

# A Linear Dose-Response Curve at the Motor Endplate

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**ABSTRACT** The motor endplate of frog sartorius muscle was voltage clamped and the peak current to different concentrations of acetylcholine and carbachol applied in the perfusing fluid was measured. Perfusing fluid was hypertonic in order to suppress contractions. Current responses were smooth and reached a peak value within 2–5 s. The dose-response curve was usually linear even with concentrations of  $10^{-2}$  M acetylcholine, indicating that the conductance change was probably proportional to the concentration of acetylcholine or carbachol. With high concentrations nonlinearity sometimes appeared but in these cases the fast onset of desensitization appeared to be preventing the current response from reaching its expected peak amplitude. When the depolarization produced by acetylcholine in a non-voltage-clamped endplate was measured the dose-response curve was hyperbolic. This relationship was imposed by the electrical properties of the endplate membrane and its surrounding sarcolemma, and could be predicted if the input resistance of the fiber was known. Experiments were also done on slow muscle fibers. Depolarizing analogues of acetylcholine had similar effects to acetylcholine. *d*-Tubocurarine reduced the proportionality constant between concentration of acetylcholine and conductance change, and this resulted in a parallel shift of the log-concentration depolarization curve. A linear dose-response curve was unexpected within the context of current theories of drug action.

## INTRODUCTION

The first attempts to quantify drug-receptor interactions were made by Clark (1933) and by Gaddum (1937). They found that the relationship between concentration and action of a drug such as acetylcholine or adrenaline was hyperbolic, i.e., the relationship could be expressed by the simple formula  $Kx = y/(A - y)$ , where  $x$  = concentration of drug,  $A$  = maximum action,  $y$  = action observed, and  $K$  = constant. In these studies, as in most subsequent studies of drug-receptor interactions, the physiological effect of the drug action was measured as the drug-evoked contraction of smooth or slow skeletal

muscle. The size of the response and the form of the dose-response curve were considered to be intimately related to an initial action of the drug at the receptor. Originally the response was thought to be directly related to the proportion of receptors occupied (Occupation Theory), the number of receptors being limited. The response has also been considered to be proportional to the rate of association of the drug with the receptor (Rate Theory, Paton, 1961). In their simplest forms these theories predicted that with increasing concentration of drug, the steady-state response would approach a maximum, resulting from maximum occupation of receptors. This prediction was not fulfilled. Both Nickerson (1956) and Stephenson (1956) found that a maximum effect could be produced by an agonist when it occupied only a small proportion of receptors, and this observation forced them to postulate the existence of "spare" receptors. This meant that the relationship between the initial reaction at the receptor and the measured response was of a more complex nature than the hitherto considered direct relationship. But this relationship has never been defined in any specific way, undoubtedly because of the complexity of the preparations of smooth muscle on which most of such experiments have been performed, and the largely unknown physiology of this muscle, in which the train of events between an initial drug action and the final contraction is not at all understood. Yet this knowledge would seem imperative, inasmuch as these factors must exert complex and variable effects on the dose-response curve, which are not related to the initial chemoreceptive action.

The physiology of the neuromuscular junction of a fast skeletal muscle fiber of the frog has been extensively studied. Therefore this system was chosen in order to analyze the functional relationships between an initial drug action and two of the subsequent physiological responses, namely, the conductance change and the potential change across the membrane. Some experiments were also done on slow muscle fibers.

#### METHODS

Sartorius muscles or strips of the rectus abdominis from winter frogs (*Rana temporaria*) were used. The muscle was bathed in hypertonic solutions (composition: NaCl = 230 mM; KCl = 2 mM; CaCl<sub>2</sub> = 1.8 mM; Na<sub>2</sub>HPO<sub>4</sub> = 1.08 mM; NaH<sub>2</sub>PO<sub>4</sub> = 0.42 mM; glucose = 1 g/ml) in order to suppress contractions (Hodgkin and Horowitz, 1957; Howarth, 1958). All experiments were done at room temperature, 18°–22°C. Recordings were made from superficial fibers with large diameters (ca. 100 μm or more). Endplates were located by probing until miniature endplate potentials with the fastest time-course were seen. The area of membrane which is sensitive to acetylcholine is about 300 μm long (Miledi, 1960), somewhat larger than the area which responds to the nerve impulse, an area about 30 μm by 15 μm. Since drug was perfused, localization of the endplate focus was not critical because the whole area received drug at virtually the same time. The bath, volume 1 ml, was continuously

perfused at a rate of 1 ml/s, and drugs were introduced in the perfusing fluid, so that concentrations in the bathing fluid at least were known and constant. An electromagnetic switching arrangement allowed changes from Ringer solution to Ringer solution containing drug. The bath could be completely flushed of methylene blue in less than 3 s. On superficial fibers the time from switching from Ringer solution to the commencement of drug action was usually between 2 and 6 s. It was apparent that the limiting factor was diffusion of the drug from the fast-flowing bathing solution to the active sites. The time to commencement of action in deeper fibers was longer, and the responses in these fibers, despite the presence of large miniature endplate potentials, were also smaller and slower, indicating the diffusion delay. Presumably some dilution of drug concentrations occurred at the endplate. However, it was felt that with this method the "dilution factor" at least would be constant from one drug concentration to another, unlike the situation if the drug was iontophoresed (and where the desired larger concentrations could not be applied) or if local perfusion methods were used.

The following drugs were used: acetylcholine perchlorate, carbamylcholine chloride, succinylcholine chloride, decamethonium chloride, *N*-methylarecoline iodide, and phenylethyltrimethylammonium iodide. The last two compounds were prepared in the department by Mr. B. Peck.

The voltage-clamp setup was essentially the same as that described by Takeuchi and Takeuchi (1959) and by Julian, Moore, and Goldman (1962). The membrane potential was measured as the potential difference between an intracellular 3 M KCl-filled microelectrode and a reference electrode placed outside the fiber. A second, current-passing microelectrode, also filled with 3 M KCl, was inserted in the same fiber approximately 50  $\mu\text{m}$  from the recording microelectrode. The resistance of the voltage electrode was 20 M $\Omega$  or more and the resistance of the current electrode 5–10 M $\Omega$ . Current was measured as the voltage drop across a 33 K $\Omega$  resistor placed between the solution in the bath and ground.

