

THE STATISTICAL
 NATURE OF THE ACETYLCHOLINE POTENTIAL AND ITS
 MOLECULAR COMPONENTS

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SUMMARY

1. When a steady dose of acetylcholine (ACh) is applied to an end-plate, the resulting depolarization is accompanied by a significant increase in voltage noise.

2. The characteristic properties of this ACh noise (amplitude and time course) are examined under various experimental conditions. The voltage noise is analysed on the assumption that it arises from statistical fluctuations in reaction rate, and in the frequency of the elementary current pulses ('shot effects') produced by the action of ACh molecules.

3. The elementary ACh current pulse (amplitude approximately 10^{-11} A), arises from a conductance change of the order of $10^{-10} \Omega^{-1}$ which lasts for approximately 1 ms (at 20° C), and produces a minute depolarization, of the order of 0.3 μ V. It is associated with a net charge transfer of nearly 10^{-14} C, equivalent to approximately 5×10^4 univalent ions.

4. At low temperature, and during chronic denervation, the duration of the elementary current pulse increases, and the elementary voltage change becomes correspondingly larger.

5. Curare has little or no effect on the characteristics of the elementary event.

6. A comparative study of ACh and carbachol actions shows that carbachol produces considerably *briefier*, and therefore less effective, current pulses than ACh.

INTRODUCTION

When a steady concentration of acetylcholine (ACh) is maintained around an end-plate, one would expect the resulting potential change to be accompanied by small 'noise' fluctuations, arising from statistical random variations in the molecular collision rate. It seemed worth searching for this phenomenon, in the hope that it might not only be detectable under

suitable conditions, but amenable to quantitative exploration which would throw some light on the underlying molecular 'shot effect'.

METHODS

Most of the experiments were made on junctional regions of frog sartorius muscle fibres, by recording potential changes which resulted from application of acetylcholine or other depolarizing drugs. The procedure was essentially the same as described in earlier papers (see e.g. Katz & Miledi, 1965*a, b*). Micro-electrodes were used for either intra- or focal extracellular recording. Acetylcholine was applied usually for relatively short periods (about 30–60 sec) by diffusion or iontophoresis from a micropipette, so that initial and final controls and test observations could be made in rapid sequence on the same end-plate without moving the recording electrodes. In other experiments, however, membrane potentials were measured by successive insertions and withdrawals of the recording electrode into several fibres, before and after the Ringer solution was changed to one containing known concentrations of ACh.

The membrane potential was recorded on a 565 Tektronix oscilloscope via two parallel channels of amplification: (a) a relatively low gain amplifier to measure the average level of depolarization and, (b) a high-gain amplifier, in most cases with condenser coupling (0.1 s time constant) to monitor voltage fluctuations.

In the earliest experiments, the root-mean-square (r.m.s.) noise voltage (i.e. the s.d. of the membrane potential) was determined by manual measurements of tracings of the enlarged oscilloscope records. In all the later experiments, the oscilloscope output was led into a magnetic tape recorder, covering a frequency band from zero to about 1200 Hz or in some experiments to 2500 Hz, and the average membrane potential as well as the r.m.s. amplitude and the power density spectrum (i.e. the spectral distribution of the variance) were determined by feeding the outputs of the tape recorder into a LINC-8 computer where the voltage records were digitized at suitable sampling intervals (each complete sample containing 256 intervals) and then analysed. The whole system was calibrated by sine-wave oscillations of known frequency and amplitude. A further test which was frequently employed was to check against one another the r.m.s. values of the recorded membrane voltage obtained by the two routine programmes of which (a) computed the variance directly, and (b) computed the power spectrum from which the variance could then be re-determined by integration. For automatic computation, it was of course necessary to select segments of the recording which were not 'contaminated' by miniature end-plate potentials (min.e.p.p.) or obvious artifacts due to occasional electrical or mechanical interference.

A possible source of artifact was the presence of unrecognized min.e.p.p.s during periods of drug-induced noise. This had to be considered in conjunction with the fact that ACh may, and occasionally does, raise the frequency of min.e.p.p.s (ACh is known to cause some depolarization in nerve endings: this does not usually suffice to evoke additional min.e.p.p.s (Fatt & Katz, 1952; Hubbard, Schmidt & Yokota, 1965), but whether this happens or not depends presumably on the initial level of the resting potential in the terminals). In some of our experiments, the ACh traces contained samples in which the noise fluctuations were so large that any small min.e.p.p.s would have been swamped, but on the same grounds they could not have seriously affected the analysis. The most direct check against possible interference by min.e.p.p.s was obtained in experiments on short-term denervated muscles in which min e.p.p.s were absent (see p. 687, Table 3, also Fig. 10).

The principal assumption which underlies all the measurements is that no interaction occurs between 'control' or 'base line noise' (i.e. the noise obtained in the absence of ACh) and 'ACh noise', in other words that the variance of the base line (and its power density spectrum) can be subtracted from the total variance (and power spectrum) of the ACh potential. Like other assumptions, which are discussed below, this is not entirely correct: a substantial component of the 'base line noise' arises probably from minute mechanical movements and, therefore, variations in the 'sealing' resistance around the intracellular micro-electrode. This component may well be somewhat reduced during a superimposed ACh-potential, but such an effect would introduce only a small error which need not seriously concern us at the elementary stage of analysis aimed at in the present paper.

RESULTS

Qualitative observations

The basic phenomenon is illustrated in Fig. 1. During continuous application of ACh to an end-plate one observes, with intracellular recording, not only a steady depolarization – 8.5 mV in this particular example – but also an excess of voltage noise. There are large quantitative variations depending on several factors discussed below (such as temperature, fibre size, type of depolarizing drug, etc.), but the basic phenomenon was seen in every experiment provided enough amplification was used. In fact, once one decides to look for it, 'ACh noise' is easy enough to detect. It is, of course, also easy to overlook, for by comparison with a discrete phenomenon such as miniature e.p.p.s (Fig. 1), the appearance of noise is unimpressive. The following observations, however, assured us that we were looking at something of physiological importance.

(1) The 'extra noise' was associated with ACh-induced potential changes, but did not occur when similar potential changes were produced by direct electric current or by the shunting effect following the insertion of the second electrode. This control experiment was made on several occasions, using the ACh pipette to enter the muscle fibre and electrotonically displace the membrane potential by the same or greater amount. No increase in noise accompanied this procedure.

(2) As will be shown later (see also Katz & Miledi, 1971), there are characteristic differences between the noise accompanying ACh and carbachol potentials of the same magnitude, which were seen very clearly when the two drugs were applied alternatively from different barrels of a multiple micropipette.

(3) ACh noise is not affected when the distance between drug pipette and end-plate surface is altered, in spite of large changes in diffusion delays and, therefore, in the times of rise and fall of the depolarization (cf. pp. 687–688 below).

In the example of Fig. 1, the ACh pipette had been placed at some distance, in the fluid above the end-plate, so that several seconds elapsed before the membrane potential began to change in response to suddenly starting or stopping the efflux of ACh. Under such conditions transient resistance changes of the pipette could have contributed nothing to the recorded noise. It was, however not always practicable to keep the drug pipette so far away, and in some experiments artifacts due to slow fluctuations in ACh efflux may have introduced an error. But whenever a quantitative test was made, by comparing the ACh noise during a given depolarization, produced from a 'near' or 'far' position of the same drug pipette (or produced by different pipettes), no change indicative of a pipette artifact was observed.

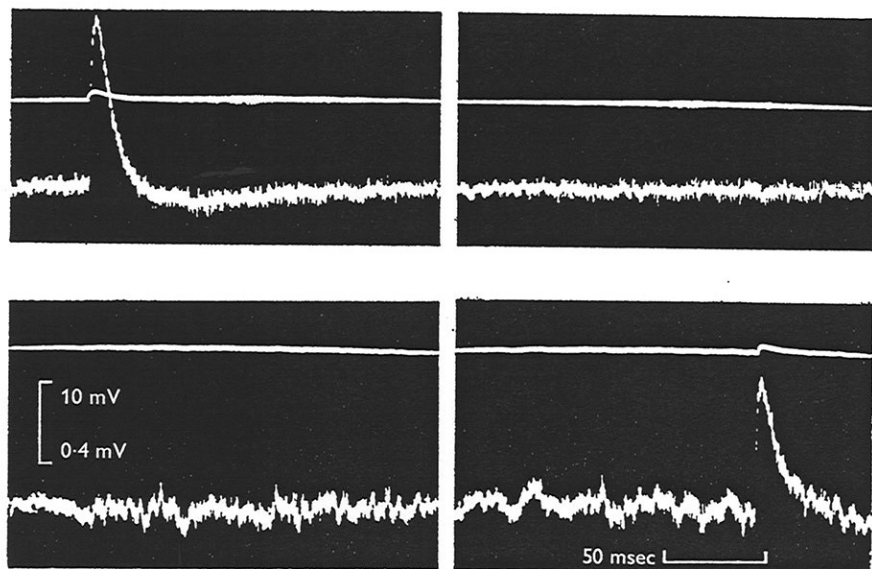


Fig. 1. Intracellular recording from an end-plate in frog sartorius. 21° C. In each block, the upper trace was recorded on a low-gain d.c. channel (scale 10 mV); the lower was simultaneously recorded on a high-gain condenser coupled channel (scale 0.4 mV). The top row shows controls (no ACh); the bottom row shows membrane noise during ACh application, by diffusion from a micropipette. In the bottom row of records, the increased distance between the low and high gain traces is due to upward displacement of the d.c. trace because of ACh-induced depolarization. Two spontaneous min.e.p.p.s are also seen.

(4) ACh noise is observed whether the drug is applied topically through a micro-pipette, or all over by replacing the bath solution with one containing a known concentration of ACh (see p. 682 below).

These experiments showed that the 'extra noise' arises from the action of ACh on end-plate receptors and not from an artifact due to possible vibrations, or resistance changes in the tip of the drug pipette.

Quantitative observations

To evaluate the ACh noise, its *amplitude* and *temporal characteristics* were determined. As a conventional index of amplitude, the root mean square value of the voltage fluctuation (i.e. the *standard deviation* of the depolarization) was measured. As an index of time course, the 'power density spectrum' (i.e. the spectral frequency distribution of the voltage *variance*) was determined. This was done in preference to the alternative procedure of using the autocorrelation function, because a more convenient computer program for the former was at hand.

The following symbols will be used.

Depolarization at time t : V_t .

Mean depolarization: V .

Variance of V_t : $\overline{(V_t - V)^2} = \overline{E^2}$.

Standard deviation of V_t : $[(\overline{(V_t - V)^2})^{\frac{1}{2}}] = \overline{E}_{rms}$.

