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VOLTAGE DEPENDENCE OF AGONIST EFFECTIVENESS AT THE FROG NEUROMUSCULAR JUNCTION: RESOLUTION OF A PARADOX

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SUMMARY

1. End-plate currents produced by nerve-released acetylcholine and iontophoretically applied acetylcholine and carbachol have been recorded from voltage-clamped frog cutaneous pectoris neuromuscular junctions made visible with Nomarski differential interference contrast optics.

2. The effectiveness of agonists – that is, the end-plate conductance change produced by a given dose – has been determined as a function of post-junctional membrane potential.

3. As the post-junctional membrane potential is made more negative, nerve-released acetylcholine becomes less effective whereas iontophoretically-applied agonists become more effective.

4. This voltage dependence of agonist effectiveness is mediated neither by end-plate current iontophoresis of agonist into the cleft nor through electric field effects on the esterase.

5. Influences of membrane potential on the opening and closing of end-plate channel gates can account quantitatively for the voltage-dependent effectiveness of both nerve-released and iontophoretically applied agonist.

INTRODUCTION

Several workers have found a non-linear relationship between end-plate current (e.p.c.) amplitude and membrane potential at the voltage-clamped frog neuromuscular junction (Kordas, 1969; Magleby & Stevens, 1972*b*): at the more hyperpolarized membrane potentials, the peak e.p.c. is smaller than one would expect from a linear relationship fitted to the peak currents around the reversal potential. In other words, the peak conductance change produced by nerve released acetylcholine (ACh) is not

constant, but becomes smaller as the post-junctional membrane potential is made more negative.

We have also noticed that the amplitude of e.p.c.s produced by iontophoretically applied agonists depends non-linearly on membrane potential, but the curvature of this current–voltage relation is opposite that seen with nerve-released ACh.* That is, when ACh is applied iontophoretically, the end-plate conductance change produced by a fixed concentration of agonist becomes progressively larger for more hyperpolarized membrane potentials. Similar observations have been made by Rang (1973*a*) on end-plate and J-P. Changeux & H. A. Lester (personal communication) on electroplax.

It appears, then, that the effectiveness of ACh at the frog neuromuscular junction, as measured by the magnitude of the conductance change produced by the application of a fixed quantity of agonist, depends upon membrane potential. Further, the direction of this voltage dependence of agonist effectiveness varies according to the mode of application. If ACh is applied by nerve-evoked release from the axon, agonist effectiveness decreases as the membrane is hyperpolarized, whereas, with iontophoretic application, effectiveness increases with membrane hyperpolarization.

We report here our analysis of this paradoxical voltage dependence of agonist effectiveness.

The data presented here support the hypothesis that the voltage dependence of ACh effectiveness derives from membrane potential influences on the opening and closing of end-plate channels. We have carried out our analysis in terms of the theory (Magleby & Stevens, 1972*b*) that a channel has only two conductance states, open and closed, and that transition between these states is governed by voltage-dependent rate constants α (closing) and β (opening). The essential difference between nerve- and iontophoretically applied ACh then lies in the duration of agonist action. Nerve-applied ACh is present for only a short time relative to the duration a channel remains open: thus the amplitude of the resulting conductance will depend on the total number of channels that can open during the brief increase in cleft ACh concentration. The peak conductance change therefore will depend on the opening rate constant β but not upon the closing rate α because so few channels will have time to close during the ACh transient. On the other hand, iontophoretically applied ACh is

* In some instances it will be necessary to maintain a distinction between e.p.c.s produced by nerve-released ACh and those which result from the iontophoretic application of agonists. Wherever this distinction is desirable and may not be clear from context, we shall indicate the source of the agonist by the subscript N for nerve and I for iontophoresis. Thus, for example, an e.p.c. produced by iontophoretically applied carbachol would be abbreviated e.p.c.$_I$.

present for a long time relative to the lifetime of an open channel. The amplitude of the resulting steady-state conductance will reflect the equilibrium population of open channels, and therefore will depend on the ratio of the opening to the closing rate constants, β/α. Both opening and closing rates decrease as the membrane potential moves more negative, but the rate constant for channel closing depends more strongly upon voltage than the rate for opening (Magleby & Stevens, 1972*b*) so that the ratio β/α increases with hyperpolarization. Thus, as membrane potential is made more negative, the nerve-evoked peak end-plate conductance diminishes in proportion to the decrease in β, whereas the conductance change produced by iontophoretically applied agonist increases in proportion to the ratio β/α.

METHODS

All observations reported in this paper were made on cutaneous pectoris muscles obtained from small specimens of *Rana pipiens*. Our techniques for preparing this muscle are similar to those described recently by Dreyer & Peper (1974). The muscle was removed from the frog and placed external surface up in a small plastic chamber (volume, 4 ml.) with a glass bottom 200 μm thick which allowed close approach of the microscope condenser for transillumination; the muscle was held flat against this bottom glass plate by two spring clips which pressed on a small strip of attached skin and the cartilage removed with the muscle. After the muscle was in place in the chamber, successive layers of muscle fibres – the muscle is perhaps ten muscle fibres thick – were removed until only a single layer of muscle fibres remained. Generally ten to twenty of these fibres had intact innervation and end-plates visible on the upward-facing surface. In some cases the muscle was then pre-treated with 400 mM glycerol in frog Ringer for 40 min (Howell & Jenden, 1967; Gage & Eisenberg, 1967). During experiments the muscle was maintained in frog Ringer with the composition (mM): NaCl, 117·2; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·16; and NaH_2PO_4, 0·85, to which 100 nM tetrodotoxin had been added; pH was adjusted to 7·2.

The preparation in its chamber was placed on the stage of a modified Zeiss–Nomarski microscope similar to that described by McMahan, Spitzer & Peper (1972). Cool water circulated through the stage of this microscope and the experimental chamber rested on two Peltier coolers that could transfer heat between the chamber and the stage. Temperature was measured at the edge of the preparation with a thermistor probe and could be controlled at this probe to values between freezing and room temperature by altering current through the Peltier devices. At the colder temperatures (near 0° C) a several-degree gradient existed between the probe and the point at which micro-electrodes were inserted into the muscle fibre. Generally, temperature was maintained close to 14° C, but we have made observations at temperatures ranging from 5 to 18° C; temperatures maintained during specific experiments are reported in the text. Most investigations were carried out with a Zeiss 16× long working distance objective and 10× or 15× eyepieces; this arrangement enabled the end-plates and the various micro-electrodes used to be clearly seen.

The two-electrode voltage clamp was used for the studies reported here; this equipment is essentially similar to that described earlier (Magleby & Stevens, 1972*a*) but has been modified in two important respects. Firstly, instead of employing the virtual bath-ground current measuring system as for earlier studies, we measured

currents as the voltage drop across a 1 MΩ resistor in series with the current-passing electrode. Secondly, we improved the voltage-clamping amplifier to provide better frequency response and membrane potential control by having the feed-back amplifier gain increase inversely with frequency below about 5 kHz.

ACh was applied directly by the iontophoretic technique (Nastuk, 1953) to end plates viewed through the microscope. Micro-electrodes were filled with a nominal 2 M acetylcholine chloride (Sigma) solution and had, when measured in saline, resistances in the range 20–100 MΩ, more generally toward the lower end of this range. Backing current in the range 5–20 nA was used, and this was more than sufficient to reduce the resting efflux of ACh from the micropipette to insignificant levels. Backing was considered adequate if the iontophoretic electrode could be moved to within 1 or 2 μm of the voltage-clamped end-plate without giving rise to detectable e.p.c.s due to the leakage of ACh. A feed-back circuit, similar in principle to that employed for voltage clamping, was used to maintain constant current through the ACh iontophoretic electrodes, and the iontophoretic circuit was optically isolated from the bath reference ground.

We wished for this study to ensure that the quantity of ACh released by the nerve was constant and that the release occurred at a relatively well defined location close to the voltage-clamping electrodes. In some instances the frog neuromuscular junction extends over many hundred μm (McMahan *et al.* 1972; Kuno, Turkanis & Weakley, 1971), and we have found that degradation can occur in high frequency components of e.p.c.s arising more than about 200 μm from the point of voltage control. To meet these requirements of a consistently uniform quantity of transmitter released at a known location, we used locally stimulated miniature end-plate currents (m.e.p.c.s). The use of m.e.p.c.s had the further advantage of minimizing the duration of ACh release (e.g. Katz & Miledi, 1965; Barrett & Stevens, 1972) and thereby improving the accuracy of the approximations used in the derivation of eqn. (8) on page 255.

