The Time Course of Miniature Endplate Currents and Its Modification by Receptor Blockade and Ethanol

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ABSTRACT  Miniature endplate currents (MEPCs) recorded from mouse diaphragms with a point voltage clamp, without inhibition of acetylcholinesterase (AChE) and in the absence of any drug, showed in their decay phase consistent deviations from an exponential time course, consisting of (a) "curvature," a progressive increase of decay rate during most of the decay phase, followed by (b) "late" tails. Both phenomena persisted when MEPCs (and channel lifetime) were prolonged by ethanol. Curvature was increased by muscle fiber depolarization and decreased by hyperpolarization. Receptor blockade by (+)-tubocurarine, α-bungarotoxin, hexamethonium, or myasthenic IgG accelerated the decay of the main part of MEPCs and eliminated curvature; the time constant of MEPCs became close to the channel time constant. We conclude that curvature arises from repeated action of ACh with cooperativity in ACh-receptor interaction; the voltage sensitivity of curvature follows from the voltage sensitivity of channel closing. Ethanol, in addition to its effect to prolong channel lifetime, enhances the tendency of ACh to act more than once to open channels before being lost to the system. Analysis of the rising phase of the MEPC, in terms of driving functions, also indicated that ethanol promotes channel opening by ACh; this action can account for a substantial increase of MEPC height by ethanol when MEPCs are made small by receptor blockade. Driving functions were also voltage sensitive, in a manner indicating acceleration of channel opening, but reduction of channel conductance, with hyperpolarization. Poisoning or inhibition of AChE prolonged MEPCs without altering the duration of ionic channels. Since ethanol caused further prolongation of MEPCs after poisoning of AChE, with little change in MEPC height, we conclude that the extension of mean channel lifetime by ethanol is accompanied by a similar extension of ACh binding to receptors. After poisoning of AChE, MEPCs became very variable in time course and the decay rate (r⁻¹) was correlated with MEPC height with a slope of log r vs. log height of 0.77 for MEPCs of >60% mean size. This slope is larger than expected from cooperativity in ACh-
receptor interaction. Correlation of $\tau$ and height of MEPCs also exists when AChE is intact; the slope of log $\tau$ vs. log height was 0.12 with or without prolongation of MEPCs by ethanol.

**INTRODUCTION**

At the neuromuscular junction of vertebrate skeletal muscle, the miniature endplate current (MEPC) represents the response to a single quantum of acetylcholine (ACh) released by the nerve terminal and consists in the abrupt opening and rather slower closing of postsynaptic ionic channels (Fatt and Katz, 1952; del Castillo and Katz, 1956a, b; Takeuchi and Takeuchi, 1959, 1960; Magleby and Stevens, 1972a, b; Landau, 1978).

The time course and height of the MEPC are implicitly functions of a rather complex system in which the transmitter diffuses within the synaptic cleft, interacts with receptors to open ionic channels, and eventually is hydrolyzed by acetylcholinesterase (AChE) or diffuses out from the cleft. Any comprehensive description of this system will therefore have to account for the real MEPC and how it is modulated by factors such as postsynaptic transmembrane potential, activity of AChE, and drugs that influence the system. Our aim in this paper is to describe the MEPC in mouse diaphragm and its alteration by membrane potential, receptor blocking agents, AChE poisoning, and ethanol, which is known to prolong the lifetime of channels (Gage et al., 1975; Quastel and Linder, 1975; Bradley et al., 1980). Preliminary reports of some of the results have been given (Quastel and Linder, 1975; Linder et al., 1979; Pennefather and Quastel, 1979a, b, 1980a, b).

**METHODS**

The methods used in the present experiments were in most respects the same as described by Pennefather and Quastel (1981). Experiments were performed on mouse hemidiaphragms superfused (Cooke and Quastel, 1973) at 25–27°C with solution containing (usually) (mM): 150 Na⁺, 2 Ca²⁺, 1 Mg²⁺, 5 K⁺, 24 HCO₃⁻, 1 H₂PO₄⁻, 135 NO₃⁻, and 11 glucose. The substitution of NO₃⁻ for Cl⁻ served to improve the signal/noise ratio of the MEPCs (Linder and Quastel, 1978). Control experiments showed no effect on MEPC height or time course with this substitution. Tetrodotoxin ($2 \times 10^{-8}$ g/ml) was added to solutions to prevent the occasional muscle twitching, which otherwise occurred when miniature endplate potentials (MEPPs) were made large by ethanol or poisoning of AChE. In some experiments, [K⁺] was raised to 10 mM in order to increase MEPC frequency and therefore permit faster acquisition of data without altering the MEPC time course or amplitude (Linder and Quastel, 1978).

**Voltage Clamp**

The two-electrode point voltage clamp was conventional (Takeuchi and Takeuchi, 1959), using microelectrodes filled with 3 M KCl; the electrode tips were placed no more than ~20 μm from one another. During operation of the voltage clamp, with a total gain of the feedback loop usually ~4,000:1 (DC–1 kHz), the MEPPs were always obliterated and the membrane potential was maintained within 1 mV of the specified holding potential.

**Recording of MEPCs**

Before A/D conversion the signal from the current monitor was put through a running-average filter suitable for the chosen rate of A/D conversion, then amplified and filtered
further with simple R/C filters set at 0.1 Hz and 3 kHz or 1 kHz. In some experiments MEPCs were recorded D/C coupled. MEPCs were then recorded on digital tape using a PDP-12 computer (Digital Equipment Corp., Marlboro, MA); the program excluded MEPCs unless there was a gap of at least 50 ms from a previous MEPC and the decay phase was uncontaminated by a second MEPC. Each individual MEPC was stored in a total of 128 points, of which the first 28-30 were baseline. The necessity for an “intelligent” program for computer identification of MEPCs limited A/D conversion to a maximum of 10 kHz. The records were subsequently examined visually to identify (and exclude from averages) artifacts, multiple MEPCs, abnormal and sub-MEPCs (Cooke and Quastel, 1973; Kriebel and Gross, 1974), and any MEPCs that were superimposed on a baseline with a significant trend.

**Averaging of MEPCs (Off-Line)**

To exclude systematic distortion in an average (i.e., a generation of features not present in individual MEPCs), MEPCs were aligned with respect to the time of the steepest part of the rising phase of the MEPC, determined by cross-correlation of points with a ramp function (0, 0, ... 0, 1, 2). This gave MEPC averages with total rise time slightly less and peak amplitude slightly greater than other methods (Pennefather and Quastel, 1981). The time constants (see below) of decay of an MEPC average were essentially identical to the averages of the time constants of individual MEPCs, except where AChE was poisoned. In the latter case sequential MEPCs varied greatly in time course and overall averages had a time course unlike that of any individual MEPCs, unless averages were made only for groups of MEPCs with similar time course.

**Calculation of MEPC Parameters**

Calculations were done on the PDP-12 computer from the MEPCs and/or MEPC averages stored on digital tape. Before further calculations were made, the baseline was subtracted and all points were corrected for high-frequency filtering using (a) replacement of each value \( x_i \) by \( (x_i - z \cdot x_{i-1})/(1 - z) \), where \( z = \exp(-\Delta t/\tau_f) \), \( \Delta t \) is the time between samples, and \( \tau_f \) is the time constant corresponding to the high-frequency filter, or (b) replacement of each value \( x_i \) by \( x_i + \tau_f(x_{i+1} + x_{i-1})/(2\Delta t) \). Method b introduced smoothing and was used only when parameters were determined on individual MEPCs. Method a was also used to deconvolute MEPC averages. To correct for the low-frequency (0.1-Hz) filter, each value \( x_i \) was replaced by \( x_i + zZ/\tau_l \), where \( z = \exp(-\Delta t/\tau_l) \), \( \tau_l = 1.592 \) s, and \( Z \) is the accumulated sum of previous corrected \( x_i \).

Data were accepted as being from a focally placed clamp only if the MEPC average, after correction for filters, showed a maximum rate of rise at least 67% of peak height in 0.2 ms (10-kHz sampling) or 80% of peak height in 0.4 ms (5-kHz sampling). After AChE poisoning the rise time of MEPCs was somewhat prolonged; 66% of peak height in 0.4 ms was considered acceptable. These criteria were determined by examination of records of extracellularly recorded MEPPs, which are of necessity focal. In addition, preliminary experiments showed that within these limits there were no MEPC averages with both lower than average height and greater than average time to peak, as would be expected if high-frequency components of the MEPC were lost because of nonfocal placement of electrodes, and as was observed with deliberately nonfocal recording. The same criteria were used for MEPCs recorded in the presence of (+)tubocurarine (dTC) (or after irreversible receptor blockade) on the basis of experiments where MEPCs were followed (at the same junction) as dTC was applied. For all conditions, application of these criteria resulted in the rejection of no more than 40% of MEPC averages, from endplates where the clamp was deemed focally placed on the basis of initial appearance of MEPPs.