Noise amplitude (\overline{E}_{rms})

During the initial series of experiments, the voltage fluctuations were measured by hand. It was apparent from records such as shown in Fig. 1 that the extra noise introduced during the action of ACh is confined to a low frequency spectrum, and it was therefore sufficient to trace the envelopes of the enlarged oscilloscope records and measure them at sampling intervals of 0.5–1 ms. The length of the individual sample was usually 0.2 s (with variations in different experiments between 0.1 and 0.26 s). This, in conjunction with the condenser coupling, resulted in loss of noise at very low frequencies and a possible underestimate of \overline{E}_{rms} by as much as 20%, but it would have been impractical to use very much longer samples (with d.c. coupling) because of inherent slow drifts and mechanical instabilities of the recording system. In the experiment illustrated in Fig. 1, several oscilloscope traces, each of 0.2 s length, were measured at 0.75 ms intervals. The 'base line', before and after ACh application, gave a noise value of 13.1 μV r.m.s. During ACh action, an average depolarization of 8.5 mV (from 93 to 84.5 mV) was registered. This was accompanied by a noise level of 32 μV (total r.m.s.), the net r.m.s. value of extra ACh noise amounting to 29.2 μV .

In all the later experiments, noise amplitudes were obtained by automatic computation which was not only much quicker but also a more accurate procedure, because many more samples could be taken and averaged. Fig. 2 illustrates the results of one such computation. The two histograms represent the distribution of variances ($\overline{E^2}$ in μV^2) from (i) a

control block of 108 'base line' samples (each approximately 0.25 s long with 1 ms sampling intervals), and (ii) a test block of 100 'ACh' samples (10.4 mV average depolarization). Mean noise levels were 19.4 and 47.7 μV respectively, giving 43.5 μV for the ACh 'net noise' (the latter would be reduced to 43.2 μV , if the single 'off-side' value at the extreme end of the histogram is omitted). It will be noted that the ACh samples are much more widely dispersed than the controls; the probable significance of this consistent observation will be discussed later (p. 684).

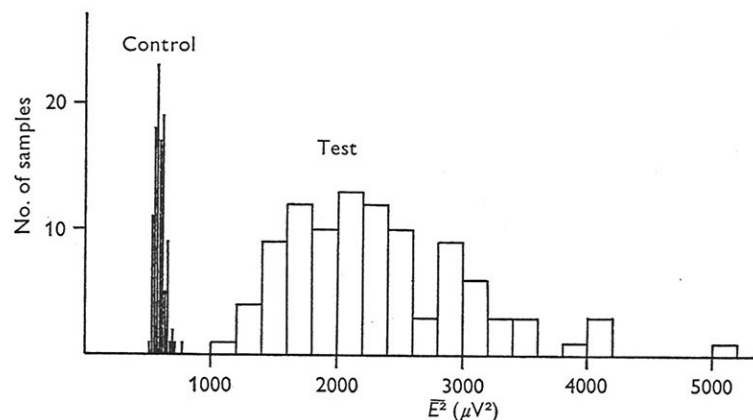


Fig. 2. Distribution of noise variances ($\overline{E^2}$ in μV^2) in control and ACh samples from a single run. The average depolarization during 'test' was approx. 10 mV. Abscissa: noise variance, in μV^2 . Ordinate: number of samples (of 0.25 s length). Temp. 2° C. Control samples were collected, as usual, before as well as after the ACh test.

Relation between ACh-potential and ACh-noise

If a linear dose/response relation obtained throughout, that is between ACh concentration and the rate of drug/receptor reaction, and between reaction rate and resulting depolarization, then, on simple Poisson statistics, the noise variance should be directly proportional to the mean ACh potential, and the r.m.s. value should increase as the square root of the mean depolarization. A significant departure from linearity is known to occur because depolarization is not proportional to the ionic conductance change induced by ACh, but is limited by a hyperbolic approach to the 'reversal potential' at about -15 mV (del Castillo & Katz, 1954; see Figs. 3 and 4A and eqn. (7)). This will be considered in more detail in the theoretical section. There is also some evidence for a relatively slight deviation from linearity in the opposite direction, in the initial portion of the dose-response curve (see Katz & Thesleff, 1957b). This will be ignored in the following treatment. The analysis is based on the assumption that,

in our experimental range, ACh concentration is linearly related to ionic conductance change, and that the only significant departure from linear summation arises in the transfer from conductance to potential change V . Examination of the dose/response curve will be deferred to later study; the present purpose is to investigate the depolarization/noise ($V/\overline{E^2}$) relation.

Theoretical

Suppose the ACh potential V is made up of linearly additive elementary 'shot effects' $f(t)$ which occur at random intervals with mean frequency n . Then according to Campbell's theorem (Rice, 1944)

$$V = n \int f(t) dt \quad (1)$$

and the associated noise variance

$$\overline{E^2} = n \int f^2(t) dt. \quad (2)$$

With intracellular recording, $f(t)$ may reasonably be assumed to be similar in shape to a miniature e.p.p., but much smaller in amplitude and probably of shorter rise time. The simplest assumption is to regard it as a minute 'blip' of instantaneous rise to amplitude a and exponential decay with a time constant τ characteristic of the min.e.p.p., i.e.

$$f(t) = ae^{-t/\tau}. \quad (3)$$

From eqns. (1), (2) and (3) one obtains

$$V = nar \quad (4)$$

and

$$\overline{E^2} = \frac{na^2\tau}{2} \quad (5)$$

and, by substitution,

$$\overline{E^2} = \frac{Va}{2}. \quad (6)$$

The meaning of these equations may be appreciated best by applying the numerical examples already given. Although the values need correction, especially for non-linear summation, the order of magnitude will not be affected by using the uncorrected raw data. For a 10 mV depolarization (V), \overline{E} was found to be of the order of 30–50 μV . From eqn. (6), the amplitude a of the elementary shot affect ($2\overline{E^2}/V$) is then calculated to be 0.18–0.5 μV . Thus, the molecular shot effects are about three orders of magnitude below the size of the min.e.p.p.; they are too small to be recorded individually, but during a high frequency random series fluctuations occur

which involve several hundreds of them at a time, and this can be observed with our methods.

As an estimate of τ , 10 ms may be taken as appropriate for frog muscle. The mean frequency n of elementary events, producing an average depolarization of 10 mV at a single end-plate would then be V/τ , i.e. $2-5.5 \times 10^6 \text{ s}^{-1}$.

The consequences of some of the theoretical simplifications must now be considered.

(a) *The 'shape factor'*. The assumption that the elementary shot effect follows a simple exponential time course (eqn. (3)) is, of course, oversimplified. The result (eqn. (6)) would, however, not be greatly altered if one substituted various alternative forms. For example, if one chose a square pulse of amplitude a and duration τ , eqn. (6) would change to $\overline{E}^2 = Va$, i.e. by a factor of 2. The alteration would be even less if more realistic assumptions are made, e.g. for a double-exponential time course $f(t) = a_1(e^{-t/\tau_2} - e^{-t/\tau_1})$. In the special case $f(t) = ae(t/\tau_1)e^{-t/\tau_1}$, i.e. a double-exponential blip of amplitude a and rise time τ_1 , eqn. (6) would become $\overline{E}^2 = Vae/4$.

The basic assumption, viz. that the shot effect is a brief monophasic pulse can hardly be questioned in view of the strictly monophasic character of the min.e.p.p. itself. In most of our experiments, however, a small diphasic artifact is imposed by the condenser coupling of the high-gain recording channel, the purpose being to eliminate the mean depolarization V from this channel, but with the unavoidable effect of attenuating very low frequency components (below 2 Hz) of the noise.

(b) *Effect of variable shot amplitude*. If the value of a itself is a random variable, this will contribute to the recorded noise so that eqn. (5) becomes

$$\overline{E}^2 = n\overline{a}^2 \frac{\tau}{2} = n(\overline{a})^2 \frac{\tau}{2} \left[1 + \frac{\text{var } a}{(\overline{a})^2} \right] = \frac{V\overline{a}}{2} [1 + (c.v._a)^2], \quad (7)$$

where \overline{a} is the mean amplitude, \overline{a}^2 the mean square, $\text{var } a$ the variance of a , and $c.v._a$ its coefficient of variation.

By using eqn. (6), i.e. assuming an exponential shape and ignoring $(c.v._a)^2$, one tends to over-estimate the shot amplitude a , while the low-frequency attenuation inherent in the recording and sampling procedure constitutes a balancing factor. No attempt is made in this paper to allow or correct for these effects.

(c) *Effect of non-linear summation of depolarization*. This is a well known effect which has been extensively discussed (Martin, 1955; Hubbard, Llinás & Quastel, 1969, pp. 66-68; Auerbach & Betz, 1971) and for which an approximate correction can be made. Indeed, without such a correction

calculations by eqn. (6) would lead to progressive underestimates of a as the depolarization V is increased.

Consider the circuit diagram in Fig. 3; the muscle fibre, with -90 mV resting potential and input resistance R , is shunted by an ACh-induced conductance g in series with a reversal potential of -15 mV . The observed depolarization V is related to g by the following equation

$$\frac{V}{V_0} = \frac{gR}{1 + gR}, \quad (8)$$

where V_0 is the maximum theoretical attainable depolarization $\epsilon - E_r = 75 \text{ mV}$. In general, we take V_0 as being equal to the measured resting potential less 15 mV.

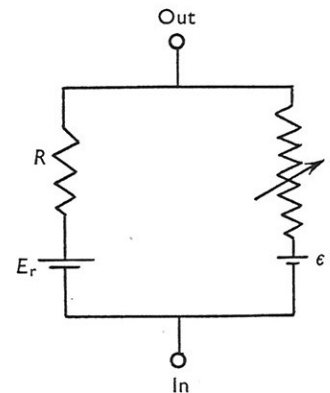


Fig. 3. Circuit diagram representing input resistance (R) and resting potential (E_r) at end-plate region of muscle fibre shunted by variable ACh-induced conductance path (g), with 'equilibrium potential' ϵ .

We assume (see p. 670) that, over the range of ACh concentrations used in these experiments, g is directly proportional to $[\text{ACh}]$, in fact, that g is the linear sum of a large number of parallel elementary conductances each of which, by itself, produces a depolarization a . Their individual contributions to the membrane potential change, however, do not summate linearly, but in the hyperbolic fashion described by eqn. (8) (see Fig. 4A). We want to correct for this non-linearity and convert the observed V to a value directly proportional to g , i.e. to the number of ionic channels. This is done by multiplying V by the factor $(1 + gR)$ which is equal to $V_0/(V_0 - V)$ (see Martin, 1955).