The relationship between  $E$ , the command signal, and the steady-state membrane potential,  $V (=IR_p)$ , was derived by Hagiwara and Saito (1959) as

$$E = I(R_p + R_p/\mu + R/\mu), \quad (1)$$

where  $R_p$  is the input resistance of the fiber as defined by the ratio ( $dE/dI$ ), where  $dE$  is the potential change caused by the constant current  $dI$ , for small displacements around the resting potential.  $\mu$  is the open loop gain of the control amplifier (10,000) and  $R$ , the series resistance, is essentially the resistance of the current-carrying electrode.  $I$  is the current supplied by the control amplifier. Normally, the input resistance of the fiber was about 200 K $\Omega$ , but with drug present the input resistance of the fiber could fall below about 10–20 K $\Omega$ , and the deviation of the membrane potential from the control voltage could be 5% or more.

The equipment could be operated in one of two modes: constant current mode, where the current-carrying microelectrode was connected via a 50 M $\Omega$  resistor to the output of a Tetrax Series 162 Pulse Generator (Tetrax, Inc., Beaverton, Ore.), or constant voltage mode, when the feedback loop was closed.

## THEORY

Acetylcholine acts only on the endplate membrane. It is not necessary to consider changes in the ionic conductances of the neighboring sarcolemma since these are normally functions of the membrane potential,  $E_m$ , which in this case is held constant at the resting potential,  $E_r$ . When the voltage between the inside of the fiber and the outside is prevented from changing, the current,  $I_{\text{epd}}$ , which flows during drug action, depends only upon the unchanged driving force on the ions involved and the conductance,  $g$ , of the endplate membrane. Hence, in the presence of drug, at time,  $t$ , current,  $I_{\text{epd}}$ , through the resistance,  $1/g$ , of the endplate membrane is

$$I_{\text{epd}(t)} = g(t)(E_m - E_{\text{epd}}), \quad (2)$$

where  $E_{\text{epd}}$  is the value of the membrane potential such that for  $g \gg 0$ ,  $I_{\text{epd}} = 0$ , and can be considered as the equilibrium potential for the ionic mechanism that generates the endplate currents. Since  $g$  is independent of the transmembrane potential (Fatt and Katz, 1951; Takeuchi and Takeuchi, 1960 *b*) and, furthermore, since for any  $g$ , the relationship between  $E_m$  and  $I_{\text{epd}}$  is linear (Takeuchi and Takeuchi, 1959) and independent of the external acetylcholine concentration, then at constant  $E_m$ ,  $I_{\text{epd}}$  must be directly proportional to  $g$ .

## RESULTS

*Measurements of Current Changes*

The form and time-course of responses obtained in this system are shown in Figs. 1 and 6. In Fig. 1 recordings of acetylcholine-induced endplate currents ( $I_{\text{epd}}$ ) in a voltage-clamped endplate are shown, and in Fig. 6, the changes in potential ( $\Delta E$ ) produced by acetylcholine in a nonclamped endplate are shown. The time-course of action is determined mostly by the perfusion rate in such a preparation. Responses where the upstroke, decline, and recovery from desensitization were approximately three times faster, were obtained with local perfusion from a pipette located upstream from the microelectrodes, but this method was not used because of uncertainty of the local concentrations. In the experiments reported here, the time from onset of action to the peak of either a current or a voltage response, varied from 2 to 5 s. Hydrolysis by cholinesterases did not appear to limit the time-course of action of acetylcholine in these experiments since the presence of cholinesterase inhibitors did not affect the form of the response. Moreover, equipotent doses of acetylcholine and carbachol caused responses which were indistinguishable in time-course.

Both the current and the voltage responses in Figs. 1 and 6, as in nearly

all records, had started to return towards the resting level before the drug was removed from the perfusing fluid. This decline marked the onset of a refractory state known as desensitization (Katz and Thesleff, 1957). After exposure to drug, an endplate had to be washed by Ringer for about 5 min before an identical response to that concentration of drug could be obtained. If the washing time was less after withdrawal of drug, subsequent responses were smaller in amplitude and slower in time-course, as if a smaller concentration of drug had been applied. That is, the membrane was desensitized. The rate of recovery of the response, i.e. the rate of offset of desensitization, was exponential (Katz and Thesleff, 1957; Harrington, 1967), and in these experiments in fast fibers, the rate constant was approximately 120 s. Rate of offset was independent of the concentration of drug. However, the rate of onset of desensitization and the amount of desensitization produced, increased exponentially with concentration (Harrington, 1967). A very approximate measure of the rate of onset of desensitization was given by the rate of repolarization or the rate of decline of the current response during maintained drug perfusion. Therefore it was necessary to wait at least 5 min, or longer with higher concentrations, before introducing another test concentration of drug. Because of these long periods between exposure to drug, endplate currents to a maximum of only four different concentrations of drug, were ever recorded in any one endplate.

**DOSE-RESPONSE CURVE IN A VOLTAGE-CLAMPED ENDPLATE** Fig. 1 A-C shows the responses of a voltage-clamped endplate to three different concentrations of acetylcholine. The peak currents are plotted against concentration of acetylcholine in Fig. 1 D and are directly proportional to the concentration, as must also be the conductance changes (see Theory). The straight line was drawn by eye. In Fig. 1 E, the peak conductances calculated from currents recorded in another experiment in a different frog, are plotted against concentration. Fig. 2 shows the results of an experiment using carbachol. The plot is log-log for convenience and when plotted linearly the straight line does pass through the origin.

It appears then that the conductance "inserted" across the endplate membrane by the drug (either acetylcholine or carbachol) is directly proportional to the concentration of drug, i.e.  $g = pC$ , where  $p$  is a proportionality constant and  $C$  is the concentration of drug. In 14 experiments on 13 frogs, the mean value of  $p$  was  $2.83 \pm 0.64$  (SE of the mean)  $\times 10^{-3}$  mho-liter/mol, range  $0.90$  to  $5.70 \times 10^{-3}$  mho-liter/mol. In three frogs in 30% normal sodium Ringer, where  $p$  might be expected to have the same value although the recorded currents were reduced,  $p$ 's of  $1.20$ ,  $2.00$ , and  $0.85 \times 10^{-3}$  mho-liter/mol were found. Three experiments using carbachol gave the following values:  $0.54$ ,  $0.90$ , and  $0.45 \times 10^{-3}$  mho-liter/mol.

