Relation Between Subsynaptic Receptor Blockade and Response to Quantal Transmitter at the Mouse Neuromuscular Junction

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ABSTRACT When a quantum of transmitter is released into a synaptic cleft, the magnitude of the subsynaptic response depends upon how much transmitter becomes bound to receptors. Theoretical considerations lead to the conclusion that if receptor density is normally high enough that most of the quantal transmitter is captured, subsynaptic quantal responses may be insensitive to receptor blockade. The effectiveness of receptor blockers in depressing the subsynaptic response should be diminished by interference with processes that normally dispose of transmitter, but increased if receptor density is reduced. In conformity with equations derived from a simple mathematical model, the apparent potency of (+)-tubocurarine (dTC) to depress the peak height of miniature end-plate currents (MEPCs) in mouse diaphragm was substantially reduced by poisoning of acetylcholinesterase (AChE) and increased by partial blockade of receptors by immunoglobulin G from patients with myasthenia gravis or α-bungarotoxin. We calculated from the data that normally capture of quantal acetylcholine (ACh) by receptors is ~75% of what it would be if there were no loss of ACh by hydrolysis or diffusion of ACh from the synaptic cleft. This fraction is increased to ~90% by poisoning of AChE. Conversely, it normally requires blockade of ~80% of receptors—and after AChE poisoning, ~90% of receptors—to reduce ACh capture (and MEPC height) by 50%. The apparent potency of dTC to alter MEPC time-course (after AChE poisoning) and to depress responses to superfused carbachol was much greater than its apparent potency to depress MEPC height, but corresponded closely with the potency of dTC to block receptors as calculated from the action of dTC on MEPC height. These results indicate that the amplitude of the response to nerve-applied acetylcholine does not give a direct measure of receptor blockade; it is, in general, to be expected that an alteration of subsynaptic receptor density may not be equally manifest in responses to exogenous and endogenous neurotransmitter.

INTRODUCTION

Katz and Miledi (1973 a) and Hartzell et al. (1975) have pointed out that when acetylcholine (ACh) is released by the motor nerve terminal and enters
the junctional cleft, a large fraction of the ACh may become bound to receptor. A simple consequence that has received little attention is that inactivation, blockade, or loss of receptors should not entail a proportional reduction in the response to ACh released by the nerve terminal. This arises because the ACh molecules in the synaptic cleft are rapidly partitioned, resulting in several possible fates. Some encounter acetylcholinesterase (AChE) and are hydrolyzed, some escape by diffusion, and some become attached to receptor. The net effect of partial blockade of receptors on the postsynaptic response to a quantum of ACh, i.e., on the miniature end-plate current (MEPC), must depend on the relative weights of the different probabilities governing this partitioning. For example, when hydrolysis is removed as a route of ACh disposal by poisoning of AChE, it should require more blockade of receptors to produce a given depression of the MEPCs. Indeed, to take a reductio ad absurdum, if there were no loss or escape of ACh by hydrolysis or diffusion and the affinity of receptors for ACh were sufficiently high, the MEPC would remain the same in size, despite an increasing receptor blockade, for as long as there remained sufficient receptors to capture the ACh in a quantum. It also follows that with an irreversible blockade of a high proportion of the postsynaptic receptors and consequent impairment of the efficiency of ACh capture, any further blockade of receptors by a reversible agent should be more directly reflected in depression of the response to endogenous transmitter. In these experiments we have measured the effects of (+)-tubocurarine (dTC) on amplitude and time-course of MEPCs. The apparent potency of dTC is indeed altered by poisoning of AChE and by irreversible blockade of receptors in the same fashion as predicted. Quantitatively, the data indicate that normally most of the ACh released into the synaptic cleft is rapidly captured by receptor, and that the fraction captured is higher than the fraction of ACh apparently bound to receptors as it diffuses out from the synaptic cleft during the decay phase of MEPCs (Katz and Miledi, 1973a). Moreover, the response of end-plates to a grossly applied cholinergic agonist such as carbachol is much more readily blocked by dTC than the response to endogenous quantal ACh; this is compatible with equal blockade by dTC of receptors involved in responses to exogenous or endogenously released cholinergic agonist.

THEORY

Here we show that elementary theoretical considerations imply that MEPC height should not simply be proportional to density of functional postsynaptic receptors; sensitivity of MEPC height to postsynaptic blockade depends upon the normal efficiency of ACh capture by receptors. This conclusion is unaltered by consideration of a variety of relatively complex (and more realistic) models of postsynaptic events. The equations that are derived provide a basis for estimating the normal efficiency of ACh capture by receptors from observation of the sensitivity of MEPC height to manipulation of this efficiency by receptor blockade and inhibition of ACh hydrolysis (poisoning of AChE).
A Simple Model and Its Predictions

We will begin by considering the simplest possible model of events immediately after release of a quantum of ACh into the synaptic cleft, treating the latter as a single homogeneous compartment in which ACh may be hydrolyzed, escape by diffusion (or perhaps other mechanisms), or bind to receptors. The rate of binding to receptor will be proportional to the subsynaptic receptor density \( R \). Thus, as a first approximation, the fraction of ACh that becomes bound \( F \) will be

\[
F = \frac{aR}{an + B} = \frac{1}{1 + \frac{B}{aR}}. \tag{1}
\]

Here \( a \) is the onward rate constant for combination of ACh with receptor, and \( B \) is a constant that includes the rates at which ACh is hydrolyzed, diffuses, or, perhaps, becomes bound to sites other than activated receptors. On the assumption that synaptic conductance is proportional to the amount of ACh that is bound in such a way as to open ionic channels (cf. Katz and Miledi [1973a]), the conductance and current produced by a single quantum of ACh, i.e., the miniature end-plate current (MEPC) should be related to the receptor density in the following way:

\[
g = g_m F = \frac{g_m}{1 + \frac{B}{aR}}. \tag{2}
\]

Here \( g \) designates the conductance or current at the peak of the MEPC and \( g_m \) represents what this value would be if there were no loss of ACh by hydrolysis or diffusion, i.e., if all the ACh became associated with activated receptor. It follows that if \( aR \) is normally much more than \( B \), so that most of the ACh is quickly bound, \( R \) can be considerably reduced with relatively small change in \( g \). This is true either for a reversible or irreversible reduction of \( R \). Conversely, reduction of \( B \) will also have little effect on MEPC height \( g \) if \( aR \) is normally much more than \( B \). The percentage increase in MEPC size that results from reducing \( B \) by poisoning of AChE will be greater the less the efficiency of ACh capture by receptors. Thus, there are two methods by which efficiency may be estimated:

(A) From the increase of MEPC height by AChE poisoning:

Writing \( \beta_a \) for \( B \) when hydrolysis does not occur, \( \beta_h \) for \( (B - \beta_a) \) and \( g_a \) and \( g_d \) for the MEPC height before and after poisoning AChE, then from Eq. 2

\[
g_a/g_n = \frac{(aR + \beta_h + \beta_a)}{(aR + \beta_a)}. \tag{3}
\]

Rearranging, then substituting for \( aR \) one obtains

\[
g_a/g_n = \frac{(\beta_h + \beta_a)}{\beta_a[\beta_a/g_m]} \tag{3}.
\]

Thus, when \( g_a/g_m \) is reduced, by reversible or irreversible blockade of receptors, the percentage increase of MEPC size produced by poisoning of AChE should be made larger. A graph of \( (g_a/g_n)^{-1} \) vs. \( g_a \) should be linear, with an intercept at \( g_a = 0 \) of \( \beta_h/(\beta_h + \beta_a) \) and the extrapolated value of \( g_a \) at \( g_a/g_n = 1 \) should give \( g_m \).
(B) From the altered apparent potency of a reversible receptor antagonist when \( \beta \) and/or \( R \) is altered, either by poisoning AChE or by irreversibly blocking receptors:

On the assumption that the reversible antagonist will occupy available receptors to an extent that is unaffected by changes in \( \beta \) or \( R \), the degree of receptor blockade will be a reproducible function of \( I \), the concentration of antagonist. For an antagonist for which the rate of dissociation from the receptor is slow relative to the duration of the MEPC one may write

\[
R = R_0/[1 + f(I)],
\]

where \( I \) is the concentration of antagonist, and \( R \) is the effective receptor density during the time that quantal transmitter is present in the cleft. Here, \( R_0 \) is the density of unblocked receptors in the absence of antagonist, and \( f(I) \) is a function of \( I \) that depends upon the mode of interaction of \( I \) with receptor. Only for the particular case of an antagonist that occupies receptor on a one-to-one basis is \( f(I) \) equal to \( I/K_1 \), where \( K_1 \) is the antagonist dissociation constant.