Now consider a given small fluctuation in the number of ionic channels and, therefore, in the conductance (δg). The corresponding fluctuation in potential δV will suffer substantial diminution as g increases and as V approaches V_0 , for

$$\frac{\delta V}{\delta g} = \frac{V_0 R}{(1 + gR)^2}. \quad (9)$$

To correct for this effect, and convert any observed δV (e.g. the r.m.s. noise voltage) into a function proportional to δg , we must multiply the former by $(1+gR)^2$, or $[V_0/(V_0-V)]^2$, i.e. the square of the previous correction factor.

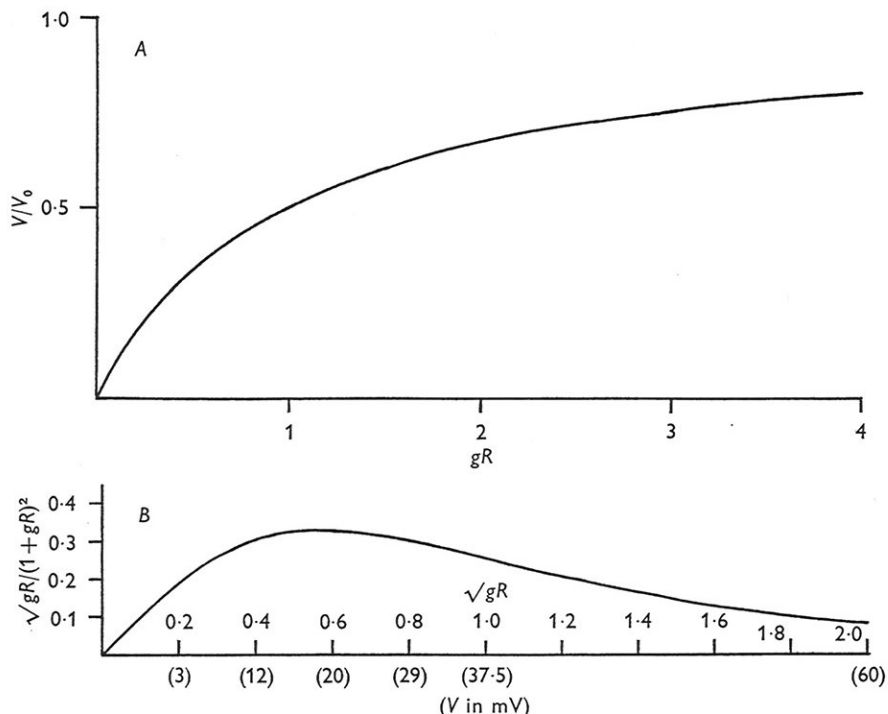


Fig. 4. *A*, theoretical relation between ACh-induced conductance (g) and end-plate depolarization (V). Cf. text, eqn. (8). *B*, theoretical relation between ACh-induced depolarization and ACh-noise (cf. eqn. (10)). Abscissae: values of \sqrt{gR} ; underneath (in brackets) are corresponding values of V (mV), for $V_0 = 75$ mV. Ordinates: values of $\sqrt{gR}/(1+gR)^2$. To obtain \bar{E}_{rms} (ACh noise value), multiply ordinate by the constant coefficient $\sqrt{(V_0 a/2)}$.

Eqn. (6) should, therefore, be rewritten as

$$\bar{E}^2 = \frac{V_0 g R a}{2(1+gR)^4} = \frac{V a}{2(1+gR)^3}. \quad (10)$$

Thus, to obtain consistency when calculating a at different levels of V , the value of $2\bar{E}^2/V$ should be multiplied by the third power of the conventional correction factor, i.e. by $[V_0/(V_0-V)]^3$. This procedure has been adopted throughout. It could be argued that, for rapid transients, the simple resistive circuit of Fig. 3 is not strictly applicable and tends to lead

to overcorrection (cf. Hubbard *et al.* 1969), but it was felt that a correction for non-linearity was important and further elaboration was not warranted at present.

If one plots the relation described by eqn. (10) (cf. Fig. 4*B*), one finds that with increasing V , \bar{E}_{rms} passes through a flat maximum when $1/g = 3R$ (and $V = V_0/4$) and then declines gradually towards zero. It follows that, with V_0 about 75 mV, the noise reaches a maximum when V is 15 to 20 mV, and there is little to be gained by depolarizing more than about 10 mV. In fact, during maintained application of large or even moderate doses of ACh, other errors may be introduced as discussed in the following paragraph.

(*d*) *Effects of desensitization and 'retention' of depolarization.* It might be thought that desensitization, i.e. gradual failure of receptor response during a maintained dose of ACh, would not vitiate our calculations, because V and g might be reduced without change in their relation. Unfortunately this is not always the case: during prolonged depolarization of frog muscle, whether caused by ACh or by other means, chloride ions gradually enter the fibres and, because of the peculiar differences between potassium and chloride permeabilities (Hodgkin & Horowitz, 1959, p. 140), the excess of internal chloride is removed only slowly which in turn leads to the retention of a variable part of the depolarization after its initial cause, e.g. ACh/receptor action, has disappeared. It has been shown by Mantley (1966) and by Nastuk & Parsons (1970) that the decline of depolarization is not a reliable index of receptor desensitization, and that the conductance g falls more rapidly than the potential change V . Related observations were made in the course of the present experiments, viz. that during prolonged ACh application, the extra noise tended to subside, while the depolarization showed relatively little or no decline. Other aspects of this phenomenon are illustrated in Table 1 and Fig. 5, namely that during gradual application and withdrawal of ACh, by slow iontophoresis or bath perfusion, the value of V lagged behind the noise level \bar{E} . The experiments most seriously affected by such 'hysteresis' and retention of V are those in which the drug was applied by a bath change, and the micro-electrode had to be re-inserted in several fibres. In such experiments, not only was the period of application much longer than desirable, but larger doses had to be given because of the low accuracy inherent in measuring V , as the difference of two membrane potentials, obtained by repeated insertions. It is not surprising, therefore, that in the 'bath application' experiments, the observed noise level associated with a given depolarization was generally lower than in the iontophoretic experiments. In summary, 'noise desensitization' together with partial retention of V tends to produce an underestimate of a as calculated by the above equations. With relatively brief

TABLE 1. A. Effect of desensitization during slow iontophoretic application and withdrawal of ACh. 11° C. Prostigmine (10^{-6} g/ml.) present. Resting potential approx. 80 mV

Time after onset of ACh(s)...	17.5		31.5	45.5	59.5	89.5
Period of observation(s) ...	17.5		14	14	14	30
Depolarization V (mV)	3.84	5.84	3.83	2.48	1.9	1.38
$\bar{E}_{r.m.s.}$ (observed)(μV)	32.5	36.5	26.5	16.9	11.2	7.1
a (uncorr.) (μV)	0.55	0.485	0.358	0.23	0.132	0.076
a (corr.) (μV)	0.66	0.66	0.43	0.25	0.143	0.081

B. Bath perfusion experiment 20° C. Resting potential 87 mV. Prostigmine (10^{-6} g/ml.) throughout. ACh (10^{-7} g/ml.) applied during two successive runs (a and b)

(a) First run	Depolarization V (mV)	a (μV)
During ACh	Initial 3.5	0.265
	Average 7.35	0.23
After ACh	'Retention' 7.65	(0.064)*
(b) Second run		
During ACh	Initial 1.23	0.285
	Average 5.3	0.175†
After ACh	'Retention' 3.2	(0.1)*

* During this period, most of the ACh must have diffused away, but a substantial depolarization remained.

† There was more rapid desensitization, during ACh, in the second run.

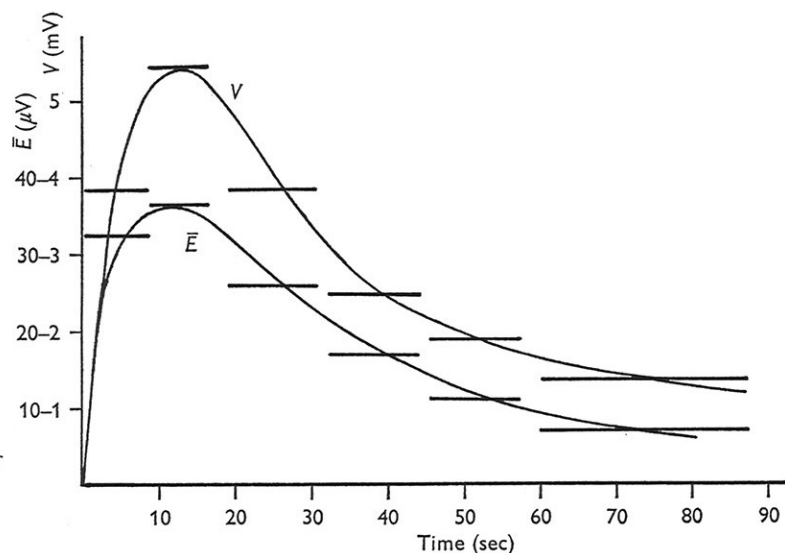


Fig. 5. ACh-induced depolarization (V in mV) and noise ($\bar{E}_{r.m.s.}$ in μV) during gradual increase and withdrawal of the drug. Temp. 11° C. Abscissae: time from start of ACh application. See also Table 1. Horizontal bars show the average levels of V and $\bar{E}_{r.m.s.}$ during individual sampling periods.

iontophoretic applications, however, and making appropriate allowance for any small retention of V at the end of the run, the error is not likely to be very disturbing. Whether 'noise desensitization' is associated with any change in the characteristics of the elementary event cannot be ascertained from the present observations.

Results

Fig. 6 shows the relation between observed ACh noise $\bar{E}_{r.m.s.}$ and depolarization V in an experiment in which the drug was applied iontophoretically in gradually increasing doses. The filled circles show the same results

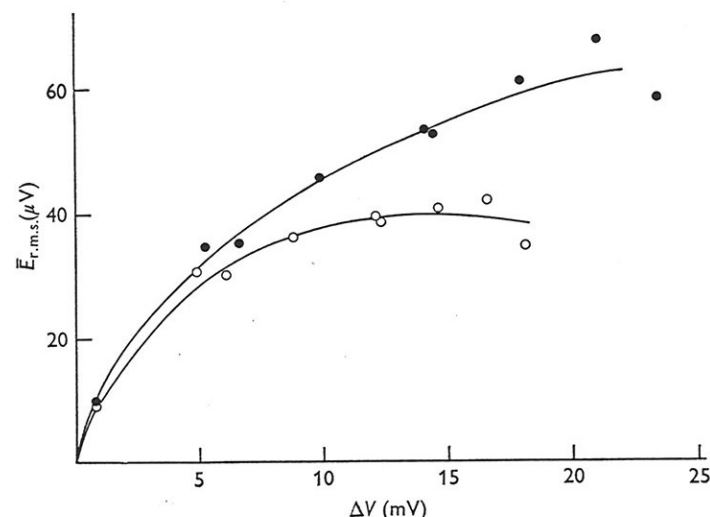


Fig. 6. Relation between ACh potential (V in mV) and ACh noise ($\bar{E}_{r.m.s.}$ in μV). Temp. 4° C. Open circles: uncorrected values. Filled circles: values corrected for non-linear summation (using eqns. (8) and (9)).

after applying the appropriate correction factors for non-linear summation (cf. eqns. (8) and (9)). On replotting the corrected values of \bar{E} against \sqrt{V} an approximately linear relation is obtained (Fig. 7) whose slope ($\sqrt{[0.195 \mu V]}$), according to eqn. (10), is equal to $\sqrt{(a/2)}$. While no high degree of accuracy is claimed, the predicted proportionality between the corrected values of \bar{E} and \sqrt{V} was adequately obeyed in several other experiments of this kind.

