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THE KINETICS OF TUBOCURARINE ACTION AND RESTRICTED DIFFUSION WITHIN THE SYNAPTIC CLEFT

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SUMMARY

1. The kinetics of tubocurarine inhibition were studied at the post-synaptic membrane of frog skeletal muscle fibres. Acetylcholine (ACh) and (+)-tubocurarine were ionophoresed from twin-barrel micropipettes, and the membrane potential of the muscle fibre was recorded intracellularly. Tubocurarine–receptor binding was measured by decreases in the response to identical pulses of ACh.

2. The responses to both ACh and tubocurarine had brief latencies and reached their maxima rapidly. It is suggested that under these conditions the kinetics of tubocurarine action are not slowed by diffusion in the space outside the synaptic cleft.

3. After a pulse of tubocurarine, recovery from inhibition proceeds along a roughly exponential time course with a rate constant, $1/\tau_{off} \simeq 0.5$ sec $^{-1}$. This rate constant does not depend on the maximal level of inhibition and varies only slightly with temperature ($Q_{10} = 1{\cdot}25$).

4. After a sudden maintained increase in tubocurarine release, the ACh responses decrease and eventually reach a new steady-state level. Inhibition develops exponentially with time and the apparent rate constant, $1/\tau_{on}$, is greater than $1/\tau_{off}$. When the steady-state inhibition reduces the ACh response to $1/n$ of its original level, the data are summarized by the relation, $1/\tau_{on} = n(1/\tau_{off})$.

5. When the ACh sensitivity is reduced with cobra toxin, both $1/\tau_{on}$ and $1/\tau_{off}$ increase. Thus, the kinetics of tubocurarine inhibition depend on the density of ACh receptors in the synaptic cleft.

6. After treatment with collagenase, part of the nerve terminal is displaced and the post-synaptic membrane is exposed directly to the external solution. Under these circumstances, $1/\tau_{off}$ increases more than tenfold.

7. Bath-applied tubocurarine competitively inhibits the responses to brief ionophoretic ACh pulses with an apparent equilibrium dissociation constant, $K = 0{\cdot}5\ \mu$M.

8. In denervated frog muscle fibres, extrasynaptic receptors have a lower apparent affinity for tubocurarine. After a pulse of tubocurarine, inhibition decays tenfold more rapidly at these extrasynaptic sites than at the synapse.

9. It is suggested that each tubocurarine molecule binds repeatedly to several

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ACh receptors before escaping from the synaptic cleft and that the probability of this repetitive binding is enhanced because the nerve terminal presents a physical barrier to diffusion out of the cleft. Consequently, the receptors transiently buffer the concentration of tubocurarine in the cleft, and the macroscopic kinetics of inhibition are much slower than the molecular binding rates for tubocurarine.

INTRODUCTION

Tubocurarine inhibits transmission at nicotinic synapses by combining with acetylcholine (ACh) receptors (Bernard, 1857; Langley, 1905). At equilibrium tubocurarine competitively inhibits ACh-induced depolarizations and conductance increases (Jenkinson, 1960; Adams, 1975; Lester, Changeux & Sheridan, 1975). Fluctuation analysis has revealed that tubocurarine reduces neither the elementary conductance nor the lifetime of individual ACh channels in the membrane (Katz & Miledi, 1972). Instead, tubocurarine reduces the frequency of channel opening (Sheridan & Lester, 1975, 1977). Elapid α-toxins bind specifically to the ACh receptor protein, and this binding also is inhibited competitively by tubocurarine (Moody, Schmidt & Raftery, 1973; Weber & Changeux, 1974; Brockes & Hall, 1975; Colquhoun & Rang, 1976). At high negative potentials, tubocurarine can also antagonize ACh action by binding to and blocking the open ion channel (Ascher, Marty & Neild, 1978; Manalis, 1977; Colquhoun, Dreyer & Sheridan, 1978). However, at physiological potentials tubocurarine binds predominantly to the agonist binding site in a reversible, voltage-independent bimolecular reaction (Lester *et al.* 1975; Colquhoun *et al.* 1978).

There have been no direct studies of the molecular rate constants for tubocurarine binding to the agonist site. Because tubocurarine does not open channels its binding cannot be measured directly with electrophysiological techniques but is inferred from changes in the response to a constant dose of agonist. Del Castillo & Katz (1957) introduced a technique for ionophoresing brief pulses of both agonist and antagonist from twin micropipettes onto the same localized population of receptors. They observed that tubocurarine's inhibitory effect decayed over several seconds, much more slowly than expected from the diffusion of tubocurarine in solution. They suggested that this slow recovery represented the molecular rate of dissociation of the tubocurarine–receptor complex.

Other experiments with frog muscle suggest that tubocurarine dissociates from the receptors rapidly enough to be displaced significantly during brief ionophoretic pulses of ACh (Blackman, Gauldie & Milne, 1975). Further information was obtained from voltage-jump relaxation experiments on *Electrophorous* electroplaques (Sheridan & Lester, 1977). The frequency of channel openings is measured by the dependence of relaxation rate constants on agonist concentration. Elapid α-toxins eliminate part of the receptor population but the opening frequency remains unchanged for the unblocked receptors. In the presence of tubocurarine, on the other hand, the opening frequency decreases for each receptor. This suggests that individual receptors experience a decreased average availability to agonist molecules and that tubocurarine binds to, and dissociates from, the receptor on a *millisecond* time scale (Sheridan & Lester, 1977).

Thus there are apparent discrepancies among the various estimates of tubocurarine binding rates. However, a drug's macroscopic rates of action will be slower than its molecular binding rates if its rate of diffusion is slowed by binding to its own receptors (Crank, 1956; Rang, 1966; Thron & Waud, 1968; Colquhoun, Henderson & Ritchie, 1972; Silhavy, Szmelcman, Boos & Schwartz, 1975). For instance, in the absence of acetylcholinesterase at the nerve-muscle synapse, repetitive binding to receptors reduces the rate of diffusion of ACh from the cleft (Katz & Miledi, 1973; Magleby & Terrar, 1975; Colquhoun, Large & Rang, 1977). This repetitive binding is not surprising considering the high density of ACh receptors in the synaptic cleft (Matthews-Bellinger & Salpeter, 1978); and given the receptor's much higher affinity for tubocurarine (Jenkinson, 1960), its diffusion in the cleft should be slowed substantially more by such repetitive binding (Adams, 1975; Sheridan & Lester, 1977). Therefore, we have investigated how the time course of tubocurarine action is determined by simultaneous diffusion and binding within the synaptic cleft of motor end-plates on frog skeletal muscle. Some of these results have already appeared in abstract form (Armstrong & Lester, 1977).

METHODS

Preparation. Sartorius and cutaneus pectoris muscles were dissected from adult *Rana pipiens* throughout the year. In a few cases, the muscles were denervated 2–6 weeks before the experiment. For denervation, animals were anesthetized in a solution of 5% ether in water, and a few millimetres of the nerve were excised where it entered the muscle. The frogs were kept at room temperature and were fed meal worms.

Muscles were pinned with tungsten wire to a layer of Sylgard (Dow Corning, 184) in the recording chamber. The preparation was 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 cutaneus pectoris fibres was prepared (Dreyer & Peper, 1974*a*) and viewed with Nomarski differential interference contrast optics (McMahan, Spitzer & Peper, 1972).

