20
0
20
40
60
80
100
120
140
(nC)^-1
10/26/76

Extrasynaptic ACh receptors on denervated muscle fibers

Bath-applied dTC also competitively inhibits iontophoretic ACh responses from extrasynaptic receptors on denervated membrane but with a lower apparent affinity (Fig. 12). After a pulse of dTC, recovery from inhibition proceeds tenfold more rapidly at these extrasynaptic sites than at unperturbed synapses on innervated fibers (Fig. 14). On three fibers, the mean recovery rate, $1/\tau_{off}$ = 8.1 sec^{-1} (6.5, 7.2, 10.7 sec^{-1}). Presumably both the absence of a nerve terminal on extrasynaptic membrane and the lower density of receptors reduce the extent to which dTC's diffusion is buffered.

Fig. 14. Recovery from dTC inhibition of extrasynaptic receptors on a denervated fiber. Each ACh pulse was delivered in a separate episode with a variable delay after the same dTC pulse. O, control response with no dTC.


1 mV
40 nA
0.1 sec
4/23/78

DISCUSSION

Are the kinetics determined by binding or buffered diffusion?

We have investigated how the time course of inhibition by dTC is determined by simultaneous diffusion and binding within the synaptic cleft of motor nerve terminals on frog skeletal muscle. With the iontophoretic technique, both dTC and ACh were applied rapidly to the same localized population of receptors in the cleft. We have considered two explanations for the slow recovery from inhibition following brief pulses of dTC. Both hypotheses assume that dTC binds to the nicotinic ACh receptor in a reversible bimolecular reaction:

$$D + R \underset{k_-}{\overset{k_+}{\rightleftharpoons}} DR$$

for which the equilibrium dissociation constant, $K_D = k_-/k_+$. Del Castillo and Katz (1957a) suggested that this slow recovery reflects a slow molecular rate of dissociation of dTC from the receptor. Alternatively, the molecular rate of dissociation might be very rapid, but dTC is retained in the cleft by rebinding to receptors, so inhibition persists.

In order to differentiate between these two hypotheses, we have perturbed the synapse in three ways: with temperature, with cobra toxin, and with collagenase. The first two perturbations are very specific and eliminate the molecular hypothesis. The molecular dissociation rate should have a higher temperature coefficient and should not depend on the receptor density. Collagenase action allows one to remove the AChE from the synaptic cleft (Hall and Kelly, 1971) and eventually allows one to remove the nerve terminal so that the synaptic cleft is exposed directly to the bath (Betz and Sakmann, 1973). These last two perturbations can be

separated temporally with purified collagenase (Fig. 10) as evidenced by the simultaneous absence of AChE activity and presence of mepps. Both consequences of collagenase action increase the rate of recovery from inhibition. This could be explained by some discrete proteolytic alteration of the receptor although the ACh sensitivity is not reduced. However, taken together the results of these perturbations strongly suggest that the nerve terminal obstructs dTC's diffusion out of the synaptic cleft and consequently each dTC molecule binds repeatedly to several ACh receptors before escaping. Therefore, the macroscopic kinetics of inhibition are much slower than the molecular rates of binding. Such buffered diffusion may explain the paradox between competitive inhibition at equilibrium and the slow rates of inhibition for many antagonists of synaptic receptors (Gaddum, 1937).

In retrospect, it seems evident that dTC molecules will diffuse more slowly within the synaptic cleft because they rebind to receptors. The synaptic cleft is a narrow tunnel densely packed with ACh receptors and esterase, and given their high affinity for dTC, the probability must be small that a dTC molecule could escape this gauntlet without rebinding. The synaptic cleft may be compared to a dialysis bag which is loaded with protein and consequently retains dTC molecules that otherwise would diffuse away freely (Silhavy, Szmelcman, Boos and Schwartz, 1975). These are particular examples of the more general case of the absorption of one substance by another through which it can diffuse and with which it can also react chemically. When the chemical complex is immobilized, the diffusion rate of the ligand will be slowed by a factor $1 + (R/K_D)$ (Crank, 1956; Silhavy et al., 1975; Colquhoun et al., 1977) where R is the concentration of binding sites and $K_D = k_-/k_+$, the equilibrium dissociation constant. Thus, on the average,

each dTC molecule will bind k_+ R times, each time for $1/k_-$ seconds. For dTC binding to AChE, R/K_D = 70 μM/30 μM ≈ 2, and dTC inhibition recovers twofold faster after removing the AChE from the cleft. Although the ACh receptors are confined in the postsynaptic membrane, they effectively equilibrate with dTC molecules in the cleft on a time scale of several microseconds because the nerve terminal obstructs diffusion out of the synaptic cleft. Thus, for dTC binding to the ACh receptor, R/K_D = 300 μM/0.5 μM ≈ 600, and dTC's diffusion should be slowed substantially by rebinding. However, after cobra toxin eliminated more than 99% of the receptors, the recovery rate increased maximally 20 fold, far less than expected.