The corrected value of a was determined in a large number of experiments. For ACh the grand average of 137 values was $0.42 \mu V$, with variations extending from 0.064 to $1.54 \mu V$. This, however, covers a wide range of temperatures (1.3 – $25^\circ C$), fibre sizes (as judged from the amplitudes of min.e.p.p.s; see Katz & Thesleff, 1957a), and also some chronically denervated preparations. These are factors which influence the value of a ,

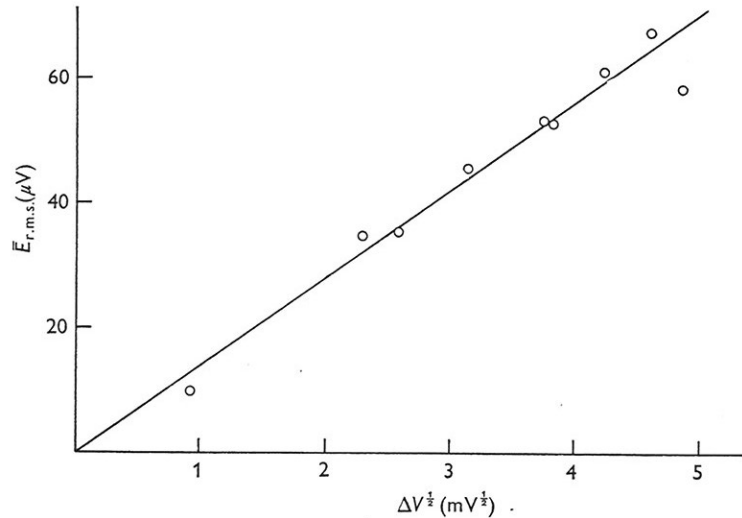


Fig. 7. Replotting the corrected values of Fig. 6. Ordinates: ACh noise values ($\bar{E}_{r.m.s.}$ in μV). Abscissae: square root of ACh potential ($mV^{\frac{1}{2}}$).

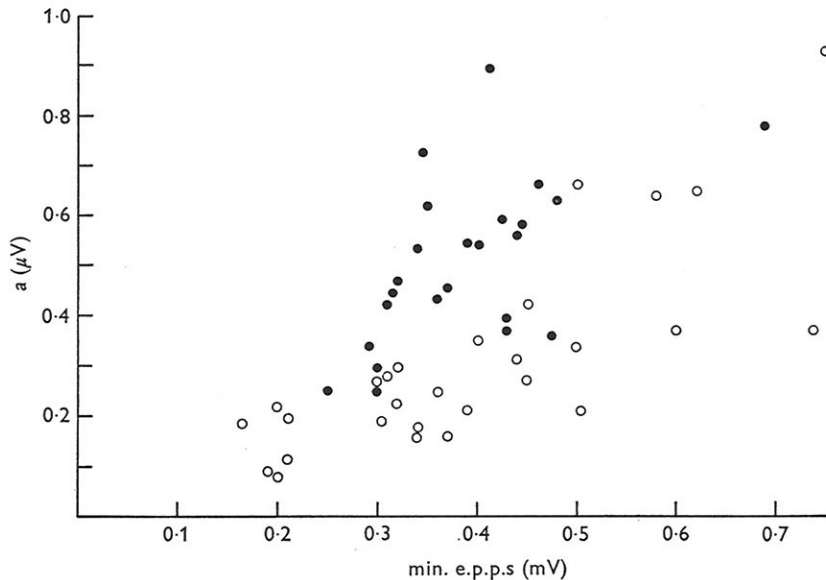


Fig. 8. Relation between mean amplitudes of min.e.p.p.s (abscissae) and values of a (ordinates). Results have been separated into two groups, high temperature (open circles) and low temperature experiments (filled circles). See also Table 2. In both groups there is a highly significant correlation between the two variates (correlation coefficients are $+0.595$ in twenty-four low temperature experiments, and $+0.801$ in twenty-nine high temperature experiments).

and by separating them the range of variability can be narrowed down considerably.

This is brought out in Figs. 8 and 9 which show (*a*) that the amplitude a of the elementary shot effect in different fibres is correlated with the size of the min.e.p.p., (*b*) that the calculated value of a increases as the temperature is reduced. The effect of temperature is shown more clearly in Table 2 in which results at high and low temperatures are compared from individual end-plates.

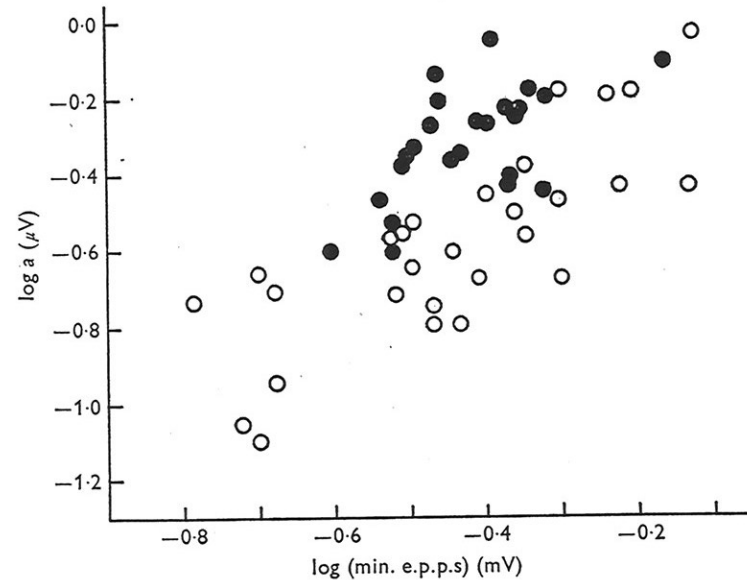


Fig. 9. Results from Fig. 8 plotted on double-logarithmic scales.

In five experiments, a increases on the average nearly threefold when the temperature is lowered by $20^{\circ} C$, while there is little change in the size of the min.e.p.p. The significance of this finding will be discussed in a later section, when the time course of the shot effect has been examined.

That there should be a correlation, among different fibres, between min.e.p.p. and shot amplitude is, of course, to be expected, for both potential changes must vary in a similar way with the input resistance R (see Fig. 3; $\delta V \doteq V_0 \delta g R$), and R is inversely related to fibre size (Katz & Thesleff, 1957*a*). After allowing for the effects of temperature and input resistance, there still remains a considerable scatter of the a values, over an approximately threefold range. A large part of this residue may be due to experimental error.

The largest values of a were obtained from muscles which had been denervated several weeks earlier (see Fig. 10). After 1 week, there was no

significant change from normal, the average values of ten experiments at 22° C being 0.25 μ V, and of twelve experiments at 3° C, 0.53 μ V which is well within the usual range (cf. Tables 2 and 4). After 2 weeks, ten experiments at 22° C gave a mean of 0.45 μ V, after 6 weeks denervation, the average of nine experiments at 25° C was 0.65 μ V. Two experiments were done at lower temperature, after 4 weeks denervation, giving 0.78 μ V

TABLE 2. Effect of temperature on ACh noise and min.e.p.p. amplitudes (five paired experiments)

Expt.	Value of a (μ V)		Ratio a_{22°/a_{2°	Min.e.p.p. (mV)		Ratio $\frac{\text{m.e.p.p.}_{22^\circ}}{\text{m.e.p.p.}_{2^\circ}}$
	22° C	2° C		22° C	2° C	
1	0.315	0.78	2.5	0.44	0.69	1.57
2	0.162	0.56	3.45	0.34	0.44	1.3
3	0.212	0.545	2.6	0.39	0.39	1.0
4	0.192	0.444	2.3	0.304	0.315	1.04
5	0.21	0.59	2.8	0.505	0.425	0.84
Mean	0.246	0.584	2.73	0.396	0.452	1.15
(Mean ratios corrected for changes in resting potential)			(2.86)			(1.21)

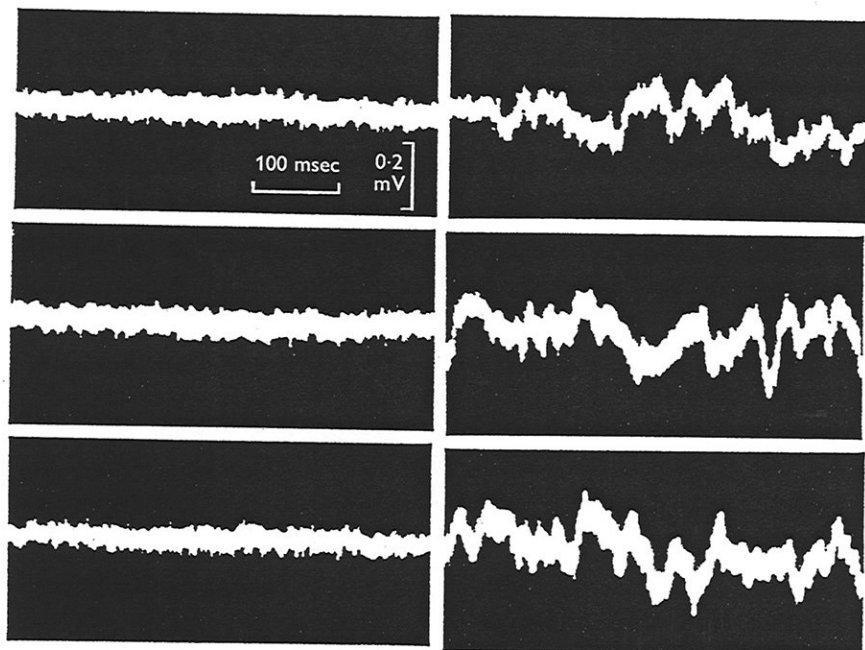


Fig. 10. An example of large, slow ACh noise, from a 4 weeks denervated end-plate. Magnetic tape recording. Temp. approx. 10° C. Left side: control records. Right side: during ACh application (approx. 10 mV depolarization).

(about 10° C) and 1.54 μ V (2°) respectively. Some of this increase can be attributed to the increased input resistance of denervated muscle fibres (Nicholls, 1956; Miledi, 1960), but an additional factor is probably an increased duration of the underlying elementary conductance change (see below).