The standard Ringer solution had the following composition (mM): NaCl, 110; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 4; Na-HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid), 2mM (pH 7–7·2). For bath-applied drugs, we used a push-pull perfusion system made from syringes. Dye measurements showed that the drug concentration in the bath was changed to within a few per cent of that in the reservoir in about 90 sec, usually without disturbing the electrodes. Except where noted, the experiments were conducted at room temperature (19–22 °C). For temperature studies, cool or warm Ringer solution was introduced from the reservoir; data were recorded as the temperature returned to ambient levels.

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

The acetylcholinesterase activity was irreversibly blocked in some experiments by incubating the muscle for 30 min in methanesulphonyl fluoride (5 mM) (Kordaš, Brzin & Majcen, 1975), followed by washing. In other experiments, acetylcholinesterase was removed from the synaptic cleft by applying 50–100 µl. *purified* collagenase (Boehringer Mannheim, 5 mg/ml.) onto the muscle surface (Hall & Kelly, 1971; Betz & Sakmann, 1973). After 20–30 min, the muscle was washed thoroughly with Ringer solution. Histochemical staining (Karnovsky & Roots, 1964) confirmed that esterase activity was eliminated at the synapse.

Electrical arrangements. The membrane potential of the muscle fibre was recorded with conventional intracellular techniques. The intracellular micro-electrodes were filled with 3 M-KCl and had resistances between 5–15 MΩ. ACh and (+)-tubocurarine were ionophoresed from twin-barrel micropipettes (del Castillo & Katz, 1957). Controlled currents were passed

through the ionophoretic pipettes by a circuit containing a high-voltage operational amplifier. The current through each barrel was the sum of a steady braking current and two command pulses with independently variable amplitude and temporal characteristics. A virtual ground circuit maintained the bath electrode (an agar bridge leading to a choride-treated Ag wire) at ground potential and measured the currents flowing through the ionophoretic pipettes.

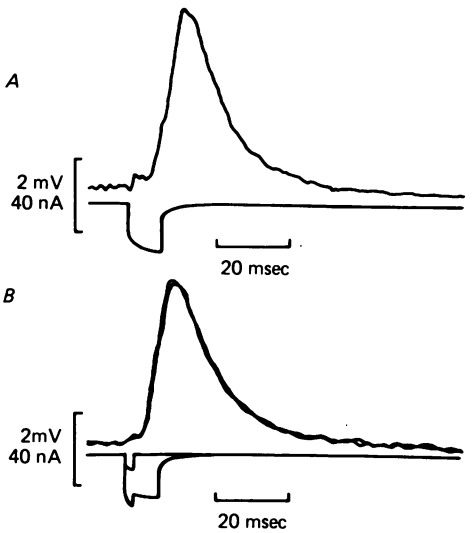

Fig. 1. Electrical coupling between the two barrels of an ionopheretic micropipette filled with glucosamine and ACh. *A*, a pulse of outward current (30 nA × 10 msec) through the glucosamine barrel also releases enough ACh to depolarize the cell. *B*, two superimposed traces. The glucosamine pulse occurs during one sweep; a much smaller ACh pulse (10 nA × 2 msec) occurs during both. A small (3 nA) balance current is applied to the ACh barrel during the glucosamine pulse. The two ACh responses superimpose, showing that the coupling has been balanced out. Records traced from the original photographs.

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 ionophoretic current pulses. For clarity, positive current flowing out of the ionophoretic pipette is displayed as a downward deflexion. The upper traces show the changes in membrane potential in response to these pulses.

Ionophoresis. We have used the high-resolution ionophoretic technique developed at the frog nerve-muscle synapse (Peper & McMahan, 1972; Dreyer & Peper, 1974*b*, *c*; Kuffler & Yoshikami, 1975). Ionophoretic micro-electrodes were constructed from fused twin-barrel capillaries containing a glass fibre in each barrel. One barrel was filled with a solution of ACh chloride (2 M); the other barrel was usually filled with a solution of (+)-tubocurarine chloride (25 mM) and KCl (0·1 M). Although the KCl presumably reduced the tubocurarine's transport number out of the pipette, the lower electrode resistance allowed large currents to be passed reliably. Electrodes were accepted for use if the ACh barrel's resistance was 120–250 MΩ, if the tubocurarine barrel's resistance was 300–600 MΩ and if each barrel reliably passed 100 nA for 50 msec.

Often there was capacitative or resistive coupling between the two barrels of the ionophoretic pipette: an outward current pulse through the tubocurarine barrel also released enough ACh to depolarize the muscle fibre. Coupling was negligible in the opposite direction because the ACh pulses were small and the tubocurarine solution was dilute. The coupling was studied by replacing the tubocurarine solution with D-glucosamine hydrochloride (25 mM) to eliminate inhibitory effects caused by binding of tubocurarine to the receptors (Fig. 1*A*). We found that it was possible to eliminate the coupling artifact by delivering an inward pulse of current to the ACh barrel simultaneously with the glucosamine pulse. This *balancing* current was adjusted to

eliminate the coupling exactly (Fig. 1*B*). Electrical coupling was balanced out in about half the experiments. In other experiments, the ionophoretic electrodes showed negligible interbarrel coupling, and such electrodes gave the same results as those requiring balance currents.

In experiments with single-barrel pipettes, tubocurarine itself produced no change in membrane potential (del Castillo & Katz, 1957). Katz & Miledi (1977) observed that a massive ionophoretic dose of tubocurarine produced a small (< 0.1 mV) local hyperpolarization at end-plates of frog muscle treated with anticholinesterase. In the present study, the largest tubocurarine pulses were 100-fold smaller than those which produced hyperpolarization in the experiments of Katz & Miledi (1977).

Conduct of the experiments. When an apparent nerve terminal had been located visually and the fibre had been impaled, the ionophoretic pipette was positioned until brief pulses (2–5 msec) of ACh produced responses that began with brief latencies (< 5 msec) and rose rapidly to their maxima ($\leqslant 15$ msec) (see Fig. 4). Initially, the braking currents were set at 3–4 nA on each barrel, but at this point they were reduced to the point where leakage of either ACh or tubocurarine was just prevented. For responses between 1 and 10 mV in amplitude, there was always a roughly linear relation between ACh dose (nC passed through the ionophoretic pipette) and response amplitude (mV). ACh sensitivity was measured by the slope of this relation (Kuffler & Yoshikami, 1975); control sensitivities ranged from 200 to 1200 mV/nC depending on the accuracy with which the pipette was positioned.

In some experiments, a portion of the nerve terminal was displaced so that the post-synaptic membrane could be exposed directly to the external solution (Peper & McMahan, 1972; Kuffler & Yoshikami, 1975). For these studies, partially purified collagenase (50–100 μl. of 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 washed through the chamber to prevent interference from neurally released ACh. Even after the preparation was washed thoroughly with Ringer solution, the enzymes continued to act: first the spontaneous miniature end-plate potentials became larger as acetylcholinesterase activity decreased in the cleft. Eventually the miniature end-plate potentials disappeared altogether, signalling that synaptic disjunction had occurred (Betz & Sakmann, 1973). The ionophoretic pipette was used to displace part of the nerve terminal mechanically and was then repositioned to produce responses with brief latencies (< 5 msec).

RESULTS

The kinetics of tubocurarine action

Recovery from inhibition. In the present experiments we have confirmed the observation that several seconds are required for the disappearance of inhibition after ionophoretically applied pulses of tubocurarine (del Castillo & Katz, 1957; Waud, 1967). In the experiment of Fig. 2*A*, for example, a single brief pulse of tubocurarine rapidly produces inhibition; recovery then proceeds along a roughly exponential time course with a time constant, $\tau_{off} \simeq 2{\cdot}5$ sec. At twenty-five synapses, τ_{off} equalled $2{\cdot}07 \pm 0{\cdot}76$ sec (mean $\pm$ S.D.).