To evoke release of transmitter quanta, we made the bathing solution hypertonic in one small region of the end-plate (Fatt & Katz, 1952) by pressure ejecting a 2·5 M sucrose solution from a micropipette which had a tip diameter of approximately 5 μm. By adjusting the position of this micropipette relative to the end-plate we could easily produce any desired rate of quantal release; most usually, a rate between 10–100 m.e.p.c./sec proved convenient for the studies reported here. The critical positioning required for this sucrose-filled electrode suggests that the evoked m.e.p.c. release occurred over a quite restricted region. The m.e.p.c.s were sampled with a digital computer and recorded in a circular buffer such that a triggering criterion reached on the rising phase of the m.e.p.c. allowed the earlier part of the rising phase as well as the preceding base line to be saved.

Proper analysis of the effects under investigation requires the ability to deliver repeated identical doses of agonist to the post-junctional membrane. To evaluate the adequacy of our techniques for applying agonist iontophoretically, we have made use of an ion-sensitive micro-electrode, developed in our laboratory for other studies, which responds to the concentration of quaternary ammonium compounds much as a standard glass pH electrode reports hydrogen ion concentration. As reported by Baum (1970), the ion exchange resin no. 477317 (supplied by Corning) is sensitive to quaternary ammonium ions. The relative selectivity of this ion exchanger for ACh over potassium is approximately 100:1. By properly adjusting the reference solution on one side of the exchanger, the non-Nernstian responses previously reported (Baum, 1970) can be corrected and the response time of the electrode minimized. Thus, suitably constructed liquid membrane micro-electrodes much like those described by others (e.g. Walker & Brown, 1970; Walker, 1971) can be used for the detection

of ACh and other quaternary ammonium compounds. Specifically, we used standard glass micropipettes (which, if filled with 3 M-KCl, would have a resistance in Ringer of approximately 2–8 MΩ, and filled the tip approximately 250 μm with the ion exchanger. The reference solution in the electrodes combined 2 M-KCl with 1·2–1·5 M (nominal) AChCl. The internal connexion was an Ag:AgCl wire. Voltage was recorded from the ion-sensitive micro-electrode with a MOSFET negative capacity pre-amplifier (Neher & Lux, 1973) which gave a response time constant of approximately 5–10 msec with the typical 500 MΩ electrode resistance.

In calibration experiments with ACh or carbamylcholine chloride (carbachol, Nutritional Biochemical Corp.), it has been found that the electrode voltage varies approximately as the logarithm of the quaternary ammonium ion concentration as low as 10^{-5} M when no other ions compete. Under the conditions of our experiments, however, sodium and potassium ions are present in concentrations which are high compared to those of ACh. These ions are transported through the membrane to some extent, and result in voltages appearing across the membrane. In the presence of fixed concentrations of sodium and potassium (and other cations) the electrode voltage depends on ACh concentration according to the relation

$$V - V_0 = A \log_e(B + C),$$

where $V - V_0$ is the electrode voltage (the voltage difference across the electrode ion exchange membrane), A is a temperature-dependent parameter, B is a factor which involves the activities of other ions present (together with their relative selectivity) and C is the quaternary ammonium ion concentration (see Sandblom, Eisenman & Walker, 1967, for a theoretical treatment of electrode operations). For low concentrations of ACh, this relationship may be linearized to give

$$V \simeq \frac{A}{B} C + (V_0 + A \log_e B).$$

Thus, for low ACh concentrations in saline the electrode response is simply proportional to concentration. Under the conditions of our experiments, this linear relationship is an adequate approximation (within the measurement errors) up to about 0·5 or 1 mM ACh, with the constant A/B equal to approximately 5 mV/mM.

Operation of this quaternary ammonium ion-sensitive electrode has been evaluated in two main ways. First, we have after each use measured a calibration curve with the appropriate ion over a range of steady-state concentrations much wider than those encountered during the experiment. To evaluate the ability of the electrode to measure concentration transients, we have positioned the ion-sensitive electrode at various distances from an iontophoretic electrode and applied square pulses of iontophoretic current to eject the contained ions. The rise and fall of concentrations at distances around the iontophoretic electrode should, for distances large compared to the tip diameter, follow the diffusion equation for fluxes originating from a point source, and the ion-sensitive electrode should then give responses which fit the diffusion equation. A single parameter in these solutions to the diffusion equation is the diffusion constant and this should have the same value at all spacings between the iontophoretic electrode and the concentration-detecting electrode. We have found that solutions for the diffusion equation do adequately fit our ion-sensitive electrode responses at all distances between 5 and 35 μm. The diffusion constant extracted from such measurements for carbachol is about 5×10^{-6} cm²/sec. Thus, the ion-selective electrode does indeed report concentrations accurately and responds to these concentrations with a time constant which is rapid compared to the fastest responses we have investigated (one-half rise-time of about 50 msec).

Data collection and analysis were carried out with the aid of a digital computer. Voltages led from the preparation were suitably amplified, then sampled at rates specified in connexion with the various experiments using an analogue to digital converter with eleven bits of accuracy. The level of pre-amplification is selected so that a large part of the total converter range is used and digitizing errors are thereby minimized.

RESULTS

Agonist effectiveness is defined here, for a particular agonist concentration, as the ratio of e.p.c. to the membrane potential maintained by voltage-clamp control; effectiveness of an agonist, then, is measured in terms of the conductance change it causes. Several authors have demonstrated that the effectiveness of nerve-released ACh decreases with hyperpolarization (Kordaš, 1969; Magleby & Stevens, 1972*b*; see Fig. 4*c*). The reasons for the conclusion that hyperpolarization increases the effectiveness of iontophoretically applied ACh are shown in Fig. 1, where $e.p.c._I$

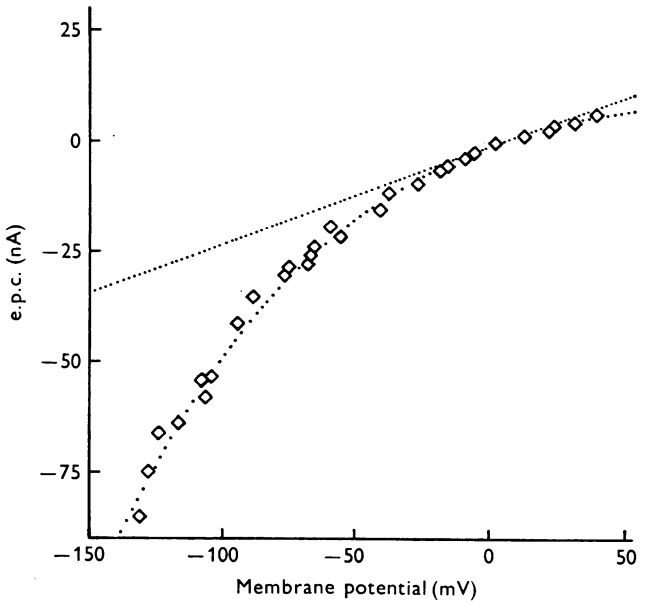

Fig. 1. Amplitude of e.p.c. as a function of membrane potential for a fixed ACh dose. As the membrane is hyperpolarized the e.p.c. resulting from a constant-current iontophoretic application of ACh is seen to increase in magnitude non-linearly and in a manner not approaching saturation. The dotted straight line is fitted to the data points near $V_{eq} = 0$ mV and provides a linear current–voltage curve for comparison. The dotted curved line is fitted from eqn. (8) discussed in the text with the parameter values indicated below. The preparation was treated with glycerol. Temp. = 14° C.

is plotted as a function of membrane potential for a constant dose of ACh from the iontophoretic micropipette. The effect is, as seen in the Figure, a rather large one: e.p.c.s twice or more what would be expected if conductance were voltage-independent occur at the more negative membrane potentials. In the following consideration of the various mechanisms which might account for this effect, much of the discussion deals, for simplicity, primarily with the voltage-dependent agonist effectiveness seen for iontophoretic agonist application.

Part I. Theoretical background

The effectiveness of ACh could depend on membrane potential as a result of at least three classes of mechanisms: (1) end-plate channels could rectify; (2) the actual concentration of ACh in the cleft could depend on voltage or, through voltage, on e.p.c.; and (3) agonist–receptor interactions or channel properties (for example, the probability that a channel opens) could vary with membrane potential. For the voltage ranges and ionic compositions of bathing media used in our experiments, however, channels show no detectable rectification (Magleby & Stevens, 1972*b*). We have therefore investigated the magnitude of effects of voltage on dose effectiveness in both of the two remaining classes.