Determination of the time constant of MEPCs was a major problem since (a) it was
considered important to avoid any method that introduced bias stemming from the varying signal/noise ratio of the recorded signals, and (b) it was soon discovered that the time course of decay of the MEPC differs substantially from a simple exponential (see Results). For example, simple least-squares fitting to the logarithms of points in the decay curve gave artifacts arising from the non-normal distribution of the logarithms of normally distributed numbers. The method finally used was the following: (a) find points between defined fractions of the MEPC peak amplitude (e.g., \(e^{-0.5}\) and \(e^{-1.5}\), etc.); (b) fit these points to a linear regression with time; (c) obtain a best-fitting rate constant (and hence time constant) for the defined period by multiplying the ratio of slope to mean height of the regression line by a correction factor dependent only on the number of points used. These factors and the lack of bias of the procedure were determined by applying the method to a computer-generated series of points with added noise of various magnitudes. The time constants determined in this way from points between \(e^{-0.5}\) and \(e^{-1.5}\) of the peak have been referred to as \(\tau_1\) in the Results.

For correlation of \(\tau_1\) and MEPC height, it was also important to avoid any artifact arising numerically. In particular, any error in estimating the baseline for the MEPC would alter in parallel the estimated values of \(\tau\) and height. The method adopted was to use the baseline before the MEPC in the determination of \(\tau_1\) and the baseline after the MEPC for the height; in simulated noisy MEPCs this procedure eliminated correlation between \(\tau_1\) and height.

**Fluctuation (Noise) Analysis**

In the few experiments in which noise analysis was employed to estimate the time course of ionic channels, standard techniques (Bendat and Piersol, 1971; Anderson and Stevens, 1973) were used. Data were stored digitally in continuous series of 4,096 points and fast Fourier analysis was performed on stretches of record (512 points) unambiguously devoid of MEPCs, and the outputs were averaged in the usual way. In each case the average spectrum of baseline noise, from before and after superfusion with ACh, was subtracted point by point from the spectrum obtained in the presence of ACh. Smoothed spectra were then compared with computer-generated Lorentzians to obtain the time constant of the ionic channels.

**Driving Functions and Spreading Functions**

Characterization of the rising phase of MEPCs was limited by recording noise and the maximum A/D sampling rate of 10 kHz; it was in principle and in practice not possible to align exactly the corresponding points on the rising phase of the MEPC. The consequent jitter or spread in the averaging is equivalent in net effect to a smoothing procedure and therefore produces little distortion in the falling phase of the MEPC but gives quite substantial distortion in the rising phase and therefore in the driving functions of the MEPCs (see Results). To determine the extent of jitter, known waveforms similar to MEPCs, superimposed on pre-recorded noise, with varied height (i.e., signal/noise ratio) and varied rate constant of rising phase, were recorded and averaged by the same programs as used for real MEPCs. The spreading functions were then obtained as the functions, which, convoluted by the ideal waveforms, gave the (distorted) average. Empirical equations were then determined for the relation between each point in a spreading function and the two variables, (a) signal/noise ratio and (b) rate constant. Thus, for any average of MEPCs, an estimate of its spreading function could be obtained on the basis of the actual signal/noise ratio (which has some error in its determination) and a preliminary estimate of the rate of rise, and the estimate could be improved by iteration.

Ideally, to obtain true driving functions it is merely necessary to deconvolute the
apparent driving functions by the spreading functions. In practice, however, all attempts to perform this operation on individual MEPC averages produced time series with large oscillations as expected (a) from the presence of noise, and (b) if the spreading function employed is not exactly correct for the particular group of data. It was therefore not possible to obtain the true driving function in this way. Eventually, we assumed (see below) that each true driving function took the form of an exponential falling from an initial peak with rate constant $\beta$, and then we found, by trial and error, a value of $\beta$ that produced the waveform (by convolution of the spreading function by $\beta$) that correlated best (minimal least squares) with the observed driving function. This method was used for each average of MEPCs.

**Autocorrelation Functions**

In principle, if the driving function is an exponential, its autocorrelation function will also be an exponential, with the same rate constant. The following rather time-consuming procedure was therefore used on some of the data. (a) Deconvolute individual MEPCs with a rate constant close to that of channel closing (1.15 $\tau_1^{-1}$ was used); (b) calculate the autocorrelation function for the resulting (very noisy) driving function of each MEPC and average over the set of MEPCs; and (c) subtract the average of the autocorrelation function of the baseline noise. This procedure indeed produced sets of points following an exponential decline except for a low flat tail at $t > 0.6$ ms. The rate constants of decline were defined within $\pm 10\%$ and were not significantly different from the value of $\beta$ determined by the previous method for the same MEPCs.

**Use of Ethanol**

As will become clear in the Results, prolongation of MEPCs produced by ethanol (Quastel and Linder, 1975; Gage et al., 1975), in addition to being quickly reversible (within a few seconds at superficial endplates), was not associated with any reduction of rate of rise or of amplitude, such as was found in exploratory experiments using low temperatures. Ethanol was therefore used in preference to low temperature for improvement of the signal/noise ratios of points in the decay phase of the MEPC. In point of fact, all features of the MEPC that were visible in MEPCs recorded in the presence of ethanol could also be found in the absence of ethanol.

**Use of Extracellular MEPCs**

In the present experiments no systematic search was made for extracellular MEPPs or MEPCs, since (a) they lack information with regard to height, and (b) the transmembrane potential cannot be manipulated. Nevertheless, such signals were not infrequently encountered and recorded. As expected (Hodgkin and Rushton, 1946), these MEPCs had a late tail $\sim 2-3\%$ of peak height, which presumably reflects current flow secondary to change of intracellular potential. Apart from this, the time course was identical, as far as could be determined, to that of focal MEPCs recorded with the voltage clamp. We have included some data for extracellular MEPCs in the results, with respect to the rising phase of the MEPC.

**RESULTS**

**Modification of MEPCs by Ethanol, AChE Poisoning, and Receptor Blockade**

Typical examples of individual MEPCs recorded by the computer are shown in Fig. 1. In the presence of 0.4 M ethanol (Fig. 1B) MEPCs were prolonged and
little altered in amplitude, and they remained very similar to one another in time course. In contrast, the prolonged MEPCs recorded in the presence of 2 μM neostigmine or after paraoxon (Fig. 1C) showed considerable variation in time course, as well as the usual variation in height (cf. Hartzell et al., 1975). The effect of neostigmine to prolong MEPCs was only slowly and incompletely reversible, and a brief exposure to paraoxon was more convenient to use and without the complication of a possible local-anesthetic-like action on MEPCs (Kordaš et al., 1975). Also shown, in Fig. 1D, are examples of unusual MEPCs of the kind excluded from averages. These particular examples were recorded with AChE poisoned, but abnormal MEPCs such as these (except rarely so

![Figure 1](image.png)

**Figure 1.** Examples of MEPCs recorded digitally at 10 (A) or 5 kHz (B–D). Filters 0.1 Hz and 3 kHz. The noise level in B is most typical. Note uniform prolongation of MEPCs by 0.4 M ethanol in contrast to variable decay rate after poisoning AChE by paraoxon. Unusual MEPCs were most common after AChE poisoning or inhibition but also occurred under control conditions (A).

prolonged) could usually be found scattered among the main population under control conditions (e.g., last MEPC in Fig. 1A). Of these abnormal MEPCs the most frequently encountered were "monsters," i.e., MEPCs larger than usual with a greatly prolonged rising phase, but we also found small MEPCs with a very slow rising phase and normal-sized or large MEPCs with rapid rise but very slow fall. In the presence of 0.4 M ethanol, which always accelerated MEPC frequency about fourfold (Gage, 1965; Quastel et al., 1971), there were relatively few abnormal MEPCs. After inhibition of AChE by neostigmine or paraoxon, it was often noted that the MEPC frequency tended to be somewhat raised and abnormal MEPCs were more abundant; these phenomena were not studied in detail.
Individual MEPCs recorded in the presence of dTC were unremarkable in appearance, except for their reduced amplitude, and the same was true of MEPCs recorded in the presence of hexamethonium, or after prior treatment of the animal with α-bungarotoxin (αBuTX) or immunoglobulin from a patient with myasthenia gravis (Pennefather and Quastel, 1980a, 1981).

![Figure 2](image)

**Figure 2.** (A) Averages of MEPCs recorded from one junction at -40, -60, -120 mV in control solution (left) and from another junction at -20, -60, -120 mV in 0.4 M ethanol (right), plotted linearly (above) and semilogarithmically (below) vs. time. Note downward concavity in most of the semilog plots. Filters 0.1 Hz and 3 kHz. (B) Averages of MEPCs after AChE poisoning, grouped according to $\tau_1$, from one junction at -60 mV, plotted semilog vs. time. For clarity, the plots have been shifted arbitrarily on the time axis. Note the increase of average height with $\tau_1$ and maintenance of relative rate of decay late in MEPCs, despite the increase of downward concavity with the increase of $\tau_1$.

Typical averages of MEPCs (control and 0.4 M ethanol) and the modulation of their time course with membrane potential are shown in Fig. 2A. The MEPCs, especially those recorded in 0.4 M ethanol, show slight downward concavity when plotted semilogarithmically vs. time, to an extent that varies with holding
potential. As previously noted by Dwyer (1981), overall averaging of MEPCs recorded after poisoning of AChE gave rise to a nearly straight exponential decay, although individual MEPCs often showed a marked downward concavity in a semilog plot. For the averages shown in Fig. 2B, \( \tau_1 \) was determined for each individual MEPC in a series recorded at one junction, and MEPCs with similar \( \tau_1 \)'s were averaged. The semilog plots demonstrate three and perhaps four distinct phenomena: 

(a) persistence of differences in decay rate beyond the region \( (e^{-0.5} - e^{-1.5}) \) of peak height for which \( \tau_1 \) was determined, 

(b) modulation of downward concavity with \( \tau_1 \), 

(c) correlation of height with \( \tau_1 \) (Hartzell et al., 1975), and 

(d) a hint of a tail. In this figure, the average of the most prolonged MEPCs is reminiscent of the flat-topped extracellular MEPPs reported by del Castillo and Katz (1956a) and Katz and Miledi (1973), although obviously flat-topped MEPCs were in this example treated as unusual and excluded from averages.