Fig. 3 shows the results of all the experiments using acetylcholine. It in-

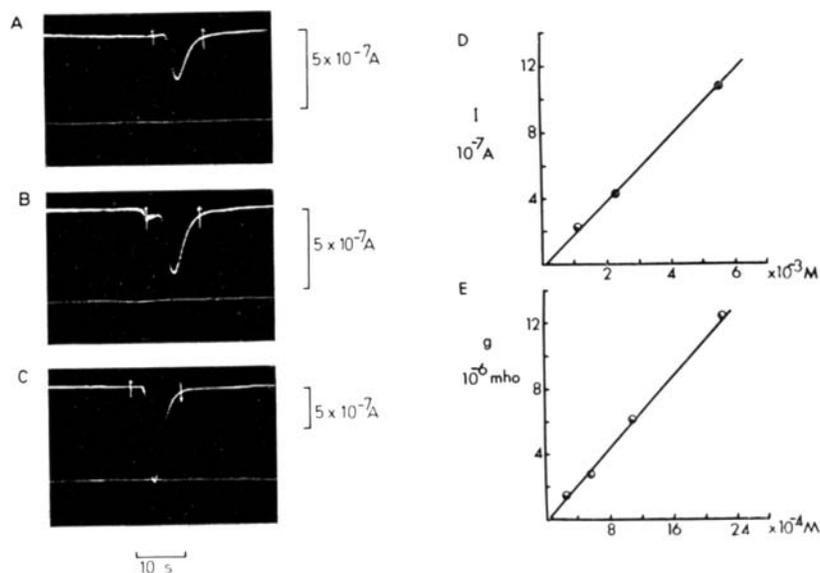


FIGURE 1 A-C. Responses of a voltage-clamped endplate to three different concentrations of acetylcholine: A =  $1.12 \times 10^{-3} \text{ M}$ ; B =  $2.24 \times 10^{-3} \text{ M}$ ; and C =  $5.56 \times 10^{-3} \text{ M}$ . The arrows mark the time during which the drug was perfused. Upper trace, current; lower trace, potential. D. Plot of the peak currents shown in A-C. E. Plot of the results of another experiment using acetylcholine. Conductances were determined assuming that  $E = 60 \text{ mV}$ . The slope was  $5.7 \times 10^{-3} \text{ mho-liter/mol}$ .

cludes both the full runs, where three or four measurements of current were obtained on a preparation, and runs where fewer than three measurements were obtained. The variation at each concentration is somewhat large, but this is not surprising since there were many factors that tended to affect the peak current for any one concentration, particularly from one endplate to another. These might include: (a) variations of the resting potential; (b) distance of the recording electrode from the endplate focus; (c) the superficiality of the endplate (see Methods); (d) age of the preparation—responses to drug generally diminished during a day's experiment, particularly if high doses were being used; (e) desensitization—responses which were obviously desensitized were omitted, i.e., responses which were smaller than a preceding response to a smaller dose of drug; (g) small responses to small concentrations may not have been detected, depending on the sensitivity settings of the equipment. Most of these effects would tend to reduce the peak current recorded, and more so with the larger responses. Despite these variations this figure shows that the relationship between conductance and concentration, at least up to concentrations of acetylcholine as high as  $2 \times 10^{-2} \text{ M}$ , is more likely to be linear than anything else.

In some runs the curve relating conductance to concentration flattened at

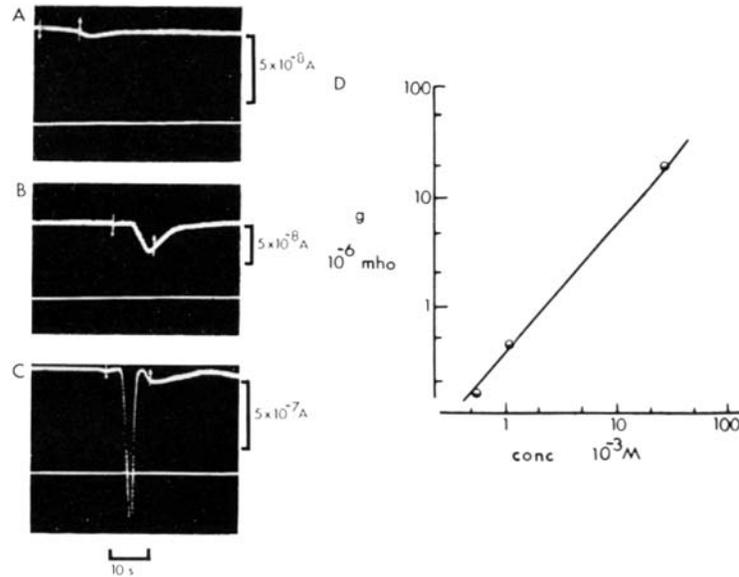


FIGURE 2 A-C. Responses of a voltage-clamped endplate to three different concentrations of carbachol: A =  $5.56 \times 10^{-4} \text{ M}$ ; B =  $1.12 \times 10^{-3} \text{ M}$ ; and C =  $2.78 \times 10^{-2} \text{ M}$ . The arrows mark the time during which the drug was perfused. Upper trace, current; lower trace, potential. D. Plot of the peak conductances determined from A-C, assuming  $E = 60 \text{ mV}$ . The slope was  $0.45 \times 10^{-3} \text{ mho-liter/mol}$ .