By substitution in Eq. 2

\[
g/g_m = (1 + [1 + f(I)]\beta/\langle\alpha R_0\rangle)^{-1}. \tag{4}
\]

Writing \( g_0 \) for the height of the MEPC in the absence of reversible antagonist and \( g_I \) for the height when the antagonist is present, the relative depression of the MEPC height by the antagonist will be related to \( \beta \) and \( R_0 \) as follows:

\[
\frac{g_0}{g_I} = \frac{1 + [1 + f(I)]\beta/\langle\alpha R_0\rangle}{1 + \beta/\langle\alpha R_0\rangle} = 1 + f(I) - f(I)/[1 + \beta/\langle\alpha R_0\rangle].
\]

From Eq. 2,

\[
[1 + \beta/\langle\alpha R_0\rangle]^{-1} = g_0/g_m,
\]

hence,

\[
g_0/g_I = 1 + f(I) (1 - g_0/g_m) = 1 + (R_0/R - 1) (1 - F_0) \tag{5}
\]

\[
f(I) = (g_0/g_I - 1)/(1 - g_0/g_m) \tag{5a}
\]

\[
R/R_0 = [1 + f(I)]^{-1} = (g_0^{-1} - g_m^{-1})/(g_I^{-1} - g_m^{-1}). \tag{5b}
\]

From Eq. 5, it is again evident that the effectiveness of the antagonist to reduce transmitter capture, and hence MEPC height is dependent on the initial efficiency of capture \( (g_0/g_m) \). The particular \( I \) at which MEPC height is reduced by 50%, i.e., its IC50, will increase with \( g_0 \). When \( f(I) \) is simply \( I/K_1 \), the IC50 will be inversely proportional to \( 1 - g_0/g_m \). This relationship might then be used to estimate \( g_m \) from the graph of \( (IC50)^{-1} \) vs. \( g_0 \) when \( g_0 \) is varied. However, the functional relation between \( I \) and \( f(I) \) need not be known to estimate \( g_m \) from alteration of \( g \) by the antagonist. Writing \( g_0' \) and \( g_I' \) and \( g_0'' \) and \( g_I'' \) for the MEPC heights at two different \( \beta \) and/or \( R \) in the absence of antagonist and at any defined concentration of the antagonist, one
has, from Eq. 5,
\[
\frac{g_0/g'_1 - 1}{g''/g''_1 - 1} = \frac{1 - g_0/g_m}{1 - g''/g''_m},
\]
and hence,
\[
g''_0(g_0/g'_1 - 1) - g_0(g''_0/g''_1 - 1) = g_m(g_0/g'_1 - g''_0/g''_1).
\] (6)

The same expression applies if \( g_0 \) and \( g''_0 \) are MEPC heights at one concentration of inhibitor and \( g'_1 \) and \( g''_1 \) the heights at another concentration of inhibitor. From this equation, a graph of the expression on the left vs. the expression in brackets on the right should be linear with a slope of \( g_m \). Once \( g_m \) is known, use of Eq. 5a allows determination of \( f(I) \) as well as the normal efficiency of ACh capture.

It should be noted that the validity of Eq. 6 depends only on (a) the validity of Eq. 2, i.e., that the inverse of MEPC height be linearly related to the inverse of the density of available receptors, and (b) the validity of the assumption that the interaction of antagonist with receptors is not influenced by changes in \( B \) (i.e., by poisoning of AChE) or by changes in \( R \) produced by an irreversible antagonist.

**More Complex Models**

The derivation of Eq. 2 represents a drastic simplification of the actual events that occur after discharge of transmitter into the synaptic cleft. In particular, it ignores (a) the dynamics of ACh association and dissociation from receptor during the rising phase of the MEPC, (b) diffusion of ACh within the cleft, (c) the possibility of local saturation of receptors, (d) cooperativity in ACh-receptor interaction, and (e) the possibility that ACh may be captured by receptors that are unable to open ionic channels. We have therefore calculated (using numerical integration of differential equations on a computer) the binding of ACh to receptors and generation of MEPC with models of increasing complexity, incorporating the above factors.

In general, we have found that when any or all the complicating factors are taken into account, and over a wide range of arbitrarily chosen parameters, the relation between MEPC amplitude and effective receptor density always remains very close to that given in Eq. 2. With variation of \( R_0 \) (partial irreversible blockade of receptors), variation of \( R \) (additional blockade by a reversible inhibitor), and reduction of \( B \) (poisoning of AChE), graphs of \( 1/g \) vs. \( R_0/R \) always give straight lines that meet close to \( R_0/R = 0 \) and \( 1/g = 1/g_m \). Fig. 1 shows such graphs for three models. In Fig. 1A, a single-compartment model in which ACh combines with receptor reversibly (to form acetylcholine receptor [AchR]), and either diffuses away or is hydrolyzed is shown. The total amount of receptor is presumed to be so great that it is not changed as a result of combination with ACh. This model can be solved explicitly. In Fig. 1B, an eight-compartment model in which ACh abruptly enters a central compartment and then diffuses outwards is shown. In each compartment, it interacts reversibly with a limited quantity of receptor. In
FIGURE 1. The relation between the inverse of computed MEPC peak height and $R_0/R - 1$ is plotted for three different models of the junction: A, B, and C. (A) One compartment, reversible attachment of ACh to receptor to form active complex, AChR*, no receptor saturation. (B) Eight concentric compartments with ACh diffusing from center, interacting with receptor in every compartment to form AChR*—receptors can become saturated. (C) Same model as B except active form of receptor is ACh2R*. In each case the lower pair of lines (○, ●) represent calculated points for control conditions and the upper pair of lines (□, ■) values calculated using the same parameters except receptor density ($R_0$) reduced to 25 of control. $R_0/R$ is presumed to be varied by a reversible antagonist with high affinity for the receptor. The open symbols represent values calculated assuming hydrolysis of transmitter in the cleft (AChE intact); filled symbols represent values calculated with transmitter loss from the cleft only by diffusion, i.e., corresponding to AChE poisoned. The abscissa is $R_0/R - 1$ rather than $R_0/R$ because values of $R_0/R < 1$ cannot be arrived at by altering $R$. Note that in the case of an inhibitor (I) that blocks receptors on a one-to-one basis $R_0/R - 1 = I/K_i$, where $K_i$ is the dissociation constant of the inhibitor-receptor complex.
this case it is assumed that attachment of only one ACh molecule is sufficient to activate receptor. Fig. 1 C shows the same as Fig. 1 B except that two ACh molecules are needed to activate receptor. In this last model, which, of the three, is the only realistic one for the neuromuscular junction (cf. Rosenberry [1979]; Dreyer et al. [1978]; Sheridan and Lester [1977]), each ACh molecule interacts with one subunit of the receptor and an isomerization occurs, leading to an open ionic channel, only when both subunits are occupied. This stabilizes the binding of ACh so that the dissociation rate becomes equivalent to the rate of channel closing.