The graphs shown in Fig. 3 summarize the actions of dTC and ethanol on MEPC height and \( \tau_1 \). After exposure to paraoxon the apparent potency of dTC to reduce MEPC height was diminished (Pennefather and Quastel, 1981), and the same was true for ethanol (Fig. 3A); i.e., when MEPC height was reduced by dTC an effect of ethanol to increase the height became quite prominent (Fig. 3C). This contrasts with controls where there was a small (<15%) increase of MEPC height by ethanol, virtually complete at 0.2 M; at junctions where AChE was poisoned (no dTC), ethanol caused little if any increase in mean MEPC height. The enhanced effectiveness of ethanol to increase MEPC size when MEPCs were made small by dTC was also seen with MEPCs made small by previously injecting animals with \( \alpha \)-BuTX or myasthenic IgG (Pennefather and Quastel, 1981). Whereas the increase of MEPC size by 0.4 M ethanol averaged 14 ± 3% (mean ± SEM) at 26 control junctions where MEPCs were recorded both in the absence and presence of ethanol, the increase was 33 ± 7% \((n = 8)\) in 0.2 \( \mu M \) dTC, 28 ± 3% \((n = 12)\) for myasthenic IgG, and 36 ± 6% \((n = 7)\) for \( \alpha \)-BuTX. These treatments each reduced MEPC amplitude by about half. Thus, the enhanced effect of ethanol to increase MEPC height, seen with dTC, cannot be attributed to a change in the affinity of receptors for dTC.

The time constants \( \tau_1 \) of MEPC decay were consistently diminished by dTC (Fig. 3B); the effect was close to maximal once MEPCs were reduced to ~50% of control height. Both in control solution and in 0.4 M ethanol the limiting time constant was ~20% smaller than \( \tau_1 \) in the absence of dTC and close to values for the time constant of channels obtained by fluctuation (noise) analysis. The latter values (each a mean of seven determinations) were obtained with underweighting of the points for the lowest frequencies in the power spectrum, which were usually higher than consistent with a simple Lorentzian curve (cf. Colquhoun et al., 1977). Values of channel \( \tau \) obtained after AChE poisoning were the same as without AChE poisoning (cf. Katz and Miledi, 1973). With AChE intact, either with or without ethanol, in all but one case channel \( \tau \) was smaller by 10–30% than \( \tau_1 \) for the MEPCs, at the same junction, recorded before and after application of ACh. These results suggest that with reduction of MEPC height by receptor blockade the rate of decay of the MEPC becomes essentially identical.
to the channel closing rate, unless AChE is poisoned. The prolongation of channel duration by ethanol concurs with previous reports (Gage et al., 1975; Quastel and Linder, 1975; Bradley et al., 1980).

In considering the effect of ethanol on MEPC height it is useful to recall that

\[ y(t) = \frac{e^{-\alpha t} - e^{-\beta t}}{\alpha - \beta}, \quad (1) \]

where \( \alpha \) and \( \beta \) are the rate constants of the falling and rising phases, respectively.
and $A_{df}$ is the area of the driving function; this area corresponds to the total conductance of channels opened and is what the height of the MEPC would become if $\alpha$ were reduced to zero. It can readily be calculated that once $\alpha$ is much less than $\beta$ (e.g., at $>0.2$ M ethanol), the peak height of the MEPC becomes insensitive to further reduction of $\alpha$. Since there is no large effect of receptor blockade to reduce $\beta$ (see below), the large increases of height that can occur with ethanol when most receptors are blocked (by dTC or an irreversible agent) cannot be explained solely in terms of a reduction in $\alpha$ (increase of $\tau_1$ and channel lifespan).

The graph in Fig. 3D illustrates that although ethanol had hardly any effect on MEPC amplitude after poisoning of AChE, it continued to increase $\tau_1$. Since the decay rate of MEPCs after AChE poisoning is controlled by buffered diffusion (Katz and Miledi, 1973), the fact that ethanol prolongs MEPCs after AChE poisoning implies that ethanol increases the fraction of ACh in the synaptic cleft that is bound to receptors.

Graphs of time constants of decay ($\tau_1$) vs. holding potential (Fig. 4) showed the same exponential relation as observed by others (Kordaš, 1969; Magleby and Stevens, 1972a; Colquhoun et al., 1977). The slope of log $\tau_1$ vs. membrane potential was altered little if at all by dTC, paraoxon, or 0.2 and 0.4 M ethanol; in 0.8 M ethanol the slope was reduced from $-7.3 \ V^{-1}$ to $-5.5 \ V^{-1}$. This reduction in slope and the data of Fig. 3D are suggestive of a process limiting
the duration of the MEPC; that is, insensitive to ethanol and less sensitive to transmembrane potential than normal channel closing.

Curvature: the Decay of MEPCs Deviates from an Exponential

It has been noted that the MEPC decay tended to be concave downward in a semilog plot vs. time (see Fig. 2). Quantitatively, this phenomenon also appeared as a progressive increase in decay rate, i.e., a decline of time constant during the decay phase of the MEPC. Thus, time constants determined between $e^{-1.5}$ and $e^{-2.5}$ of the peak were consistently smaller than $\tau_1$ in controls (ratio $0.95 \pm 0.01$, mean $\pm$ SEM, $n = 59$) and in 0.4 M ethanol (Pennefather and Quastel, 1980a). However, the deviation from a single exponential was not large and in any one average of MEPCs could easily escape observation. Fig. 5A shows a particularly clean example of curvature. The average is of 360 MEPCs from one junction, recorded in 0.4 M ethanol. The solid line represents values expected for an exponential decay, i.e., an exponential drawn tangent to the decay curve through points near $e^{-1}$ of the peak, with a slope determined from points between $e^{-0.5}$ and $e^{-1.5}$ of the peak. Observed data points fall below this line both early and late in the decay phase. Thus, data points expressed as a fraction of expected form a curve with values less than unity except near $t = \tau_1$. The averages (with standard errors) of such fractions are shown plotted semilogarithmically vs. $t/\tau_1$ in Fig. 5B. Each such graph is in effect a semilog plot, pivoted counterclockwise about the point where the MEPC has reached $e^{-1}$ of the peak. In each case the second point corresponds to the peak of the MEPC and, to facilitate comparison, the time base is different for each plot. In the presence of 0.2 and 0.4 M ethanol $\tau_1$ was increased two- and fourfold, while the initial rate of rise of MEPCs was very similar to the controls. It follows that for the data from 0.2 and 0.4 M ethanol the differences between observed and expected points early in the decay phase are only to a small extent secondary to incompleteness of the processes governing the rising phase of the MEPC. The rising phase in the plots in Fig. 5B at $t < \tau_1$ represents a rate of decay initially less than that at $t/\tau_1 = 1$, while the subsequent trend downward in most of these plots indicates a gradual increase in the MEPC decay rate ($\alpha$). The tendency of $\alpha$ to increase would appear to be the same with 0.2 or 0.4 M ethanol as in the controls, but graded with the transmembrane potential. There appears also to be a late increase of values at $t/\tau_1 > 2.5$ and $-120$ mV, in controls and with 0.4 M ethanol, which suggests the existence of a late tail in the MEPC (see below).

Modification of Curvature by Receptor Blockade

The most obvious explanation for the observed deviation of the MEPC decay from an exponential time course was that it reflects repeated action of ACh in the synaptic cleft to open new channels, i.e., reverberation (Katz and Miledi, 1973), which would be associated with downward concavity (Magleby and Terrar, 1975) because the opening of a channel requires more than one ACh molecule (e.g., Dreyer et al., 1978); the extent of reverberation should progressively diminish as the concentration of free ACh in the cleft goes down. Receptor blockade would be expected to reduce reverberation by lowering the probability
that ACh freed by dissociation of the ACh-receptor complex will once again come into contact with unblocked receptor before being hydrolyzed or otherwise lost.

It has already been seen that $\tau_1$ was consistently reduced by dTC, as is to be expected if reverberation is normally substantial (Mallart and Molgö, 1978; Katz and Miledi, 1978), and similar results were also obtained with hexamethonium
(at concentrations much lower than causes channel blockade [Milne and Byrne, 1979]), α-BuTX, and myasthenic immunoglobulin (Pennefather and Quastel, 1980a). As shown in Fig. 6, receptor blockade was also associated with the disappearance of curvature. The MEPCs now conformed closely to an exponential decay down to $t = 2\tau_1$, where there appeared upward deviations that were very similar to those appearing only at $-120$ mV in the controls (Fig. 5B). The virtual obliteration of curvature by 0.1 μM dTC, which reduced MEPC height by $<50\%$ (Fig. 5), militates against the possibility that curvature is primarily the result of appreciable depletion of local [Na⁺] outside opened channels (Attwell and Iles, 1979; Jachter and Sachs, 1982), as does the finding that curvature is reduced by hyperpolarization (which would increase Na⁺ depletion).