the higher concentrations. However, the following factors must be considered before entertaining any type of relationship other than the linear one. *Firstly*, high concentrations of drug greatly reduced the input resistance of the fiber. It can be seen from Eq. 1 that with a feedback amplifier gain of 10,000, and taking an average value of  $10 \text{ M}\Omega$  for the resistance of the current-carrying electrode, when the input resistance fell below about  $20 \text{ K}\Omega$ , the membrane potential fell short of the command potential by 10% or more, with consequent reduction of the true peak current. *Secondly*, the effects of desensitization became more significant at the higher concentrations in at least two ways. The effect of desensitization resulting from incomplete recovery from a previous dose was more apparent when a very high dose was applied. Also, with the higher concentrations, the onset of desensitization was much faster, since the rate of onset of desensitization is exponential with increasing concentrations, and often the maximum expected current would not be achieved. This could be indicated by reduced times from onset of action to peak response, as shown in Fig. 4. The time to peak action for the three smaller concentrations was approximately the same, being about 3 s. For the highest concentration, the time taken was 2 s, the current recorded was  $1 \times 10^{-6} \text{ A}$  and the current-concentration curve (dashed line in Fig. 4) was flattened. But if the upstroke was extrapolated so that the time to peak was approximately

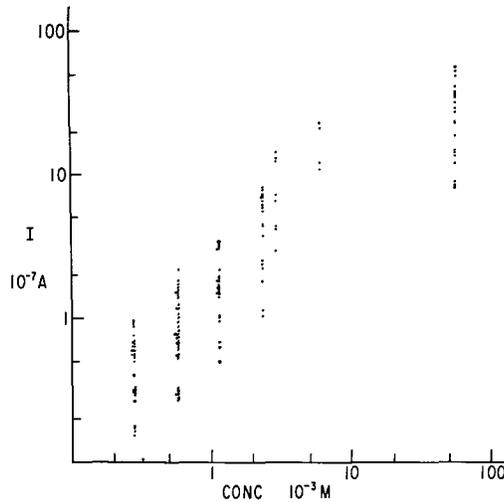


FIGURE 3. Collective results of all applications of acetylcholine to clamped endplates, from both complete and incomplete runs.

the same, the peak current was  $2.5 \times 10^{-6}$  A and the current-concentration curve was linear over the whole dose range tested. This was equivalent to measuring the rate of change of current, which, however, was not measured electronically. This effect of desensitization would have been complicated by the further observation that the duration of the upstroke of the response was slower with smaller concentrations and faster with larger concentrations. Hence desensitization effects of this kind apparently did not occur in all experiments, for example, the experiment with carbachol shown in Fig. 2. Since these two effects acted so as to reduce the recorded peak current, it was extremely likely that they alone could account for any observed deviations from linearity, at least for concentrations of acetylcholine as high as  $2 \times 10^{-2}$  M, the highest concentration routinely used. That these effects were not interfering with the responses would have to be ascertained before the linear hypothesis could be rejected in favor of some other hypothesis, at least over the concentration ranges involved.

#### *Measurements of Potential Changes*

DERIVATION OF THE RELATIONSHIP BETWEEN CONDUCTANCE AND DEPOLARIZATION Fig. 5 is a schematic diagram of the endplate membrane and the surrounding sarcolemma. It is assumed that a transmitter receptor site with its associated ionic "channels" cannot also be an ionic "channel" for electrical activation. The omission of capacitive circuit elements is equivalent to assuming that the time-course of chemical transmission is long compared with the electrical time constant of the membrane. This is not normally true, but in the case of the relatively slow application of acetylcholine in these ex-

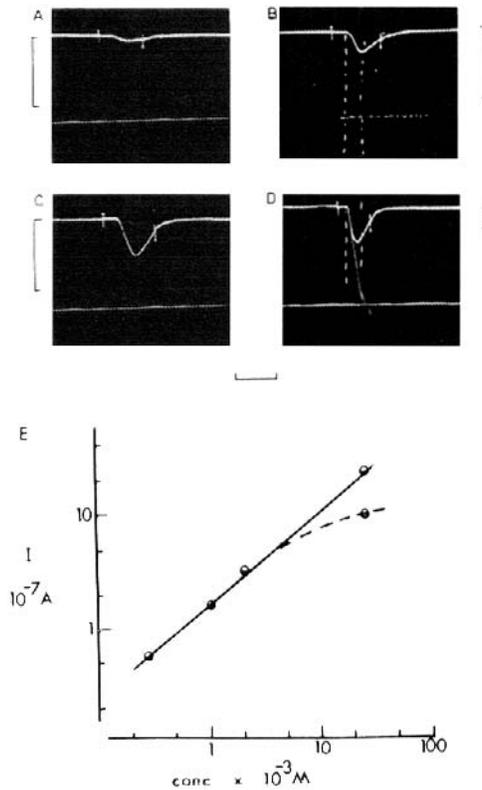


FIGURE 4 A-D. Responses of a voltage-clamped endplate to four different concentrations of acetylcholine: A =  $2.78 \times 10^{-4}$  M; B =  $1.12 \times 10^{-3}$  M; C =  $2.24 \times 10^{-3}$  M; and D =  $2.78 \times 10^{-2}$  M. Upper trace, current; lower trace, potential. Vertical scales =  $5 \times 10^{-7}$  A; horizontal scale = 5 s. E. Plot of the above results. The dashed line passes through the recorded current for the highest concentration. The smooth line passes through the value that might have been expected if the response had not desensitized.

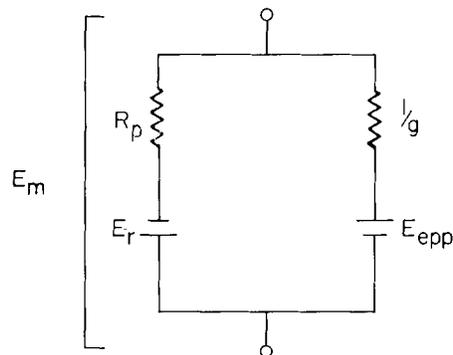


FIGURE 5. Simplified circuit diagram of the endplate membrane (right-hand side) and the surrounding sarcolemma (left-hand side). For further details see text.