The latter two models were essentially similar to those considered by Rosenberry (1979) except for the employment of more compartments for the simulation and with the last model calculated MEPCs were essentially the same as those of Wathey et al. (1979). The values shown in Fig. 1 C are from model MEPCs that roughly approximated true MEPCs, and the parameters used were close to those employed by Wathey et al. (1979); the receptor target radius was 1.1 μm, i.e., any transmitter diffusing >1.1 μm from its point of origin was assumed lost to the system. In this case, the rate constants were such that binding rather than isomerization was rate-limiting. However, very similar plots were obtained with models in which isomerization was rate-limiting (cf. Adams [1980]), and, in general, the form of plots such as those in Fig. 1 C was quite insensitive to the parameters that were chosen, over a very wide range. In particular it may be noted that there was little effect of altering the rate constants of the first ACh receptor interaction, provided these were sufficiently rapid, i.e., the extent of binding of ACh in a transitory form to receptor had little influence. With all the models tested the increase of MEPC height produced by eliminating AChE always conformed closely to Eq. 3, i.e., the percentage increase of height was made larger when MEPCs were made smaller by reduction of receptor density, and plots of \( g_a / g_d \) vs. \( g_a \) were linear. However, derived values of \( \beta / (\beta_h + \beta_a) \) did not correspond in any simple way to the relative importance of hydrolysis and diffusion in disposing of the transmitter, with models incorporating cooperativity. Moreover, the extrapolated maximum possible MEPC height, \( g_{m} \), was not the same as the number of transmitter molecules divided by the number necessary to activate a receptor. Typically, for models in which two ACh molecules are needed to open a channel, \( g_{m} \) (by extrapolation) was at most 3,500 channels, for a quantum containing 10,000 molecules of ACh, (Hartzell et al., 1975). This difference arises because any set of parameters that can produce an MEPC that is rapidly rising but not overly prolonged relative to channel duration also implies that at the time of the MEPC peak there is still a considerable quantity of ACh attached to receptor in a closed conformation, i.e., AChR or ACh2R in a form not yet isomerized to the active form. Values of \( g / g_{m} \) are close to, but not synonymous with, the fraction of released transmitter that is bound to activatable receptors at the peak of the MEPC.

We have also calculated, with the above models, the net effect on MEPC height (and time-course) of (a) a competitive antagonist that dissociates very rapidly from the receptor, and (b) what would be expected if there were
transmitter-binding sites that were not activatable receptors. In general, either modification made little difference. Provided the affinity of receptor for antagonist is sufficiently high that at equilibrium most of the total antagonist in the cleft is attached to receptors, the behavior is virtually identical to that found with a slowly dissociating (or irreversible) antagonist. This occurs because no substantial displacement of antagonist from receptor can take place without a large consequent increase in free antagonist in the cleft. In the case of extra binding sites for ACh, if the rate constant of dissociation was made rapid relative to dissociation of transmitter from the active form of the receptor, the net effect was equivalent to an increase of $\beta$ in Eq. 1. In the case of binding sites that interact with transmitter and antagonists in the same way as receptors but do not act to open channels (i.e., "silent" receptors) the net effect was merely a constant fractional reduction in the MEPC.

It may also be remarked that with all these models one-half-maximal reduction of the time constant of the decay phase of MEPCs (after AChE poisoning) by receptor blockade was associated with one-half-maximal occupation of receptors by the blocker (see Eq. 8, below).

Still More Complex Models

In the models already considered, the number of ACh-binding sites on a receptor was the same as the number of ACh molecules required to initiate the conformational change associated with opening of a channel, and in the presence of an antagonist, the receptor was either blocked or unblocked. It is possible, however, that the number of binding sites is more than the number of ACh molecules needed to activate them, and that an antagonist may combine with one or more of these sites without entirely excluding activation of the receptor by ACh. In such a case, one loses the simple distinction between blocked and unblocked receptors. Numerical evaluation of the consequences of such models is extremely slow because of the large number of receptor species in each compartment and the many ways in which cooperativity may be specified. Nevertheless, from those cases for which we have created models, we feel that it can be stated that the model complexity does not result in behavior substantially different from that of simpler models in that Eqs. 2–6 remain applicable.

METHODS

All experiments were performed on mouse hemidiaphragms, mounted on Sylgard (Dow Corning Corp., Midland, Mich.). End-plate regions were directly superfused (Cooke and Quastel, 1973) with solution bubbled with 95% O2-5% CO2, at 25–27°C. The standard solution used had the following composition: Na+ (150 mM), Ca2+ (2 mM), Mg2+ (1 mM), K+ (5 mM), HCO3- (24 mM), H2PO4- (1 mM), NO3- (135 mM), glucose (11 mM). NO3- was employed instead of Cl-, to improve the signal-to-noise ratio of the MEPCs (Linder and Quastel, 1978). In addition, tetrodotoxin (Sankyo Co. Ltd., Tokyo; 2 × 10^-6 g/ml) was used to eliminate the occasional muscle twitching that otherwise occurred when miniature end-plate potentials (MEPPs) were made large by inhibition of AChE. In many of the experiments, [K+] was raised to 10 mM, to increase MEPP frequency and therefore permit faster acquisition of data. Control
experiments showed that neither this nor the substitution of NO$_3^-$ for Cl$^-$ had any effect on MEPC time-course or amplitude (Linder and Quastel, 1978).

Voltage Clamp

The techniques employed for the two-electrode point voltage-clamp were essentially conventional (Takeuchi and Takeuchi, 1959) using microelectrodes filled with 3 M KCl. That both electrode tips were located in the same muscle fiber and close to an endplate was judged from the appearance on the records from both electrodes of identical, fast-rising MEPPs. During operation of the voltage clamp, the total gain of the feedback loop was usually ~4,000:1 (direct current~1 kHz), and there were never any detectable voltage signals corresponding to the MEPCs recorded by the current monitor. Membrane potential was always maintained within 1 mV of the holding potential.

Recording

To improve the signal-to-noise ratio of MEPCs and therefore to permit the computer to identify and store MEPCs without selection as to amplitude (except for the very smallest population—cf. Cooke and Quastel [1973]; Kriebel and Gross [1974]), the signal from the current monitor was put through a running-average (modified Paynter) filter; filters constructed for averaging times of 0.1 ms, 0.2 ms, and 0.5 ms were used in conjunction with analog-digital (A-D) conversion at frequencies of 10 kHz, 5 kHz, and 2 kHz, respectively.

Before going to the A-D converter of a PDP-12 computer (Digital Equipment Corp., Maynard, Mass.) the current signal was amplified and filtered further with simple resistance-capacitance filters set at 0.1 Hz (lower limit) and either 3 kHz, 1 kHz, or 0.3 kHz (upper limit). In nearly all experiments, the upper limit was set at 3 kHz or 1 kHz—the 1-kHz setting reduced the noisiness of the signal but introduced a need for appreciable digital correction of the records (see below). The 0.3-kHz filter quite severely distorted the rising phase of MEPCs, but was occasionally useful for the recording of MEPCs that would otherwise have been too small to be identified as MEPCs by the computer program.

Averaging of MEPCs

The major problem encountered in averaging MEPCs (at least 25 and usually ~40 in each group) was to establish a time reference for each signal in such a way as to superimpose the steepest (i.e., initial) portion of the rising phases as closely as possible. The method finally adopted was to find the point of maximal cross-correlation with a ramp function (0,0,...0, 1, 2). This gave MEPC averages with total rise time slightly less than, and peak amplitude slightly greater than, those obtained by using either of two other fairly satisfactory criteria for definition of the time reference: (a) the pair of
points in the rising phase with maximum difference, or (b) the group of points (three or four) in the rising phase with maximum variance.

The height, rate of rise, and rate of decay of an MEPC average were essentially identical to the averages of the same parameters of individual MEPCs, except where AChE had been poisoned, in which case MEPCs varied greatly in time-course and time-course and height were positively correlated (Hartzell et al. 1975).

**Calculation of MEPC Parameters**

All calculations were done on the PDP-12 computer directly from the MEPCs and/or from averages of MEPCs stored on digital tape. Always, before further calculations were made, the baseline (an average of 25 points before the rise of the MEPC) was subtracted and all points were corrected for the high-frequency filtering employed during recording, using either of two algorithms: (a) replacement of each value $X_i$ by $(X_i - Z \cdot X_{i-1})/(1 - Z)$, where $Z = \exp (-\Delta t/\tau_f)$, $\Delta t$ is the time between samples, and $\tau_f$ is the time constant corresponding to the high frequency filter, or (b) replacement of each value $X_i$ by $X_i + \tau_f(X_{i+1} - X_{i-1})/(2\Delta t)$. The result of the second method was about equivalent to that of the first followed by a running two-point smoothing and was employed when parameters were to be determined on individual MEPCs. With individual MEPCs recorded after poisoning AChE, the height was defined as the peak average of three sequential points; with these MEPCs, time constants of decay were sufficiently long relative to the frequency of A-D sampling that the MEPC peak corresponded to a plateau of at least three nearly equal points.