The MEPC Tail

The existence of a tail in the normal MEPC was confirmed by recording MEPCs at $-60$ and $-80$ mV (where noise is minimal), at a sampling frequency of 5 kHz (rather than 10 kHz), and under control conditions (AChE intact, no drugs, 10 mM K⁺). Grand averages are shown in Fig. 7. Here the data are from 33 and 38 endplates, respectively, and represent averages in each case of $\sim 1,500$ individual MEPCs. The standard errors (which correspond to a recording noise of $\sim 0.12$ nA rms) emphasize that the tail is so small as to be only just within the resolution possible with the technique. Fitting each tail to an exponential gives time constants of 7.4 and 5.3 ms for $-60$ and $-80$ mV, respectively, with an intercept at $\sim 0.7\%$ of the peak height. Graphs of “fraction of expected” (as in Figs. 5B and 6), shown in the insets, illustrate that on such a plot the tail becomes apparent at times (e.g., 3–5 ms) much earlier than where the tail is evident on the simple semilog plot.

The Rising Phase of MEPCs: Driving Functions

With the exception of MEPCs recorded after poisoning of AChE, and unusual MEPCs, the rising phase of MEPCs was in these experiments nearly complete.

Figure 5 (opposite). (A) Linear (below) and semilog (above) plots of average of 360 MEPCs from one junction, at $-60$ mV in presence of 0.4 M ethanol, corrected for recording filters. Lines are drawn horizontally through semilog plot at $e^{-0.5}$, $e^{-1}$, and $e^{-1.5}$ of the peak. The tangent line was drawn with $\tau_1$ determined by least-squares fitting of points between $e^{-0.5}$ and $e^{-1.5}$ of the peak. Note that the MEPC is below this tangent both early and late in the MEPC. (B) Semilog plots of fraction of expected height vs. time, for 0, 0.2, and 0.4 M ethanol, at $-40$ to $-120$ mV. Each graph represents the mean (± SEM) of values obtained by expressing every point in each average as a fraction of the value expected for an exponential curve with time constant $\tau_1$, determined for that particular average, passing through points close to $e^{-1}$ of the peak. Standard error bars shown only where they extend beyond plotted points. Most graphs represent means from 6–9 averages (19 in the case of $-60$ mV, control). Data for 0.2 M ethanol are from six junctions where MEPCs were recorded at each holding potential. Curves (theoretical) above each set of plots represent how such plots would appear for the function $y = (e^{-at} - e^{-bt})$ with $\beta = 5\alpha$, 10$\alpha$, and 20$\alpha$ plotted above data for 0, 0.2, and 0.4 M ethanol, respectively.
within 0.4 ms. Under control conditions, the peak of the MEPC generally occurred later when decay was prolonged by ethanol; this was especially evident with MEPCs made small by receptor blockade, with dTC (Fig. 8A), α-BuTX, or myasthenic IgG. Receptor blockade did not itself cause any obvious change in the fast part of the rising phase; averages of extracellular MEPCs recorded from the same endplate before and during application of dTC showed similar rising phases when scaled to the same maximum height (Fig. 8A).

As pointed out by Magleby and Stevens (1972b) and Cohen et al. (1981), even if the amount and time course of channel opening were identical under two different conditions, the rising phase of the MEPC would perforce vary with the rate of channel closing. The time course of opening of channels \( U(t) \) during an EPC or MEPC, which is the driving function of the observed waveform \( y(t) \), may be determined provided the rate of channel closing \( \alpha_o \) is known, since at any instant the rate of change is:

\[
\frac{dy(t)}{dt} = U(t) - \alpha_o y(t); \quad (2)
\]

\[
U(t) = \frac{dy(t)}{dt} + \alpha_o y(t). \quad (2a)
\]

For an MEPC following Eq. 1, of course, application of Eq. 2a, with \( \alpha = \alpha_o \), will give: \( U(t) = \beta A_{ut} e^{-\beta t} \).

In the present experiments the rate "constant" of decay \( \alpha \) was observed to increase throughout the decay phase (until the late tail was manifest); the driving function therefore must include late components representing repeated channel
opening. With $a$ as a function of $t$, better written as $a(t)$, one has

$$a(t) = -d\ln[y(t)]/dt = -[1/y(t)] \cdot dy(t)/dt = a_0 - q(t);$$

$$dy(t)/dt = y(t) \cdot q(t) - a_0 \cdot y(t),$$

where $q(t)$ is the component of $a(t)$ that varies with time. Thus, during the decay phase of the MEPC, by substitution for $dy(t)/dt$ in Eq. 2a,

$$U(t) = y(t) \cdot q(t).$$

That is, the driving function itself has a tail, extending throughout the decay phase of the MEPC, with a decay rate somewhat faster than the decay rate of the MEPC itself. When AChE is poisoned, of course, $q(t)$ is close to $a_0$ [since $a(t)$ is much smaller than $a_0$] and the tail of the driving function is virtually identical in time course to the MEPC itself.

If one applies Eq. 2a using a value for $a_0$ different from true $a_0$, say $a_0 - \epsilon$, Eq. 2a becomes,

$$\text{apparent } U(t) = dy(t)/dt - (a_0 + \epsilon) \cdot y(t)$$

$$= U(t) - \epsilon \cdot y(t).$$

Thus, the "error" consists in addition or subtraction of the MEPC itself,
multiplied by $\varepsilon$; this error is small in the rising phase of the MEPC, where $dy(t)/dt$ is much larger than $\varepsilon \cdot y(t)$. The real tail in the driving function can therefore arbitrarily be exaggerated or eliminated by choosing values of $\alpha_o$ higher or lower than the true rate of channel closing.

In Fig. 8, B and C, are shown averages of driving functions for MEPCs with AChE intact, calculated using the approximation

$$U_i = (y_i - y_{i-1})/\Delta t + \alpha_1(y_i + y_{i-1})/2,$$

where $U_i$ and $y_i$ are values of $U(t)$ and $y(t)$ at the $i$th sample point. The use of $\alpha_1$ (i.e., $\tau_i^{-1}$) rather than $\alpha_o$ (which is greater than $\alpha_1$) serves to reduce the contri-
bution of (presumed) repeated ACh action to the driving function. When similar calculations were made (for the MEPCs with no dTC) using 1.2 $\alpha_1$ as an estimate of $\alpha_n$ instead of $\alpha_1$, the driving functions were virtually identical for points down to <10% of the peak of the driving function. Even using $\alpha_1$ itself, it is apparent in the driving functions of normal MEPCs in Fig. 8B that the tail was not entirely suppressed by the use of $\alpha_1$ rather than the true channel closing rate.

The interpretation of these driving functions is complicated by the fact that it is intrinsic in the method of recording and of averaging that there be some jitter or spread in allocating points in the MEPC to time bins in the average; a calculated driving function represents the convolution of the true driving function by the spreading function for that particular MEPC average. Examination of how the computer programs treated artificial MEPCs superimposed on records of real noise (see Methods) showed that the spreading function varies with the signal/noise ratio and the rate constant of rise of the MEPC. Above each of the driving functions in Fig. 8B and C, is shown the mean of the spreading functions estimated to be appropriate to each of the particular MEPC averages from which the driving functions were calculated. As will become evident below, the very obvious modulation of the driving function by holding potential seen in Fig. 8B was in fact mainly secondary to systematic alteration in the spreading function associated with alteration of the signal/noise ratio.

**Rate Constant of the Rising Phase of MEPCs**

The results shown in Fig. 8, B and C, suggested that the main part of the true driving function might indeed fit an exponential of the form $U(t) = \beta A_{eq} e^{-\beta t}$, as would be expected (a) if ACh combination with receptors is very fast and the driving function reflects isomerization of the activated receptor channel to the open channel state (Adams, 1981), or (b) if the rate-limiting step is a combination of ACh with receptors, and the local concentration of ACh falls primarily because of uptake by receptors (Land et al., 1980; Pennefather and Quastel, 1981). By either mechanism the rate constant $\beta$ represents the rate at which the number of channels that have been opened approaches its maximum. It is also the rate
of loss of the immediate precursor of the open channel state. That \( U(t) \) did indeed fit an exponential, at least to a first approximation, was verified in two ways: (a) driving functions of individual MEPCs (rather than averages) were analyzed in terms of their autocorrelation functions (see Methods); (b) graphs of the integral of the driving function (of averages) showed an exponentially asymptotic approach to a maximum.

With MEPCs recorded in the presence of receptor blockade method (b) showed an excellent fit to a single exponential, but otherwise (controls, ethanol) the contribution of the tail of the driving function was such as to preclude accurate estimation of \( \beta \) by this method. Method a was too time-consuming (on the PDP-12 computer) to be practicable for most of the data, but served as a check on the values obtained by the method finally chosen for estimation of \( \beta \). This was to correlate the actual driving functions for each MEPC average with theoretical driving functions generated on the basis of the known signal/noise ratio and various specified \( \beta \)'s; trial and error gave a \( \beta \) with the best least-squares fit (see Methods). This method always gave a well-defined \( \beta \) and an extrapolated value for \( A_{df} \) that was close to the true area of the driving function.