periments, the error in making this assumption is negligible. The muscle fiber (left-hand side) is represented by a resistance,  $R_p$ , (the input resistance) in series with a battery,  $E_r$ , that generates the resting potential.  $R_p$  is normally voltage dependent. However, except maybe for responses which reach their peak within 2 or 3 s, the effects of nonlinearities in  $R_p$  due to the voltage dependency of the membrane resistance of the sarcolemma could be neglected without serious error.<sup>1</sup> When acetylcholine acts on the endplate membrane, another resistance,  $1/g$ , in series with a battery,  $E_{ep}$ , is inserted in parallel with the input resistance of the fiber. Since the batteries  $E_r$  and  $E_{ep}$  are not the same, current flows and the recorded transmembrane potential,  $E_m$ , approaches more closely  $E_{ep}$ .  $E_m$  is given by  $(E_r + \Delta E)$ , where  $\Delta E$  is the amplitude of the drug-induced depolarization, i.e., the endplate potential. Hence, the potential difference across  $R_p$  is  $\Delta E$  and across  $1/g$  is  $(E_m - E_{ep}) = (E_r - E_{ep} + \Delta E)$ . At equilibrium the currents through these resistors are equal and opposite, and

$$\Delta E/R_p = g(E_{ep} - E_r - \Delta E). \quad (3)$$

As both  $E_r$  and  $E_{ep}$  depend only on the ionic environment,  $E$  may be substituted for  $(E_{ep} - E_r)$  giving on rearrangement:

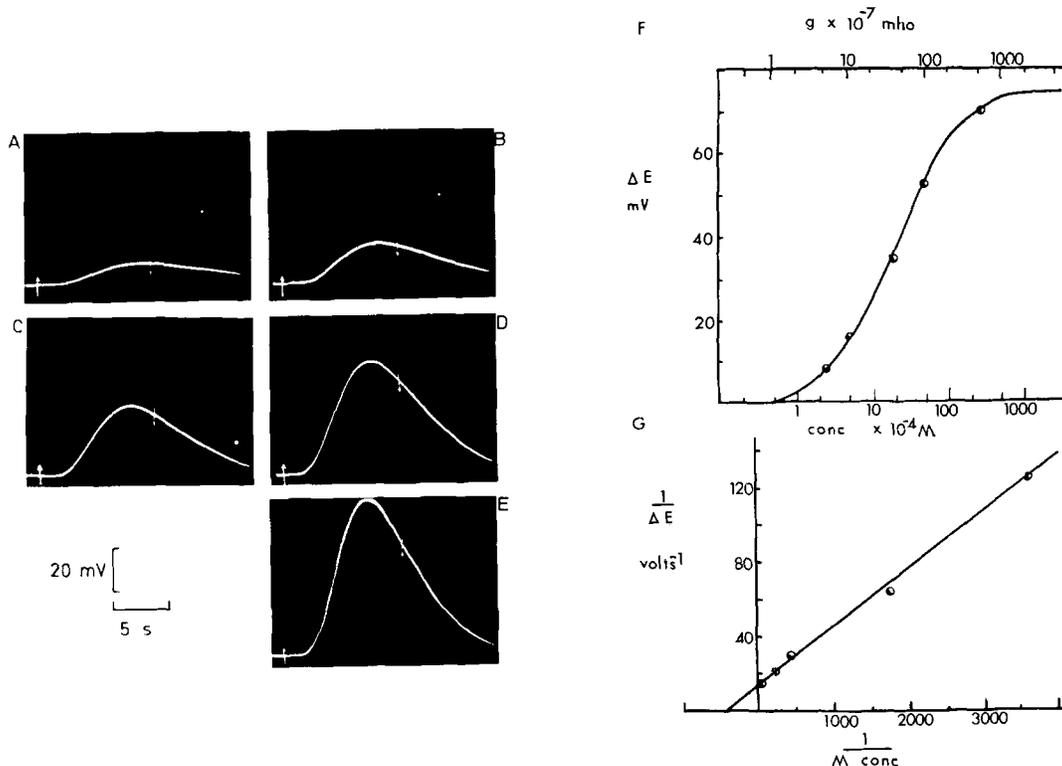
$$\Delta E/(E - \Delta E) = R_p g \quad (4)$$

The relationship between  $\Delta E$  and  $g$  is given then by a section of a rectangular hyperbola. This derivation is essentially the same as that given by Martin (1955) when he wished to examine the nonlinear summation of endplate potentials.

Since it has already been shown that  $g = \mu C$ , this value of  $g$  may be inserted into Eq. 4 to give the relationship between concentration and potential change which will have the same form as that between conductance and potential change. Consequently, provided that the relationship between concentration and conductance is linear, a hyperbolic relationship between concentration and depolarization exists, provided that  $R_p$  is constant, and, conversely, if a hyperbolic relationship between depolarization and concentration is found, the relationship between conductance and concentration *must* be linear, at least over the concentrations involved.

<sup>1</sup> Transient changes in  $R_p$ , occasionally seen as action potentials, were superimposed upon the slower endplate depolarization. Effects of changes in  $R_p$  due to the voltage-dependent increase in potassium conductance, i.e. delayed rectification (Jenerick, 1959; Burke and Ginsborg, 1956), could be significant (a calculated reduction of the maximum response by about 5 mV), but the voltage-dependent potassium inactivation system (Nakajima et al., 1962; Adrian et al., 1966) with its time constant in the order of a second, would reduce the effect of delayed rectification.

**DOSE-RESPONSE CURVE** Fig. 6 A-E shows the responses of an endplate to five different concentrations of acetylcholine. The resting potential of this fiber was  $-85$  mV, and the resting input resistance was  $230$  k $\Omega$ . These membrane characteristics were substituted into Eq. 4, and assuming a value of



**FIGURE 6 A-E.** Depolarizations in an endplate, produced by five different concentrations of acetylcholine: A =  $2.78 \times 10^{-4}$  M; B =  $5.56 \times 10^{-4}$  M; C =  $2.24 \times 10^{-3}$  M; D =  $5.56 \times 10^{-3}$  M; and E =  $2.78 \times 10^{-2}$  M. The resting membrane potential was  $-85$  mV. The input resistance of the fiber was  $230$  k $\Omega$ . The arrows mark the time during which the drug was perfused. F. Plot of the peak depolarizations shown in A-E. The circles are the experimentally obtained values plotted against the logarithm of the concentration. The smooth curve is the solution of Eq. 4 for  $R_p = 230$  k $\Omega$ ,  $E_r = -85$  mV, and  $E_{\text{ep}} = -10$  mV.  $\Delta E$  is plotted against the logarithm of  $g$ . The proportionality constant is  $1.95 \times 10^{-3}$  mho-liter/mol. G. The same results plotted on reciprocal coordinates. The value of the intercept is  $-450$  M $^{-1}$ , which is equivalent to  $230$  k $\Omega$  as explained in text.