Onset of inhibition. When tubocurarine is released at a constant rate for a few seconds, the ACh response is eventually inhibited to a steady-state level. The onset of tubocurarine inhibition is faster than recovery. In Fig. 2*B*, for example, inhibition is established along a roughly exponential time course; but the time constant, τ_{on}, is 120 msec, more than tenfold faster than recovery at that synapse. Such kinetics cannot be measured accurately with trains of identical ACh pulses because desensitization reduces the response to successive pulses at frequences higher than 4 Hz. Instead, data are recorded in a series of episodes. Episodes are repeated at intervals of several seconds. During each episode, one antagonist pulse is delivered at a fixed time, followed after a variable delay by a single ACh pulse. Before and

after the series, control ACh responses are measured with no tubocurarine pulse to verify that the ACh sensitivity recovers completely between episodes and does not change because of electrode movement during the series.

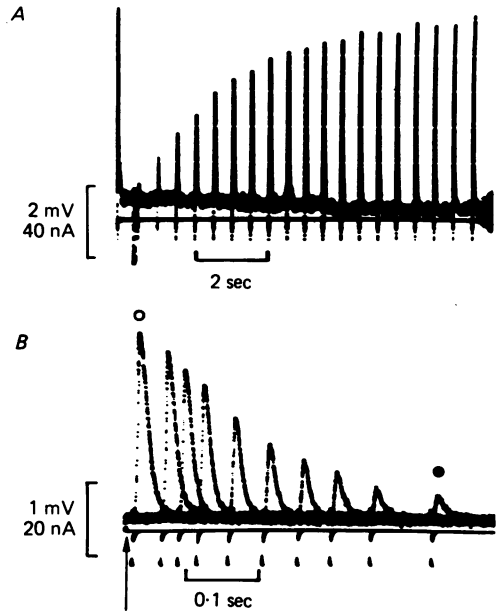

Fig. 2. *A*, recovery from tubocurarine inhibition. One pulse of tubocurarine (40 nA × 50 msec) was delivered during a train of identical brief ACh pulses. Note that on this time scale, the ACh responses appear as vertical lines. *B*, onset of tubocurarine inhibition following a sudden maintained increase in tubocurarine release. The tubocurarine pulse began at the arrow and lasted 1 sec. Episodes were repeated at intervals of 20 sec (see text). ○, control response to ACh pulse alone; ●, response after maintaining tubocurarine release for 20 sec.

In preliminary experiments we recorded ACh responses with longer rise times (> 40 msec), presumably because the ionophoretic pipette released the drugs rather far from the receptors. At these sites, the onset of inhibition was slower than recovery, as in the experiments of Waud (1967). Waud's ionophoretic pipettes were probably also some distance from the receptors, judging by his low ACh sensitivities (~ 4 mV/nC).

Two observations show that the slow inhibitory kinetics do not result artifactually from the use of twin-barrel ionophoretic micropipettes. First, when separate ACh and tubocurarine ionophoretic pipettes are positioned individually at the synapse with Nomarski optics, very similar kinetics are observed. Secondly, another antagonist, hexamethonium, shows much faster kinetics when tested with twin-barrel micropipettes (Fig. 3). After a pulse of hexamethonium, recovery from inhibition proceeds with a time constant of 26 msec, or roughly 100 times faster than after a similar pulse of tubocurarine. In fact the time course of recovery after the hexamethonium pulse is similar to the wave form of the ACh potentials themselves. For events as rapid as this, temporal resolution is limited by the passive time constants of the muscle membrane.

Access to the synaptic cleft

Our experiments are designed to investigate the processes that govern the kinetics of tubocurarine action within the synaptic cleft. This study could be complicated by two kinds of diffusional artifacts associated with the ionophoretic method. First if the ionophoretic pipettes released tubocurarine rather far from the cleft, the kinetics of inhibition would be slowed by the diffusion of tubocurarine through the space

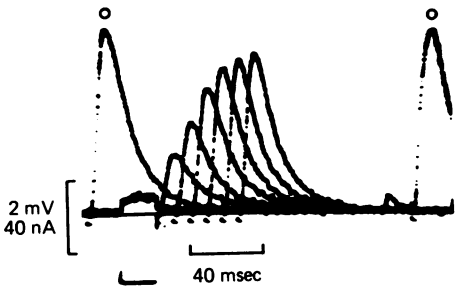

Fig. 3. Recovery from hexamethonium inhibition. Episodes were repeated at intervals of 5 sec. Open circles (○) indicate control responses to the same ACh pulse before and after the series. Note the much briefer time scale than in Fig. 2*A*.

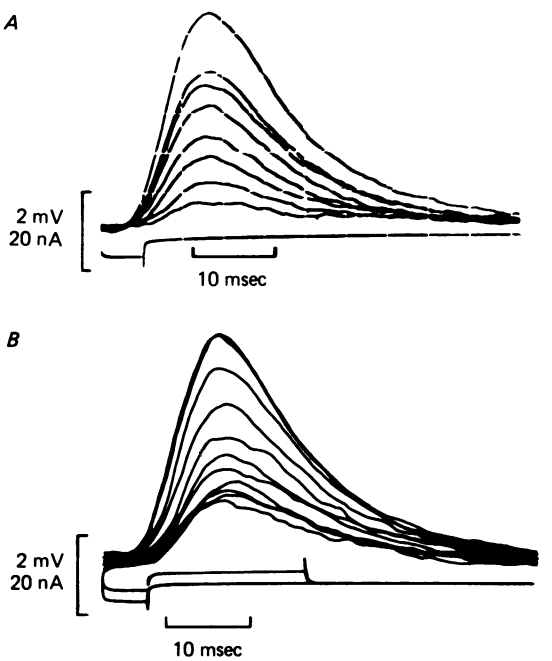

Fig. 4. Wave forms of ACh pulses during tubocurarine action. Two series from the same cell. *A*, recovery from inhibition. Identical ACh pulses were delivered with a variable delay (0, 0·25, 0·5, 1·0, 1·5, 2·0, 2·5, 15 sec) after the end of a long (15 sec) tubocurarine pulse. Episodes were repeated every 30 sec. The smallest and largest responses are steady-state responses in the presence and absence of tubocurarine. τ_{off} = 2·2 sec. *B*, onset of inhibition during a train of eleven ACh pulses at 4 Hz. About 20 msec after the second ACh pulse, tubocurarine release began (5 nA) and continued for the rest of the series. Note that the ACh response reached a steady-state level of inhibition. τ_{on} = 0·67 sec.

outside the cleft. However, with the improved visibility afforded by Nomarski optics, the ionophoretic pipette can be positioned accurately within a few micrometres of the synaptic cleft. To verify this positioning, we have examined the wave forms of individual ACh responses during the onset and recovery from inhibition (Fig. 4). The ACh responses all reach their peaks within 15 msec after the ACh pulse starts.

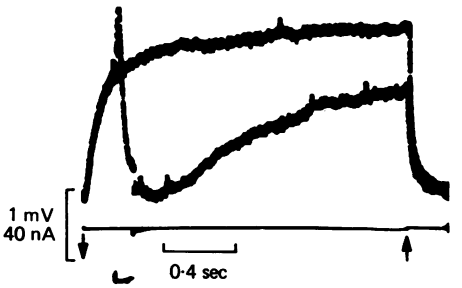

Fig. 5. Action of tubocurarine during prolonged ACh application. Two episodes showing the depolarizing response to a 1 nA reduction of the ACh braking current for about 2 sec (at arrows). In one episode, a pulse of tubocurarine was delivered during ACh application. The small transient depolarization at the beginning of the tubocurarine pulse arose from interbarrel coupling which was not balanced out in this experiment (see Methods). Direct-coupled recording.