Data were accepted as being from a focally placed clamp only if the maximum rate of rise of the average of the MEPCs in a sequence, corrected for filtering, was at least two-thirds of the peak height in 0.2 ms, when MEPCs were sampled at 10 kHz, or 80% of peak height in 0.4 ms, when sampling was 5 kHz. When AChE was poisoned, the rise time of MEPCs was somewhat prolonged; MEPCs were recorded at 5 kHz and were accepted as focal if two-thirds of peak height was achieved in 0.4 ms. These limits excluded ~25% of records that had been tentatively accepted as “focal” at the time of recording.

The rate of decay of MEPCs was determined by a least-squares procedure applied to points of the MEPC lying between $e^{-0.5}$ and $e^{-1.5}$ of the MEPC peak amplitude. The lack of bias of this procedure was verified by applying it to computer-generated series of points following predetermined rates of exponential decay with added white or filtered noise of various magnitudes.

**Responses of End Plates to Carbachol**

In some experiments carbachol was applied (by local superperfusion) to endplates and the ensuing depolarization ($\Delta V$) measured. Experiments were limited to junctions that gave a prompt response to carbachol and returned to control value upon withdrawing carbachol. To correct for nonlinearity of the postsynaptic response, the following equation was used

$$\Delta G/G = \frac{-\Delta V}{V_m + \Delta V} \exp \left[ 0.0075 (80 + V_m + \Delta V) \right].$$

Here $V_m$ is the control membrane potential. This equation corresponds to the correction of Martin (1955) with the assumption of a transmitter equilibrium potential of 0 (Linder and Quastel, 1978) and with the addition of a term to adjust for voltage sensitivity of the mean duration of the ionic conductance channels (Anderson and Stevens, 1973); it was assumed that channels opened by carbachol have the same
voltage sensitivity as those opened by ACh. In principle, the result of this correction should be proportional to the conductance that would have been measured if the muscle fiber had been clamped at $-80 \text{ mV}$.

RESULTS

Preliminary Observations

The theoretical treatment presented above made several predictions capable of experimental verification: (a) The apparent potency of a slowly reversible antagonist should be reduced by poisoning of acetylcholinesterase (AChE), and increased by irreversible blockade of receptors (Eq. 5). (b) The increase of MEPC height produced by poisoning of AChE should increase when MEPCs are made small by either a reversible or irreversible receptor blocker (Eq. 3). (c) Eq. 6 should apply. It was therefore necessary to find agents capable of producing uncomplicated AChE poisoning, and reversible and irreversible receptor blockade.

For poisoning of AChE we found the irreversible agent paraoxon (4 #M applied for a period of 4 min) to be suitable; this yielded MEPCs prolonged in time-course and no further prolonged by application of Prostigmin (or more paraoxon), and that were unchanged for the duration of an experiment. Frequency of MEPCs was increased about twofold, and higher concentrations or more-prolonged exposure to paraoxon caused muscle contracture presumably related to partial depolarization of muscle fibers (cf. Laskowski et al., [1975]; Laskowski and Dettbarn [1979]). Prostigmin itself we found to be only slowly reversible and to give no practical advantage.

As a reversible competitive inhibitor we chose (+)-tubocurarine (dTC), since the dissociation constant of the receptor dTC complex is known to be $\sim 100 \text{ nM}$ (Lu, 1970; Waud et al., 1973). Since the “concentration” of ACh receptors is $\sim 1 \text{ mM}$ (cf. Wathey et al. [1979] and Discussion) the great majority of dTC molecules in the synaptic cleft at concentrations $<< 1 \text{ mM}$ must be attached to receptors. Moreover, dTC is known not to have an appreciable local-anesthetic-like action at concentrations $< 1 \mu\text{M}$ (Katz and Miledi, 1978; Colquhoun et al., 1979). Preliminary experiments showed that the reduction of MEPC amplitude produced by superperfusion with dTC was maximal within a few minutes. At $\leq 0.5 \mu\text{M}$ dTC we could find no alteration of voltage sensitivity of MEPCs and no alteration of MEPC time-course not attributable simply to receptor blockade.

For effectively irreversible blockade of receptors, our initial choice was $\alpha$-bungaratoxin ($\alpha$-BuTX) (Chang and Lee, 1963; Katz and Miledi, 1973b; Magleby and Terrar, 1975), which almost certainly has the same binding site as ACh (Colquhoun and Rang, 1976). However, superperfusion with $\alpha$-BuTX produced very uneven blockade; MEPCs at different junctions varied between normal in size and nearly invisible. Injection of mice with $\alpha$-BuTX was rather unpredictable in effect, giving either too little or too much blockade. The results with $\alpha$-BuTX shown below are from one diaphragm, from a mouse injected with $\alpha$-BuTX, where it was possible to record uniformly small MEPCs before and after poisoning of AChE and in the presence of dTC.
As an alternative to α-BuTX we tried injection of mice with immunoglobulin G (IgG) from patients with myasthenia gravis, which had been reported to give rise to MEPPs of about one-half normal size (Toyka et al., 1975); it was found that after repeated daily injections for a few days (from a sample given to us by Dr. D. B. Drachman) MEPPs and MEPCs in mouse diaphragms were uniformly moderately reduced in size and responded to poisoning of AChE or dTC much as predicted for an irreversible receptor blockade. However, the rate of decay of MEPCs after AChE poisoning was not as fast as in MEPCs reduced to a similar height by dTC (Pennefather and Quastel, 1980). This was interpreted as indicating some continued ability of blocked receptor to bind ACh, because adding dTC brought the rate of decay to the same limiting rate as observed with dTC in control diaphragms, and it is known that the IgG binding to receptor does not necessarily preclude binding of α-BuTX (Lindstrom and Lambert, 1978; Mittag et al., 1978). As pointed out in Theory, such transmitter binding does not negate the applicability of Eq. 2, provided the complex is short-lived.

Modification of MEPCs by Receptor Blockade and AChE Poisoning

Table I lists the mean amplitudes and time constants of decay of MEPCs, recorded at −80 mV, in control diaphragms, diaphragms from animals

| Table I | MEPC AMPLITUDE, "TIME CONSTANT" OF DECAY, AND SENSITIVITY TO (+)-TUBOCURARINE: MODIFICATIONS BY POISONING OF AChE, IgG FROM MYASTHENIA PATIENTS, AND α-BuTX |
|---------|---------------------------------------------------------------------------------------------------------------|
|         | IgG from myasthenia patients                                                                                     | Untreated  | 2 d  | 4 d  | α-BuTX (5 μg i.v.) |
| MEPC amplitude |                                                                                                               |            |     |     |                   |
| Control       |                                                                                                               | 4.03 ± 0.06 nA (119) | 3.11 ± 0.14 nA (11) | 2.17 ± 0.08 nA (8) | 2.96 ± 0.13 nA (11) |
| After paraoxon |                                                                                                               | 4.67 ± 0.13 nA (24) | 3.90 ± 0.13 nA (7) | 3.06 ± 0.19 nA (9) | 3.12 ± 0.12 nA (13) |
| MEPC "time constant" |                                                                                                               | 1.13 ± 0.02 ms (119) | 1.02 ± 0.03 ms (11) | 0.96 ± 0.04 ms (8) | 0.85 ± 0.03 ms (11) |
| Control       |                                                                                                               | 4.17 ± 0.17 ms (24) | 3.86 ± 0.25 ms (7) | 3.71 ± 0.11 ms (9) | 3.31 ± 0.17 ms (13) |
| After paraoxon |                                                                                                               | 1.02 ± 0.03 ms (11) | 0.96 ± 0.04 ms (8) | 0.85 ± 0.03 ms (11) |                   |
| IC50 for (+)-tubocurarine |                                                                                                               | 166 ± 9 nM | 107 ± 32 nM | 73 ± 17 nM | —                   |
| Control       |                                                                                                               | 339 ± 26 nM | 106 ± 7 nM | 109 ± 9 nM | 109 ± 4 nM |
| After paraoxon |                                                                                                               | 166 ± 9 nM | 107 ± 32 nM | 73 ± 17 nM | —                   |

All values ± SEM. Number of junctions in parentheses. Holding potential, −80 mV.

pretreated with IgG from myasthenia patients, and in a diaphragm from an animal pretreated with α-BuTX, in each case before and after poisoning of AChE by paraoxon. In conformity with the findings of Katz and Miledi (1973a and 1978), Magleby and Terrar (1975), and Mallart and Molgó (1978), presumptive receptor blockade by dTC, α-BuTX, or IgG from myasthenia patients caused acceleration of the decay of MEPCs both before and after treatment with paraoxon. There were also other effects on time-course,
produced by all three agents, consisting in slight prolongation of the rising phase and alteration of the falling phase to approximate more closely a simple exponential decay. These findings will be described in detail in a subsequent paper.