$-10$  mV for  $E_{\text{ep}}$ , values of  $\Delta E$  were calculated for various values of  $g$ . The resulting curve relating  $\Delta E$  and  $g$  was superimposed over the experimentally determined points as in Fig. 6 F. A concentration of  $2.78 \times 10^{-4}$  M acetylcholine was equivalent to a conductance of  $5.4 \times 10^{-7}$  mho, and  $p$ , the proportionality constant, was  $1.95 \times 10^{-3}$  mho-liter/mol. These curves are

plotted in Fig. 6 F on a semilog scale merely for convenience. The smooth curve represents the theoretical relationship and the circles the experimental points.

The proportionality constant was not normally determined by this method. Consideration of Eq. 4 shows that the relationship between  $1/\Delta E$  and  $1/g$  is linear, the intercept on the ordinate being  $1/E$ , and the intercept on the abscissa being that value of  $-1/g$  equal to  $-R_p$ . Hence, a plot of the reciprocal of the concentration ( $1/C$ ) against  $1/\Delta E$  will be linear provided that concentration is proportional to conductance.  $E_{o,p}$  need not be known. The proportionality constant is given by the ratio of the measured value of  $R_p$  and the value of the intercept on the abscissa expressed as  $1/C$  (= liter per mole). This is shown in Fig. 6 G. The mean value of the proportionality constant obtained by these methods from 13 experiments on 10 frogs was  $3.70 \pm 0.93$  (SE of the mean)  $\times 10^{-3}$  mho-liter/mol, range 1.14 to 7.70. A second group of frogs which came from a different supplier were more sensitive to acetylcholine. The mean value from eight experiments on seven frogs was  $18.6 \pm 3.3$  (SE of the mean)  $\times 10^{-3}$  mho-liter/mol, range 11.4 to  $37.0 \times 10^{-3}$  mho-liter/mol.

If the experimentally determined potential changes were not described by a section of a hyperbola, either the relationship between concentration and conductance would not be one of direct proportionality, or  $R_p$  in Eq. 4 would not be a constant over the voltage ranges encountered. Changes in the other parameters would affect the maximum response or the shape and slope of the dose-response curve. Good fits to the predicted hyperbolic relationship were found. Small deviations that may have existed around the resting potential (Katz and Thesleff, 1957; Podleski and Changeux, 1970) were not looked for.

It should be noted that different values of the proportionality constant result in parallel shifts of the theoretical curve along the abscissa, when it is plotted on a semilog scale. The corollary to this is that the parallel shifts of semi-logarithmically plotted dose-response curves that occur in the presence of so-called competitive antagonists such as curare (Jenkinson, 1960), do not imply at all that the antagonism is "competitive" in the classical sense. The only information that is given by such parallel shifts is that the proportionality constant between concentration and conductance is decreased in some unknown manner by the presence of the antagonist. But as long as the dose-response curve is hyperbolic in the presence of the antagonist the relationship between concentration of acetylcholine and conductance must still be linear, provided, of course, that  $R_p$  has not been changed by the antagonist, since different values of  $R_p$  also cause parallel shifts of the theoretical curve. In the case of curare, this is not so (del Castillo and Katz, 1957), and it is also known that curare acts by reducing the sum of ( $\Delta g_{Na} + \Delta g_K = g$ ) without affecting the ratio  $\Delta g_{Na}/\Delta g_K$  of the endplate membrane (Takeuchi and Takeuchi,

1960 *a*). None of the above results and conclusions deny that the conductance-concentration relationship might not eventually with very high concentrations of acetylcholine become nonlinear, and presumably flatten out, and indeed with high concentrations of curare ( $1.25 \times 10^{-5}$  M) some indication of this was found. Paton and Waud (1967) who measured a response with external electrodes, also found that the dose-response curve flattened with the higher concentrations of the antagonist, and hence that the maximum effect could not be obtained with the agonist, i.e., the antagonism seemed unsurmountable. Experiments with curare which theoretically should have enabled explorations of the higher dose ranges, gave equivocal results. The responses produced by the very high concentrations of acetylcholine necessary when high concentrations of *d*-tubocurarine were present all repolarized very rapidly, indicative of a rapid onset of desensitization, so that it was often impossible to see the "proper" response. As these experiments were not done in voltage-clamp mode, the rate of change of the upstroke of the current surge could not be estimated, though this might have been profitable.

These results have confirmed the existence of a hyperbolic relationship between concentration and potential change. The theoretical calculations show that for such a relationship to exist the underlying relationship between concentration and conductance must be linear. And the voltage-clamp experiments indicated that this relationship is in fact linear.

#### *Other Compounds*

If another compound acts in the same way as acetylcholine, the same qualitative relationships between concentrations and conductances or depolarizations should exist. For less potent compounds the proportionality constant should be less. The effects of carbachol on a voltage-clamped endplate have already been shown (Fig. 2) to be linear. The other compounds tested, namely, succinylcholine, decamethonium, *N*-methylarecoline, and phenylethyltrimethylammonium, were examined on unclamped endplates, and in all cases the predicted hyperbolic relationship was found. This indicates that with all these drugs, the conductance changes produced were directly proportional to the concentration of drug. Fig. 7 A-F shows the effects of four different concentrations of phenylethyltrimethylammonium and two concentrations of acetylcholine on the same endplate. The microelectrode became dislodged with the second dose of acetylcholine. The time-courses of the responses to the two compounds were the same. The peak depolarizations are plotted in Fig. 7 G on reciprocal coordinates.  $R_p$  had not been measured in this fiber so the individual values of the proportionality constant could not be determined. The potency, expressed as the molar ratio, is given by the ratio of the intercepts on the abscissa, namely,  $5,100/2,500 = 2.04$ . The potencies measured this way for all drugs tested, are given in Table I.