Since the diffusion constant for ACh is about 1 μm^2/msec (Dreyer & Peper, 1974*c*), this implies that at its peak most of the ACh response is produced by receptors within 4μm of the ACh pipette. Although tubocurarine is released from the adjacent barrel of the twin pipette, inhibition develops and decays over several seconds. If the time course of inhibition were determined by diffusion through the space outside the synaptic cleft, tubocurarine's diffusion constant would have to be roughly 100 times smaller than that of ACh. But, in fact, the two drugs' diffusion constants differ by less than twofold (del Castillo & Katz, 1957).

Secondly, as inhibition develops near the twin pipette, an increasingly large fraction of the ACh response might be contributed by more distant uninhibited receptors (Feltz & Mallart, 1971). This would slow the apparent onset of inhibition. However, within each series, all the ACh responses begin with uniformly brief latencies (< 5 msec) and take the same time to peak (Fig. 4). One possible explanation might be that tubocurarine speeds the diffusion of ACh; however, this effect is small when the acetylcholinesterase is active (Katz & Miledi, 1973). Thus, ACh is not spreading to more distant uninhibited receptors. Instead, during inhibition, the amplitude of the ACh responses changes as tubocurarine slowly takes its effect over a localized population of receptors.

It would be helpful to have a rough estimate of the fraction of receptors activated by our ACh pulses. At the peak of the response, ACh has diffused less than 4 μm in both directions along a linear terminal, covering a total population of less than 10^5 receptors (Matthews-Bellinger & Salpeter, 1978). Each receptor contributes about 0.25 μV of depolarization (Katz & Miledi, 1972); therefore responses of 1–10 mV peak amplitude involve at least 4–40 % of the nearby receptors.

We have examined the wave form of the response to a tubocurarine pulse against the background of a steady ACh response (Fig. 5). Inhibition begins with a brief latency and rapidly reaches its maximum. Nevertheless, recovery still proceeds

slowly; much more slowly, in fact, than does repolarization at the termination of the much longer ACh pulse. Notice also that at its maximum the tubocurarine completely inhibits the ACh response. Evidently, tubocurarine and ACh are acting on the same localized population of receptors. Furthermore, both drugs begin acting at these receptors within a few milliseconds after their release from the ionophoretic pipette.

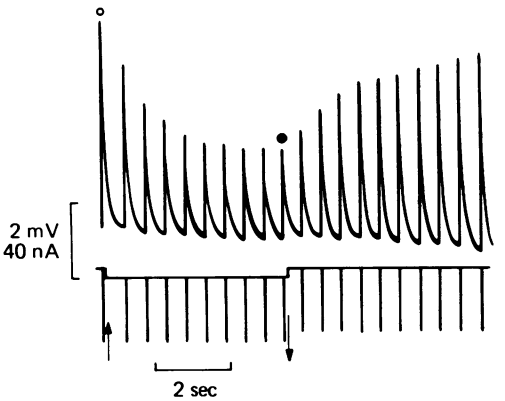

Fig. 6. Time course of inhibition. A train of identical brief ACh pulses is delivered at two pulses per second. A long tubocurarine pulse (↑↓) begins just after the first ACh pulse and lasts 5 sec. ○, ΔV_o; ●, ΔV_∞ (see text). Figure traced from the original photograph.

Most of these receptors must be in the synaptic cleft because ACh receptor density is reduced over two orders of magnitude within just a few microns of the cleft (Kuffler & Yoshikami, 1975; Matthews-Bellinger & Salpeter, 1978). In summary, the inhibitory kinetics we observe are governed by processes within the synaptic cleft.

Relation between the rates and steady-state level of inhibition

We have examined the relationships among the apparent rate constants for onset ($1/\tau_{on}$) and recovery ($1/\tau_{off}$) and the steady-state level of inhibition. Steady-state inhibition is defined as $n = \Delta V_o/\Delta V_\infty$, where ΔV_o and ΔV_∞ are, respectively, the steady-state amplitudes of ACh potentials before and during maintained release of tubocurarine (Fig. 6). That is, tubocurarine reduces the ACh response by n-fold. Presumably n increases with the tubocurarine concentration near the receptors. After the tubocurarine pulse ends, the ACh responses recover to their original amplitude. Semilogarithmic plots of the data (Fig. 7) yield values for $1/\tau_{on}$ and $1/\tau_{off}$.

In general we find that $1/\tau_{off}$, the rate constant for recovery, does not depend on n, the steady-state level of inhibition (Figs. 7*A* and 8*A*). On the other hand, the rate constant of onset, $1/\tau_{on}$, increases with n. The relation is roughly linear, and the constant of proportionality is $1/\tau_{off}$. Over all of the eight synapses studied with these procedures, $1/\tau_{off}$ varies by almost threefold (Fig. 8*B*); but the ratio of $(1/\tau_{on})/n$ to $1/\tau_{off}$ is 0.97 ± 0.15 (mean $\pm$ S.D. of an observation). Therefore, for each synapse the data conform to the empirical relation

$$1/\tau_{on} = n(1/\tau_{off}). \quad (1)$$

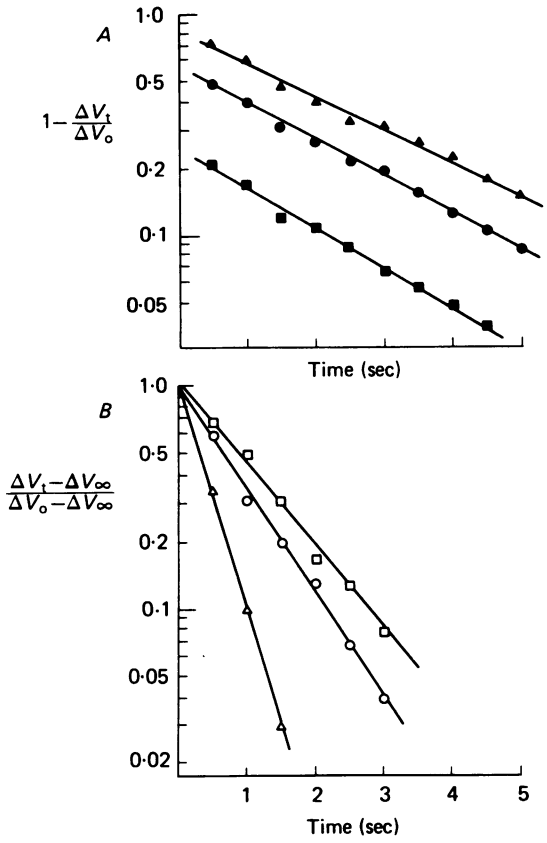

Fig. 7

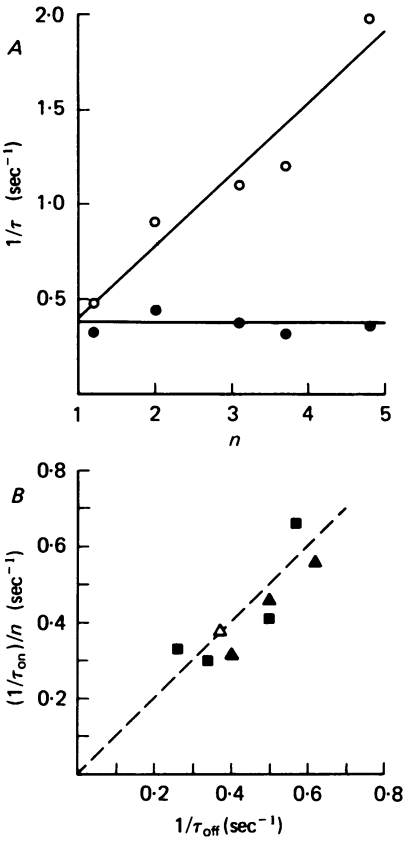

Fig. 8

Fig. 7. Semilogarithmic plots of inhibition versus time for three different levels of tubocurarine release. *A*, recovery plotted as the remaining fraction of steady-state inhibition versus the time after the end of the tubocurarine pulse. *B*, onset of inhibition plotted as the normalized fraction of the original response versus the time after the beginning of the tubocurarine pulse.