Results obtained in this way are listed in Table IA together with values for the area of the driving function, for MEPCs recorded at least at \(-80 \) mV and one other holding potential, in control conditions and in \(0.2\) M ethanol. The average value of \( \beta \) in control solutions at \(-80\) mV \((7.8 \pm 0.2/\text{ms})\) corresponded well with values of \( \beta \) for three averages of extracellularly recorded MEPCs, namely, \(7.6/\text{ms}, 7.8/\text{ms}, \) and \(8.2/\text{ms}\). In both series there was a small but significant modulation of \( \beta \) with holding potential. The differences in slope of log \( \beta \) vs. voltage are not significant and the combined data gave a slope of \(-1.7 \pm 0.3/\text{V} \) (56 degrees of freedom; \( r = 0.407, t = 3.93, P < 0.001 \)); i.e., \( \beta \) (the rate constant for channel opening) was slightly but consistently increased by hyperpolarization and reduced by depolarization.

Because of the substantial \((\pm 15\%)\) variation of MEPC height from one average to another and the implicit correlation of area of driving function \( (A_{df}) \) with height (big MEPCs have a large \( A_{df} \)), values for \( A_{df} \) were normalized with respect to MEPC height and then, as with \( \beta \), expressed as a fraction of the value at \(-80\) mV at the same junction. The data in Table IA indicate a clear, though small, modulation of this parameter with holding potential both in the controls and in \(0.2\) M ethanol. It should be noted that it is implicit in Eq. 1 that a reduction of \( \alpha_0 \), as occurs with ethanol or hyperpolarization, will entail an increase of MEPC height. Thus, a voltage sensitivity of the ratio of \( A_{df} \) to height might be expected simply from the voltage sensitivity of \( \alpha_0 \). However, net MEPC height (expressed as conductance) was actually constant with holding potential (Table IB, control), or declined with hyperpolarization \((0.2, 0.4, \) and \(0.8\) M ethanol). Thus, these data indicate a significant positive correlation of \( A_{df} \) (i.e., total conductance opened) with holding potential. Under control conditions, the decay of the MEPC is sufficiently fast that the voltage sensitivity of the decay rate counteracts this effect and leads to an MEPC height (in nanosiemens) that is nearly independent of holding potential. The trend of the change of \( A_{df} \) with holding potential coincides with that observed by Magleby and Stevens (1972b) at frog
endplates. It is most easily explained as reflecting a reduction of channel conductance with hyperpolarization (see Discussion).

The values of $\beta$ in 0.2 M ethanol varied over a rather wide range (6/ms–12/ms), and the difference from controls in Table IA is not significant. However, in a separate series (16 junctions, 2 extracellular), where at each junction MEPCs were recorded both in control solution and in 0.4 M ethanol, mean $\beta$ was raised

| Voltage Dependence of Driving Function (DF) and Height of MEPCs |
|---------------------------------------------------------------|
| (A) | $\beta$ (ms$^{-1}$) | Area DF/MEPC height |
|     | (mV) | Control | 0.2 M ethanol | Control | 0.2 M ethanol |
|     |      |         |              |         |              |
| $mV$ |      |         |              |         |              |
| -40 | 0.94±0.02 | (9) | 0.96±0.04 | (6) | 1.05±0.01 | (9) | 1.08±0.02 | (7) |
| -60 | 1.00±0.03 | (11) | 0.90±0.05 | (7) | 1.03±0.01 | (11) | 1.05±0.01 | (7) |
| -80 | [7.8±2.0] | (11) | [9.1±2.7] | (9) | [1.42±0.013] | (11) | [1.26±0.03] | (9) |
| -100 | 0.97±0.03 | (8) | 1.12±0.07 | (8) | 0.97±0.01 | (8) | 0.96±0.01 | (8) |
| -120 | 1.05±0.04 | (6) | 1.11±0.09 | (5) | 0.92±0.006 | (6) | 0.94±0.010 | (5) |
| Slope | -0.9±0.4 | -2.7±0.8 | +1.53±0.15 | +1.84±0.19 |
| $r$ = 0.30, $t$ = 2.1 | $r$ = 0.51, $t$ = 5.4 | $r$ = 0.85, $t$ = 10.7 | $r$ = 0.85, $t$ = 9.5 |
| $P < 0.05$ | $P < 0.01$ | $P < 0.001$ | $P < 0.001$ |

| MEPC height (nS) |
|------------------|
| (B) | Control | 0.2 M ethanol | 0.4 and 0.8 M ethanol |
|     | (mV) |         |              |         |              |
| $mV$ |      |         |              |         |              |
| -40 | 1.14±0.05 | (14) | 1.15±0.04 | (13) | 1.09±0.02 | (19) |
| -60 | 1.06±0.02 | (17) | 1.08±0.02 | (14) | 1.05±0.01 | (22) |
| -80 | [47.2±2.1] | (66) | [51.2±2.3] | (15) | [55.5±2.1] | (23) |
| -100 | 1.04±0.03 | (12) | 0.96±0.02 | (14) | 0.98±0.02 | (16) |
| -120 | 1.02±0.04 | (7) | 0.95±0.02 | (9) | 0.95±0.02 | (11) |
| Slope | +0.53±0.48 | +2.2±0.4 | +1.7±0.3 |
| $r$ = 0.15, $t$ = 1.1 | $r$ = 0.58, $t$ = 5.02 | $r$ = 0.59, $t$ = 6.07 |
| $P < 0.001$ | $P < 0.01$ |

In each case, all values except those at $-80 \text{ mV}$ are expressed as means ± SEM of ratio of observed value at $-80 \text{ mV}$ at same junction. For $-80 \text{ mV}$ actual means ± SEM are given in square brackets. The number of observations is in brackets. Slopes are slopes of ln values vs. holding potentials in volts for the range $-40$ to $-120 \text{ mV}$ in $A$ and $-60$ to $-120 \text{ mV}$ in $B$. The slopes of $\beta$ vs. $V$ are not significantly different for 0 and 0.2 M ethanol. The values for height include data from more junctions than $A$, since good values for height, but not $\beta$, could be obtained from averages that were relatively noisy, or recorded at 5 kHz instead of 10 kHz (0.4 and 0.8 M ethanol). For the correlation of MEPC height with holding potential, data for $-40 \text{ mV}$ were omitted because there may have been a small tendency for relatively small MEPCs to be overlooked by the computer program that identified MEPCs.

by ethanol from $7.7 ± 0.3/\text{ms}$ to $9.95 ± 0.60/\text{ms}$, an increase of $28 ± 5\%$ ($t = 6.0, P < 0.001$). At the 14 of these junctions that were clamped at $-60$ or at $-80 \text{ mV}$, the total area of the driving function was reduced by $5.7 ± 2.2\%$ ($t = 2.53, P < 0.05$). Thus, the net increase of MEPC height ($14.3 ± 2.3\%$) by 0.4 M ethanol resulted from the interplay of increase of $\beta$, slowing of decay rate (reduced $a_0$), and reduction of total conductance opened in the early part of MEPC. At seven junctions where MEPCs were recorded in the presence of 0.4
μM dTC, and in the absence and presence of 0.4 M ethanol, β appeared to be reduced, from 7.9 ± 0.8/ms to 5.9 ± 0.2/ms, but the difference was not significant because of the high spread of values. The area of the driving function was increased by 26 ± 5% (P < 0.01). Together with the reduction of α₀, this accounted for the net rise in MEPC height of 43 ± 5%.

Parameters of Reverberation

It is intuitively obvious that if more than one ACh molecule must combine with a receptor in order to open a channel, the chance of any one ACh molecule in the cleft participating in a channel opening will not be constant but will at any moment depend upon the local concentration of ACh. The extent of repeated ACh action will therefore depend upon the rate at which ACh is being freed by dissociation of ACh-receptor complexes and the rate at which it is lost by hydrolysis and diffusion from the cleft. This reasoning may be formalized in terms of a simplified model of events in the synaptic cleft.

Treating the cleft as a single, well-mixed homogeneous compartment, with two species of receptor, R and AᵣR (the open channel form), with

\[ \lambda \leftarrow nA + R \rightleftharpoons AᵣR \]

the rate of change of AᵣR at any moment will be

\[ \frac{d[AᵣR]}{dt} = -\alpha(t)[AᵣR] = -\alpha₀[AᵣR] + K[A]^n[R] \]

and thus,

\[ \alpha(t) = \alpha₀ - K[A]^n[R]/[AᵣR], \]  \hspace{1cm} (3)

where K is a constant, [A] is the concentration of ACh, n is the number of ACh molecules combining to open a channel, and \( \alpha(t) \) is \(-d\ln[AᵣR]/dt\), the instantaneous rate constant. If we further simplify by assuming that during the decay of the MEPC there is a balance between the rate at which ACh is released into the cleft by dissociation of AᵣR, and the net rate at which ACh is lost by hydrolysis and diffusion, with combined rate constant \( \lambda \), then

\[ \lambda[A] = n\alpha(t)[AᵣR]. \]

Substituting for [A] in Eq. 3, and using \( n = 2 \),

\[ \alpha(t) = \alpha₀ - 4[\alpha(t)]^2K[R][AᵣR]/\lambda^2. \]

Since the actual rate constant \( \alpha(t) \) is not very far from constant during the main portion of the MEPC decay, and with the further assumption that \([R]\) and \( \lambda \) are effectively constant, this equation predicts a more or less linear relation between \( \alpha(t) \) and MEPC height at time \( t \), with Eq. 3 becoming

\[ \alpha(t) = \alpha₀ - \alpha₁^2 \cdot C \cdot \gamma(t)/y_p \]  \hspace{1cm} (3a)

where \( \gamma(t)/y_p \) is the height of the MEPC relative to its peak, \( \alpha₁ \) is \( \tau₁^{-1} \), and \( C (= 4K[R]/\lambda^2) \) is a constant.