Effect of curare. The most widely accepted interpretation of curare action is that it is a competitive blocking agent, which prevents the access of ACh to the receptor molecules. If this is correct, then the effect of curare, in the presence of a given dose of ACh, should be to reduce the frequency but not the amplitude of the molecular ACh 'shot effect'. In other words, V would be greatly reduced (or a much larger dose of ACh required for a given depolarization) while the value of a should remain unchanged.

The effect of curare (D-tubocurarine chloride in concentrations of 1.5×10^{-6} g/ml.) was examined in fifteen experiments (2.5–14° C). These doses of curare reduced the amplitudes of min.e.p.p.s to less than 1/10 so that in most cases they could no longer be recognized. To overcome the possible (though far-fetched) objection that under these conditions ACh-induced noise might not be distinguished from a rise in frequency of unrecognizable min.e.p.p.s, six of the fifteen experiments were made on 8-day denervated muscle fibres, which showed no spontaneous end-plate activity. The mean values for a in all fifteen experiments were 0.48 μ V before, and 0.45 μ V after curare treatment (appropriate correction having been made for any change in resting potential). The mean ratio (before/after) determined in individual fibres was 1.11 ± 0.07 . In the six denervated cases, the averaged ratio was 0.97, while in the nine other experiments it came to 1.21. A 20% change may well be within the error limits of the present method: it may in fact have been caused by the very difficulty which has just been mentioned, viz. how to detect the occasional small min.e.p.p.s among relatively large ACh noise. Even the smallest min.e.p.p.s can usually be recognized, and eliminated, during the control runs, but not always during the ACh test. This might conceivably introduce a small error and over-estimate of a which would not be encountered after curare treatment.

However that may be, it is clear that curare, in doses which greatly reduce the amplitude of the min.e.p.p., and which require a much higher dose of applied ACh to produce a given depolarization, has little or no effect on the accompanying ACh noise and, therefore, on the calculated value of a . This is indeed the result that would be expected if a is a measure of the individual molecular effect of the ACh/receptor reaction.

Effect of prostigmine. In twelve experiments (22° C; nine on normal, three on 7-day denervated fibres), the effect of prostigmine (neostigmine methylsulphate 10^{-6} g/ml.) was investigated. Mean values of a were 0.28 μ V before, and 0.33 μ V after prostigmine treatment, the mean increase being $18 \pm 6\%$.

The amplitude of the min.e.p.p. increased, on the average, by 55%. The effect on the noise level is so small that its interpretation is difficult and even its reality remains in doubt. A further complication was that in four fibres, in which a much larger dose of prostigmine (2×10^{-5} g/ml.) was applied, both min.e.p.p.s and a were reduced in amplitude. It may finally be mentioned that in five fibres after 2 weeks denervation, prostigmine (10^{-6} g/ml.) produced no change in a .

On our interpretation of the ACh noise, at least in its simplest form, one would not expect enzymic hydrolysis, or its inhibition by anti-esterases, to alter the magnitude of the shot effects produced by molecular drug/receptor action. It was, therefore, somewhat disappointing that prostigmine did not produce a clear-cut result, and further work will be needed to clarify this point.

Effect of varying the longitudinal distance between ACh and recording sites. The standard procedure in most experiments was to keep the intracellular recording electrode close to the region of ACh application, usually within less than 100 μm of fibre length. In two experiments, at 4.5° C, the recording pipette was inserted near one end of the junction, and the ACh tests were made, first with the drug pipette nearby (within 50–80 μm), then moving it 0.6–0.65 mm away, to the other end of the long junctional region, without displacing the recording electrode so that now both, the mean depolarization and the accompanying ACh noise, were recorded with some electrotonic decrement. The calculated value of a declined to about one half, from 0.62 to 0.3, and from 0.9 to 0.52 μV respectively. A relatively large decrement would be expected as the noise variance $\overline{E^2}$, and especially its higher frequency components (see Fig. 13 below), are more severely attenuated than the steady average depolarization V (cf. Falk & Fatt, 1964 and Katz & Miledi, 1968).

Effects observed during bath application. That ACh noise can be observed without resorting to iontophoresis, has already been mentioned, and Table 1B showed results of an experiment in which the bath was perfused with an ACh solution. Several experiments were made in which membrane potential and noise were recorded by repeated electrode insertion before and after the bath solution was changed to one containing ACh, $1\text{--}5 \times 10^{-7}$ g/ml. in the presence of prostigmine. Although ACh noise was clearly observed in every case these experiments suffered, for quantitative purposes, from the lower accuracy inherent in measuring differences in repeated insertions and, more seriously, from the desensitization and retention phenomena discussed earlier. The values of a calculated from several such 'bath experiments' came to 0.13 μV at 20° C (mean of two), and 0.29 μV at 6° C (mean of seven), which is well below the average of the main group of experiments in which short-period iontophoresis was used

(mean of forty-four experiments at 22° C was 0.32 μV , while forty experiments at 4° C gave 0.47 μV).

Temporal characteristics of ACh noise

It was obvious from inspection of the records that the ACh noise, seen with an intracellular electrode, is confined to a rather low frequency spectrum. This is, of course, bound to be the case, for the fibre membrane across which the voltage fluctuations are recorded, acts as a low pass filter with time constant of the order of 10 ms. If the elementary 'shot effect' followed an exponential time course as described by eqn. (3), then the 'power density spectrum' of the noise voltage, that is the spectral distribution of the variance $\overline{E^2}$, would follow the equation

$$\frac{\overline{w}(f)}{\overline{w}_0} = \frac{1}{1 + 4\pi^2 f^2 \tau^2}, \quad (11)$$

where f is frequency in Hz, $\overline{w}(f) = d\overline{E^2}/df$, \overline{w}_0 the maximum value (for $f = 0$), and τ the membrane time constant. Plotted on double-log scale, eqn. (11) gives the interrupted curve in Fig. 12. The 'half power' point is reached when $1/2\pi f = \tau$, and the final slope would be -2 (or -1 when plotted on a twice-expanded log-frequency scale as in the present illustration). The maximum value, \overline{w}_0 at $f = 0$, is given by

$$\overline{w}_0 = 2Var \quad (12)$$

(see Rice, 1944). Thus, an alternative way of determining τ would be to find the approximate asymptotic maximum of $\overline{w}(f)$ and divide it by four times the total noise variance (eqn. (6)).

However, while this is a convenient approach, the real situation is more complex, and the assumption of an exponential elementary blip is clearly oversimplified. In the first place, the recording and sampling procedure imposes a cut off at very low, as well as at high frequencies (an example of very low frequency attenuation is shown in Fig. 13; in the other graphs frequencies below 3 Hz are not shown). More important deviations are to be expected on the two following grounds: (a) the 'shot effect' has presumably a finite rising phase which would tend to increase attenuation and the negative slope at the high frequency end of the $\log \overline{w}(f)/\log f$ relation; (b) the relaxation of a brief voltage transient at the cable input would not be strictly exponential, but have an initial phase of faster decline, and spectral power density would decline *less* steeply than according to eqn. (11), in fact with only half its final logarithmic slope.

It may be surmised that under conditions in which the rising phase of the shot effect is very brief (e.g. at high temperature and recording very close to the origin of the ACh-potential), a deviation of type *b* would pre-

dominate, whereas at low temperature when the duration of the underlying elementary conductance change may become appreciably longer, or when recording at some distance from the site of ACh action, the opposite divergence (type *a*) might become apparent.

An incidental point of interest is that a knowledge of the approximate duration, or time factor of decay, of the shot effect enables one to predict the 'confidence limits' of the noise level determined from a given record length. If the shot effect is of the exponential type (eqn. (3)), then the expected variance of the individual values of $\overline{E^2}$, determined in a large number of samples each of record length T , is given approximately by

$$\text{var } (\overline{E^2}) / \text{mean } (\overline{E^2}) = 2\tau/T \quad (13)$$

(cf. Bendat, 1958, p. 268). In the experiment illustrated in Fig. 2 (2° C), the sample length T was 256 ms, while the coefficient of variation of the ACh noise (taken from the histograms) was 0.314. From eqn. (13), $0.314^2 = 2\tau/T$, hence $\tau = \frac{1}{2}(256 \times 0.098) = 12.5$ ms. The corresponding half-power point $1/2\pi\tau = 12.7$ Hz, which is very close to the one observed in this experiment (13 Hz). This close agreement, however, is somewhat fortuitous because the higher frequency part of the spectrum departed from the theoretical curve, the attenuation factor for $\overline{w}(f)$ from 10 to 100 Hz being 105 (instead of 39 for an exponential with $\tau = 12.5$ ms). Nevertheless, the theory provides a reasonable explanation for the wide dispersion of the histograms of the ACh noise in Fig. 2. The 'control noise' shows much less variability: its coefficient of variation was only 0.113. This is not unexpected because the control noise extends to a much higher frequency spectrum and has components of much briefer time course than the ACh noise.

Examples of 'power spectra' of ACh noise are shown in Figs. 11–13. The ordinates ($\overline{w}(f)$) are in arbitrary units (or log units), while the abscissae (f) are in Hz (or log Hz). There was no evidence, within the limits of our experiments, for a significant change in the spectral distribution of $\overline{w}(f)$ when the ACh dose was varied. The absolute values of $\overline{w}(f)$ depend, of course, on the ACh dose and are related to V in the way described earlier.

The curves conform in general to the low frequency pattern to be expected from the characteristic input impedance of a muscle fibre, with a τ of the order of 10 ms. There are significant deviations from the simple low-pass filter noise (eqn. (11)) shown clearly in Fig. 12, in which experimental results are plotted together with a theoretical curve for a simple RC input of 10 ms time constant. In seventy-seven experiments which included ACh noise spectra at temperatures between 1.5 and 24° C, from normal and denervated fibres, the average '½ power' frequency was 14 Hz (range 8–27 Hz). On the exponential hypothesis this would correspond to $\tau = 11.5$ ms.

On grouping the results, and excluding long denervations, the mean '½ value' in sixteen experiments at high temperature (19–24° C) was 19.5 ± 1.44 Hz; at low temperature (1.5–14° C), the mean of forty-one experiments was 13 ± 0.38 Hz. This relatively small effect corresponds to

the low temperature coefficient of the membrane resistance and can therefore be explained by the lengthening in the time constant τ_m as the temperature is reduced (cf. del Castillo & Machne, 1953).

