ACETYLCHOLINE-INDUCED CURRENT FLUCTUATIONS IN TISSUE-CULTURED MUSCLE CELLS UNDER VOLTAGE CLAMP

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ABSTRACT Acetylcholine applied ionophoretically to chick skeletal muscle cells grown in tissue culture produces membrane current fluctuations. Cells treated with vinblastine are transformed to a roughly spherical shape. Such transformed cells can be voltage-clamped with microelectrodes. The frequency spectrum of the current fluctuations at fixed voltage obeys a relation of the Lorentz form. From analysis of the current noise, the conductance of a single ionic channel is estimated to be 39 pmho at a temperature of 25°C, and increases with increasing temperature, exhibiting a Q_{10} of 1.7. The relaxation time for the channel conductance is more sharply temperature dependent, showing a Q_{10} of approximately 5. These results are in agreement with the picture of acetylcholine-activated ionic channels determined from experiments on frog end plate (Anderson and Stevens, 1973). The relaxation time for carbachol activation is shorter than for acetylcholine, and appears to be more temperature sensitive.

INTRODUCTION

Steady application of acetylcholine (ACh) to a motor endplate generates electrical noise because of the random activation of ion-conducting pathways. ACh noise in the frog endplate has been studied extensively by Katz and Miledi (1972, 1973) and by Anderson and Stevens (1973). As was first shown by Hagins and Srebro (Hagins, 1965), shot-noise analysis can be applied to membrane current fluctuations to estimate the elementary conductance unit activated during excitation. Anderson and Stevens (1973) used an analysis of current noise under voltage clamp conditions to estimate a value for the conductance of frog endplate ionic channel (γ = 32 pmho). We (1973) measured ACh current noise on a different preparation—chick skeletal muscle cells grown in tissue culture—and obtained a value of conductance consistent with the values found for frog endplate. The tissue culture cells possess ACh receptors spread about the entire surface of the cell but at a much lower density than found in an endplate region (Sytkowski et al. 1973); thus the unit channel conductance does not vary markedly with channel density.

The initial experiments on the tissue cultured cells by conventional electrical filtering

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methods led to values of channel conductance and channel relaxation time comparable to those found for the frog end-plate. In the present work, we have extended these measurements, using computer Fourier analysis methods, to obtain a more precise value of the channel conductance and to determine the temperature-dependence of conductance relaxation. In addition, current-fluctuation spectra were obtained with carbachol as an agonist.

To obtain cells of shape amenable to voltage clamp, we treated the muscle cells with vinblastine. This had the dual effect of transforming the cells to a roughly spherical shape and reducing contractility. In our experiments and in the work of Fukuda et al. (1976*a*, *b*), the alkaloid treatments did not alter excitability. This type of treatment may allow the extension of noise experiments to other excitable cells in tissue culture. Since the shape of the cells can be modified by chemical treatment (Fukuda, et al., 1976*a*, *b*), it may be possible to study varieties of excitation in cells transformed in shape to permit better space clamp. In addition such cells can be studied in different states of development and after genetic modification (Nelson, 1975). However, very small cells are accessible only to high-impedance microelectrodes, which introduce spurious electrical noise, some of which cannot be eliminated. In a later section, we give a detailed circuit analysis of the voltage-clamp noise experiment in which particular attention is given to noise sources common in microelectrode experiments on small cells.

A preliminary account of some of this work was presented at the 1975 Biophysical Society Meeting (Sachs and Lecar, 1975).

METHODS

Cells were prepared by the method of Fukuda et al. (1975*a*). Pectoral muscles from 11- or 12-day chick embryos were dissected under sterile conditions, cut into small pieces, and triturated in about 6 ml of Ca- and Mg-free Hanks' solution (10 mM HEPES) with a 12-ml disposable syringe. The cell suspension was left in the syringe for a few minutes to let large undissociated clumps settle. A sample was counted with a hemocytometer, and the cells were then plated on collagen-coated dishes (Falcon Plastics, Div. of Bio Quest, Oxnard, Calif.) at a density of 1–1.5 $\times$ 10^6 cells/60-mm dish. The culture medium formulation was 500 ml Dulbecco's modified Eagle's medium, 50 ml heat-inactivated horse serum, 10 ml embryo extract, 1 mM L-glutamate, and 100 U/ml of penicillin and streptomycin. All components of the media were obtained from Grand Island Biological Co., Grand Island, N.Y. The cells were cultured in 5% CO_2-air at 37°C.

After 3 days in culture the medium was changed to a medium containing 10 nM colchicine or vinblastine to form rounded "myoballs" from the young myotubes (Fukuda et al., 1975*a*). After 2–3 days in the alkaloid medium, the normal medium was restored and the cells remained round for about 1 wk afterwards, with media changes every 3 days.