Mechanisms involving cleft agonist concentration. Voltage might influence cleft ACh concentration by altering the hydrolytic rate of the acetylcholinesterase in the cleft. Thus, a given dose of ACh would produce a greater cleft concentration if the esterase turn-over number were decreased. Conceivably, post-junctional hyperpolarization could modify the rate of activity of membrane-bound esterase and thereby make the agonist more effective at hyperpolarized voltage levels. The magnitude of this effect can be evaluated by examination of the voltage dependence of agonist effectiveness for situations in which the esterase plays no role. The experimental possibilities thus are to poison the esterase or to use an agonist which is little hydrolysed. Because of complications introduced by anticholinesterase treatment, we have chosen to substitute carbachol for ACh instead of poisoning the esterase. We have found, as will be described (see page 258), that iontophoretically applied carabachol has a voltage-dependent effectiveness quantitatively similar to that of ACh, so that voltage-dependent esterase activity, although it may in fact exist, is not a suitable explanation in the present context.

A second factor determining the cleft concentration of agonist applied iontophoretically is the flux of agonist into the cleft, a mechanism first suggested by Takeuchi & Takeuchi (1959) in a different context. E.p.c.s must move, by iontophoresis, a certain additional fraction of ACh into or out of the cleft. Although this effect is probably not a large one, the inward currents associated with more negative membrane potentials might be sufficient to increase agonist cleft concentrations and therefore produce greater effectiveness. The magnitude of the perturbation caused by iontophoresis of ACh depends jointly on the $e.p.c._I$ magnitude and upon the concentration of ACh in the bathing medium, as described below. The $e.p.c._I$ depends upon the end-plate conductance change, which is itself a function of bath ACh

concentration. It follows that the relative perturbation of ACh cleft concentration is determined by both ACh concentration and voltage. Hence, the result of this iontophoretic mechanism is that the function which relates ACh effectiveness to membrane potential should depend on bath ACh concentration. The operation of agonist iontophoresis by e.p.c.$_I$s can thus be detected by investigating the concentration dependence of the voltage-agonist effectiveness relationship. We have found (see page 260) that the voltage dependence of ACh effectiveness does not depend on concentration. Although the iontophoresis of ACh must occur, it does not contribute importantly to the ACh response under the conditions of our experiments.

Test for assessing the importance of agonist iontophoresis by e.p.c. In assessing the sources for voltage dependence of agonist effectiveness, one may distinguish two general classes of mechanisms. First are mechanisms (Class 1) for which the shape of the e.p.c.–membrane potential relationship (Fig. 1) changes with agonist concentration, and second are those mechanisms (Class 2) for which the shape of this relationship is the same for all concentrations of a given agonist. It will be seen that the iontophoresis mechanism discussed above falls into Class 1, whereas voltage-dependent gating, considered later (see page 254), is an example of a theory in Class 2.

It will be seen that there is an easy test for distinguishing between Class 1 and 2 mechanisms: if the e.p.c.-membrane potential relation does not change its shape as agonist concentration is varied, then a plot of e.p.c.s for various voltages at one concentration as a function of e.p.c.s for the corresponding voltages at a different concentration should be linear; for Class 1 mechanisms, however, such a plot will be non-linear. In the following paragraph we show that the iontophoresis mechanism does indeed fall into Class 1, and we derive expressions which allow us to predict the curvature of the e.p.c. (one concentration) *vs* e.p.c. (a second concentration) plot.

Iontophoresis, a Class I Mechanism. The perturbation of cleft ACh concentration by e.p.c. iontophoresis may be assessed quantitatively in terms of the treatment which follows; the conclusions of this simplified development have been confirmed by more detailed calculations which take into account the physical mechanisms underlying the iontophoretic effect and also the agonist concentration gradients which occur along the end-plate. We have, for convenience, assumed that end-plate conductance is proportional to the square of ACh concentration – this assumption is in accord with our preliminary assessments of the dose–response curve – but the theory to follow makes no essential use of this. Several approximations are made in the derivation, and the adequacy of these approximations has in each case been verified for the range of variables over which the final equations are applied.

For the relatively low concentrations of ACh that obtain in our experiments, the cleft concentration of ACh, C in the absence of e.p.c., is linearly related to the bath concentration C_o, and the perturbation by iontophoresis of the cleft concentration is proportional to the e.p.c.$_I$ magnitude (I):

$$C = C_o (k_o + k_1 I). \tag{1}$$

The parameter k_o may be obtained from solutions of the diffusion equation with the appropriate boundary conditions (which incorporate esterase activity), and the parameter k_1, which governs the strength of the iontophoretic effect, depends (according to the flux equation) on the mobility of ACh but not significantly upon ACh concentration itself.

The dose–response curve relates end-plate conductance g to the cleft ACh concentration:

$$g = QC^2.$$

Q is a constant which characterizes, for a given situation, agonist efficacy. The variable, e.p.c.$_I$, measured in our experiments is related to conductance by

$$I = g(V-V_{eq}) = QC^2(V-V_{eq}),$$

where V is the end-plate membrane potential and V_{eq} the end-plate equilibrium potential. Substitution from eqn. (1) yields

$$I = QC_o^2(k_o+k_1I)^2(V-V_{eq}),$$

which, for appropriately small perturbations, may be approximated by

$$I = QC_o^2k_o^2(V-V_{eq})+QC_o^2 2k_ok_1I(V-V_{eq}).$$

This last relation may be rearranged to give

$$I = \frac{I_o}{1-\frac{2k_1I_o}{k_o}}. \tag{2}$$

The e.p.c.$_I$ which would result in the absence of any ACh iontophoresis has been denoted $I_o = Qk_o^2C_o^2(V-V_{eq})$, and is, for a constant ACh concentration, simply proportional to V.

Eqn. (2) should, over an appropriate range of ACh concentrations and membrane potentials, account for the deviations from linearity of the e.p.c.$_I$–membrane potential relationship if the iontophoretic mechanism is the dominant source of these deviations. The term 2 (k_1/k_o) I_o determines the deviation from linearity of the current–voltage relation, and the essential character of this type of theory is that the magnitude of the deviation depends non-linearly upon the unperturbed current I_o; thus, the relative deviation from linearity $(I-I_o)/I_o$ varies with I_o and can be written

$$\frac{I-I_o}{I_o} = \frac{2k_1I_o}{k_o-2k_1I_o}. \tag{3}$$

Class 2 mechanisms. Alternatively suppose that agonist effectiveness depends on voltage only through membrane potential and does not depend upon cleft ACh concentration. Instead of eqn. (3), then, we have

$$\frac{I-I_o}{I_o} = f(V),$$

where $f(V)$ is some function of membrane potential, but not of agonist concentration. (Eqn. (8), to be presented later (page 11), can be reduced to this form by the identification $I_o = MC^n(V-V_{eq})$.) This last equation may be rearranged to give

$$I = (1+f(V))I_o. \tag{3a}$$

The practical test. In practice, a convenient way of detecting a significant participation of the agonist iontophoresis mechanism is to plot the e.p.c.$_I$ measured at a variety of membrane potentials and resulting from the application of one agonist dose as a function of the e.p.c.$_I$ measured at the same voltages but with a different agonist concentration. This plot should be linear for mechanisms of Class 2 (e.g. voltage sensitivity of end-plate channel gating; see below) but curved for Class 1 (e.g. e.p.c. iontophoresis of ACh).

This test to distinguish between Class 1 and 2 mechanisms may be derived as follows. Let $I(1, V)$ and $I(2,V)$ be the e.p.c.$_I$s measured at voltage V and two different ACh concentrations specified by the indices 1 and 2; further, let $I_o(1, V)$ and $I_o(2,V)$ be

the corresponding unperturbed e.p.c.$_{\text{I}}$s (found by fitting a linear relation to I as a function of V around $V-V_{eq}$) and suppose that concentrations 1 and 2 are arranged so that $I_o(2, V)$ is D times greater than $I_o(1, V)$:

$$I_o(2, V) = DI_o(1, V).$$

If iontophoresis of applied agonist were operating, then, from eqn. (2),

$$I(2, V) = \frac{I_o(2, V)}{1-(2k_1/k_o)I_o(2, V)}; \quad I(1, V) = \frac{I_o(1, V)}{1-(2k_1/k_o)I_o(1, V)}.$$

These relationships, together with the requirement that $I_o(2, V) = DI_o(1, V)$, yield

$$I(2, V) = \frac{DI(1, V)}{1+(2k_1/k_o)(1-D)I(1, V)}. \tag{4}$$

Thus, when $I(2, V)$ is plotted as a function of $I(1, V)$, the relation should deviate from linearity to an extent determined by $2(k_1/k_o)(1-D)I(1, V)$. Since D is under experimental control and k_1/k_o and $I(1, V)$ can be determined from the data, it is possible to determine the extent to which the iontophoresis hypothesis can account for voltage-dependent effectiveness.