A more striking difference between high and low temperature results was found in the rate of attenuation at the high frequency end. This is seen in Fig. 12, where the low temperature curve lies *below*, and the high temperature relation *above* the theoretical curve. This was a consistent finding

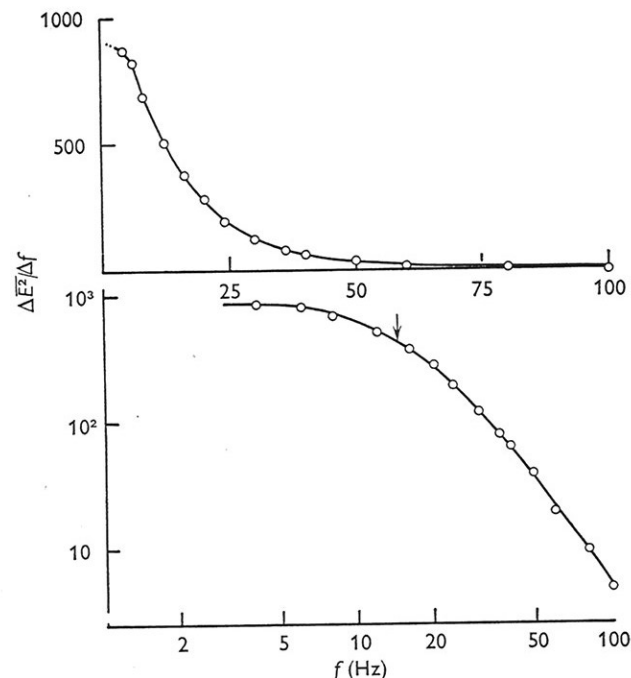


Fig. 11. 'Power spectrum' of intracellularly recorded ACh noise. Temp. 5.5° C. Upper part: linear plot (ordinates: $w = \Delta\overline{E^2}/\Delta f$ in relative units; abscissae: frequency in Hz). Lower part: same results in double-logarithmic co-ordinates.

which can be summarized by saying that whereas at high temperature the value of $\overline{w}(f)$ fell to roughly 10% of its maximum at 100 Hz, at low temperature it dropped to about 1%. Taking the attenuation factor of $\overline{w}(f)$, from 10 to 100 Hz, as an empirical measure, its mean value was 10.2 ± 0.9 (twenty-one experiments) at high temperature, and 88 ± 5.9 (forty experiments) at low temperature.

Our tentative interpretation of these results is that the most important change at low temperature consists in a lengthening of the rising phase of the elementary potential, due to a prolongation of the drug/receptor interaction, or at any rate of the elementary conductance change. This would

increase attenuation in the higher frequency range in the way observed. The declining phase and total duration of the potential change would be less affected by temperature (cf. Eccles, Katz & Kuffler, 1941; del Castillo & Machne, 1953), which is probably reflected in the smaller change of the $\frac{1}{2}$ power frequency in the lower part of the spectrum. As regards the departures of the final slope from the theoretical curve in Fig. 12, we would interpret this in the way already outlined, viz. that at high temperature

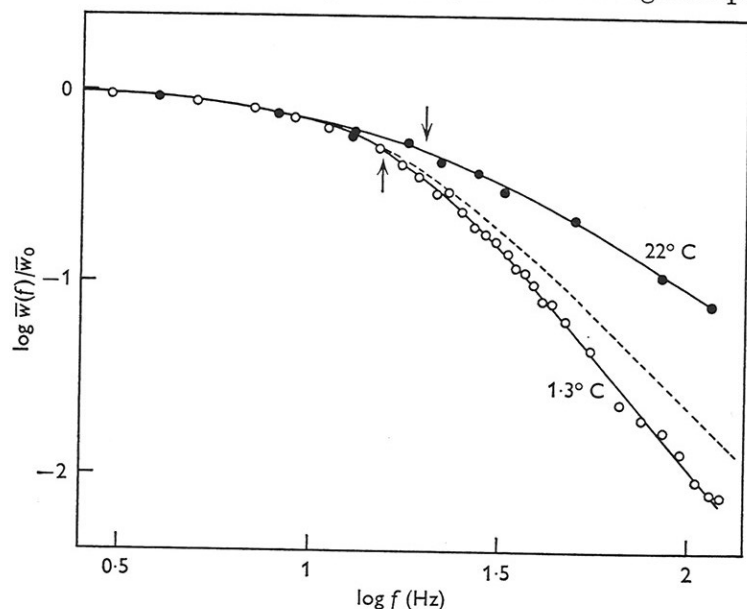


Fig. 12. Showing effect of temperature on shape of power spectrum. Filled circles: 22°C; open circles: from same end-plate at 1.3°C. The curves have been brought to a common plateau by vertical displacement. (The actual plateau was approx. 6 times higher during the low temperature run.) The dashed line is a theoretical curve, from eqn. (11), for $\tau = 10$ ms.

the attenuation in the range of 10–100 Hz depends mainly on the frequency characteristics of the cable input impedance, while at low temperature the lengthening of the elementary current pulse becomes the more important factor and would have the effect of adding an extra filter section to the cable input.

This interpretation is strengthened by the results of two experiments, at 4.5°C, in which the 'cable length' between site of ACh application and of recording was increased, from less than 100 to over 600 μm (Fig. 13). This caused not only the value of a to diminish (see above), but the final slope and the 10/100 Hz attenuation to increase (from 71 to 118, and from 80 to 214, in the two cases).

Effects of denervation. Chronic denervation, for 2 weeks or more, had a significant effect on the spectral noise distribution (Table 3). After 1 week, the results were within normal range; but after 2–6 weeks, the $\frac{1}{2}$ -power points had fallen to a lower frequency, while the attenuation factor (10/100 Hz) had increased considerably, indicating that the ionic conductance

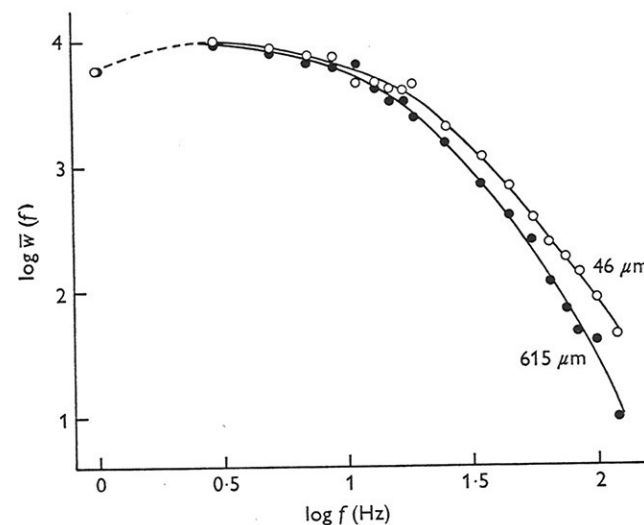


Fig. 13. Power spectra with different 'cable length' between points of ACh application and of intracellular recording. Temp. 4.5°C. Longitudinal separation: 46 and 615 μm respectively.

TABLE 3. Temporal characteristics of ACh noise in denervated muscle fibres

A. High temperature (20–25°C). Values are mean \pm s.e. of mean			
Period of denervation	7 days	2 weeks	6 weeks
No. of expts	6	8	7
$\frac{1}{2}$ -power frequency (Hz)	24 \pm 1.1	13 \pm 0.9	12 \pm 0.8
Attenuation (10/100 Hz)	7.8 \pm 0.9	39.4 \pm 4.1	47.3 \pm 3.4
B. Low temperature			
Period of denervation	8 days	4 weeks	
No. of expts. (temp.)	4 (3°C)	1 (10°C)	1 (2°C)
Half-power frequency (Hz)	12 \pm 0.9	9.5	6.7
Attenuation (10/100 Hz)	108 \pm 2.9	117	380

channel and the elementary current flow persisted for a longer period. This could well be responsible in part for the large increase in noise amplitude which was observed concurrently (p. 679).

A check on pipette artifacts. It has been mentioned on (p. 667) that occasionally checks were made against possible fluctuations in the drug

pipette, by moving it up and down, closer to the fibre surface or farther away. These conditions are associated with large changes in the time course of the ACh potentials, and it was of interest to see whether the noise characteristics are affected. If an appreciable part of the ACh noise were due to pipette artifacts, such as fluctuations in resistance and therefore in drug efflux, this would clearly be more noticeable when the pipette is brought close, and conversely 'smoothed out' and greatly diminished when it is moved higher up in the fluid. The following are results of four of these experiments (two from normal muscle at low temperature, and two from denervated muscle at high temperature): the mean amplitude a was $0.54 \mu\text{V}$ in the *near*, and $0.58 \mu\text{V}$ in the *far* position, the mean ratio being 1.07 ± 0.04 . In three experiments the power spectrum was analysed: mean ' $\frac{1}{2}$ -points' were 13.3 Hz in the *near*, and 14.2 Hz in the *far* position; their mean ratio was 1.08 ± 0.04 . Finally, the mean values for $10/100 \text{ Hz}$ attenuation were 37 in the *near*, and 43 in the *far* position, with a mean ratio of 1.22 ± 0.19 . These results were satisfactory not only in confirming the genuine nature of the ACh noise, but they bring out the important point that the characteristic features of the ACh noise, and the size and time course of its analysed components, are *not* diffusion-dependent, which sets it apart from other forms of ACh potentials that have been recorded.

Actions of curare and prostigmine. It was reported above (cf. p. 681) that curare and prostigmine, in doses of 10^{-6} g/ml. , had very little effect on the calculated size of a , curare sometimes reducing it slightly, and prostigmine possibly increasing it by about 20%. Similarly doubtful changes were observed when the time characteristics were analysed in the same experiments, the $\frac{1}{2}$ -power frequency being slightly reduced and the attenuation factor ($10/100 \text{ Hz}$) slightly increased after application of *either* of these drugs.

Thus, in four paired experiments at 21° C , the $\frac{1}{2}$ -power frequency was $21 \pm 2.6 \text{ Hz}$ (mean \pm s.e.) before and $19 \pm 1.6 \text{ Hz}$ after prostigmine treatment, the mean ratio (prostigmine/normal) being 0.92 ± 0.034 . The attenuation factors were 9 ± 1.5 before, and 11 ± 1.5 after, their mean ratio being 1.3 ± 0.041 .

In five experiments with curare, at 4.5° C , the $\frac{1}{2}$ -power frequency was $13 \pm 0.54 \text{ Hz}$ before and 11 ± 0.46 after tubocurarine application, the mean ratio being 0.85 ± 0.06 . Attenuation factors were 91 ± 5.6 before, 97 ± 6.3 after, and their mean ratio 1.07 ± 0.013 .

It remains uncertain whether these small changes were actually related to the drug action, especially as no return series to normal Ringer had been made in these experiments.