The increase in MEPC amplitude produced by AChE poisoning in control diaphragms was 16 ± 4%. The same average value was obtained from five junctions, at each of which it was possible to record MEPCs before, during, and after application of paraoxon. This value is close to that reported for AChE blockade in snake (Hartzel et al., 1975) and rat (Colquhoun et al., 1977). This result in itself suggests that normally capture of transmitter by receptors is nearly complete (cf. Hartzell et al. [1975])—only then can blockade of hydrolysis have little effect. In terms of Eq. 1 (Theory), poisoning of AChE is tantamount to reduction of \( \beta \); a small action on \( F \) in the control situation indicates that normally \( aR \) is substantially more than \( \beta \). In the “myasthenic” diaphragms AChE poisoning caused somewhat greater increases in MEPC size (25 ± 6% for 2-d treatment, 43 ± 10% for 4-d treatment). The same was true for the diaphragm treated with \( \alpha \)-BuTX (32 ± 9%), and the effect of AChE poisoning to increase MEPC height was also enhanced by receptor blockade by dTC (Fig. 2 A).

Increase of MEPC Amplitude by AChE Poisoning

Fig. 2 B and C shows graphs of the increase of MEPC height produced by poisoning of AChE with paraoxon, at various concentrations of dTC, in control diaphragms and in diaphragms from animals treated with \( \alpha \)-BuTX or with IgG from myasthenia patients. Also included here are data from a diaphragm from an animal treated for 3 d with IgG from myasthenia patients; in this diaphragm no recordings were made with added dTC. In all cases the percentage increase of MEPC height was greater when MEPCs were made smaller by receptor blockade and the effect was independent of whether the MEPCs were made small using reversible blockade by dTC, or irreversible blockade by \( \alpha \)-BuTX or IgG from myasthenia patients, or a combination of both. The inverse plots (graphs of control MEPC height divided by MEPC height after paraoxon vs. control height) show a good fit to a straight line, in conformity with Eq. 3. The intercept on the ordinate gives a value of \( \beta_d / (\beta_d + \beta_h) \) of 0.46; from computer simulation of MEPCs (Theory), this intercept does not necessarily give the relative importance of diffusion and hydrolysis in disposing of transmitter. The line gives, by extrapolation, the value of \( g \) at which poisoning of AChE would produce no further increase of MEPC height (i.e., \( g_m \)); this is 5.1 ± 0.6 nA (at -80 mV, i.e., 64 ± 7 nS). Given that a single channel has a conductance of 20–25 pS (reviewed by Colquhoun, 1978) this corresponds to the activation of ~3,000 channels. If this value for \( g_m \) is taken to correspond to 100% uptake of quantal ACh by receptor (Theory), then the fraction that becomes bound to receptor normally and after the various treatments can easily be calculated; the data indicate that, under normal conditions, 79% of the ACh in a quantum is captured by receptor, whereas after AChE is inhibited, 92% is captured.
FIGURE 2. (A) Bar diagram showing heights of MEPCs (at -80 mV) recorded before and after poisoning of AChE by paraoxon. Within each bar, the broken line below the top of the bar indicates the height before poisoning of AChE. In control diaphragms (Con) and in diaphragms from mice previously injected with α-BuTX (α-BUTX) or IgG from myasthenia patients (Myasthenic IgG), with no dTC and with 0.1 μM dTC, and also with 0.4 μM dTC in control diaphragms only. Number of junctions is shown in parentheses. Note that the percentage reduction in MEPC height by dTC was increased by α-BuTX or IgG from myasthenia patients and reduced by poisoning of AChE. Bars, ± SEM. (B) Enhancement of MEPC height by poisoning AChE. Plot of ratio of height after paraoxon (gα) to height with AChE intact (g0) vs. height with AChE intact (g0) at various concentrations of dTC. (♦), control diaphragms; (■), 4-d IgG from...
Modification of MEPC Amplitude by (+)-Tubocurarine

As already shown in Table I and Fig. 2, the apparent potency of dTC in reducing MEPC height was reduced by poisoning of AChE and enhanced by irreversible receptor blockade by α-BuTX and IgG from patient with myasthenia. Fig. 3 shows a graph of all the data plotted according to Eq. 6; each point is derived from averages of MEPC size with and without dTC or at two different levels of dTC, and with two different starting levels of MEPC height (produced by poisoning of AChE, and/or pretreatment with α-BuTX, or IgG from myasthenia patients). Despite some scatter, the points (44 in all) fit well to a straight line that passes through the origin (intersect on abcissa = −0.01 ± 0.08) with a slope, corresponding to $g_m$, of 5.35 ± 0.17 nA. This is not significantly different from the value of $g_m$ estimated from the graph in Fig. 2C.

Once $g_m$ is known, it is in principle possible to determine the relationship between concentration of dTC and $f(I)$, since from Eq. 5

$$f(I) = \frac{(g_0/g_I - 1)}{(1 - g_0/g_m)}.$$
Thus, from each observation of MEPC size at a given $I$ (and the value when $I = 0$) a value for $f(I)$ is obtained. Fig. 4 shows the relation between calculated $f(I)$ and $[\text{dTC}]$ for the data obtained in control diaphragms, before and after poisoning of AChE, with $[\text{dTC}]$ between 40 and 800 nM. This graph suggests simple proportionality between $f(I)$ and $I$, as expected if there is simple one-to-one binding of dTC to receptor. However, previous observations on the blockade of responses to cholinergic agonists suggest a one-to-one
competition of dTC with ACh, of which probably two molecules interact with receptor to open an ionic channel (Discussion). On this basis, one would expect that dTC can attach to either of two subunits of the receptor and therefore that the fraction of receptors free of at least one dTC molecule would be given by $1/(1 + I/K_i)^2$; this would cause an upward curve (and slope greater than unity) in the graph in Fig. 4. Whatever the model chosen, the data indicate that at 100 nM [dTC], at least $65\%$ of receptors are effectively blocked; at this concentration of dTC MEPC height is reduced by $36\%$ when AChE is intact and by $28\%$ when AChE is poisoned (Fig. 2, Table I).

In conformity with a linear relation between $f(I)$ and [dTC], the depression of MEPC peak amplitude produced by varied concentrations of dTC in every case fitted a linear relation between the inverse of MEPC height ($g^{-1}$) and [dTC]. This is shown in Fig. 5 A for the control and 4-d "myasthenic" diaphragms, before and after treatment with paraoxon. The slope of the line relating $g^{-1}$ and [dTC] was decreased by poisoning of AChE and increased in the "myasthenic" diaphragms, and all lines appear to meet in a region close to [dTC] = $-40$ nM and $g^{-1} = 0.2$, i.e., $g_m \approx 5$ nA. In Fig. 5 B and C are shown the values of IC50 for dTC, derived from least-squares fitting to the lines in Fig. 5 A (as the ratios of intercept to slope), plotted vs. $g_0$. In conformity with Eq. 5, the IC50 increases with reduction of $g_0$ (Fig. 5 B) and the graph of $(IC50)^{-1}$ vs. $g_0$ fits a straight line (Fig. 5 C), confirming that Eq. 5, with $f(I)$ set proportional to $I$, provides a good description of all the data; data from the diaphragms previously treated with $\alpha$-BuTX and for 2 d with IgG from myasthenia patients are also included here. By extrapolation, 50% of receptors were blocked at [dTC] = $40 \pm 2$ nM, and $g_m$, the apparent maximum possible value for $g$, is $5.04 \pm 0.26$ nA. Recalculations using the new estimate of $g_m$ did not change the linear relation between $f(I)$ and [dTC] as shown in Fig. 5.