Electrical measurements were usually done in a bicarbonate-free Hanks' solution buffered with 10 mM HEPES containing 5% heat-inactivated horse serum. The serum appeared to help maintain the preparation stable for longer periods of time and made penetrations more reliable. The medium had the following ionic concentration (in millimoles): Na^+, 146; K^+, 5.8; Mg^{++}, 0.9; Ca^{++}, 1.3; Cl^-, 151.

The microscope stage was modified to include a thermally insulated stage plate cooled or heated by an annular thermoelectric device (Cambion, Cambridge Thermionic Corp., Cam-

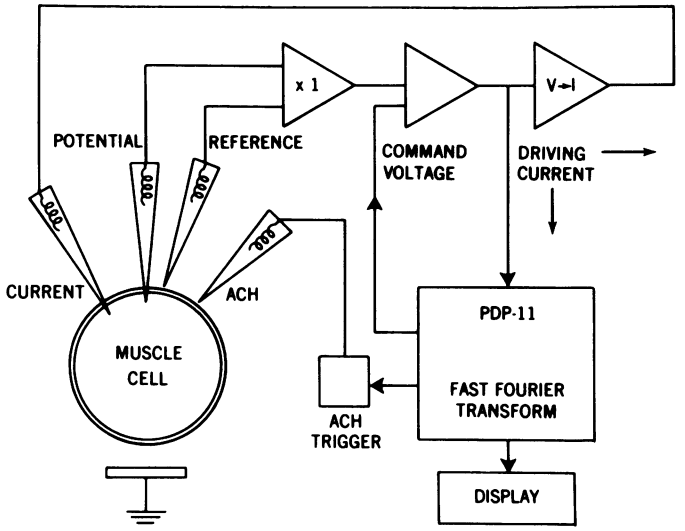

FIGURE 1 Schematic diagram of experimental arrangement. See text for details.

bridge, Mass.). The bath temperature was controlled by a servo system with a stability of better than 0.2°C.

Electrical Recording System

The essential elements of the system are shown in Fig. 1. The voltage clamp was a standard two-microelectrode system. Current was supplied by a voltage-to-current (V-I) converter (100 nA/V) (Colburn and Schwartz, 1972).

The current electrode was filled with 4 M K acetate + 100 mM KCl, and the voltage electrode with 3 M KCL. The electrodes were beveled on an Arkansas stone (Barrett and Graubard, 1970), and had resistances of 10–50 MΩ. The fluid level in the dish was kept to a minimum level (about 1 mm) to reduce stray capacity.

The current and voltage electrode amplifiers incorporated negative capacity adjustments set to minimize the rise time of the amplifier without producing ringing. A minimal effective capacity of the current electrode is necessary to reduce errors in the power spectra (see Appendix).

Data Recording

The input of the *V-I* converter was amplified and filtered (Krohn Hite Corp., Avon, Mass., model 3202, 40 db/octave, maximally flat setting) and sent to a 12-bit direct memory access interface on a PDP-11/20 computer (Digital Equipment Corp., Maynard, Mass.). The computer provided clamp commands and drug release trigger pulses, stored data, and presented the data analysis on-line.

The basic program functions were to take a control record (all records were 1,024 points in length), apply the drug, allow time (about 15 s) to reach steady state, and then take another record. The computer then calculated power spectra of both records with a fast Fourier transform and computed the difference spectra. The cycle was then repeated to average difference spectra. The spectra were displayed in log-log form on a Tektronix 4010 graphics terminal (Tektronix, Inc., Beaverton, Ore.), with a fitted curve (described below) and relevant constants such as resting potential, holding potential, gain, temperature, etc., and the parameters of the

theoretical fit. Frequency smoothing (Hanning average) was sometimes used to make the data clearer. Records of the voltage and mean currents during the experiment were logged on a strip chart recorder.

Two types of voltage command sequences were used. The first was a three-level sequence in which the background noise was measured at the holding potential (typically −50 mV), then 20 and 40 mV hyperpolarized (the steps lasted 5–10 s).

ACh was then applied (usually for about 15 s before the noise was recorded) and the voltage steps were repeated in the presence of ACh. This sequence was designed to minimize the effect of drift on the voltage dependence of the preparation, since ensemble averages were made with the different voltage levels closely spaced in time. But the sequence required the use of a fast-recovery high-gain amplifier since capacity transients due to the voltage steps caused blocking. The fast recovery amplifier was noisier than the PAR 113 (Princeton Applied Research, Princeton, N.J.) used for constant-voltage experiments.

The second sequence simply held the command at a fixed level and averaged the responses to successive drug doses. This method was preferred when the currents were small. It had the disadvantage of measuring the responses to different potentials at widely different times.