Focal extracellular noise analysis

The results so far described were all obtained with intracellular recording of membrane potentials. The noise, in this case, is limited to the very low frequency band in which the membrane of the muscle fibre does not act as a short-circuit. To obtain more direct information on the time course of the elementary *conductance* change and the associated ionic current pulse, voltage clamping, or extracellular recording from locally activated regions of the end-plate is needed (cf. del Castillo & Katz, 1954; Katz & Miledi, 1965*a*). This is technically more difficult, but has the advantage of enabling one to overcome the limitation imposed by the membrane time constant. With focal external recording, the power spectrum would represent the time characteristics of the elementary *current* pulses, that is of a much faster event than the membrane potential change.

Although only rather few extracellular experiments have been made so far, their results are sufficiently interesting to be reported. In some of the experiments, low resistance NaCl or KCl filled micropipettes, in others platinized indium electrodes (Katz & Miledi, 1965*a*) were used. Regions with relatively large external min.e.p.p.s had to be located in the way previously described (Katz & Miledi, 1965*a*). The ACh pipette was applied nearby, at fairly close range, in order to confine the drug action to a small surface area and so avoid the need for excessive depolarization of parts of the fibre which would not, in any case, contribute to the focal noise record. Whenever practicable, the depolarization was monitored with a separate intracellular electrode. No attempt was made to work out a value of a for extracellular noise, because the amplitude dependence on electrode position would have made it meaningless; moreover, the value of V_{ext} was small and difficult to determine with any degree of accuracy. Our main concern was the analysis of the time characteristics.

The experiments were made at high temperature (21 – 25° C). The ACh noise (cf. Fig. 14) was obviously 'faster', extending into a higher range of frequencies than that observed with intracellular recording. Its amplitude was generally small and, of course, critically dependent on the position of the micro-electrode. Moreover, it tended to decline rather quickly during prolonged ACh application, which is a further indication of the difficulty in achieving optimal conditions when one wishes to record from, and apply drugs to, the same focal spot.

The noise records were analysed using small sampling intervals (0.15 – 0.5 ms). Examples of extracellular power spectra are shown in Figs. 14 and 16. It is clear that they are 'flat' over a much wider range than their intracellular counterparts. In four experiments the decline to $\frac{1}{2}$ -power occurred at frequencies of 100 , 125 , 120 and 180 Hz , i.e. about 10 times

higher than for internal noise recording. This would suggest that the 'relaxation time' of the elementary ACh current pulse is of the order of 1 ms, though it must be admitted that this is a very rough estimate. In the case of the membrane potential noise, the assumption of an approximately exponential shape is reasonable; to make similar assumptions for the underlying elementary current pulse seems more arbitrary and, indeed, unlikely. The opening and closing of an individual ionic channel is more probably a sudden on/off event, but it might be argued that if the duration of individual 'on' states varies in random fashion, the final result might approximate to that for an exponential average shape.

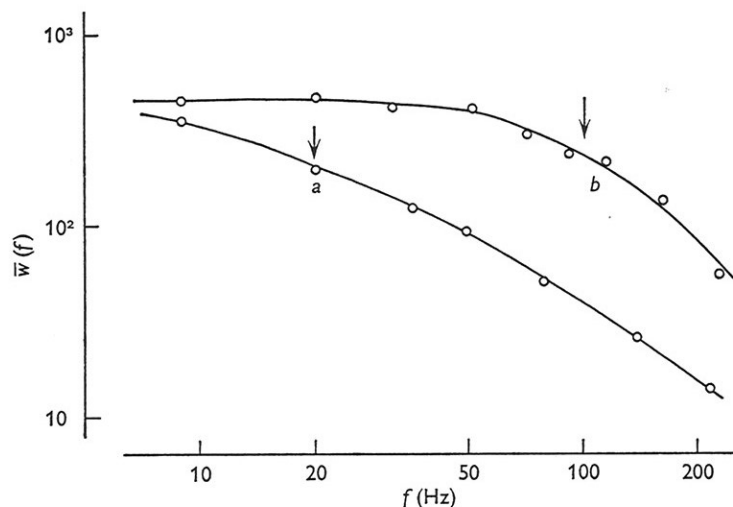


Fig. 14. Power spectra of intracellular (a) and focally recorded extracellular (b) ACh noise. Temp. 22 and 25° C respectively. Half power points indicated by arrows.

Although the power spectra of the external noise could not be determined as completely nor with the same accuracy as those of the intracellular records, use can be made of the two alternative ways of calculating τ outlined above. It was found that the $\frac{1}{2}$ -power frequency ($\tau = 1/2\pi f$) gave nearly twice as large a value as the ratio of $\bar{w}_0/4\bar{E}^2$ ($\tau = 2V\alpha\tau/2V\alpha$). Thus in four experiments, the first method gave a mean value of 1.14 ms, the second method gave 0.67 ms, which shows once more that the hypothesis of a simple exponential shape does not fit the facts. But it also suggests that, as an order of magnitude for the average 'life time' of the ACh channel, 1 ms is probably not far out.

Carbachol and acetylcholine: a comparative study

We had been concerned, especially in view of the somewhat inconclusive prostigmine experiments, to obtain further evidence whether the presence of active ACh esterase, in close proximity to the receptors, is able in any way to interfere with, and perhaps limit the duration of, individual ACh/receptor actions. This seems a priori unlikely, but not impossible. It was thought that one way of approaching this problem was to make a comparative analysis of ACh and carbachol actions, with the drugs applied to the same spot from a double-barrel pipette. If the esterase was able to curtail

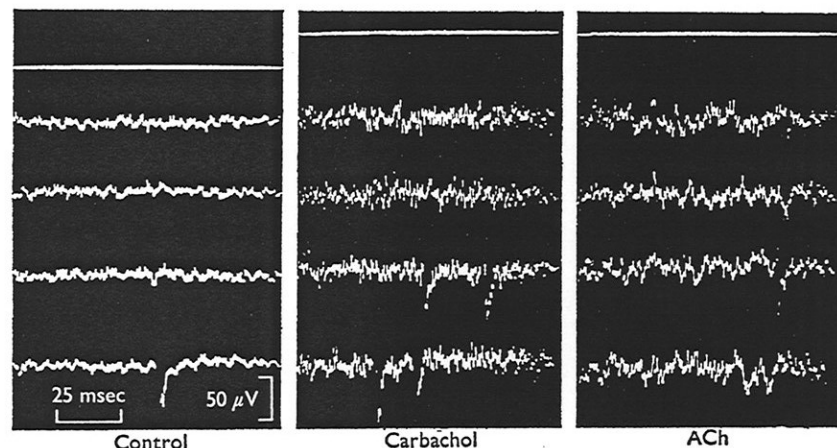


Fig. 15. Sample records (magnetic tape playback) of focal extracellular noise during Carb and ACh applications. Temp. 21° C. Membrane depolarization (about 5 mV) was monitored by intracellular recording and is indicated by the upward displacement of the horizontal line at the top of each column. The very large rapid deflexions (e.g. in the bottom traces of control and Carb records) arose from external min.e.p.p.s which saturated the tape amplifier.

the ACh action, then one would expect to find evidence for a more prolonged shot effect for the current generated by a similarly active, but non-hydrolysable agent like carbachol. To our surprise the opposite effect was found, as illustrated in Figs. 15 and 16.

The externally recorded carbachol noise was consistently 'faster' than that due to ACh, and the quantitative analysis indicated that the 'time factor' for the molecular carbachol action was 2.5–3 times shorter than for ACh. That is to say, it appeared that the ion channel if opened by the carbachol molecule stays open for only 0.3–0.4 ms, by comparison with 1 ms when activated by ACh.

Having found this interesting difference between ACh and carbachol

(Carb) we returned to the easier technique of intracellular recording in order to check the results in another way. The argument was that a shorter duration of the carbachol current should be reflected in a smaller amplitude of the elementary potential change a when recording intracellularly. This was indeed the case: at 24° C, in three experiments, the mean value of a_{ACh} was 0.41 μV , that of a_{Carb} 0.12 μV , the mean ratio being 3.2 ± 0.27 . In three other experiments at low temperature (3° C), the difference in amplitudes was smaller, the mean ratio being only 1.8 ± 0.23 . This would

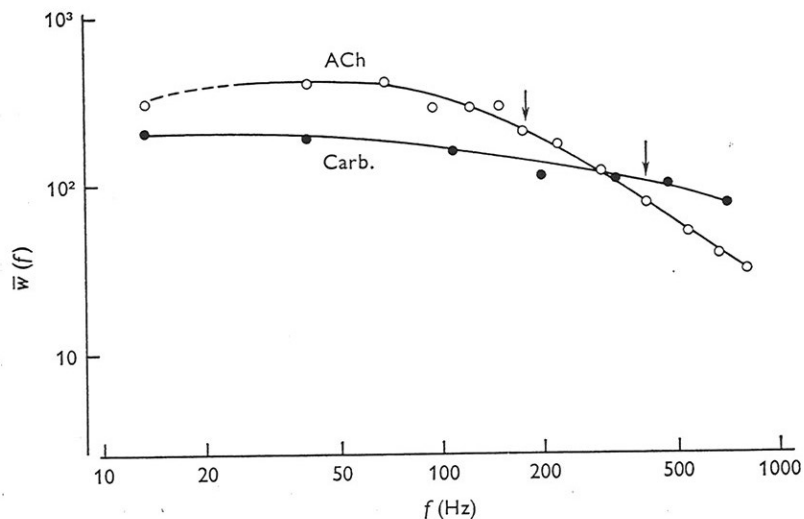


Fig. 16. Extracellular noise power spectra ACh (open circles) and Carb (filled circles). Temp. 24° C. Half-power points (at arrows): 180 and 410 Hz, for ACh and Carb respectively.

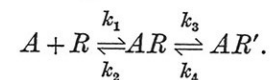
be in keeping with the fact that at low temperature, both ACh and Carb currents increase in duration, for the difference in the size of the potential change would then become less striking. The form of the power spectrum ($\frac{1}{2}$ -point and 10/100 Hz attenuation) did not differ noticeably for the two drugs at high temperature, but in the low temperature experiments there was a very clear difference in the attenuation factors: in the three ACh curves $\bar{w}(f)$ declined from 10 to 100 Hz by a factor of 155, 145 and 110 respectively, while the corresponding values for carbachol were much lower, 30, 22 and 17.5, indicating again a shorter duration of the carbachol induced conductance channels.

DISCUSSION

In the present work an attempt has been made to introduce a new electrophysiological approach, namely membrane noise analysis, to the problems of drug/receptor interaction. The experiments were of an exploratory kind, and it must be emphasized that the quantities which have been extracted from the data by analysis will need revision when more accurate results and a more refined method of analysis are available. Indeed, our basic assumption namely that the same elementary event underlies both noise and average depolarization remains a working hypothesis, direct evidence for which will be difficult to procure. Meanwhile, the orders of magnitude of the calculated 'shot effect', of its size and time course, are of considerable interest and provide a basis for discussing certain questions which seemed previously not to be open to experimental attack. Among these are: the number of ionic gates involved in the production of a miniature e.p.p.; the absolute conductance of a single ion gate opened by ACh molecules; the duration of the gating action and the total transfer of charge through the ion channel; the relation between the time course of the elementary current and the kinetics of drug/receptor action; the probability of single or repeated action of individual ACh molecules during normal transmission, etc.