On the other hand, if a mechanism of the second class is operating (for two different agonist concentrations, as before):

$$I_o(2, V) = DI_o(1, V)$$

and

$$\begin{aligned} I(2, V) &= (1+f(V))I_o(2, V) \\ &= (1+f(V))DI_o(1, V), \end{aligned}$$

so that

$$I(2, V) = DI(1, V).$$

Thus, for Class 2 mechanisms, as membrane potential is varied e.p.c.$_{\text{I}}$s at one concentration are always proportional to e.p.c.$_{\text{I}}$s at a different agonist concentration.

Mechanism involving voltage-sensitive gating. The second class of explanations for voltage dependence of ACh effectiveness relate to electric field effects on channel behaviour. The kinetics of channel gating is known to depend on post-junctional membrane potential, and the implications of voltage-dependent gating for agonist effectiveness can be assessed through the already available quantitative treatment of this phenomenon (Magleby & Stevens, 1972*b*; Anderson & Stevens, 1973). According to this view, n molecules of ACh (T) combine with a receptor (R) to form an acetylcholine-receptor complex (T_nR) which can undergo a conformational change to open a channel:

$$nT + R \overset{K}{\longleftrightarrow} T_nR \underset{\beta(V)}{\overset{\alpha(V)}{\rightleftarrows}} T_nR^*. \tag{5}$$

Binding, which is assumed to be rapid, is described by the equilibrium dissociation constant K, while α and β, which depend on membrane potential V, specify the rates of transition between the closed conformation T_nR and the open conformation T_nR^*. The voltage-dependent rate constants are specified by

$$\alpha(V) = e^{A_1V+B_1}, \quad \beta(V) = e^{A_2V+B_2}. \tag{6}$$

When the iontophoretic micro-electrode for application of ACh is more than a few μm from the post-junctional membrane, concentration is slowly varying compared to the rates for opening and closing channels, so reaction (5) may be considered to be in equilibrium. For two-state channels with an open conductance γ and a closed

conductance 0, the equilibrium relation between end-plate conductance, g_s, and ACh concentration C is given by

$$g_s = \frac{(\beta/\alpha)\gamma N C^n}{K + [(\alpha+\beta)/\alpha]C^n}, \tag{7}$$

where N is the total number of channels available. The factor n is the number of ACh or carbachol molecules that must be bound to a receptor in order for the associated channel to open; the value of n is not yet known but is probably close to 2 (for carbachol: Rang, 1971, 1973*b*; Jenkinson & Terrar, 1973; for ACh: V. E. Dionne & C. F. Stevens, unpublished observations) under the conditions of our experiments. If only a small fraction of the total available number of receptors has bound ACh, that is, if the limit of low ACh concentration applies, this relationship becomes

$$g_s = \left(\frac{\beta}{\alpha}\right)\frac{\gamma N C^n}{K}.$$

Because e.p.c.$_I$ rather than conductance is directly measured, it is more convenient to substitute this equation into the relationship $I_s = g_s (V - V_{eq})$, where V_{eq} is the end-plate reversal potential, and I_s is the e.p.c.:

$$I_s = \left(\frac{\beta}{\alpha}\right)\frac{\gamma N C^n}{K}(V - V_{eq}).$$

Finally, with substitution from eqn. (6), for the rate constants, we have an expression which relates e.p.c.$_I$ to ACh concentration and post-junctional membrane potential.

$$I_s = MC^n(V - V_{eq})\, e^{(A_2 - A_1)V}, \quad M = \frac{\gamma N}{K} e^{B_2 - B_1}. \tag{8}$$

It should be noted that, under the conditions of our experiments, concentration is not uniform over the end-plate because the ACh is applied iontophoretically from what is effectively a point source. Hence, C in eqn. (8) is not constant but varies with distance from the ACh electrode. A more complete derivation takes this position dependence into account. The resulting equation, however, has precisely the same form as that given here with concentration at an arbitrary point replacing the C of eqn. (8).

The voltage dependence of the rate constants α and β which appear in expression (6) may be evaluated from the behaviour of e.p.c.$_N$s. The end-plate conductance change resulting from application of a single quantum of transmitter is given, according to the scheme (5) above, by (Magleby & Stevens, 1972*b*; Anderson & Stevens, 1973)

$$g_1(t) = \frac{\beta N}{K}\int_0^t C_1^n(\tau)\, e^{-\alpha(t-\tau)} d\tau.$$

In this equation $g_1(t)$ is the conductance change due to application at zero time of one quantum of transmitter, α, β and K are the parameters as in the scheme (5) above, and $C_1(t)$ is the concentration of ACh resulting from the release of one quantum of transmitter. The preceding equation assumes the low concentration limit of agonist.

It should be noted that, in the equations (preceding paragraph and eqn. (8)) which apply to the case of low ACh concentrations, the equilibrium dissociation constant K always appears as dividing the opening rate constant β. This means that, unless ACh doses approaching receptor saturation are used, voltage-dependent ACh-receptor binding cannot be distinguished from membrane potential influence on the

conformational change which, we assume, is responsible for opening an end-plate channel.

Since the transient of cleft ACh concentration is, according to this formulation, very brief on the time scale determined by the rate constant α, the integral above differs little from

$$g_1(t) \simeq \frac{\beta N \mathrm{e}^{-\alpha t}}{K} \int_0^t C_1^n(\tau)\,\mathrm{d}\tau,$$

which for times after the brief rising phase of the m.c.p.s. is approximately

$$g_1(t) \simeq \frac{\beta N W \mathrm{e}^{-\alpha t}}{K} \quad (t > 0), \tag{9}$$

where

$$W = \int_0^\infty C^n(\tau)\,\mathrm{d}\tau.$$

Thus, for m.e.p.c.s which rise rapidly to their peak and then decay relative slowly, the peak conductance is very nearly $g_1(0) = (\beta/K)\,NW$. The peak miniature end-plate conductance therefore reflects the rate constant β and m.e.p.c.s have a decay governed by the rate constant α. To the extent that our formulation is correct, the α and β obtained from measurement of m.e.p.c.s should, when substituted into eqn. (8), account for the voltage dependence of effectiveness for iontophoretically applied ACh.

Part II. Experimental analysis

Documentation of voltage-dependent ACh effectiveness. E.p.c.s induced by a constant iontophoretic ACh dose are recorded as a function of time for various voltages and illustrated in Fig. 2 for a non-glycerol-treated end-plate. The ACh concentration as a function of time near the ACh-filled iontophoretic micro-electrode is shown in the upper traces. It is evident that, although the magnitude of the e.p.c.$_I$ changes considerably as a function of membrane potential, the simultaneously recorded ACh concentration transient did not vary significantly over trials. Fluctuations in e.p.c.$_I$ amplitude due to non-repeatable agonist delivery are less than about $\pm 10\,\%$. Thus, our iontophoretic delivery is repeatable and changes in e.p.c.$_I$ amplitude as a result of trial-to-trial variations in bath agonist concentration are small compared to the effects of membrane potential on e.p.c.$_I$. The e.p.c.$_I$s induced by a constant iontophoretic current as a function of time are shown for a glycerol pre-treated muscle in Fig. 3.

The standard procedure for these experiments was to apply a 0·5 sec iontophoretic pulse once every 10 sec and to alternate trials with voltage at the holding potential (-70 mV for Figs. 1 and 3) and trials at various other membrane potentials. The magnitude of e.p.c.$_I$s was measured at the time indicated by the vertical line in Fig. 3 and plotted as a function of membrane potential in Fig. 1. Although ACh concentration transients did not vary significantly from trial to trial, the curvilinear relationship demonstrating voltage-dependent ACh effectiveness clearly exists between e.p.c.

and membrane potential. A similar relationship has been observed in measurements made on twenty end-plates.