**Modification of MEPC Time-Course by Receptor Blockade**

Katz and Miledi (1973a) pointed out that dTC should and does accelerate the decay of MEPCs after poisoning of AChE by increasing the fraction of ACh in the synaptic cleft that is not bound to receptor and is therefore free to diffuse; they used this phenomenon to estimate the fraction of ACh bound to receptor during the decay phase. Following their argument, the rate of decay of MEPCs will be proportional to the fraction of ACh not bound to receptor $(1 - P)$ at the time when the decay rate is measured, and to the rate of diffusion ($D$) of unbound ACh from the cleft, i.e.,

$$\tau^{-1} = D(1 - P),$$

where $\tau$ is the MEPC time constant. It follows that

$$\tau/\tau_0 = (1 - P_0)/(1 - P),$$

where $\tau_0$ is the control time constant and $P_0$ is the proportion of ACh in the cleft bound to receptor during the decay phase of the control MEPC. With the same simplifying assumptions as used by Colquhoun et al. (1977), namely, (a) that each of the binding sites buffering ACh in the cleft usually binds only
one ACh molecule or dTC molecule; (b) binding and dissociation of ACh occur sufficiently rapidly so that there is effective equilibrium at all times; (c) there is no saturation of these receptors by ACh; and (d) the concentration of
ACh is insufficient to alter binding of dTC, one may write

\[
[ACh]/[AChR] = \frac{K_a}{R}
\]

\[
R = \frac{R_0}{1 + I/K_i}
\]

\[
P = \frac{[AChR]/[ACh] + [AChR]}{[AChR]/[ACh] + 1} = \left[1 + \frac{K_a}{R_0} \left(1 + \frac{I}{K_i}\right)\right]^{-1},
\]

where \(R_0\) is the receptor concentration in the absence of antagonist. Combining this with Eq. 7, one obtains

\[
\tau = \tau_0 \left[1 - P_0/(1 + K_i/I)\right].
\]

Thus, the effect of dTC to accelerate decay of MEPCs after poisoning of AChE should give a measure of the dissociation constant \(K_i\) for dTC of those sites involved in binding ACh during the decay phase of the MEPC. The graph in Fig. 6 shows averages of time constant determined on individual MEPCs plotted vs. [dTC]; the curve is drawn according to Eq. 8 with parameters derived by nonlinear least-squares fitting to this equation. The best fitting value of \(K_i\) for dTC is 53 ± 17 nM. The apparent fraction of ACh in the cleft bound to receptor in the absence of receptor blockade \((P_0)\) is 0.43 ± 0.03.

Models of postsynaptic events described in Theory that incorporate cooperativity in ACh and/or dTC binding to receptor also predict that the time constant of MEPCs, when AChE is poisoned, will be reduced to halfway between control and minimum (when binding is zero) when the density of
binding sites is reduced by one-half. The data show that this occurs in the neighborhood of 40–100 nM. Whatever the intricacies of dTC interaction with the receptor(s) (i.e., whether or not assumption a above is valid) it is evident that at 100 nM dTC the MEPC time constant was at least one-half-maximally reduced, although the height of the same MEPCs was reduced by only 28% (see Table II).

**Blockade of Postsynaptic Response to Carbachol by dTC**

To obtain another independent estimate of the receptor blockade produced by dTC, on the tentative assumption that all postsynaptic receptors have the same affinity for dTC, we employed superperfusion of the end-plate region with carbachol in the absence and presence of dTC.

**TABLE II**

| Test                        | Response (percent control) | Calculated percent reduction in free receptors |
|-----------------------------|---------------------------|-----------------------------------------------|
|                             | 40 nM dTC  | 100 nM dTC  | 40 nM dTC  | 100 nM dTC  |
| 10 μM carbachol             | 53±5 (11) | 31±3 (10)  | 47±5        | 69±3        |
| 20 μM carbachol             | 54±6 (12) | 34±4 (11)  | 46±6        | 66±4        |
| MEPCs AChE intact           | 80±5 (7)  | 64±3 (19)  | 50±8        | 70±3        |
| MEPCs AChE poisoned         | 88±5 (8)  | 72±4 (6)   | 52±15       | 75±10       |

Control responses to superperfused carbachol were: 10 μM, ΔG/G = 0.132 ± 0.005 (71 observations); 20 μM, ΔG/G = 0.344 ± 0.022 (22 observations). In each case ΔG/G was derived from the observed depolarization using the formula given in Methods; with the carbachol tests, the percent reduction in free receptors was calculated simply as equal to percent reduction of ΔG/G (see text). For MEPCs, the percent reduction in free receptors was calculated from Eq. 5b, using 5.35 nA as the value of g∞. All values ± 1 SEM. Number of observations in parentheses.

In principle, by any model of the antagonist-receptor interaction, the response (conductance change) to a given dose of agonist is simply proportional to the fraction of receptors that are not occupied and thereby blocked by the antagonist. When the concentration of the agonist is low relative to its dissociation constant, i.e., when the measured response occurs with only a small fraction of receptors occupied by the agonist, the fraction of receptors occupied by the antagonist will be virtually the same as in the absence of agonist.

Fig. 7 shows a typical result; depolarizing responses to locally superperfused carbachol (10 or 20 μM) were substantially reduced by 40 nM dTC, which had little effect on the amplitude of MEPPs. Average data are shown in Table II; these data are consistent with the same occupancy of receptors by dTC as calculated from the effect of dTC on MEPC height. The average slope of the
log-log plot of $\Delta G/G$ vs. carbachol concentration (over the range 5-40 $\mu$M) was $1.50 \pm 0.07$ in the absence of dTC and was not significantly altered by dTC.

In diaphragms from animals treated with IgG from myasthenia patients the slope of the log-log plot of $\Delta G/G$ vs. carbachol concentration and the apparent potency of dTC to depress responses to carbachol were the same as in control diaphragms.

In a few experiments, ACh was used as well as carbachol after poisoning of AChE; the potency of dTC to reduce responses to ACh was the same as its potency to reduce responses to carbachol.

**Figure 7.** Inhibition of postsynaptic depolarizing responses to superperfused carbachol (10 and 20 $\mu$M) by 40 nM dTC (right)—responses are about one-half the controls (left). MEPPs recorded just after these tests of carbachol sensitivity are shown above; there was no noticeable depression of size by the 40-nM dTC. Time scales: 1 s per division. 10 mM K$^+$ present. There was no change of resting membrane potential in the 5 min allowed for equilibration with dTC.

**DISCUSSION**

The principal results of this study may be summarized very briefly. In mouse diaphragms, the potency of (+)-tubocurarine (dTC) in depressing MEPC amplitude is substantially reduced by poisoning of acetylcholinesterase (AChE). Even when AChE is intact, the potency is much less than the potency of dTC to depress responses to superperfused carbachol. The effect of poisoning AChE to increase the peak amplitude of MEPCs is normally rather small (as in the snake [Hartzell et al., 1975]; or rat [Colquhoun et al., 1977]), but the percentage increase is made greater when MEPCs are reduced in size by dTC or by pretreatment of animals with $\alpha$-bungarotoxin ($\alpha$-BuTX) or IgG from...
patients with myasthenia gravis, both of which appear irreversibly to diminish effective receptor density. The effectiveness of AChE poisoning to increase MEPC size appears to be the same when MEPCs are made small by the irreversible (or very slowly reversible) blockers as when MEPCs are made small by dTC. The last observation makes it clear that the enhanced effectiveness of AChE poisoning when MEPCs are made small (Fig. 2) is not to be explained in terms of displacement of blockers from the receptor sites by a transient high concentration of ACh in the synaptic cleft. Moreover, the apparent potency of dTC to depress MEPCs is increased when MEPCs have already been reduced in size by α-BuTX or IgG from myasthenia patients.