Fig. 8. A, Relation between the rates and steady-state level of inhibition in the experiment of Fig. 7: rate constants of onset, $1/\tau_{on}$ (○), and recovery, $1/\tau_{off}$ (●) versus n, an index of the steady-state level of inhibition ($n = \Delta V_0 / \Delta V_\infty$; see text). Straight lines were fitted by the method of least squares. *B*, data from eight end-plates; $(1/\tau_{on})/n$ versus $1/\tau_{off}$. The dashed line indicates a slope of unity. ■, sartorius fibres; ▲, cutaneus pectoris fibres. Δ, fibre from the experiment of Fig. 8*A*.

What determines the kinetics of inhibition?

We consider two explanations for the results presented thus far. In both mechanisms, we assume that tubocurarine (I) binds to the ACh receptor (R) in a reversible bimolecular reaction,

$$I + R \underset{k_-}{\overset{k_+}{\rightleftharpoons}} IR \qquad (2)$$

and the equilibrium dissociation constant K is k_-/k_+. However, the molecular binding rates differ in the two cases.

Rate-limiting binding. If the time course of inhibition were determined by the molecular rates of tubocurarine binding to the receptor, then the macroscopic rates of inhibition would depend on tubocurarine concentration in a predictable manner (Hill, 1909). One assumes that the tubocurarine concentration in the synaptic cleft changes instantaneously on the time scale of the binding reactions. Thus the results of Fig. 2*A* would represent the response of receptors to a short pulse of tubocurarine; and during the recovery the only tubocurarine present would be bound to receptors. Tubocurarine molecules would dissociate from receptor an average of k_-^{-1} seconds after binding and would then diffuse away from the synapse without rebinding. We have

$$1/\tau_{off} = k_-. \tag{3}$$

Likewise, the results of Fig. 2*B* would represent the response of receptors to an instantaneous jump of tubocurarine concentration in the synaptic cleft. If we now assume that the ACh pulses activate only a small fraction of the receptors (see p. 372), we have

$$n = [\mathrm{I}]/K + 1, \tag{4}$$

$$1/\tau_{on} = k_+[\mathrm{I}] + k_- = n(1/\tau_{off}). \tag{5}$$

Eqn. (5) has the same form as eqn. (1) which summarizes the experimental results.

Buffered diffusion. This theory recognizes that one cannot produce a rapid concentration jump of drug near the receptors because diffusion within the synaptic cleft is slowed by binding to receptors. Even though the tubocurarine concentration can be changed rapidly in the space outside the synaptic cleft, the concentration within the cleft rises and falls with the time course of inhibition in the experiments of Fig. 2. The theory has been presented in detail by Rang (1966), Thron & Waud (1968), and Colquhoun *et al.* (1972, 1977).

In the simplest version of this theory, one assumes that the synaptic cleft can be considered a single well-stirred compartment, that the binding reactions proceed much more quickly than the observed kinetics of tubocurarine inhibition, and that the binding sites are simply the drug's receptor sites. In the complete absence of binding sites, tubocurarine would equilibrate with the external solution by diffusion with a time constant τ_D which is proportional to the diffusion coefficient. τ_D is probably of the order of one millisecond (Eccles & Jaeger, 1958; Katz & Miledi, 1973). When M moles of receptor sites are present in a volume V, each tubocurarine molecule binds at a frequency k_+M/V and remains bound for $1/k_-$ seconds. Thus, each molecule spends a fraction of its time bound and unable to diffuse. When most of the receptor sites are not occupied by tubocurarine, the general solution (Colquhoun *et al.* 1972) reduces to an exponential with time constant

$$\tau = (1 + M/KV)\tau_D. \tag{6}$$

The following calculations approximate the real situation in the synaptic cleft. There are roughly 10,000 receptors in each square micrometre of post-synaptic membrane and the height of the cleft is 50 nm (Matthews-Bellinger & Salpeter, 1978); then M/V is 300 μM. For tubocurarine, $K = 0.5\ \mu$M, so that $M/KV = 600$, and τ increases to approximately one second. If tubocurarine is present at $0.5\ \mu$M, its equilibrium dissociation constant, each molecule free within the cleft is in

equilibrium with 300 others bound to receptors. The cleft contains 300 times as much tubocurarine as it would if it had no receptors. Thus the receptors transiently *buffer* the concentration of free tubocurarine molecules in the cleft, and the concentration in the cleft equilibrates with the bulk solution 600 times more slowly than in the absence of buffering. Since innervated fibres have very few receptors outside the synaptic cleft (Kuffler & Yoshikami, 1975; Matthews-Bellinger & Salpeter, 1978), one expects little or no buffering outside the cleft.

For higher tubocurarine concentrations, some of the receptor sites in the cleft will already have tubocurarine molecules bound; therefore the buffering capacity decreases, and inhibition is established more rapidly. Under these conditions the kinetics of inhibition become non-exponential. However, these deviations are not readily apparent when less than half the receptors are occupied (Silhavy *et al.* 1975), and detailed simulations show that the empirical relation, eqn. (1), provides a good summary of the relations among $1/\tau_{on}$, $1/\tau_{off}$, and n (Rang, 1966; Colquhoun *et al.* 1972; Keynes, Bezanilla, Rojas & Taylor, 1975).

Tests for binding versus buffering

In this section we describe experiments that distinguish between the two mechanisms just presented.

The cobra toxin test. Consider the effect of decreasing the number of receptors, M, in the synaptic cleft (Thron & Waud, 1968). If tubocurarine dissociates from the receptor and leaves the cleft without rebinding, one expects no change in the kinetics of inhibition (eqns. (3) and (5)). There is a different prediction if one assumes that buffered diffusion governs the kinetics. Each tubocurarine molecule will bind to fewer receptors before escaping from the cleft; consequently, both diffusion and inhibition will become faster (eqn. (6)).

Elapid α-toxins bind irreversibly to the receptor and block both tubocurarine binding and activation by ACh (Lester, 1972; Maelicke, Fulpius, Klett & Reich, 1977). Both $1/\tau_{on}$ and $1/\tau_{off}$ always increase after exposure to cobra toxin (Fig. 9). We have used the ACh sensitivity of the post-synaptic membrane as a measure of the density of unblocked receptors (Hartzell & Fambrough, 1972; Land, Podleski, Salpeter & Salpeter, 1977). On the average the recovery rate constant increases threefold after the toxin has reduced ACh sensitivity tenfold (Fig. 10). Thus the kinetics of inhibition depend on the receptor density, in support of the buffered diffusion hypothesis.