In the first place, one might ask whether the phenomenon described as 'ACh noise' is really drug-induced, or depolarization-induced in a way analogous to the 'potassium channel noise' at the node of Ranvier (Verveen & Derksen, 1968). We think that the control experiments with direct current application, the observations on the quantitative relation between V and \bar{E}^2 , and the drug-dependent, but not depolarization-dependent, differences between ACh and Carb are sufficient evidence to show that the potassium channel noise did not play a significant part in the present experiments.

Kinetics of drug action and its relation to ACh noise. On the 'occupation' theory of drug/receptor action (an adaptation of the Michaelis-Menten hypothesis) the drug A forms an intermediate compound with the receptor R which then, via a second step, is transformed to a depolarizing conformation AR'



For a competitive inhibitor (a curare-like agent), the 'second' affinity constant k_3/k_4 - that is, the ratio AR'/AR , also called the 'efficacy' or 'intrinsic activity' of the drug/receptor compound - is very small. In equilibrium conditions,

$$\frac{AR'}{K} = \frac{A}{A(1 + k_4/k_3) + k_2 k_4 / k_1 k_3}$$

where K is a constant, equal to the total receptor concentration ($R + AR + AR'$).

For different agents of equal 'first' affinity constant k_1/k_2 , the depolarizing potency is directly related to the ratio k_3/k_4 . Thus, a lower potency may be due to a relatively low forward rate constant k_3 , or to a lower stability of AR' , that is a higher reverse rate constant k_4 .

It is tempting to suggest that the greater potency of ACh, compared with Carb, arises from greater stability (smaller k_4) of $AChR'$, as against $CarbR'$, and that this is reflected in the longer duration of the elementary ACh current pulse, leading to larger charge transfer and change of membrane potential. It is, however, not easy to decide between this and an alternative possibility, viz. that the drug/receptor complex may have a much shorter life time than the ionic channel, and that the relaxation time of the ion gate depends in a more indirect way on the preceding drug action.

One is used to regarding Carb as the more stable depolarizing agent than ACh, and it may therefore seem surprising and somewhat confusing that its molecular action is now reported to be briefer than that of ACh. There is, however, no real discrepancy: carbachol is more stable and produces more prolonged effects because it is not hydrolysed by ACh-esterase. Hence for the same applied dose, the local concentration of Carb at the receptor sites will reach a higher level and be maintained longer than that of ACh, provided of course the esterase has not been blocked. In the present work, however, quite a different aspect has been studied: we have been concerned with the time course and effectiveness of *molecular* drug/receptor action, not with the rise and fall of the local drug *concentration*. In short, while all the earlier work – including that done with close-range iontophoresis – showed up depolarizations whose time course was diffusion-limited, the properties of ACh noise are not diffusion-limited, but disclose more elementary changes, presumably at the molecular receptor level.

Properties of the 'ion gates'. The kinetic hypothesis outlined above is based on the assumption of a simple reaction between one ACh and one receptor molecule. It may be questioned whether the 'ion gate' is opened by the action of a single, or by the cooperative action of several ACh ions. This remains undecided, though the very slight initial curvature of the dose-effect relation (Katz & Thesleff, 1957*b*; Jenkinson, 1960) suggests that it is not likely to be more than two ACh molecules per receptor.

A further question is whether the elementary potential change arises from the opening and closing of a 'channel' or 'gate' of single molecular dimensions. Alternatively, it is possible that each receptor is surrounded by a pattern or 'domain' of membrane molecules, all of which are brought into action and contribute to ion permeability when one ACh molecule reacts with the centre.

There is yet another possibility, namely that each elementary potential change may be composed of a large number of much smaller 'gating actions' which are initiated by multiple collisions of an ACh molecule with a cluster of receptor molecules. This would not be inconceivable if the lifetime of ACh/receptor occupation turned out to be much briefer than the duration of the elementary conductance change.

There are no doubt many possible variants on such speculations, none of which can be ruled out at present. What can be done, however, is to derive some of the physical properties of the 'ion gate' from our observations. Thus one can calculate the order of magnitude of the elementary conductance change, and of ion transfer, from the value of a . To do this, straight extrapolation from the already available quantities associated with the e.p.p. and miniature potential (Fatt & Katz, 1951; del Castillo & Katz, 1954; Takeuchi & Takeuchi, 1960) is the simplest approach.

The 'shot effect' determined from intracellular noise records is about three orders of magnitude smaller than the min.e.p.p. and has a similar time course (considering normal muscle, at 20° C, without drug treatment). The conductance change during the rise of the min.e.p.p. is approximately $10^{-7} \Omega^{-1}$ (del Castillo & Katz, 1954; Takeuchi & Takeuchi, 1960), so that a rough estimate for the ACh shot effect would be $10^{-10} \Omega^{-1}$. From an analysis of a 25 mV e.p.p. in curarized muscle, Fatt & Katz (1951) calculated a net charge transfer of 8×10^{-10} C, equivalent to approximately 5×10^9 univalent ions. For a 0.25 μ V potential change of similar time course, the corresponding figure would be 5×10^4 ions. One arrives at a similar result by calculating that an elementary current pulse of 75 mV $\times 10^{-10} \Omega^{-1}$ lasting for 1 ms transfers a charge of 7.5×10^{-15} C which is equivalent to approximately 4.7×10^4 ions. A simple alternative approach is to relate average current flow i during a depolarization V of, say, 10 mV to the average value of n (see p. 672). For a fibre of $R = 250000 \Omega$, $a = 0.25 \mu$ V and $\tau = 10$ ms, $i = V/R = 4 \times 10^{-8}$ A and $n = V/a\tau = 4 \times 10^6$ s $^{-1}$. Hence the charge transferred per unit event is 10^{-14} C, equivalent to about 6×10^4 ions. This is a minimum net value and presents a very massive movement of ions brought about by the action of, possibly, one ACh ion. It is of interest that the channel conductance of $10^{-10} \Omega^{-1}$ is not very different from the one taken by Hille (1970) to represent the sodium gate in the activated axon membrane.

The value of 5×10^4 ions transferred in 1 ms is, however, very far from the quantities calculated by Kasai & Changeux (1971) for the Na flux across decamethonium activated membrane receptor sites. Kasai & Changeux have already commented on this discrepancy and suggested

various possible explanations to which we have little to add. Their results were obtained by determining decamethonium-induced sodium flux across the membranes of vesiculated particles separated from the electric organ of the electric eel. Having also determined the density of receptors by specific toxin-labelling, Kasai & Changeux arrived at a flux-rate of about 5×10^3 sodium ions per minute per receptor site, from which they calculated a conductance of $10^{-15} \Omega^{-1}$ – five orders of magnitude smaller than our figure. Of the possible suggestions discussed by Kasai & Changeux (1971), we mention the possibility (a) that the functional properties of the receptors may have been impaired during the preparation of membrane

TABLE 4. Relation between min.e.p.p. and 'elementary' ACh potential

Type of expt.	No. of expts.	Mean min.e.p.p. (mV)	Mean a (μ V)	Mean ratio (\pm S.E.) of (m.e.p.p.)/ $10^3 a$
High temp., no prostigmine	29	0.41	0.32	1.46 ± 0.096
High temp., prostigmine	13	0.51	0.33	1.86 ± 0.20
Low temp., no prostigmine	24	0.39	0.49	0.83 ± 0.042

fragments, and (b) that organized patterns of membrane molecules surrounding each receptor may be required which are destroyed during the homogenization procedure.

The approximately 1000-fold ratio between min.e.p.p. and ACh shot effect invites comment. In Table 4 mean values of min.e.p.p.s, calculated shot effects and ratios between them are shown for three groups of experiments, (a) at high temperature (about 22° C) without anti-esterase, (b) at the same temperature after prostigmine treatment (10^{-6} g/ml.), and (c) at low temperature without prostigmine. The curare experiments have not been included, because miniature potentials were too small to be measured, while there was little change in a (see p. 681).

These figures suggest that something of the order of 1000 ion gates are opened during the normal action of one packet of transmitter substance. In deeply curarized muscle, this number is greatly reduced because most of the normally available receptor molecules are occupied by the inhibitor. That the number increases during prostigmine treatment (cf. pp. 681–682) above) is also not surprising, for a greater proportion of transmitter molecules would become, or remain, effective instead of being hydrolysed, and many of them may now activate several ion gates in succession.

In this connexion, it may be recalled that the relaxation time of the molecular ACh action, derived from extracellular noise measurements, is of

the order of 1 ms. This does not differ greatly from the time factor of decline of the extracellular miniature potentials, at high temperature and in the absence of prostigmine (cf. Fatt & Katz, 1952; del Castillo & Katz, 1954). If this corresponds to the average life-time of the activated ACh/receptor complex, then it would follow that during normal transmission there is no time for repetitive 'gating action'. After prostigmine treatment, however, external min.e.p.p.s can become much more prolonged (see Fatt & Katz, 1952; del Castillo & Katz, 1954), presumably because individual transmitter molecules now repeat their operation and open a whole series of ionic channels in succession.

At low temperature, the intracellular noise analysis indicates that there is a substantial increase in the amplitude a and in the duration of the elementary current pulse. The increase in a may, in fact, be somewhat less than calculated in Tables 2 and 4, because the change in shape of the shot effect brought about by a lengthening of the rising phase would tend to reduce the factor 2 in eqn. (6) towards $4/e$. Nevertheless there remains a definite rise in the amplitude of the molecular ACh effect, whereas there is little increase in the size of the min.e.p.p. Assuming constancy of the quantal packet, the discrepancy may reflect a lowering of k_3 or of k_1/k_2 , i.e. of the affinity constant of the first reaction step. In this case, a release of the same quantum of transmitter would initiate a smaller number of molecular gating actions, each of which makes a longer and, therefore, more effective contribution to the potential change.

All this, unfortunately, does not tell us the number of ACh molecules contained in a quantal packet, for we do not know what proportion of the released transmitter molecules reach their molecular targets, nor their probabilities of activating ion gates, etc. Estimates of approximately 50,000 ACh molecules per quantal packet have been made on the basis of chemical and pharmacological assays (Straughan, 1960; Krnjević & Mitchell, 1960; Potter, 1970). This figure presents considerable difficulties, not only for the vesicular hypothesis, for a synaptic vesicle could barely accommodate that many ACh molecules, but for the interpretation of our present results, for it would suggest an extraordinarily inefficient mechanism of release and high wastage of transmitter molecules. It will be interesting to see how this puzzling discrepancy will ultimately be resolved.

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