Specimen records of miniature e.p.c.s (m.e.p.c.s) measured at a variety of voltages are illustrated in Fig. 4*A*. Each trace is the average of between fourteen and twenty m.e.p.c.s. The m.e.p.c.s were found to decay exponentially with a decay constant which, in turn, depends exponentially on membrane potential (Magleby & Stevens, 1972*a*; Gage & McBurney, 1972; Kordaš, 1972), as illustrated, for the Fig. 4*a* data, in Fig. 4*b*. In confirmation of earlier studies (Kordaš, 1969; Magleby & Stevens, 1972*b*), the m.e.p.c. peak amplitude varied non-linearly with membrane potential, as shown in Fig. 4*c*. The possibility that small m.e.p.c.s were not detected at voltages near V_{eq}, a source for error which could influence the degree

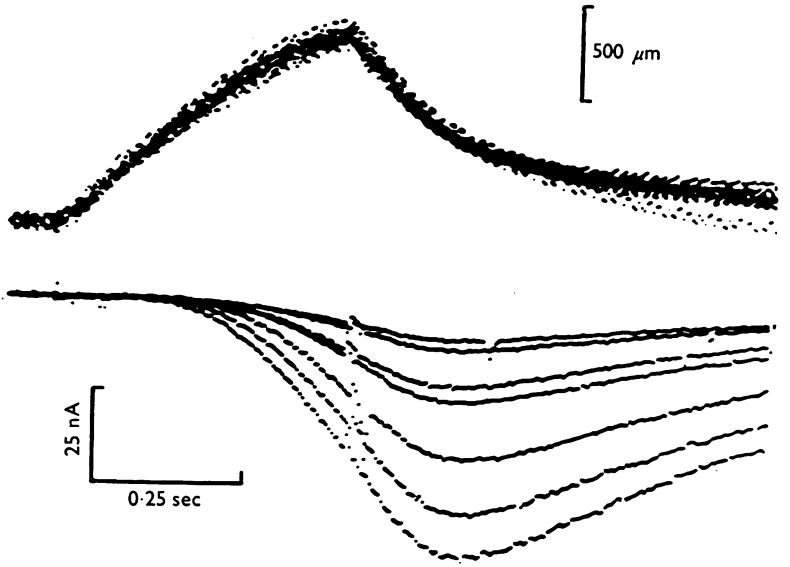

Fig. 2. Specimen records of e.p.c. (lower traces) and ACh concentration (upper traces) as a function of time for various membrane potentials. Half-second iontophoretic pulses of ACh were applied every 10 sec from a high-resistance pipette located 20 μm from the end-plate surface; the concentration at 5 μm from the iontophoretic micro-electrode tip was monitored with the ACh-sensitive micro-electrode. The upper traces in the Figure are a superimposition of the ACh-selective electrode response for the seven e.p.c.s below. (Pick-up from power lines by the 500 MΩ ACh-selective electrode has broadened the trace; an individual trace can be seen tailing below the others at the far right.) The e.p.c.s were recorded at (top to bottom) membrane potentials of −41, −49, −61, −72, −97, −113 and −123 mV. Switching artifacts in the e.p.c. traces shortly after the beginning and somewhat before the peaks delineate the time during which current was passed through the iontophoretic electrode. Membrane potential is recorded in the final digital sample of each e.p.c. trace. Temp. = 18° C.

of non-linearity, was tested by comparison of the coefficients of variation for the m.e.p.c. peaks at different membrane potentials. Because this coefficient was independent of voltage, significant systematic omission of small m.e.p.c.s did not occur. Similar results have been obtained on more than twenty-five end-plates, although the amount of curvature is minimal for some cells and pronounced for others.

We now turn to an evaluation of the relative contribution to voltage-dependent agonist effectiveness made by the various mechanisms considered above.

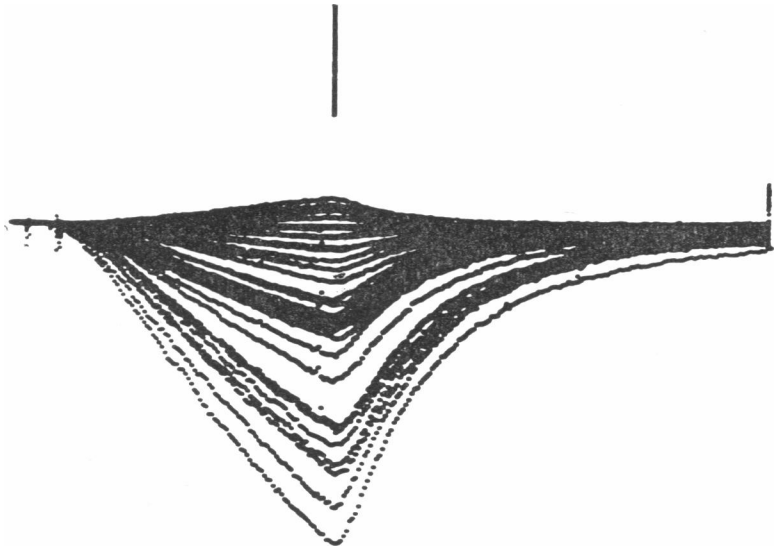

Fig. 3. The e.p.c. as a function of time for various membrane potentials and a constant dose of ACh applied iontophoretically. A constant current iontophoretic pulse 0·5 sec long was delivered during most of the e.p.c.$_I$ rising phase with the iontophoretic electrode tip positioned about 20 μm from the end-plate. At the time indicated by the vertical bar e.p.c.s were sampled and plotted as a function of membrane potential in Fig. 1. Temp. = 14° C.

Electric field effects on rate of ACh hydrolysis does not account for voltage dependence of agonist effectiveness. In order to evaluate the contribution of possible voltage-dependent esterase activity on the non-linear e.p.c.$_I$–voltage relationship, we have used carbachol in place of ACh for experiments such as that illustrated in Fig. 1. If voltage sensitivity of the esterase hydrolysis rate accounts for the non-linear e.p.c.$_I$–voltage relationship such as that shown in Fig. 1, the curvature of the relationship obtained with carbachol should be less marked. If, however, the curvature results from some other source, such as voltage sensitivity of the rate constants α and β, non-linearity in the e.p.c.–voltage relationship should

still be present. In this context it must be noted that although the values of α are different for carbachol and ACh (Katz & Miledi, 1973), this rate constant is still voltage-dependent as judged from the cut-off frequency of carbachol-induced conductance fluctuations (V. E. Dionne & C. F. Stevens, unpublished observations). It is clear from Fig. 5, where e.p.c. resulting from the iontophoretic application of carbachol is plotted as a function of

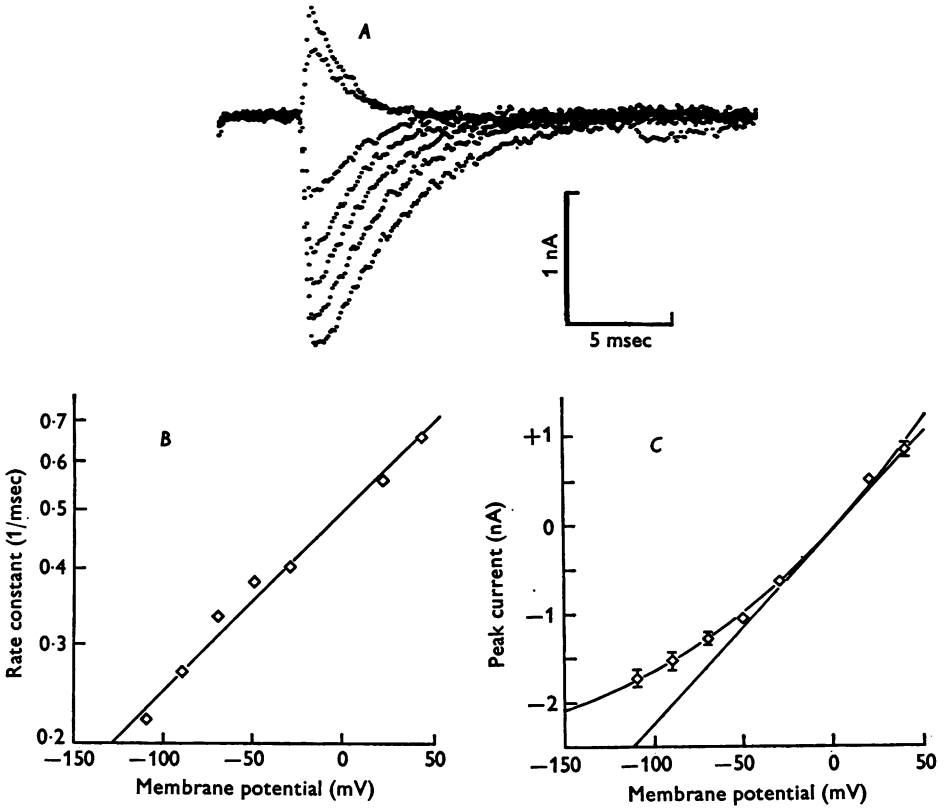

Fig. 4. Voltage dependence of m.e.p.c.s. *A*, specimen records of m.e.p.c.s at different membrane potentials are shown as a function of time. Each trace is the average of fourteen to twenty m.e.p.c.s recorded digitally at the same membrane potential. The preparation was glycerol-treated to allow records on both sides of V_{eq} to be obtained. *B*, the decay constant α of these records is plotted semilogarithmically as a function of membrane voltage and fitted by a straight line. *C*, peak m.e.p.c. plotted as a function of membrane voltage from the records in (*A*). The error bars, shown where they are larger than the plotted symbol, are the S.E. of the means. The data deviate markedly from a straight line but curve in the direction opposite that for the data illustrated in Fig. 1. The data in (*B*) were fitted by eqn. (6) and those in (*C*) by eqn. (9) with the parameter values indicated below. The straight line in (*C*) is tangent to the fitted curve at V_{eq}. Temp. = 14° C.

post-junctional membrane potential, that curvature of the relationship is still present; such behaviour of carbachol-induced e.p.c.s with voltage has previously been noted by Rang (1973*a*). In fact, not only is curvature apparent in Fig. 5, but the deviations from linearity are somewhat more pronounced than those seen for ACh. A quantitative comparison of the curvature produced by ACh and carbachol will be presented later.