In addition to the ACh receptors, other molecules in the cleft might also bind tubocurarine and slow its diffusion. An obvious candidate is acetylcholinesterase. One expects less dramatic results from blocking the tubocurarine-esterase interaction than from blocking receptors, since acetylcholinesterase binds tubocurarine roughly fifty times less tightly than do receptors (Mooser & Sigman, 1974) and is present at fivefold lower density (Matthews-Bellinger & Salpeter, 1978). When acetylcholinesterase is inactivated irreversibly with methanesulphonyl fluoride (see Methods), there is no effect on $1/\tau_{off}$. This might be expected if methanesulphonyl fluoride blocks the enzyme's catalytic activity without blocking the peripheral site where tubocurarine binds (Mooser & Sigman, 1974; Taylor & Lappi, 1975). Therefore we used another method to eliminate tubocurarine binding to the acetylcholinesterase in the cleft.

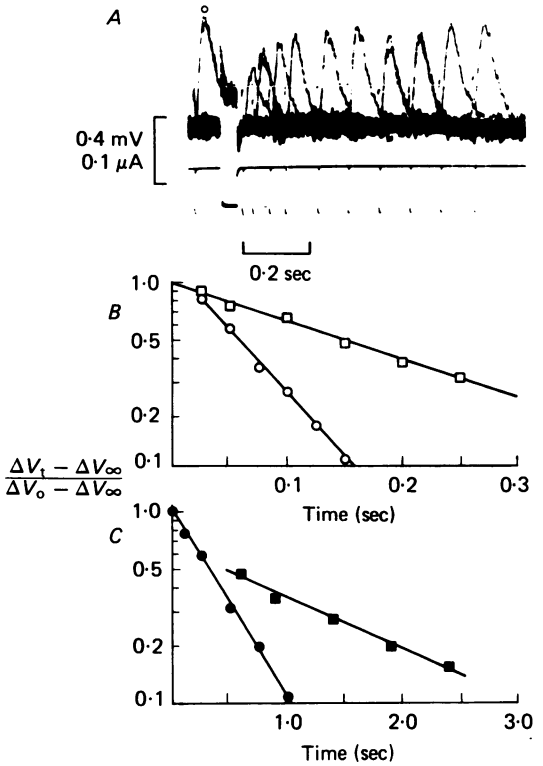

Fig. 9. Cobra toxin increases the rates of inhibition by tubocurarine. *A*, recovery from inhibition. Episodes were repeated at intervals of 30 sec. Open circle (○), two superimposed responses to control ACh pulses before and after the series of episodes. Cobra toxin had reduced the ACh sensitivity at this synapse from 330 to 3·8 mV/nC. *B* and *C*, semilogarithmic plots of the rate of onset (○, ●) and the rate of recovery (□, ■) from inhibition. Note that the lines which do not intersect 1·0 have not been normalized to the steady-state inhibition as it was not measured. *B*, rates before toxin; *C*, rates after toxin. Note the different time scales. Rate constants (sec^{-1}): □, 0·46; ○, 1·5; ■, 6·7; ●, 23.

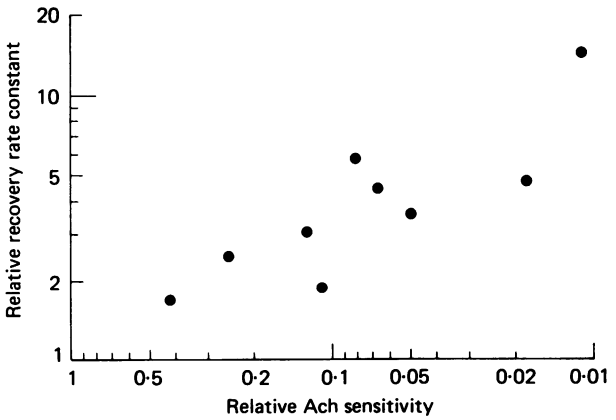

Fig. 10. The relation between receptor density in the synaptic cleft and the recovery rate constant for tubocurarine after exposing the synapse to cobra toxin. $1/\tau_{off}$ *vs.* ACh sensitivity (mV/pC), both normalized to the control values before exposure to toxin.

The collagenase tests. The acetylcholinesterase is removed from the synaptic cleft by the action of collagenase (see Methods). The synapse remains otherwise intact as judged by the presence of miniature end-plate potentials (Fig. 11*A*). The measured value of $1/\tau_{off}$ increases to $1{\cdot}0 \pm 0{\cdot}3$ sec^{-1} (mean $\pm$ S.D., five synapses), or roughly twice as great as the value for normal synapses. At this point, one cannot be certain that this small change is due to suppression of buffering by acetylcholinesterase. If collagenase also removes the basement membrane, tubocurarine's diffusion coefficient could increase.

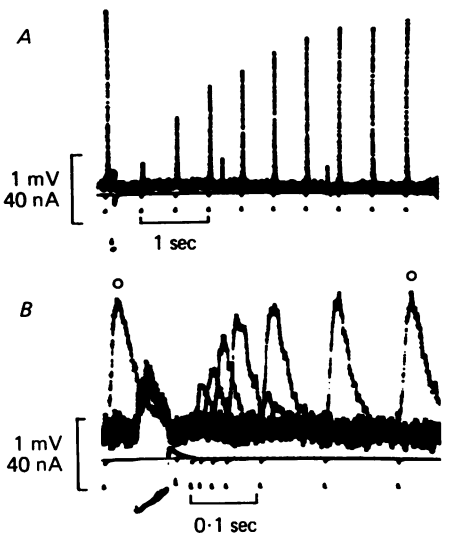

Fig. 11. The action of collagenase on recovery from inhibition by tubocurarine. *A*, recovery after a pulse of tubocurarine (50 nA × 50 msec) in the absence of acetylcholinesterase. Note spontaneous miniature end-plate potentials. *B*, recovery after removal of the nerve terminal.

When enzyme action has progressed to the point where miniature end-plate potentials disappear, it is possible to displace part of the nerve terminal with its overlying Schwann cell from above the post-synaptic membrane (see Methods). These structures present a reflective barrier to the escape of molecules from the synaptic cleft; they thus increase the probability that a tubocurarine molecule will bind to the receptors. It follows that removal of the nerve terminal causes a decrease in the effective concentration of tubocurarine receptors by increasing V, the volume in which tubocurarine is free to diffuse. Neither the protease action nor the removal of the nerve terminal reduces the ACh sensitivity of the post-synaptic membrane; in fact the sensitivity usually increases slightly to values greater than 10^3 mV/nC. However, upon displacement of the nerve terminal, $1/\tau_{off}$ suddenly increases by a further factor of five to ten (Fig. 11*B*). The mean $1/\tau_{off}$ was 9·4 sec^{-1} at four synapses (4·6, 5·4, 11·0, 16·7 sec^{-1}). This is nearly twentyfold faster than recovery at intact synapses. Like the experiments with receptor blockade and esterase removal, this observation is explained only by buffered diffusion.

The temperature test. A high temperature dependence characterizes the binding kinetics of most ligand–protein interactions. At the ACh receptor in particular,

results from several laboratores show that the lifetimes of end-plate channels depend strongly on temperature ($Q_{10} \sim 3$); channel opening rates show a similar dependence (Sheridan & Lester, 1975) although these transition rates could be governed by rate-limiting conformational changes rather than by binding steps. One expects that k_+ and k_- for tubocurarine would have a similar marked dependence on temperature. If molecular binding rates determine the kinetics of inhibition, one therefore expects $1/\tau_{on}$ and $1/\tau_{off}$ to show a high temperature dependence (eqns. (3) and (5)). On the

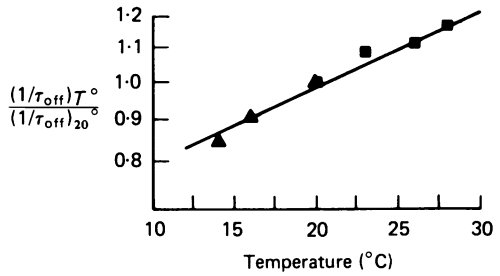

Fig. 12. Temperature dependence of recovery from inhibition. The recovery rate constant, $1/\tau_{off}$, is normalized to $1/\tau_{off}$ at 20 °C and plotted on a logarithmic scale. Line corresponds to a Q_{10} of 1·25. Data from two synapses.

other hand, in the buffered diffusion theory the rates do not depend on the molecular rate constants, but only on their ratio K; this parameter has a Q_{10} near unity (Jenkinson, 1960). Therefore, one predicts that $1/\tau$ depends on temperature only through the rather weak dependence of the diffusion coefficient. This seems to be the case, for $1/\tau_{off}$ has a Q_{10} of only 1.25 in the range from 14° to 28 °C (Fig. 12).