To apply this equation to the data it was necessary first to deconvolute the
actual MEPC by \( \beta \), the rate constant of the rising phase of the MEPC, since even if \( \alpha(t) \) were in fact constant, the MEPC would follow a time course \( y(t) = (e^{\alpha t} - e^{-\beta t}) \cdot A_m / \beta (\beta - \alpha) \), and \( d\ln[y(t)]/dt \) would be less than \( \alpha \) until times where \( t \gg \beta^{-1} \). This procedure, using the values of \( \beta \) determined for each MEPC average (see above) gave curvature for control MEPCs virtually identical to that seen with ethanol (Figs. 5 and 6); for MEPCs recorded with ethanol the curvature was unaltered.

Fig. 9 shows two examples of graphs of \( \alpha(t) \) (i.e., \( d\ln[y(t)]/dt \) for deconvoluted MEPCs) plotted vs. \( y(t)/y_p \) from the data shown in Fig. 5B. In each case, a straight line was fitted to points from at least 0.4 ms after the MEPC peak (to avoid the points sensitive to any error in \( \beta \)) down to 20% of the peak [below which \( \alpha(t) \) becomes poorly defined]. Similar linear plots were obtained for all the MEPC grand averages shown in Fig. 5B; the derived fitting parameters are listed in Table II. From these values it would appear that the modulation of curvature with holding potential, seen in Fig. 5B, for the most part follows from the voltage sensitivity of the rate of channel closing (\( \alpha_o \)), rather than a change of the parameter \( C \). However, with ethanol, prominent curvature coexists with low rates of channel closing. This implies that \( C \) is increased by ethanol, which agrees with the observation that ethanol increases \( \beta \). The data are also consistent in giving an \( \alpha_o \) [extrapolated maximum \( \alpha(t) \) and presumed channel closing rate] greater than \( \tau_i^{-1} \) by 20%, which fits well with the rate constants found when MEPCs were made small, and reverberation was presumably minimized, by receptor blockade (Fig. 3). Because of the possibility of appreciable contribution of the late tail of the MEPC (see Fig. 5B), it is not clear whether the low values of \( C \) at \(-120 \text{ mV} \) are genuine. Nor, because of the scatter of values, is it clear...
whether $C$ may be a function of holding potential. The fit to Eq. 3a, and the value of $a_0$ being more than $\tau_1^{-1}$ by 20% leads to the conclusion that normally the total area of the MEPC is augmented $\sim 35\%$ by repeated ACh action.

**Correlation of MEPC Time Course and MEPC Height**

**DECAY RATE.** It has already been noted that when AChE was poisoned, MEPCs in any one series (one junction, constant conditions) varied considerably in time course. With AChE intact, with or without ethanol, there was also variation of $\tau_1$, to an extent greater than expected from the recording noise. In Fig. 10 are shown scattergrams of $\tau_1$ vs. MEPC height at two junctions: (a) after poisoning of AChE and (b) in the presence of 0.8 M ethanol. In the latter case there is a relatively low slope of $\tau_1$ (or log $\tau_1$) vs. MEPC height (although the correlation is highly significant statistically), and none of the scatter of $\tau_1$ characteristic of AChE poisoning (Fig. 10A; Katz and Miledi, 1973; Hartzell et al., 1975).

**Table I**

| Parameters of Reverberation |
|-----------------------------|
| Control                     |
| 0.2 M ethanol               |
| 0.4 M ethanol               |
| $\tau_1$ as % $\alpha_1$   |
| C                           |
| $\tau_1$ as % $\alpha_1$   |
| C                           |
| $\tau_1$ as % $\alpha_1$   |
| C                           |

| $\mu V$ | $\tau_1$ | $\alpha_0$ | $C$ | $\tau_1$ | $\alpha_0$ | $C$ | $\tau_1$ | $\alpha_0$ | $C$ |
|---------|---------|------------|-----|---------|------------|-----|---------|------------|-----|
| -20     | 0.80    | 137±8      | 0.8±0.1 | -20     | 0.80    | 137±8      | 0.8±0.1 | -20     | 0.80    | 137±8      | 0.8±0.1 |
| -40     | 0.90    | 122±4      | 0.6±0.1 | -40     | 0.90    | 122±4      | 0.6±0.1 | -40     | 0.90    | 122±4      | 0.6±0.1 |
| -60     | 0.99    | 121±5      | 0.6±0.1 | -60     | 0.99    | 121±5      | 0.6±0.1 | -60     | 0.99    | 121±5      | 0.6±0.1 |
| -80     | 1.25    | 126±4      | 0.9±0.1 | -80     | 1.25    | 126±4      | 0.9±0.1 | -80     | 1.25    | 126±4      | 0.9±0.1 |
| -100    | 1.44    | 109±4      | 0.4±0.1 | -100    | 1.44    | 109±4      | 0.4±0.1 | -100    | 1.44    | 109±4      | 0.4±0.1 |

Despite the scatter of values for $\tau_1$, after AChE poisoning, there is evident in this example a very significant correlation of $\tau_1$ with MEPC height. This was true for all sets of MEPCs recorded after AChE poisoning by paraoxon or inhibition by neostigmine. For the full range of MEPC heights the best correlation was usually between log $\tau_1$ and log height; the slope of the log-log regression line varied between 0.43 and 1.01, with a mean of 0.685 (SD = 0.14, SEM = 0.02) for 49 sets of >25 MEPCs; in all but 5 sets the slope was >0.5. At any one junction addition of ethanol (which further prolonged MEPCs) did not significantly alter the slope of log $\tau_1$ vs. log height. With dTC, points relating $\tau_1$ to height fell close to the same line as before or after dTC; the slope of log $\tau_1$ vs. height was unaltered, but the slope of log $\tau_1$ vs. log height was reduced, in the same way as observed with spontaneously small MEPCs (Fig. 10C).

With AChE intact, a correlation between MEPC time constant ($\tau_1$) and height could be observed not only whenever large numbers of MEPCs were recorded in sequence at a single junction (with conditions held constant), but also when data from a number of junctions were analyzed in such a way as to eliminate variance due to different recording conditions, i.e., by normalizing $\tau_1$ and height in terms of the mean $\tau_1$ and mean height for the particular set of MEPCs. For
example, at seven junctions where MEPCs were recorded in standard solution at -40 to -120 mV the slope of log $\tau_1$ vs. log height varied between 0.081 ± 0.028 and 0.177 ± 0.045 (± SEM) at the different junctions (with 200–300 MEPCs at

![Graphs of log $\tau_1$ vs. log height](image)

**Figure 10.** (A and B) Scattergraphs of log $\tau_1$ vs. height for two junctions, at -60 mV after paraoxon (A) and at -80 mV in 0.8 M ethanol with AChE intact (B). (C) Graphs of log $\tau_1$ vs. log height in controls (C), after AChE poisoning (●), and in presence of 0.4 M ethanol after treatment with X-537A, AChE intact (■). For the first two plots, height and $\tau_1$ for each MEPC were normalized in terms of mean height and mean $\tau_1$ for the set of MEPCs. Points are plotted in such a way that average $\tau_1$ and average height are at actual averages for -60 mV. Points for controls represent 1,563 MEPCs recorded at -40 to -120 mV, from seven junctions. Data for after AChE poisoning are from 3,600 MEPCs, from 19 junctions recorded at -60 and -80 mV. Data for 0.4 M ethanol are from 2,485 MEPCs, all recorded at -60 mV at 14 junctions without normalization. Plotted at left are $\tau$ for ACh noise in 0.4 M ethanol (■) and no ethanol (●; with or without AChE poisoning). Standard errors are shown when larger than the plotted points.
each junction). Grouping values according to holding potential showed no significant voltage dependence of this correlation. For the whole group (1,563 MEPCs) the slope was 0.116 ± 0.020, for the log-log correlation of normalized \( \tau_1 \) vs. normalized height. Fig. 10C shows a graph of these data with mean (± SEM) of log-normalized \( \tau_1 \) plotted vs. log-normalized height. Similar treatment of the data from junctions where AChE was poisoned (Fig. 10C) showed that linearity between log \( \tau_1 \) and log height obtained only for MEPCs of >60% mean height; unusually large MEPCs fit well on the extrapolated portion of the best-fitting line for log \( \tau_1 \) vs. log height (determined using only MEPCs between 60 and 180% mean height), which had a slope of 0.77 ± 0.01. The other graph in Fig. 10C is for MEPCs recorded in the presence of 0.4 M ethanol, after exposure of the preparation for a few minutes to the nonselective ionophore X-537A (Pressman, 1973) at 5 \( \mu \)M. This treatment was previously observed to provoke the appearance of relatively many small MEPCs (McCort and Quastel, unpublished; cf. Kriebel and Gross, 1974), for up to several hours after exposure. Here, 2,485 MEPCs from 14 junctions recorded (at -60 mV) in one afternoon have been treated as though they were from one junction. The slope of log \( \tau_1 \) vs. log height was 0.123 ± 0.010.

**RATE OF RISE** For the series of MEPCs recorded after X-537A (and in 0.4 M ethanol) there was a small positive correlation of the maximum rate of rise of the MEPC relative to its peak (i.e., fraction of peak height in two sample points, determined for each MEPC) with MEPC height, with a slope (log-log) of 0.034 ± 0.005. That is, relatively large MEPCs tended to be associated with a faster than average approach to their peaks. This correlation is the opposite of what would be expected (see Eq. 1) from the positive correlation of \( \tau_1 \) with height.