All the above results are explained quite simply if it is supposed that normally the subsynaptic receptor density and onward rate constants for ACh to combine with receptor to form a relatively stable conformation are sufficiently high that most of the ACh in a quantal package quickly becomes captured by receptors. From examination of various models (Theory), this explanation remains valid whatever the intricacies of ACh-receptor interaction. The MEPC is normally insensitive to receptor blockade simply because when ACh encounters blocked receptors, the ACh is able to diffuse further away from the point of its release, eventually to encounter and be captured by unblocked receptors. Only when receptor density is reduced to the point at which the rates of ACh loss from the cleft by hydrolysis and diffusion become much larger than the rate of attachment to receptor will MEPC height become close to proportional to effective receptor density. Poisoning of AChE has normally little effect on MEPC height merely because normally most of the ACh in the package becomes attached to receptor even when AChE is intact. When the density of functional receptors has been reduced, there is a lower efficiency of capture; poisoning of AChE permits ACh to diffuse further, to encounter and be captured by unblocked receptors, and MEPC amplitude is thereby more greatly enhanced. In terms of the schematic view of the distribution of activated receptor at the peak of the MEPC shown by Hartzell et al. (1975), the effect of receptor blockade is to reduce the height and to increase the spatial spread; this arises not only because the peak height occurs later, but because the net rate of diffusion of transmitter is faster when most receptor sites are occupied, by transmitter as well as by antagonist. It may be noted that near the point where the quantum of transmitter is released activatable receptors must be nearly saturated; this is a general feature of models in which the transmitter diffuses from a point source and much of the transmitter becomes bound to receptor (cf. Matthews-Bellinger and Salpeter [1978]; Wathey et al. [1979]; Adams [1980]; Land et al. [1980]).

It should be emphasized that the above interpretation of the data does not depend upon any assumptions regarding the mode of interaction of dTC with receptor or upon the assumption that dTC dissociates slowly from the receptor. Nor does it depend on any assumption that the action of IgG from myasthenia patients (or α-BuTX) consists solely of simple irreversible blockade of the receptors. For example, the same interpretation would hold if IgG from patients with myasthenia were to act to prevent the conformational change leading to an open channel and consequent stabilization of the ACh-receptor
complex rather than to prevent access of ACh to binding sites; this would explain the relatively prolonged time-course of "myasthenic" MEPCs (after AChE poisoning), in relation to the extent of apparent receptor blockade (cf. Table I and Fig. 6). However, it is evident that a reduced binding (either in extent of duration) of ACh by those receptors that are rendered inactive is fundamental to the enhanced effect of AChE poisoning to increase MEPC height, found with dTC, α-BuTX, and IgG from myasthenia patients. A blockade in which inactivated receptors bound ACh in the same way as active ones would simply result in reduction of MEPC height, with no alteration of time constants or of potency of dTC, or change in effect of AChE poisoning.

The relation between MEPC height and fraction of receptors free to respond to ACh can be inferred from the present data and is shown in Fig. 8. Here the extent of receptor blockade at various concentrations of dTC was obtained using Eq. 5b and a value of 5.1 nA for \( g_m \). The degree of blockade with α-BuTX and IgG from myasthenia patients are derived from the observed IC50s for dTC in these diaphragms (Theory) and not directly from MEPC height.
With the latter agents, as with dTC, the percentage receptor blockade is of course not necessarily synonymous with percentage occupancy of receptor sites. If, for example, there are two receptor sites per receptor, occupied at random by α-BuTX (or IgG from myasthenia patients) then, if x were the fraction of sites occupied, \((1 - x)^2\) would be the fraction of receptors with no receptor site occupied and still capable of responding to ACh. This model would give a nearly linear relation between height of MEPC and fraction of binding sites occupied by toxin (Ito et al., 1978; Land et al., 1980; see also Albuquerque et al. [1973]).

In man the plasma concentration of dTC that results in partial paralysis is between 0.4 and 0.8 μg/ml or 0.6 and 1.1 μM (Stanski et al., 1979; see also Wingard and Cook [1978]). From the present data, a concentration of 0.8 μM would be expected to produce an MEPC of one-fifth the normal amplitude, which corresponds to the reduction in size of MEPPs observed in biopsied muscle from myasthenia gravis patients with partial muscular paralysis (Elmqvist et al., 1964). From the present results it is evident that to achieve this 80% reduction in size and to cause marginal paralysis, all but 5% of the receptors must be blocked. In the presence of receptor blockade poisoning of AChE becomes less able to prolong the MEPC (Fig. 6), but the reduced effect on time-course is countered to a certain extent by the larger effect to increase MEPC size. Because the relatively low time constant of a muscle fiber the height of an MEPP is about proportional to the area of a MEPC, it follows that the effect of an anticholinesterase to increase height of MEPPs (as opposed to MEPCs) may be much the same with or without receptor blockade. In both situations the augmenting effect of the anticholinesterase arises solely from blockade of AChE but the mechanism is somewhat different. Normally, the augmentation arises mainly from repetition of ACh action in the cleft, i.e., increased “reverberation” (Katz and Miledi, 1973a), but when most receptors are blocked, poisoning of AChE also allows ACh to diffuse further from its source to encounter and to act upon unblocked receptors before being lost to the system.

Our estimate of the fraction of total ACh bound to receptors at the peak of the MEPC (92% after AChE is poisoned) is considerably greater than the value of ~50% for the fraction of cleft ACh apparently bound to receptors during the decay phase of the MEPC, also after AChE poisoning, as deduced from the acceleration of MEPC or end-plate current (EPC) decay rate by receptor blockade (this paper; see also Katz and Miledi, [1973a]; Magleby and Terrar [1975]; Colquhoun et al. [1977]). One may doubt that the difference in ACh binding at different stages of the MEPC is so great in reality, given the approximations inherent in both of the estimates. Nevertheless, it is interesting to note that a nearly constant degree of binding is predicted only if there is no cooperation in ACh-receptor interaction. With simple, sequential models in which two receptor (R) subunits have equal affinity for ACh, but where the opening of channels is associated with an isomerization of ACh₂R to a form, ACh₂R*, which dissociates relatively slowly, i.e.,

\[2ACh + R \rightleftharpoons ACh + AChR \rightleftharpoons ACh₂R \rightleftharpoons ACh₂R*,\]
the fraction of total ACh that is free at equilibrium, in a closed system, is

\[
\frac{[\text{ACh}]}{[\text{ACh}_d]} = \left\{ 1 + 2[R_d][\text{ACh}] \right\} \cdot \frac{(1 + K[\text{ACh}] + K[\text{ACh}]L)/[(1 + K[\text{ACh}])^2 + K^2[\text{ACh}]^2L]}{(1 + K[\text{ACh}] + K[\text{ACh}]L)/[(1 + K[\text{ACh}])^2 + K^2[\text{ACh}]^2L]}^{-1},
\]

where \([\text{ACh}]\) denotes concentrations. When \(L \gg 0\), this function increases rather than decreases with falling \([\text{ACh}_d]\) (except near receptor saturation). Thus, the fraction of total ACh bound to receptors can decrease as the MEPC decays and the concentration of ACh in the cleft goes down. Eventually “buffering” of ACh in the cleft will be governed by unproductive ACh binding with a dissociation constant of \(K^{-1}\). Since the density of ACh receptor sites on the postsynaptic membrane (~3 \(\times\) 10^{-4}/\(\mu m^2\)) corresponds to an \(R_t\) of ~0.5 mM (Fambrough and Hartzell, 1972; Fertuck and Salpeter, 1974 and 1976; Wathey et al., 1979), if there are two sites per receptor a limiting fraction bound of 50% (when \([\text{ACh}_d]\) and \([\text{ACh}]\) are low) corresponds to a \(K^{-1}\) of ~1 mM. This is much greater than the apparent dissociation constant for ACh, ~30 \(\mu M\), for its action to open ionic channels (Sakmann and Adams, 1978; Adams, 1980; Sakmann et al., 1980), implying that \(L\) is indeed large or that there is positive cooperation in ACh binding before the isomerization step (which does not change the argument). To account for rapidly rising MEPCs, the onward rate constant for combination of ACh with receptor must be \(\geq 10^{-7} M^{-1} \cdot s^{-1}\) and is probably \(\sim 10^9 M^{-1} \cdot s^{-1}\) (cf. Rosenberry [1979]; Wathey et al. [1979]). If \(K^{-1}\) is ~1 mM, this gives an offward rate constant for unproductive binding of ACh of ~100/ms or 100 times faster than the rate of closing of channels. The similar affinity of receptor for dTC in terms of peak MEPC size (Figs. 4, 5) and in terms of ACh buffering during MEPC decay (Fig. 6) suggests that the identical receptors are involved.