Competitive antagonism on a millisecond time scale

In experiments where agonists and tubocurarine are both applied in the bath to a muscle or an electroplaque, tubocurarine inhibits agonist responses in a competitive fashion (Jenkinson, 1960; Adams, 1975; Lester *et al.* 1975). With bath application, the two drugs are allowed to equilibrate with the receptors for a time much longer than the molecular dissociation rates, so the two drugs compete for the same binding sites. We have presented evidence that tubocurarine dissociates from the receptor in less than 100 msec. The question now arises whether bound tubocurarine molecules dissociate from the receptor rapidly enough to be replaced by ACh during the rising phase (< 15 msec) of responses to brief ionophoretic ACh pulses. If so, one would expect to observe competitive interaction between inophoretically-applied ACh and bath-applied tubocurarine.

The clearest differences are observed between competitive and non-competitive binding when the receptors are saturated with ligand. Unfortunately in electrophysiological studies of drug antagonism, other processes saturate before agonist-receptor binding. For quantitative work the ionophoretic technique presents the further difficulty that the concentration of ACh varies in an unknown way both spatially and temporally. None the less, as expected for a competitive antagonist

(Gaddum, 1937), bath-applied tubocurarine causes a shift of the curve relating peak response and log dose ACh to higher doses; at low tubocurarine concentrations there is no change in the slope of the curves (Fig. 13). There is a small but consistent flattening of the curves at the higher tubocurarine concentrations. This ... 'itional inhibitory

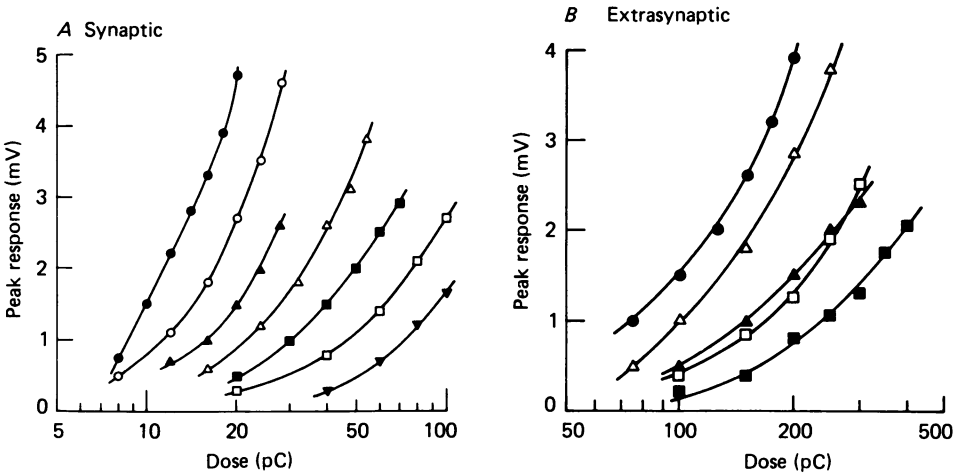

Fig. 13. Dose–response relations for ionophoretically applied ACh in the presence of bath-applied tubocurarine. *A*, synaptic receptors on innervated fibre: ●, no tubocurarine; ○, 0·25 μM; ▲, 0·5 μM; Δ, 1·0 μM; ■, 2·0 μM; □, 3·0 μM; ▼, 4·0 μM. *B*, extrasynaptic receptors on denervated fibre: ●, no tubocurarine; Δ, 2 μM; □, 4 μM; ▲, 6 μM; ■, 8 μM.

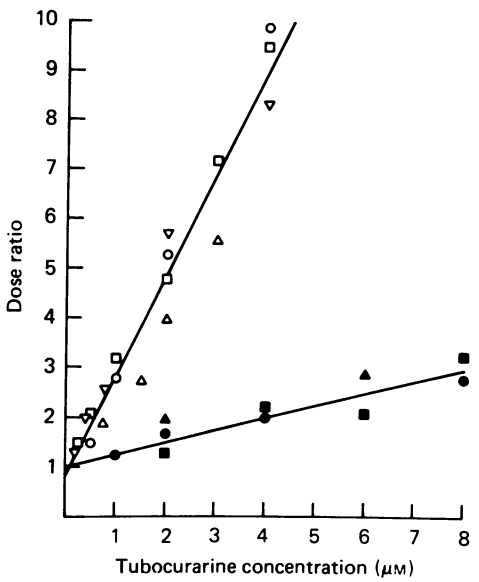

Fig. 14. Dose ratio test of tubocurarine antagonism. K = [I]/(dose ratio − 1). The ratio of ACh doses (pC) required to produce the same response with and without antagonist was measured at responses of about 2 mV. Open symbols, data for synaptic receptors from four innervated fibres: K = 0·5 μM. Closed symbols, data for extrasynaptic receptors from three denervated fibres: K = 4·0 μM. Squares, data from Fig. 13. Straight lines were fitted by the method of least squares.

effect may arise from the blockade of open receptor channels (Manalis, 1977; Colquhoun *et al.* 1978).

We have summarized the dose–response data by plotting the dose ratio (the ratio of the ACh dose, in picocoulombs, required to produce a given response in the presence of tubocurarine to that required in its absence) as a function of tubocurarine concentration (Fig. 14; Gaddum, 1937; Arunlakshana & Schild, 1959; Jenkinson, 1960). The linear plot provides further evidence for competitive inhibition and yields an apparent equilibrium dissociation constant, K, of 0·5 μM. Since this value was also obtained in experiments using bath-applied ACh (Jenkinson, 1960; Adams, 1975), our study seems to be measuring the same tubocurarine–receptor interactions as in those studies. Thus for tubocurarine concentrations up to those that reduce ACh responses by more than tenfold, the tubocurarine–receptor interaction is well described by reversible binding to a site in common with ACh. Buffered diffusion explains how such competitive binding can be reconciled with the slow macroscopic kinetics of inhibition (Gaddum, 1937).

Extrasynaptic receptors on denervated fibres

Extrasynaptic receptors on denervated muscle appear to have a higher dissociation constant, K, for tubocurarine than do synaptic receptors (Figs. 13 and 14), but there is an additional limitation on using the ionophoretic technique to study dose–response relations for extrasynaptic receptors. In such studies, one presumes that the response increases with dose because the agonist concentration increases near the receptors. However, on denervated fibres, the extrasynaptic membrane has a uniform, low sensitivity to ACh and acetylcholinesterase is absent. With ionophoretic pulses, the response might increase even though nearby receptors are saturated because larger doses of ACh spread to more distant receptors and activate them (Feltz & Mallart, 1971). We know this effect occurs in our extrasynaptic ACh responses from denervated fibres, because the times to peak increase from 25 msec in the absence of tubocurarine to 75 msec at the highest tubocurarine concentration (8 μM); and to some extent this must contribute to tubocurarine's apparently lower effectiveness at extrasynaptic receptors.