The same was true for MEPCs recorded after AChE poisoning, provided MEPCs were grouped according to \( \tau_1 \). That is, among MEPCs with similar \( \tau_1 \), the larger MEPCs approached their peaks faster. This correlation was significant (\( P < 0.01 \)) and for MEPCs with \( \tau_1 \) between 2 and 5 ms the mean slope (log-log) was 0.1. However, between groups of MEPCs with similar \( \tau_1 \), relative rate of rise was negatively correlated to \( \tau_1 \) and to height. For MEPCs recorded under control conditions (AChE intact, no ethanol), there was a slight negative correlation between relative rate of rise and peak height, perhaps because recording noise tends to give a negative correlation between these parameters.

**Reverberation in Small MEPCs**

Because the mean decay rate of MEPCs is a function of MEPC height (Fig. 10), it was considered worthwhile to examine the MEPCs recorded after treatment with X-537A (and in 0.4 M ethanol) in order to determine whether the extent of reverberation varied with spontaneous variation of height. Fig. 11 shows semilog plots of MEPC averages vs. time from the peak, rotated (as in Fig. 5B) so as to emphasize deviations from an exponential decay; the data were grouped and averaged with respect to peak height. For the smallest MEPCs (top, all MEPCs <1.1 nA, average 0.8 nA), there appears to be little if any curvature. Otherwise, (a) the influence of the tail is manifest later the larger the MEPCs and (b) early points are slightly lower the larger the MEPCs. Analysis as in Fig. 9 showed no significant difference of curvature over the range of mean peak
amplitude from 1.5 to 3.8 nA (at -60 mV). This result contrasts with what was observed with MEPCs moderately reduced in size by receptor blockade (Fig. 6), and suggests that except for very small quanta of ACh, the concentration of ACh in the cleft in the active region, at corresponding times in the decay phase, is much the same whatever the quantal size. That is, MEPCs of different amplitude normally represent different areas of subsynaptic receptor activation (rather than different densities of activation), as suggested on theoretical grounds by Land et

![Figure 11](image-url)

**Figure 11.** Graphs similar to those of Fig. 5B (but without adjustment of time base and deconvoluted with $\beta = 9/\text{ms}$) for MEPCs recorded at 14 junctions, in 0.4 M ethanol, after treatment with X-537A to produce small MEPPs. Arrows indicate $t = T_i$. Each plot is for an average of MEPCs grouped according to amplitude with means of 0.8, 1.5, 2.3, 3, and 3.8 nA from the top downward. Note the lack of modulation of curvature with height, except at 0.8 nA (range 0.5–1.275 nA). Holding potential -60 mV.

al. (1980) and by Adams (1980). The apparent modulation with height of the relative amplitude of the MEPC tail was not investigated further.

**DISCUSSION**

**Time Course of MEPC Decay**

In the present experiments, digital averaging of relatively large numbers of MEPCs permitted delineation of the time course of MEPC decay with a precision
that has not previously been attempted, either for MEPCs or for endplate currents. The data confirm previous reports in that (a) most of the decay curve is close to a simple exponential (Takeuchi and Takeuchi, 1959) with a rate constant that is positively correlated to transmembrane potential (Kordaš, 1969; Magleby and Stevens, 1972a), and (b) the decay rate of the MEPC is lower than the closing rate of channels, as determined by fluctuation analysis (Katz and Mileti, 1973; Feltz et al., 1977; Colquhoun et al., 1977; Mallart and Molgó, 1978; Gage and Van Helden, 1979). In addition, the MEPC decay shows a downward concavity on a semilog plot, i.e., a progressive increase of rate of decay with time, under control conditions as well as after poisoning of AChE (Dwyer, 1981). It was pointed out by Magleby and Terrar (1975) that, after poisoning of ACHE, such curvature was to be expected as a consequence of cooperativity in ACh-receptor interaction; in their experiments this curvature was evidently obscured because the EPC is tantamount to an average of MEPCs with differing rates of decay.

With reduction of MEPC amplitude by blockade of receptors, either by dTC or the essentially irreversible agents α-BuTX and myasthenic IgG, the MEPC decay rate becomes increased, and when AChE is intact approaches the rate of channel closing. Since this effect of dTC reaches a limit at only 0.2 μM (which reduces MEPCs to half size), it cannot be attributed to the local-anesthetic-like action evident with dTC at much higher concentrations (Manalis, 1977; Katz and Miledi, 1978). At the same time, curvature (Figs. 5 and 6) is obliterated. We tentatively conclude (in accord with the suggestion of Magleby and Terrar [1975]) that the normal curvature reflects a progressive diminution with time of the likelihood that that any one ACh molecule, when it is released by dissociation of an ACh-receptor complex, again acts to open a channel; this decline in probability arises from the requirement of two ACh molecules for channel opening, which makes the rate of channel opening proportional to the square of local ACh concentration (Katz and Thesleff, 1957; Dreyer et al., 1978; Colquhoun, 1978; Adams, 1981). On this basis, the observed modulation of curvature with transmembrane potential appears as a consequence of modulation of rate of channel closing, if channel closing is tightly linked to dissociation of ACh-receptor complexes (see below). An exact equation to describe the expected curvature is, however, not possible, and the relation (Eq. 3a) that we have derived on the basis of a simplified model can be only an approximation. Using a multicompartment model (Pennefather and Quastel, 1981) similar to that of Wathey et al. (1979), we find that curvature does indeed emerge as a consequence of cooperativity. Nevertheless, we cannot discount the possibility of other mechanisms contributing to the curvature. In particular, it is likely that the same factors are involved in normal curvature as in the reverberation following AChE poisoning; here the slope of the correlation between log τ and log MEPC height cannot be explained simply by cooperativity (see below).

Relation Between Channel Lifetime and ACh Binding to Receptor

Normally, ACh dissociation from receptors must be closely linked to channel closing, since MEPCs recorded after AChE poisoning show no initial fast falling phase (Fig. 1C; Katz and Mileti, 1973), as would occur if ACh remained
associated with receptors for long after channels closed (at ~1/ms). Meanwhile, if ACh were to come off receptors before channels closed, when AChE is poisoned there would be an overlap in the lifespans of first and second generation channels opened by the same ACh molecules, leading to an increase in MEPC height that would be large if there were a long time gap between ACh discharge and channel closing. In actuality, only a small increase (16 ± 4%) in MEPC height is observed when AChE is poisoned and this can be attributed to the improved initial capture of quantal ACh by receptors (Pennefather and Quastel, 1981). When ethanol is present, the difference in MEPC height produced by AChE poisoning is <10%. Thus, ethanol must prolong ACh attachment to receptors at least as much as it prolongs channel lifetime. Again the lack of a relatively fast initial falling phase when AChE is poisoned excludes the possibility that ethanol prolongs ACh binding more than channel lifetime.

Ethanol Promotes Opening of Channels by ACh

If the sole effect of ethanol were prolongation of channel lifespan and ACh binding, curvature would be attenuated by ethanol in the same way as seen with hyperpolarization. The persistence of curvature in the presence of ethanol thus implies that ethanol must have an additional action, which tends to enhance repeated ACh action. A simple calculation may clarify this point. In 0.4 M ethanol, the rate of channel closing is one-quarter normal. If hydrolysis and diffusion are unaltered, concentrations of free ACh in the cleft, at corresponding points in the MEPC, will also be one-quarter normal, and the rate of new channel opening (proportional to [ACh]2) should be one-sixteenth normal. It actually is, however, about one-quarter normal (note definition of time base in Fig. 5B); i.e., C (in Eq. 3a) is increased (Table II). Since even at 0.8 M ethanol there was no suggestion of the high variability of τ1 that is characteristic of AChE inhibition, it seems unlikely that this arises from inhibition of AChE by ethanol.

Two other sets of observations also indicate that ethanol acts to facilitate channel opening by ACh: (a) the substantial increase of MEPC height by ethanol when MEPCs are made small by receptor blockade, and (b) the increase by ethanol (+28 ± 5% at 16 junctions by 0.4 M) of the rate constant β.1

1 It should be kept in mind that β represents the rate of disappearance of the precursor of the open channel. To make this point clear we write the following scheme:

\[ R + \text{products} \xleftarrow{\lambda} 2A + \text{R} \]

Here λ is the combined rate of loss of ACh from the cleft by hydrolysis and diffusion. If κ is very fast, \( AR \) is quickly in equilibrium with \( A \) (free ACh), and the scheme collapses to

\[ R + \text{products} \xleftarrow{\lambda} 2A + R \]

And if \( \beta_0 \) and \( K_{-1} \) are sufficiently fast, this in turn collapses to

\[ R + \text{products} \xleftarrow{\lambda} 2A + R \]

In the last case, \( \beta \) represents \( \beta' + \lambda \), the rate constant for loss of free (and nearly free) ACh from the cleft; this could only be diminished by inhibition of AChE. Since it is unlikely that
Facilitation of receptor activation by ethanol of course predicts that the steady state response-concentration curve for exogenous ACh should be shifted by more than the factor by which channels are prolonged. In a few experiments with ACh, after AChE poisoning by paraoxon, we have searched for and did not find any such phenomenon—responses were increased only three- to fivefold by 0.4 M ethanol—this result may easily be explained by an increase of desensitization by ethanol, as can be seen with a variety of general anesthetic agents (e.g., Pennefather and Quastel, 1980b). The small reduction of area of the driving function by ethanol (no dTC) is consistent with a small effect to reduce channel conductance; higher alcohols (e.g., propanol) have a marked effect to reduce MEPC height (Quastel and Linder, 1975).