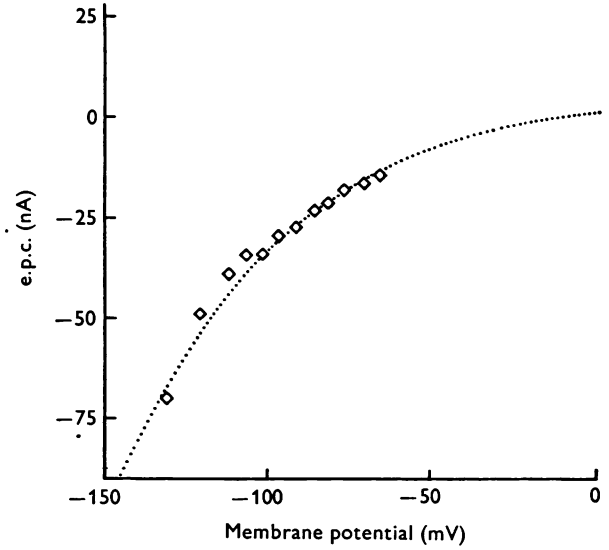

Fig. 5. Amplitude of e.p.c. as a function of membrane potential for a constant dose of carbachol. As the membrane is hyperpolarized, the e.p.c. is seen to increase in a manner similar to that for ACh (see Fig. 1). The theoretical line was generated using eqn. (8) with the parameters indicated below. Temp. = 14° C.

We conclude, then, that although the acetylcholinesterase may play a role in agonist effectiveness, this is not the predominant source of the voltage sensitivity described here.

ACh iontophoresis by e.p.c.s does not account for voltage dependence of ACh effectiveness. As was indicated earlier, one possible mechanism for voltage dependence of agonist effectiveness is the iontophoresis of agonist into or out of the cleft by e.p.c.s. Thus, hyperpolarization produces larger e.p.c.s which themselves sweep agonist into the cleft and increase the concentration, thereby augmenting the e.p.c. Analysis of this mechanism (see page 268) reveals that the shape of the $e.p.c._I$ *vs.* voltage curve should depend non-linearly on agonist concentration if iontophoresis is the source of voltage dependence. We have investigated the effect of agonist concentration on the $e.p.c._I$–voltage curve over a rather large range of concentrations (as judged from the amplitude of e.p.c.s). Experimentally we

accomplished this by plotting e.p.c.$_I$ amplitude measured near the end of the iontophoretic pulse (large ACh concentration) as a function of e.p.c.$_I$ measured either soon after onset or long after offset of the iontophoretic pulse (low ACh concentration) for a wide range of membrane potentials. If the voltage dependence of ACh effectiveness is independent of ACh concentration, e.p.c.$_I$ at one concentration should be proportional to current at a different concentration, whereas, if the iontophoretic mechanism is the source of voltage sensitivity, this relationship should be characteristically non-linear.

A plot of e.p.c.$_I$ at one agonist concentration *vs.* e.p.c.$_I$ at a different concentration, with a membrane potential as a parameter, appears in

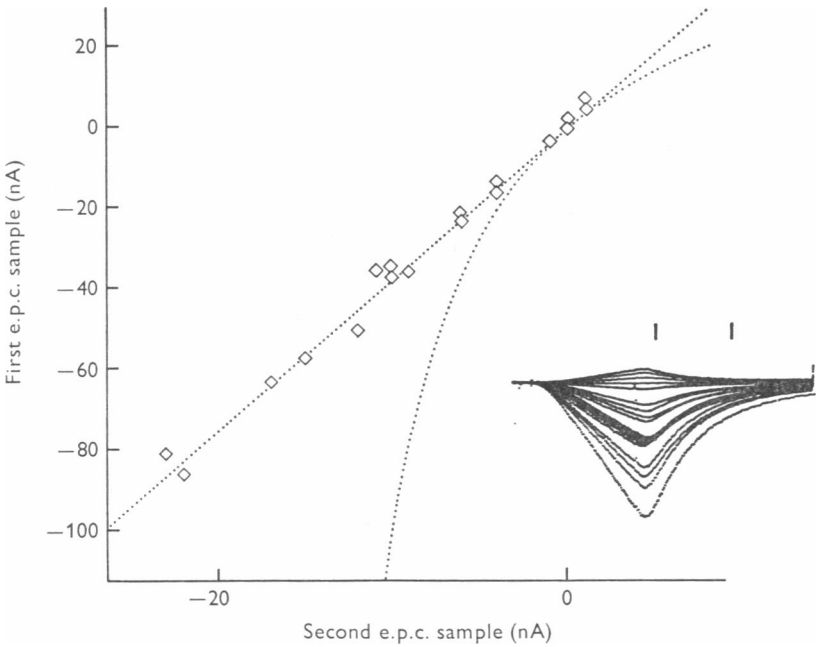

Fig. 6. The e.p.c. at one concentration plotted as a function of e.p.c. at a different concentration for a variety of membrane potentials. Iontophoretically induced e.p.c.s were recorded at different membrane voltages as discussed in the text and shown in the inset. These e.p.c.s were sampled at two values of ACh concentration (shown by the vertical lines) and one plotted as a function of the other. The curved dotted line was generated using eqn. (4) with $D = 0{\cdot}26$ and $2(k_1/k_0) = -0{\cdot}022$ obtained from the current–voltage curve measured at the higher sample concentration. Were iontophoresis to account for the voltage dependence of ACh effectiveness the data plotted should conform to the theoretical curve shown. The straight dotted line, fitted by eye to the data, was generated by a class of theories which includes voltage-dependent rate constants (see page 254). Temp. = 14° C.