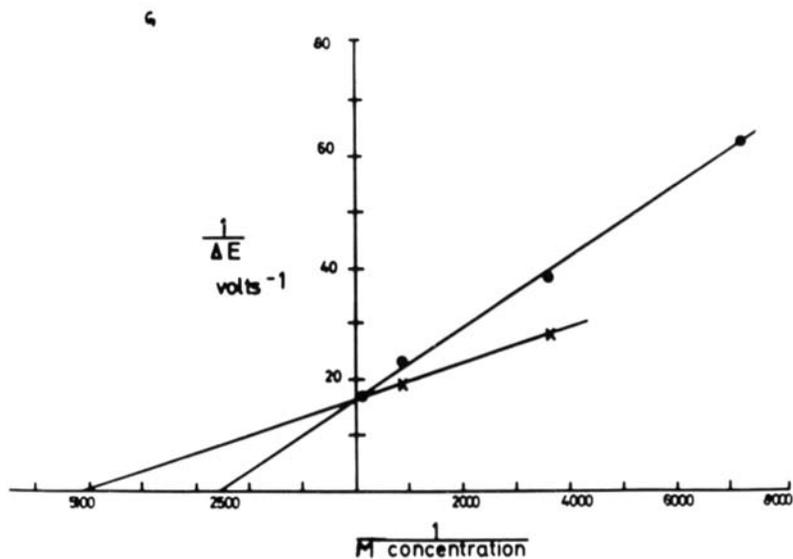
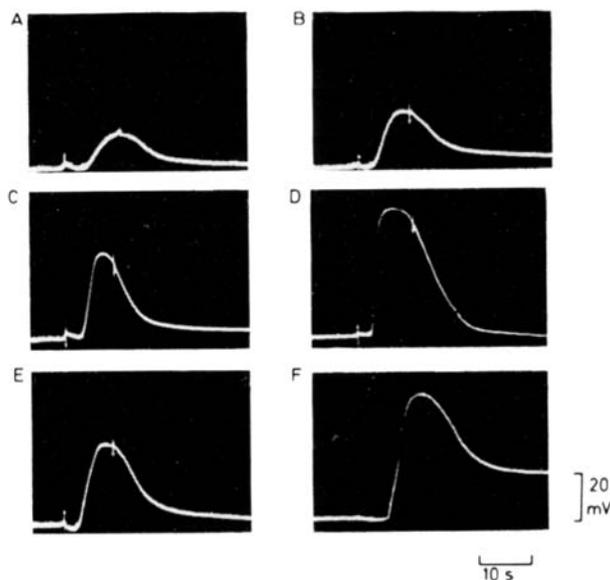


FIGURE 7 A-D. Depolarizations in an endplate produced by four different concentrations of phenylethyltrimethylammonium: A =  $1.39 \times 10^{-4}$  M; B =  $2.78 \times 10^{-4}$  M; C =  $1.10 \times 10^{-3}$  M; and D =  $5.56 \times 10^{-3}$  M. E-F. Responses in the same endplate to acetylcholine: E =  $2.78 \times 10^{-4}$  M and F =  $1.12 \times 10^{-3}$  M. G. Results of A-F plotted on reciprocal coordinates. The filled circles are the responses to phenylethyltrimethylammonium and the crosses, to acetylcholine. The molar ratio of phenylethyltrimethylammonium relative to acetylcholine is given by the ratio of the intercepts on the abscissa, i.e.  $5,100/2,500 = 2.04$ , as described in text.  $R_p$  was not measured.

TABLE I  
 DRUG POTENCIES COMPARED TO ACETYLCHOLINE ON THE  
 DEPOLARIZATION RESPONSE

Drug	Molar ratio	no. of exps	no. of frogs
Phenylethyltrimethylammonium	$1.95 \pm 0.08^*$	6	4
Succinylcholine	$3.40 \pm 0.07^*$	5	3
Carbachol	$1.80 \pm 0.15^*$	8	5
<i>N</i> -Methylarecoline	$1.77 \pm 0.05^*$	4	3
Decamethonium	$47 \pm 10^*$	9	4
	(Range 17-94)		

\* SE of the mean.

### *Slow Fibers*

A few experiments were done on slow muscle fibers to find out if the same sorts of relationships existed between dose and response as existed in the fast fibers. It is well known that the relationship between concentration of acetylcholine and the graded contracture in this muscle is hyperbolic (Clark, 1933; Paton, 1957) and it is considered that the contracture is directly related to the depolarization of the cell membrane. The endplates in slow fibers are not localized in one defined region of the fiber as in fast fibers (Kuffler and Vaughan-Williams, 1953); also, the average diameter of a slow fiber is small (Adrian and Peachey, 1965), which made it more difficult to insert a second microelectrode. Hence voltage-clamp studies were not done. Although the rectus abdominis of the frog is particularly rich in slow fibers, they still may represent less than half the total number of fibers (Kuffler and Vaughan-Williams, 1953). The criteria used for a successful penetration of a slow fiber were (a) that the resting potential was low ( $-40$  mV to  $-65$  mV); (b) that miniature endplate potentials could be obtained anywhere along the fiber—it was noticed that as the electrode was moved along a fiber the mean amplitude of the miniatures varied, indicating zones of greater or less sensitivity, although the sensitivity was never completely absent; and (c) the form of the response, which, unlike the response in the fast fibers, was bumpy, due most likely to contributions from more distant parts of the fiber. The response also lasted longer, desensitized less, and recovered from desensitization much faster.

Fig. 8 shows tracings of the potential changes in a fiber in the rectus abdominis of a frog, and a plot thereof on reciprocal coordinates. The depolarization-concentration curve was hyperbolic for both the maximum response and for the initial bumps, and there was no reason in this experiment or in others to doubt that the conductance change was linearly related to the concentration as in fast fibers. Hence, it would appear that the hyperbolic dose-response curve between contractures and the log of the dose in slow muscle fibers is imposed, not by the initial action at the receptor, but by the electrical properties of the membrane.