**Voltage Sensitivity of Channel Opening**

Our conclusions with respect to the effect of membrane potential on channel opening are different from those of Magleby and Stevens (1972b), although our results agree well with regard to the voltage sensitivity of the area of the driving function. In interpreting their data, Magleby and Stevens (1972b) assumed for mathematical simplicity that only a small fraction of released ACh becomes bound to receptors. If this were so and if receptor isomerization is not rate limiting, $\beta$ would be governed mainly by hydrolysis and diffusion ($\lambda$) and the area of the driving function would directly reflect the outward rate constant for conductance increase. In the present experiments, there was a small but significant tendency of $\beta$ to increase with hyperpolarization, i.e., an acceleration rather than slowing of channel opening by hyperpolarization. The voltage sensitivity of the area of the driving function then presumably reflects rectification by the channels, as has been observed in studies of single-channel conductance (Gage and Van Helden, 1979; Van Helden et al., 1979; Takeda et al., 1980; Horn and Brodwick, 1980). In the present experiments, this rectification appeared as a decrease in MEPC height with hyperpolarization, once the channel closing rate ($\alpha_c$) was so slowed by ethanol that the voltage sensitivity of $\alpha_c$ cannot be manifest in MEPC height (Table I). It is notable that little or no such voltage sensitivity
of total conductance was apparent in MEPCs recorded in the virtual absence of extracellular Ca²⁺ and in the presence of ethanol (Linder and Quastel, 1978).

With regard to the inferred action of ethanol and of membrane potential on channel opening, there is a rather obvious quantitative difference between effects on the parameters \( \beta \) and \( C \), both of which must reflect the same underlying processes of ACh capture and channel opening. However, these processes must be very differently weighted in \( \beta \) and \( C \). For example, any degree of saturation of receptors by ACh near the site of release (Land et al., 1980, 1981; Adams, 1980) will tend to limit \( \beta \) but will have little influence on \( C \). If isomerization is normally rate limiting, then \( \beta \) will be influenced primarily by \( \beta_0 \), while \( C \) will be relatively sensitive to \( k_1, k_2, k_{-1}, \) and \( k_{-2} \). That ethanol and membrane potential do not influence the system in exactly the same way (see Fig. 5B) does not negate the possibility that ethanol and hyperpolarization may be equivalent in terms of their action on channel closing (Gage et al., 1975; Stettmeier and Finger, 1982).

**Late Tail of the MEPC**

The late tail of the normal MEPC (AChE intact) has not previously been reported for MEPCs, but has occasionally been seen in EPCs (Magleby and Stevens, 1972b). Its persistence in the presence of ethanol and/or receptor blockade is indicated by the plots in Fig. 7. With regard to mechanism, it is not predicted by any simple scheme of ACh-receptor interaction (e.g., Wathey et al., 1978) and must represent either (a) slowly closing channels (perhaps of the extrajunctional type [Dreyer et al., 1976]), (b) a population of binding sites that constitute a reservoir for ACh and slowly release it into the cleft (see Katz and Miledi, 1975; Feltz and Trautmann, 1980), or (c) a population of receptors that hold ACh for longer than do those receptors responsible for the main part of the MEPC and occasionally open channels that are essentially normal. We find (using computer simulation) that the reservoir hypothesis cannot work unless these hypothetical binding sites capture most of the ACh in a quantum. This arises because release of ACh from binding sites at only \(~0.15/\text{ms}\) can generate only a very low concentration of ACh in the cleft when AChE is intact. The very prominent tail seen with EPPs or EPCs after inhibiting or poisoning AChE has been demonstrated to consist of channels of essentially normal amplitude and duration (Katz and Miledi, 1975).

**Correlation Between MEPC Height and Decay Rate**

In these experiments, the observation that is most difficult to explain is one that has previously been noted by Katz and Miledi (1973) and by Hartzell et al. (1975), namely, the very marked correlation that exists between MEPC decay rate and MEPC height after AChE is poisoned. However, this result cannot be ascribed to variations in the diffusion path for ACh, since even when AChE is intact MEPCs are on average nearly as large as when AChE is poisoned; i.e., MEPC height is insensitive to rate of loss of ACh. Nor can it be attributed to variation in local subsynaptic receptor density, since quite large changes in receptor density, although they cause large changes in \( \tau_1 \), have little effect on MEPC height (Pennefather and Quastel, 1981). Thus, these factors will give rise
to a scatter of values of $\tau_1$ at a nearly constant height. The primary cause of variation of MEPC height is presumably spontaneous variation in ACh per quantum, and this is associated with large variation in $\tau_1$. A rough calculation leads to an expected maximum slope of log $\tau_1$ vs. log height, as follows.

Let us suppose that all ACh is either free in the cleft or bound to receptors, and that rate constants for association and dissociation are fast relative to diffusion rate ($\lambda$). Treating the system as a single compartment, there is a quasi-equilibrium with

$$[A_nR] = K_a[R][A]^n; \quad [A] = ([A_nR]K_a^{-1}[R]^{-1})^n,$$

where $n$ is the number of ACh molecules that attach to $R$. Total ACh is

$$[A] = [A] + n[A_nR] = [A] + nK_a[R][A]^n$$

and

$$-\lambda[A] = d[A]/dt = (d[A]/dt)(1 + n^2K_a[R][A]^n).$$

Also, noting that

$$d\ln[A]/dt = n^{-1}d\ln[A_nR]/dt = -n^{-1} \tau^{-1},$$

we obtain:

$$n\lambda \tau = 1 + n^2(K_a[R])^n[A_nR]^n.$$

For MEPCs at the same fraction of peak height (e.g., at $e^{-1}$), $[A_nR]$ is proportional to peak height. Hence, the maximum possible slope of log $\tau$ vs. log height for a single compartment system is $(n - 1)/n$, where $n$ is the number of ACh molecules that cooperate to combine with the receptor to open a channel. The average observed slope of log $\tau$ vs. log height was in fact $0.685 \pm 0.02$ (mean ± SEM, $n = 49$), and was greater ($0.77 \pm 0.01$) if we exclude from the correlation those MEPCs <60% average height and apparently approaching a limiting time constant, which implies an $n$ of at least 3 and probably 4. The result is much the same if receptors may bind more ACh molecules than necessary to open a channel (cf. Dunn and Raftery, 1982); the maximum possible slope is then $(m - 1)/n$, where $m$ is the number bound and $n$ is the number causing channel opening.

To the above calculation, it may be added that the multicompartment model of Wathey et al. (1979) can give a steep relation between log $\tau$ and log height only in terms of a prolonged tail for large MEPCs, rather than early in the MEPC, and fails to predict properly the shape of MEPCs (Fig. 2). In our own version of the model of Wathey et al. (1979), we find that the above rough calculation overstates the expected slope of log $\tau_1$ vs. log height. The observed slope of log $\tau_1$ vs. log height may be generated by either (or better, both) of the following: (a) there normally exists a substantial fraction of receptors in a state (presumably desensitized) where they bind ACh well and release it slowly (cf. Katz and Thesleff, 1957; Boyd and Cohen, 1980): this mechanism acts preferentially to reduce the $\tau_1$ of small MEPCs, which fail to saturate such receptors in the vicinity of the release site; or (b) temporary conversion of receptors by a first activation to a state which is more readily activated by ACh to open channels; this tends to
prolong the larger MEPCs. The first of these hypotheses implies that after AChE poisoning, ACh action may be terminated mainly by a kind of receptor uptake rather than by diffusion out from the synaptic cleft. If these receptors occasionally open channels this mechanism also accounts for the late slow tails of MEPCs.

Conclusions

To conclude, we summarize the events in the normal MEPC as follows. Following release into the synaptic cleft, most of the ACh in a quantal package rapidly meets receptors and is captured as biliganded receptors isomerize to the open channel form; the ACh remains attached to the receptors for a time close (if not identical) to the time the channels remain open. The capture process is accelerated rather than slowed by muscle fiber hyperpolarization. The concentration of ACh in the cleft quickly falls to a level at which ACh hydrolysis by AChE proceeds faster than capture by receptors, but even when the MEPC has decayed to $e^{-1}$ of its peak, ~20% of the ACh re-released into the cleft again participates in opening channels. This reverberation progressively diminishes as the MEPC decays and the concentration of ACh in the cleft goes down. When AChE is poisoned, reverberation becomes much more prominent and varies greatly with the amount of ACh that was in the quantum, as well as with receptor density and diffusion path for ACh; this phenomenon cannot be explained solely in terms of cooperativity in binding of ACh to receptors, as was suggested by Hartzell et al. (1975). In addition, MEPCs have a low, slow tail that suggests the presence of a population of receptors (perhaps desensitized) which occasionally open channels and bind ACh for much longer than those receptors responsible for the main part of the MEPC.

We thank Mr. J. Gudaitis for his assistance in development of the computer program for online recognition and recording of MEPCs.

This work was supported by grants from the Muscular Dystrophy Association of Canada and the Medical Research Council.

Received for publication 12 April 1982 and in revised form 12 September 1983.

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