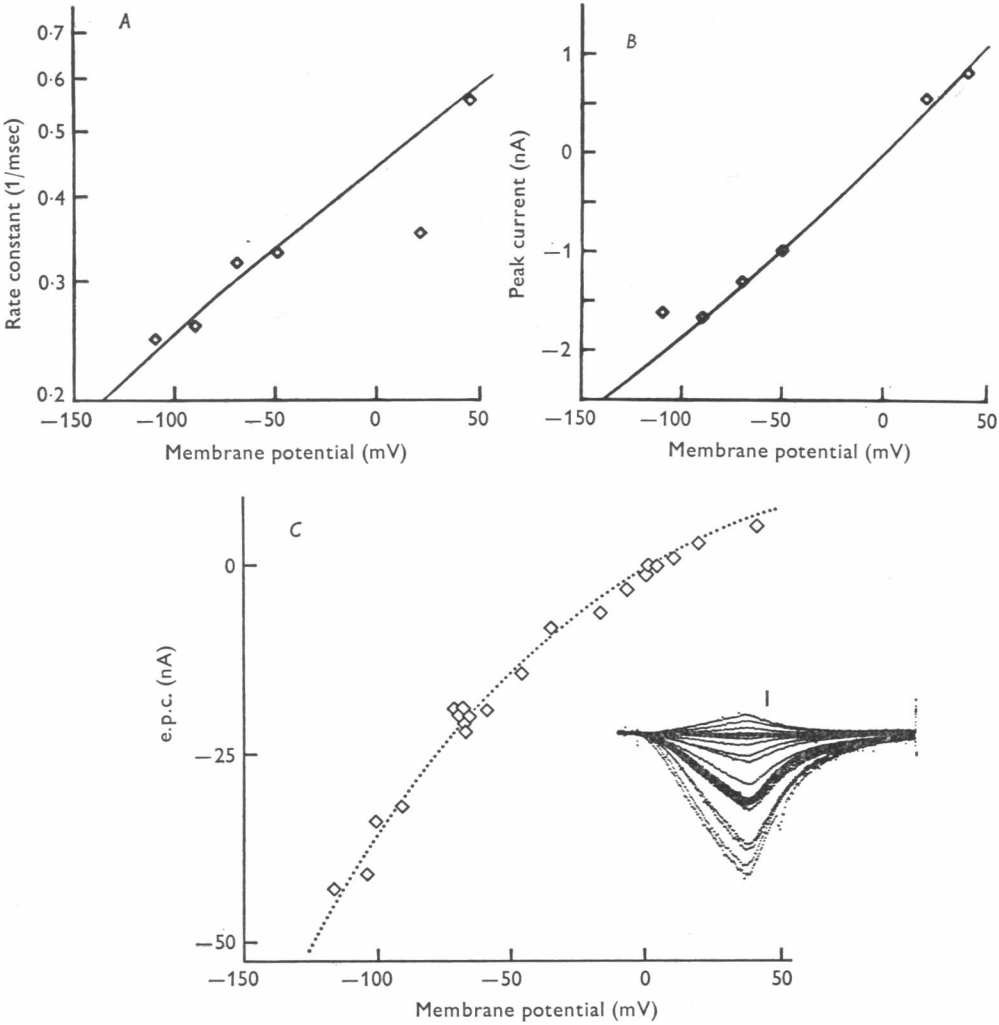

Fig. 7. Test of theory with data from one end-plate. The m.e.p.c.s and iontophoretically induced e.p.c.s both as a function of voltage, were recorded from the same cell. *A*, the decay constant of the m.e.p.c.s as a function of membrane potential is plotted and fit by eye with a straight line (the single deviant value was ignored). A value of $A_1 = 0{\cdot}0062\ \mathrm{mV}^{-1}$ in eqn. (6) was determined. *B*, the m.e.p.c. peaks are plotted as a function of membrane potential and fitted by eqn. (9) (again ignoring a single deviant point). A value for $A_2 = 0{\cdot}0010\ \mathrm{mV}^{-1}$ in eqn. (6) was obtained. *C*, the e.p.c.s shown in the inset were evaluated at the vertical bar and plotted as a function of voltage. The curved line through the data was generated by eqn. (8) using the values of A_1 and A_2 obtained in (*A*) and (*B*). The value $V_{eq} = 0$ mV was determined for this glycerol-treated cell. Temp. = 14° C.

Fig. 6. Over a range of membrane currents from +5 to −85 nA (with concentrations at the end-plate differing by twofold), the magnitude of the voltage-dependent effectiveness of ACh was uninfluenced by concentration, a fact previously reported by Rang (1973*a*) for carbachol and for decamethonium. Iontophoresis of ACh by e.p.c.$_I$s certainly must occur, but this effect cannot account for the bulk of the curvature in the current–voltage relationships presented in Figs. 1 and 5.

Membrane potential influence on gating can account for the voltage dependence of ACh effectiveness. Neither voltage dependence of acetylcholinesterase hydrolysis rates nor iontophoretic perturbation of cleft concentration by e.p.c.s can explain the dependence of ACh effectiveness on membrane potentials; it remains to evaluate contributions to this effect made by the field-sensitive gating of end-plate channels. Because the cleft agonist concentrations employed in these experiments did not, as judged by our determinations of the dose–response curve, reach the range where any detectable saturation of receptors is occurring, eqn. (8) is appropriate to describe the e.p.c.$_I$ *vs.* voltage data for a constantly applied ACh dose. This equation has been fitted to the data in Figs. 1, 5 and 7*c*, and apparently provides an adequate description of the data.

If field-dependent end-plate channel gating does in fact account for the effect under investigation here, the voltage dependence of rate constants α and β in scheme (5), estimated from m.e.p.c.s (with eqns. (9) and (6)), should with eqn. (8) fit the e.p.c.$_I$ *vs.* voltage curve. The behaviour of the rate constants α and β may be estimated from the dependences of peak m.e.p.c. and m.e.p.c. decay constant on membrane potential. Rate constant α is illustrated for a particular cell in Fig. 7*a*, where it is seen that, as reported earlier, m.e.p.c. decay depends exponentially on membrane potential. The straight line fitted to the data points in Fig. 7*A* provides an estimate of 0·0062 mV^{-1} for A_1 in eqn. (6), a value within the range of those reported in Magleby & Stevens (1972*a*).

Peak average m.e.p.c. *vs.* membrane potential is plotted in Fig. 7*b*, and from the theoretical curve drawn according to eqn. (6), the factor A_2 has been estimated to be 0·0010 mV^{-1}. In seven experiments we have found the average value of A_2 to be 0·0032 mV^{-1} with a range of 0·0010–0·0044; these values are similar to those derived from analysis of e.p.c.s by Magleby & Stevens (1972*b*).

Gage & McBurney (1972) report that the conductance change produced by one quantum of transmitter is $5{\cdot}5 \times 10^{-8}$ mho $\pm$ $1{\cdot}6 \times 10^{-8}$ (S.D. of observation). According to our findings, however, this factor should contain a specification of membrane potential: according to eqns. (6) and (9), the peak conductance $g_{peak}(V)$ at membrane potential V should be approximately

$$g_{peak}(V) = g_{peak}(0)\, e^{A_3 V}.$$

We have not systematically determined the parameters in this equation for a large number of end-plates, but for six preparations the average g_{peak} (0) was found to be 66 nΩ (range, 41–97) and the average A_2 to be 0·0034 mV^{-1} (range 0·0010–0·0044). Thus for this small sample, the single quantum end-plate conductance change is

$$g_{peak}(V) = 66\, e^{0\cdot0034V}\ n\Omega.$$

The e.p.c.s produced by the iontophoretic application of ACh to the end-plate that yielded the data in Fig. 7*a* and 7*b* are plotted as a function of membrane potential in Fig. 7*c*. The smooth curve in that Figure is drawn according to eqn. (8), with the values of A_1 and A_2 obtained from the data shown in Fig. 7*a* and *b* respectively. We have measured e.p.c.$_I$ *vs.* voltage and m.e.p.c.s at various voltages in the same cell at seven end-plates, and have found, in all cases, that the same parameters will, within our limits of experimental error, account simultaneously for the α *vs.* V (as Fig. 7*a*), the β *vs.* V (as Fig. 7*b*), and the e.p.c.$_I$ *vs.* V (as Fig. 7*c*) curves. The average values of $A_2 - A_1$ (eqn. (8)) for these curves was $-0\cdot0053$ with a range of $-0\cdot0041$ to $-0\cdot0061$ mV^{-1}. For carbachol application, eqn. (8) also fits the data with $A_2 - A_1$ equal to $-0\cdot0130$ mV^{-1} for one end-plate and $-0\cdot0105$ mV^{-1} for another.

In all instances eqn. (8) has provided a good fit to the observed e.p.c.$_I$–voltage relationships obtained with iontophoretic application of ACh and carbachol, and, whenever independent determinations of membrane potential dependence for the rate constants α and β have been made from measurements on m.e.p.c.s, the parameters thus obtained have been, as indicated above, adequate to account for the influence of voltage on ACh effectiveness. We have noted, however, that the e.p.c.$_I$–voltage relationships predicted from eqn. (8) with parameters obtained from m.e.p.c.s (via eqns. (9) and (6)) tend sometimes to be less curved than the observed relations. This tendency is obscured for any given set of observations by scatter in experimental measurements, but we believe that it is actually present. We have not, however, systematically studied these possible deviations between theory and experiment because they have been too small to accurately characterize.

DISCUSSION

Electric field sensitivity of end-plate channel gating can account quantitatively for the voltage-dependent ACh effectiveness described here, whereas the alternative mechanisms considered – rectification, voltage influence on acetylcholinesterase hydrolytic rate and iontophoresis of ACh by e.p.c.s – have been eliminated as primary explanations for the effects we have described.

Although the theory that we have used is certainly adequate to account

for the data, other alternatives are, of course, possible and could explain these and other observations equally well. For example, we have assumed, by analogy to enzyme–substrate interactions that have been studied with rapid kinetic techniques (see Eigen & Hammes, 1963; Hammes, 1968*a*, *b*; Gutfreund, 1971) that the binding step of ACh to its receptor is rapid and that the rate-limiting step is a subsequent conformational change responsible for channel opening. If, however, the opposite is assumed (namely, if the conformational change is rapid and the binding step is rate-limiting and voltage-dependent), a different dose–response curve equation (eqn. (7)) would result; nevertheless, this alternative theory would be identical to the one we have used (eqn. (8)) in the low concentration limit. Judged by the dose–response curves we have determined (V. E. Dionne & C. F. Stevens, unpublished observations) and other information (Magleby & Stevens, 1972*a*, *b*; Anderson & Stevens, 1973), the ACh concentrations used in the experiments reported here have all been far from receptor saturation so that we have no data which permit us to distinguish between the two alternatives.