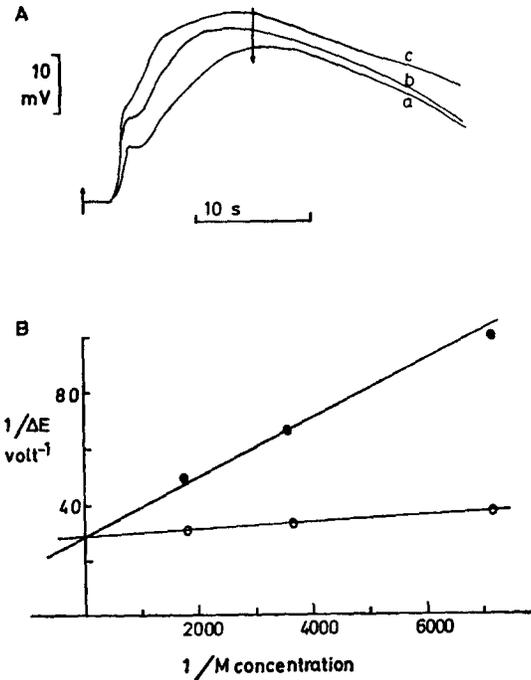
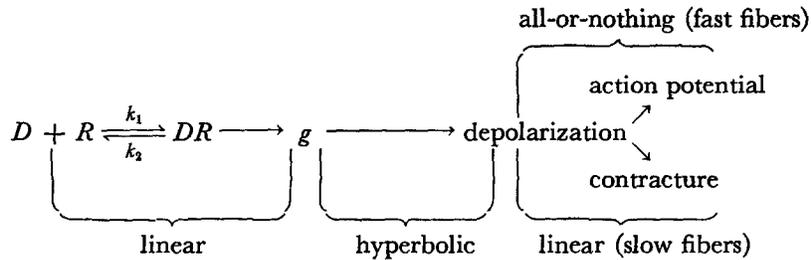


FIGURE 8 A. Tracings of the depolarizations produced by acetylcholine in a slow muscle fiber:  $a = 1.12 \times 10^{-4}$  M;  $b = 2.78 \times 10^{-4}$  M; and  $c = 5.56 \times 10^{-4}$  M. B. Plot of the results on reciprocal coordinates. The filled circles are the values recorded at the "bump" and the open circles are the peak depolarizations.  $R_p$  was not measured.

DISCUSSION

The relationship between concentration of acetylcholine and the resulting conductance change at the motor endplate was linear, over concentration ranges where the relationship between concentration of acetylcholine and the change in potential was hyperbolic. It has generally been accepted that this hyperbolic relationship is imposed, in the main, by the drug-receptor properties. The findings reported here suggest that at the motor endplate at least, this hyperbolic relationship is not imposed at the chemoreceptive level, but later, at the membrane level, by the electrical properties of the membrane. The functional relationships may be summarized as:



where  $D$  represents drug,  $R$  represents receptor,  $DR$  represents an association of the drug and a receptor, and  $g$  is the resulting conductance change.  $k_1$  and  $k_2$  are the forward and backward rate constants of an initial drug-receptor interaction.

A linear relationship between concentration and conductance, with no obvious maximum response, was unexpected within the context of current theories of drug action. However, it is now apparent that as long as a hyperbolic relationship between depolarization (or contracture) and concentration, with a consequent maximum response, is maintained, even with antagonists present, the initial linear relationship still exists. The occurrence of parallel log dose-depolarization (or contracture) curves does not necessarily imply that the antagonism is competitive. If the bimolecular reaction always postulated for drug action does occur, and the number of receptors is limited, and the measurements do reflect this reaction, then the value of the equilibrium constant,  $K_e$  ( $= k_2/k_1$  in above diagram), must be very large and the formation of the drug-receptor complex would not be favored, as the following calculation shows. From the law of mass action, and with the assumption of a limited number of receptors (without this assumption no unique solution for the equation involving the proportion of receptors occupied at equilibrium,  $y$ , could be obtained since the equation would be a quadratic in  $y$ ), the proportion,  $y$ , of receptors occupied at equilibrium is  $y = x/(x + k_2/k_1)$ , where  $x$  is the concentration of drug. If the measured response is assumed to be proportional to  $y$ , then for the response to be directly proportional to the concentration  $x$ ,  $k_2/k_1$  ( $= K_e$ ) must be very much greater than  $x$ . Thus  $K_e$  for acetylcholine could be no less than  $2 \times 10^{-2}$  mol/liter, this being the concentration of acetylcholine that consistently did not flatten the concentration-conductance curve, or the concentration with which the maximum depolarization could always be obtained with an antagonist present. If the association rate constant,  $k_1$ , is limited only by diffusion, its value is  $2.5 \times 10^9$  liter/mol-s at  $37^\circ\text{C}$  (Burgin, 1966), and the average half-life ( $= 1/k_2$ ) of any complex would be  $2 \times 10^{-8}$  s, a negligible value for any "occupation" theory. The results could also indicate some novel drug-receptor interaction for which there are endless possibilities for speculation.

However, there is no reason a priori for assuming that the number of receptors is limited. Mathematically it is not even necessary to postulate a receptor inasmuch as a unimolecular reaction appears to be taking place. If a bimolecular reaction is occurring, the simplest explanation is that the receptor is in excess, and one is reminded of Clark's (1933) original idea of physical adsorption at a surface. Even if there are specialized molecular regions which, of course, there must be, recent work using bungarotoxin and autoradiographic techniques shows that there are in kinetic terms many of these areas. Miledi et al. (1971) estimated approximately  $1 \times 10^4$  active sites

per  $\mu\text{m}^2$  of receptive surface of *Torpedo* electric tissue, Barnard et al. (1971) found a density of  $1.2 \times 10^4$  per  $\mu\text{m}^2$  in mouse skeletal muscle, and in frog skeletal muscle, Miledi and Potter (1971) estimated  $1 \times 10^5$  receptors per  $\mu\text{m}^2$ . Hence, use of kinetics based on a bimolecular reaction involving a limited number of receptors would seem unwarranted.

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