If the above reasoning is correct, the relatively small fraction of ACh bound to receptor during the decay phase of the MEPC (after AChE poisoning) reflects both cooperativity in ACh-receptor binding associated with channel opening, and a relatively faster rate of dissociation of ACh from the labile ACh-receptor complex(es) not associated with an open channel. In terms of physiological function, these factors will minimize trapping of ACh by receptor in the labile forms normally before channel opening and permit efficient use of ACh after its release without producing a signal that is greatly prolonged relative to the duration of the ionic channels. At the same time, a premium is put on the high concentration of transmitter in the cleft immediately after presynaptic discharge of a quantum—the “competition” of AChE and receptor for the transmitter will favor receptor binding when ACh first enters the cleft but will favor hydrolysis when the concentration of ACh in the cleft becomes low. Also, nonquantal “leak” of ACh from the nerve terminal (Katz and Miledi, 1977; Vyskočil and Illés, 1977; Vizi and Vyskočil, 1979) will cause little postsynaptic response.

The mode of interaction of dTC with receptors is not critical to the present results or to the conclusions that have been presented, but it is notable that three different actions of dTC—the effect on MEPC size, the effect on MEPC time-course (when AChE is poisoned), and the effect on responses to carbachol—are consistent in that they give similar derived values for the degree
of receptor (or binding site) blockade by dTC. From the degree of blockade, it is possible to ascribe a value for the \( K_1 \) (dissociation constant) for dTC only if a specific model of dTC-receptor interaction is chosen. Reported values for the \( K_1 \) of dTC in mammalian muscle, based on shifts of dose-response curves are close to 100 nM (Lu, 1970; Waud et al., 1973; Walker and Yeoh, 1974). The dose-ratio method (see below) employed by these investigators requires the assumption of one-to-one competition of antagonist with agonist. If channel opening usually requires the "cooperation" of two molecules of ACh, for which there is very persuasive evidence (e.g., Sheridan and Lester [1977]; Dreyer et al. [1978]), this is tantamount to the assumption that two dTC molecules can combine with the receptor and with this model the present data give a value of between 100 and 150 nM for the \( K_1 \) for dTC.

Nevertheless, our present data suggest that the degree of receptor blockade by dTC is linearly, rather than parabolically, related to dTC concentration (see Fig. 4). This agrees with the observation of Goldsmith (1963) that at concentrations of dTC considerably above its apparent \( K_1 \), the amplitude of end-plate potentials (EPPs) (in frog) was inversely related to the dTC concentration, rather than to the square of dTC concentration. These results fit most easily to a model in which only one dTC molecule can attach to the receptor, i.e., there is only one binding site per receptor (in which case the \( K_1 \) is \( \sim 40 \) nM) or there are two binding sites but binding to one site excludes binding to another (in which case the \( K_1 \) is \( \sim 80 \) nM if both sites have the same affinity). Either of these models predicts, however, that the classical method for estimating antagonist affinity—measurement of the shifts of dose-response for agonist produced by various concentrations of antagonists (Gaddum, 1937; Arunlakshana and Schild, 1959; see also Colquhoun et al. [1979])—should show that the dose-ratio (DR, the factor by which agonist concentration must be raised to compensate for the antagonist) is not linearly related to antagonist concentration. If \( n \) molecules of agonist "cooperate" to activate a receptor but one antagonist molecule is sufficient to block the receptor, then it should be \( DR^n - 1 \) rather than \( DR - 1 \) that is proportional do [dTC]. However, such studies with dTC generally show a good fit to linearity (Jenkinson, 1960; Rang and Ritter, 1969; Parker and Goldfine, 1973; Walker and Yeoh, 1974; Adams, 1975b) in mammalian as well as in amphibian and avian muscle. Indeed, the data of Colquhoun et al. (1979) show that the fit to a one-to-one ratio of dTC-agonist competition becomes even better after correction for the secondary action of dTC, to block open ionic channels (Manalis, 1977; Katz and Miledi, 1978).

Thus, results obtained from studies using equilibrium responses to cholinergic agonists lead to a model of dTC-receptor interaction different from that obtained from MEPCs (present data) or EPPs (Goldsmith, 1963). It is possible that this discrepancy can be accounted for in terms of different affinities for dTC of the two binding sites (purified rat junctional receptors [Brockes and Hall, 1975]; Torpedo electric organ membrane fragments [Weiland and Taylor, 1979; Neubig and Cohen, 1979]; cultured myoblasts [Sine and Taylor, 1979; 1980]). Such a model specifies for \( f(I) \) a value of

\[
\frac{I}{K_1} + \frac{I}{K_2} + \frac{I^2}{K_1 K_2},
\]
where $K_1$ and $K_2$ are the two dissociation constants. A $K_2$ of 1 $\mu$M, together with a $K_1$ of 50 nM, is within experimental error for the relation between $f(I)$ and $[\text{dTC}]$ shown in Fig. 4, and predicts a dose-ratio estimate of $(K_1K_2)^{1/2}$ of ~200 nM, which is at the upper limit compatible with published data for mammalian receptors (Waud and Waud, 1975). The close-to-linear relation between the inverse of EPP height and $[\text{dTC}]$ in the range 1–10 $\mu$M, as observed by Goldsmith (1963) in the frog, can fit this model only if $K_2$ is ~10 $\mu$M; this is difficult to reconcile with the data of Colquhoun et al. (1979).

Another possibility is complexity in dTC (and ACh) interaction with receptor. For example, if there are four binding sites per receptor (Khromov-Borisov and Michelson, 1966; Miller et al., 1978), one may postulate that a channel can be opened (with stabilization of the agonist-receptor complex) by agonist occupancy of any two of these sites. If attachment of dTC to one or even two of the sites does not prevent receptor activation (channel opening) but merely reduces the probability, it would also explain the observation of Sheridan and Lester (1977), subsequently confirmed by Colquhoun and Sheridan (1979), that the transition between closed and open channel states is slowed by dTC (although not by $\alpha$-BuTX) without having to postulate "astonishingly fast" association and dissociation of dTC. We find, using computer synthesis of MEPCs (Theory), that it is indeed possible with such a model to obtain MEPCs for which the inverse of MEPC height is very nearly linear with dTC concentration, as we observe (and observed for EPPs by Goldsmith [1963]), whereas equilibrium responses to agonist in the presence of dTC fit very closely to what would be expected for a one-to-one competition of dTC with agonist. Further description and discussion of this (rather complicated) model will be presented elsewhere.

In apparent contradiction to the present results, Goldsmith (1963) found that responses to 70-ms iontophoretic pulses of ACh were less sensitive to increases in the concentration of dTC than were EPPs recorded at the same junction. This can be explained as follows. In the case of the EPP or MEPP, there can be little net dissociation of dTC from receptor during the very short time of rise of the synaptic conductance. However, with a long iontophoretic pulse of ACh, a high concentration of ACh is maintained locally for a relatively long time during which there is effective displacement of dTC by the agonist. In support of this view, brief iontophoretic pulses gave rise to responses that were more similar to EPPs in their sensitivity to dTC than were responses to long iontophoretic pulses (Goldsmith, 1963). In conformity with the present results, Adams (1975b) has reported that, in the frog, responses to iontophoretic pulses of carbachol are less sensitive to blockade by dTC than are the responses to bath application of carbachol, and pointed out that from the work of Auerbach and Betz (1971) the MEPP and EPP size in frog is even less sensitive to dTC. The explanation of this phenomenon suggested by Adams (1975b) is essentially the same as the one we have adopted.

The data of Albuquerque et al. (1980) also show differences in sensitivity to dTC of responses to exogenous and endogenous ACh; reduced MEPC height to 44% of control and reduced the response produced by a 30-s iontophoretic pulse of ACh to 25% of control. A much greater difference in sensitivity was
observed when perhydrohistrionicotoxin was used as an antagonist; such a large difference cannot be explained on the same basis as the difference observed with dTC and suggests receptor heterogeneity. Because perhydrohistrionicotoxin potentiates the desensitization process (Burgermeister et al., 1977) the receptor heterogeneity may have been generated by nonuniform distribution of iontophoresed ACh over the receptor area (see Miledi, 1980), i.e., normal-size MEPCs may have been generated outside the region of desensitization.

In conclusion, it may be noted that a nonlinear relation between effective receptor density and response to endogenous quantal transmitter is implicit wherever the receptor density and rate of binding are such that normally most of the transmitter becomes captured by receptor. It follows that at many synapses, responses to exogenous (or circulating) transmitter substance should be more sensitive to receptor blockade than the responses produced by the same transmitter when it is released by the nerve terminal.

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