Our formulation throughout has been in terms of voltage-dependent conformational changes because this step, by analogy to enzyme-substrate interactions, seems most likely to be rate-limiting. If, as discussed above, the binding step were rate-limiting, then one would have to develop a theory in which binding and unbinding were voltage-dependent. We must emphasize, however, that even within the framework of our formulation, the possibility of voltage-dependent binding remains and we have no means, with the data at hand, of detecting the presence of such a process. We have assigned all voltage dependence for the channel opening step to the rate constant β, but, as noted earlier, β always appears in the low concentration limit eqns. (7) and (8) used here as divided by the dissociation constant K. Part or all of the voltage dependence assigned here to β may in fact reside in K; such voltage-dependent dissociation constants have long been known (Onsager, 1934).

More complicated kinetic schemes are also consistent with all our observations. For example, if channels closed by another route rather than in the simple way supposed in scheme (5), precisely the same equations that we have used could result. According to our observations, channels must open with one rate constant β (which depends exponentially on membrane potential) and close with another rate constant α (with the same properties) but these two rate constants need not, as in (5), connect the same pair of states. As a specific example, channels could close by going through a desensitized state, and such behaviour would be consistent with our observations.

Another alternative scheme, which could in practice be indistinguishable

from (5), arises from the following considerations: if n in scheme (5) is close to two, it would mean that approximately two ACh molecules must bind to a receptor in order to cause the associated channel to open. More likely, however, is the proposition that a channel can, for example, be opened with either one or two bound ACh's, but that the opening probabilities are much lower if one ACh is bound to the receptor than with two. According to this view, scheme (5) would be replaced by the more complicated version:

$$\begin{array}{ccccc} & & \overset{K_1}{\longleftrightarrow} & & \overset{K_2}{\longleftrightarrow} \\ 2T + R & & T + TR & & T_2R \\ & & \alpha_1 \uparrow\downarrow \beta_1 & & \alpha_2 \uparrow\downarrow \beta_2 \\ & & T + TR^* & & T_2R^* \end{array} \qquad (10)$$

where, as before, * indicates an open channel; other possible transitions not indicated above have, for simplicity, been supposed to be most unlikely. With appropriate parameters, this more complicated kinetic scheme would, within the limits of accuracy imposed by our data, reduce to the equations we have used.

Although the alternatives just enumerated may satisfactorily account for all available data as well as the model we have selected, we favour the treatment presented here because it is the simplest acceptable scheme at the same time consistent with the rapid kinetic observations made on enzyme–substrate systems.

We have considered, in addition to the mechanisms described in the Results section, various other explanations for the voltage dependence of ACh effectiveness. One possibility is that desensitization occurs during the iontophoretic application of ACh and then wears off in the interpulse interval at a rate which depends upon membrane potential. Under this hypothesis the increased effectiveness of iontophoretically applied ACh at hyperpolarized post-junctional membrane potentials would be accounted for by a postulated speeding of recovery from desensitization at more negative voltage levels; even if our non-linear e.p.c.–voltage curves were not explained totally on this basis, voltage dependence of desensitization rates could alter the shapes of the curves we have obtained. We feel, however, that desensitization did not appreciably affect our observations for several reasons. First, we interspersed observations made at a standard holding potential (usually -70 mV) between observations made at the various other potentials so that any changes in the average state of desensitization that occurred at one voltage should be reflected in the standard test applications at -70 mV. We found that the response to iontophoretic application at -70 mV did not significantly change throughout the experiment. Further, preliminary observations in this laboratory (J. H. Steinbach & C. F. Stevens, unpublished observations) have suggested that the

recovery from desensitization has a voltage dependence opposite that which would be required to account for the increased effectiveness of ACh at hyperpolarized levels. That is, with hyperpolarization, the onset of desensitization is more rapid (Magazanik & Vyskočil, 1970) and the recovery is less rapid than at the more depolarized voltage levels. Therefore, as one hyperpolarized the post-junctional membrane and applied ACh in pulses at a constant rate, it would be expected that desensitization would decrease rather than increase the amplitude of the response for more negative membrane potentials.

Another class of possible explanations is more difficult to exclude definitely because it is less precisely formulated: in general, it is possible that ACh effectiveness depends on some local ion concentration (including H^+) and that this ion concentration depends in turn upon either membrane potential or e.p.c. For example, it might be supposed that sodium competes with ACh for binding sites on the receptor, and that local depletion of sodium which occurs near the post-junctional membrane with large e.p.c.s might decrease antagonism of ACh by sodium and thereby make ACh more effective. Against all hypotheses in this class are the observations that the ACh effectiveness as a function of membrane potential had the same form independent of concentration and e.p.c. magnitude. These parameters varied naturally from preparation to preparation as well as during observations on a single preparation when the distance between the end-plate and the iontophoretic ACh electrode was varied. Finally, these explanations cannot resolve the paradox of opposite voltage dependence for agonist effectiveness for nerve-released and iontophoretically applied ACh.

We have used the agonist ACh in most of our experiments in order to facilitate comparisons with nerve-applied agonist. Specifically, because each agonist causes end-plate channels to close with different rate constants, as judged by noise analysis (Katz & Miledi, 1973), it would not be easy to justify the use of α and β determined from m.e.p.c.s to predict the voltage dependence of effectiveness for any agonist other than ACh. We have chosen, however, to investigate the participation of possible voltage-dependent esterase activity by substituting the very slowly hydrolysed carbachol for ACh rather than by poisoning esterase because anticholinesterases appear to have complicated actions which we do not, as yet, fully understand. We have found that these agents have, in addition to what appears to be a curare-like action, complicated effects upon the decay of e.p.c.s, and thus, presumably, upon the kinetics of channel closing. For example, the time constant for decay of m.e.p.c.s depends, in a prostigmine treated preparation, rather strongly upon ACh concentration, with larger ACh doses producing slower decays; D. A. Terrar & K. L. Magleby (personal communication) have independently

observed the same types of effect on multiquantal e.p.c.$_N$s, and have also discovered additional influences of desensitization and of other pharmacological agents which appear in the presence of anticholinesterases. Since we have detected no influences of carbachol upon the kinetics of e.p.c$_N$s, it seemed preferable to use this agonist, in place of ACh, to study the role played in voltage-dependent agonist effectiveness by esterases.

Within the accuracy of our observations, we feel that we have largely accounted for the voltage dependence of ACh effectiveness as a function of membrane potential. This conclusion adds further support to the theoretical framework employed here. The precision of our observations is not, however, sufficient to rule out some contributions from any or all of the effects we have considered. As pointed out earlier, we have occasionally noticed deviations of observations from theoretical lines which appear larger and more consistent than can be accounted for simply on the basis of measurement uncertainties. Specifically, with iontophoretic application of ACh, the e.p.c.$_I$–voltage relation seems to deviate more sharply from linearity than expected from the voltage dependence of the rate constants α and β estimated from m.e.p.c.s; the difference $A_2 - A_1$ in (8) sometimes seems too small. Although we have not systematically investigated this effect, it seems that the deviation between observations and theory may well involve one of the factors (such as channel rectification or iontophoresis of ACh into the cleft by e.p.c.s) that we have mentioned above. Another alternative is that the theory used may not in all instances account adequately for voltage dependence of channel kinetics. Magleby & Stevens (1972*a*) observed that departures from exponential decay sometimes occur at voltage levels more negative than -100 mV and it is possible that this behaviour, not taken into account in our calculations, could explain part of the deviations between theory and experiment. In any case, this possible discrepancy must be borne in mind in future investigations.

If our analysis is in the main part correct, we have resolved a paradox of voltage-dependent agonist effectiveness and thereby given further insight into the interactions of ACh with its receptor. Whether or not our analysis is correct, however, these studies have given quantitative definition to one aspect of drug action – the voltage sensitivity of agonist effectiveness – and have underscored the need to consider molecular events whenever the interaction of ACh and its receptor is being investigated. Further, our results emphasize the care that must be taken in interpreting agonist actions at the neuromuscular junction, and the influence techniques may have on results. For example, dose–response curves determined without use of a voltage clamp or some similar arrangement could be

erroneous because the agonist-produced depolarization could itself alter agonist effectiveness.

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