What are curare's molecular reaction rates?

We have provided evidence that the macroscopic kinetics of dTC inhibition are much slower than the molecular reaction rates of dTC binding to the ACh receptor. However, as more receptors are eliminated with toxin, the measured recovery rate, $1/\tau_{off}$, should approach the molecular dissociation rate, k_-. We estimate that k_- exceeds 10 sec^{-1}; and since $K_D = k_-/k_+ = 0.5$ μM for synaptic receptors, the bimolecular association rate constant, k_+, must exceed 2×10^7 $M^{-1}sec^{-1}$. These are both reasonable estimates of rates of ligand-protein interactions, but we suspect that these estimates are lower limits.

The recovery rate also increases about 20 fold after removing the nerve terminal on innervated fibers or extrasynaptic membrane of denervated fibers. In the latter case, there is no nerve terminal and the receptor density is lower; nevertheless, the recovery rate is only about 10 sec^{-1}. We infer that either dTC is still being buffered by rebinding at unknown sites or 10 sec^{-1} is the limit of resolution of the iontophoretic technique. In fact, both alternatives are probably

partially correct for the same reason: dTC is a very hydrophobic molecule. Therefore, it may be expected to partition in the membranes of the cleft in keeping with its notoriety for nonspecific binding. Furthermore, the concentration of dTC in the iontophoretic pipette is low because it is only sparingly soluble in water. Therefore, rather long iontophoretic pulses (50-100 msec) are required to release sufficient dTC to inhibit the ACh response and would obscure dTC action on a faster time scale.

However, other studies lead us to conclude that dTC binds to, and dissociates from, the ACh receptor on a millisecond time scale. These studies involve bath-applied dTC, so the dTC concentration near the receptors reaches a steady-state. Both the frequency of ACh channel opening during voltage-jump relaxations (Sheridan and Lester, 1977) and the peak response to brief iontophoretic ACh pulses (Figs. 11 and 13) are reduced in a competitive fashion by dTC. Thus, dTC must dissociate from the receptor with a rate constant on the order of 1 $msec^{-1}$. This implies that dTC binds to the receptor with an unusually high forward rate constant of 2×10^{9} $M^{-1}sec^{-1}$.

What is the receptor's affinity for curare?

On denervated fibers, extrasynaptic ACh responses are also competitively antagonized by dTC. But the extrasynaptic receptors have a tenfold lower apparent affinity for dTC than do the synaptic receptors (Fig. 12). On denervated rat diaphragm muscle, a similar tenfold difference in dTC affinity between synaptic and extrasynaptic receptors has been observed for dTC antagonism of iontophoretic ACh responses (Beranek and Vyskocil, 1967) and for dTC antagonism of radiolabeled toxin binding to isolated ACh receptors in vitro (Brockes and Hall, 1975). These

data have been interpreted as evidence for a structural difference between synaptic and extrasynaptic receptors.

In contrast, Colquhoun and Rang (1976) observed no difference in dTC's ability to block toxin binding to isolated synaptic and extrasynaptic receptors from the rat diaphragm. They suggested that dTC inhibits synaptic receptors more effectively because the receptors buffer the concentration of dTC in the synaptic cleft. Our results substantiate their interpretation by showing that extrasynaptic receptors recover from dTC pulses more rapidly than do receptors at the nerve-muscle synapse (Fig. 14). When the nerve terminal is removed from innervated fibers, the recovery rate increases; and the above hypothesis predicts that the affinity of these 'exposed' synaptic receptors should be reduced.

In summary, the nerve terminal's barrier to diffusion allows receptors to buffer transiently the concentration of high affinity ligands within the synaptic cleft. Thus, even after a brief pulse of ligand release, repeated binding to receptors may prolong the time course of drug action orders of magnitude longer than the molecular rate constants of binding and dissociation (cf. Parnas, Armstrong and Strumwasser, 1974). Such buffered diffusion has obvious implications for signaling at central synapses where transmitter action is terminated by diffusion out of a synaptic cleft.

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