Of particular interest is the additional finding that extrasynaptic receptors are also characterized by a rapid recovery from tubocurarine inhibition. For three denervated fibres, the mean recovery rate constant, $1/\tau_{off}$, is 8·1 sec^{-1} (6·5, 7·2, 10·7 sec^{-1}). This result is consistent with either of the two mechanisms, binding or buffered diffusion, that might determine the kinetics. In the binding mechanism, $1/\tau_{off}$ is simply k_-; and an increase in this parameter could of course underly the increase in K. Since buffered diffusion has been established for synaptic receptors, it seems more economical to account for the rapid kinetics in terms of this mechanism. With reference to eqn. (6), extrasynaptic regions may have (*a*) a larger equilibrium constant K as noted above; (*b*) a lower receptor density, therefore a lower value of M; (*c*) more exposed receptors, therefore an increase in the value of V by analogy to synapses whose terminals have been removed. The expected result is less repetitive binding, and therefore more rapid kinetics as observed.

DISCUSSION

What are tubocurarine's molecular reaction rates?

The present data show that the macroscopic kinetics of inhibition become faster as a result of experimental manipulations that facilitate the diffusion of tubocurarine near ACh receptors. One concludes that tubocurarine binds to ACh receptors with molecular rate constants substantially greater than the rate constants of its macroscopic action at normal synapses (1 sec^{-1} or so). As one performs manipulations to decrease the probability that a tubocurarine molecule will rebind after dissociating from a receptor, the measured rate constant for recovery, $1/\tau_{off}$, should approach the molecular dissociation rate constant, k_-. In these experiments, the fastest directly measured values for $1/\tau_{off}$ were 10–20 sec^{-1}. We suspect that these values represent the limits of the present technique rather than the actual value for k_-. These limits arise partially because of tubocurarine's relatively hydrophobic nature. Tubocurarine may be expected to partition non-specifically in the membranes of the cleft (Beychok, 1965); thus, the drug's diffusion might be buffered even without ACh receptors. Furthermore, tubocurarine's low aqueous solubility requires the use of low concentrations in the ionophoretic pipette. Therefore, rather long ionophoretic pulses (50–100 msec) are required to release sufficient tubocurarine for measurable inhibition; this would obscure tubocurarine action on a faster time scale. Even in the experiments where the nerve terminal was displaced, binding to the receptors alone would have transiently buffered the local tubocurarine concentration (Purves, 1977). For a receptor density of $10^4/\mu m^2$, $K = 0{\cdot}5\ \mu M$ and molecular equilibrium on a time scale of *milliseconds*, $1/\tau_{off}$ would still not exceed roughly 10 sec^{-1} (Purves, 1977; R. D. Purves, unpublished calculations).

Thus one can conclude that k_- is at least 10 sec^{-1}. Since $K = k_-/k_+ = 0{\cdot}5\ \mu M$ for synaptic receptors, the bimolecular association rate constant, k_+, must be at least 2×10^7 $mole^{-1}$ sec^{-1}. This is the same lower limit inferred for agonist binding rates (Sheridan & Lester, 1977). Other experiments suggest that k_+ and k_- for tubocurarine exceed 10^9 $mole^{-1}$ sec^{-1} and 10^3 sec^{-1}, respectively (Blackman *et al.* 1975; Sheridan & Lester, 1977). Such values are consistent with our observation that tubocurarine competitively inhibits the peak response to brief ionophoretic pulses of ACh; however, if the ACh pulses activated only a small fraction of the receptors in our experiments, tubocurarine may have equilibrated with the receptors much faster than expected from its dissociation rate constant (see Rang, 1966). New methods will be required for direct measurements of tubocurarine's molecular rate constants.

Synaptic versus extrasynaptic receptors

There are also uncertainties about the origin of the different tubocurarine sensitivities of normal and denervated fibres. On denervated fibres, the extrasynaptic receptors have an eightfold lower apparent affinity for tubocurarine than do the synaptic receptors (Fig. 14). On denervated rat diaphragm muscle, a similar difference in tubocurarine affinity between synaptic and extrasynaptic receptors has been observed for the blockade (*a*) of ionophoretic ACh responses (Beránek & Vyskočil, 1967) and (*b*) of α-toxin binding to isolated ACh receptors (Brockes & Hall, 1975).

These data have been interpreted as evidence for a structural difference between synaptic and extrasynaptic receptors. In contrast, Alper, Lowy & Schmidt (1974) and Colquhoun & Rang (1976) observed no difference in tubocurarine's ability to block α-toxin binding to isolated synaptic and extrasynaptic receptors from the rat diaphragm. The latter authors suggested that tubocurarine inhibits synaptic receptors more effectively because the receptors transiently buffer the concentration of tubocurarine in the synaptic cleft (cf. Paton & Waud, 1967). Our results substantiate their interpretation by showing that extrasynaptic receptors recover from tubocurarine pulses more rapidly than do synaptic receptors. The recovery rate also increases when the nerve terminal is removed from innervated fibres; and Colquhoun & Rang's (1976) hypothesis predicts that these *exposed* receptors should have a lower affinity for tubocurarine. However, we find the same apparent dissociation constant, K, for synaptic receptors as did Jenkinson (1960) under equilibrium conditions where buffered diffusion would be negligible. In summary, the present results do not allow us to decide whether the apparent difference in tubocurarine affinity (a) reflects a true molecular difference between synaptic and extrasynaptic receptors, or (b) arises artifactually from the ionophoretic method.

Buffered diffusion as a physiological mechanism

There are other cases where drug kinetics are altered by repetitive binding to membrane-bound receptors (Thron & Waud, 1968; Colquhoun *et al.* 1972; Keynes *et al.* 1975; Silhavy *et al.* 1975). In particular, the effect has been noted already for agonist action at the nerve-muscle synapse when acetylcholinesterase activity is blocked (Katz & Miledi, 1973; Magleby & Terrar, 1975; Colquhoun *et al.* 1977). The effects described here for tubocurarine are much larger, primarily because of tubocurarine's high affinity for the ACh receptor: roughly 100 times greater than that of ACh itself at the resting potential (Dreyer & Peper, 1975; Lester, Koblin & Sheridan, 1978). However, prolonged exposure to ACh increases the receptor's affinity for agonists by nearly this factor (Weber, David-Pfeuty & Changeux, 1975; Colquhoun & Rang, 1976; Weiland, Georgia, Lappi, Chignell & Taylor, 1977) and also causes desensitization on a time scale of seconds (Katz & Thesleff, 1957). Therefore, buffered diffusion may partially determine the observed kinetics of desensitization.

Buffered diffusion could play a role in signalling by naturally occurring transmitters or hormones. Even if transmitter were released within a few milliseconds, repeated binding to receptors may prolong its action for several seconds. A possible example concerns the putative intracellular transmitter in retinal cones. Baylor & Hodgkin (1974) found evidence that this transmitter is inactivated after blocking Na channels and that the inactivation rate constant in turn depends on the level of transmitter. This dependence could arise if the transmitter level were transiently buffered by repetitive binding to saturable receptor sites. For effects on the scale of those seen here, four factors seem necessary: (*a*) a high density of receptor molecules (roughly $10^4/\mu m^2$); (*b*) a restricted space (roughly 50 nm wide) to force repetitive interactions between the transmitter and its receptor; (*c*) a high transmitter-receptor affinity ($K < 1\ \mu M$); and (*d*) the absence of other mechanisms more rapid than diffusion for removal of the transmitter.

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