To save computer core space, a simple, nonleast squares curve fit routine was used to fit the data, on-line, to a function of the form $P(\omega) = P(0)/(1 + \omega^2\tau^2)$. The data points were integrated to form a first measure of the total power, P_t. A few of the lowest frequency points were averaged to form an estimate of $P(0)$. τ was estimated from $\tau = P(0)/(4 \times VAR)$, where $VAR = \int_0^\infty P(\omega)d\omega$ (estimated by P_t). From this value of τ, corrections to P_t for the power lost by the finite recording bandwidth were calculated. Based on the new value of τ, P_t was recalculated. Iterations were repeated until changes in the parameters were negligible. This procedure produced excellent results with pure Lorentzian inputs (filtered white noise). We made no statistical tests for goodness of fit.

The overall system accuracy was checked with a calibrated noise source (Quan-Tech Div., Scientific Atlanta Inc., Randolph Township, N.J.) Electrode and amplifier frequency responses were checked by using white noise as an input signal to the *V-I* converter and measuring the power spectrum of the output voltage. Frequency responses were usually flat in the range of interest, although clamp ringing was difficult to eliminate in experiments done at higher temperatures where the faster channel kinetics dictated a wider system bandwidth. No experiments were used in which the step response showed oscillations within the bandwidth of interest.

Reversal potentials were measured in voltage clamp by a null method that minimized the distortion produced by desensitization. The potential was set and a pulse of ACh applied. The potential was then changed until the response reversed, and by successive approximation the reversal potential was found. Current-voltage plots of the ACh response tended to be nonlinear due to desensitization and were not used for reversal potential measurements except for interpolation in the immediate vicinity of the reversal potential.

Sources of Error in the Voltage Clamp Measurements

There are two main sources of instrumental error: errors due to inadequate frequency response of the clamp, and errors due to system noise and stray capacity. The first type of error may be estimated from an analysis similar to the one given by Anderson and Stevens (1973).

Using the equivalent circuit shown in Fig. 2 (ignoring the noise sources), the transfer function of membrane current, i_m, to total current, I_{tot}, is simply calculated. The square of the absolute value transfer function, the value relevant to the power spectra, is:

$$|i_m/I_{tot}|^2 = (1 + R_I G_m/K)^2 + \omega^2 Cm^2 R_I^2/K^2 \quad (1)$$

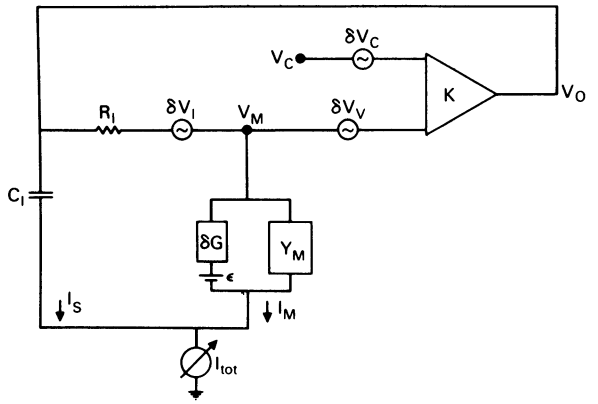

FIGURE 2 Schematic diagram of microelectrode voltage clamp circuit showing various noise sources encountered in voltage-clamp current-noise experiment. See text for definitions.

where K is the control amplifier gain, ω is the radial frequency, G_m is the cell conductance, R_I is the current electrode resistance, and C_m is the membrane capacity. The worst case error, calculated for $R_I < 5 \times 10^7\ \Omega$, $\omega < 6.28 \times 500 s^{-1}$, $C_m < 2nF$, $G_m < 10^{-6}$S, $K \approx 10^3$ is less than 20%. The error would tend to cause the high frequency response to fall off faster than expected. However, almost no spectra were observed in which the data at high frequencies decreased faster than $1/f^2$. Thus we believe that, in general, distortions of the spectra due to dynamic properties of the clamp were not important.

The second type of error mentioned above is analyzed in the Appendix. To summarize, the differential spectral density of the current, drug minus control, is given by Eq. 11A:

$$\Delta\rho_I < \rho_G(V_m - \epsilon)^2(1 + \omega^2\tau_I'^2) + \rho_{V_I}\omega^2 C_I^2 + \rho_{V_0}G_m'^2(1 + \omega^4\tau_m'^2\tau_I'^2) \qquad (2)$$

where ρ_G is the mean square drug induced conductance fluctuation, V_m is the membrane potential, ϵ is the reversal potential, τ_I' is the time constant of the current electrode, ρ_{V_I} is the mean square noise voltage across the current electrode, C_I is the effective capacitance of the current electrode, ρ_{V_0} is the mean square voltage noise of the clamp input (uncorrelated cell noise, and any amplifier noise), G_m' is the cell conductance, and C_m the cell capacitance. Primed quantities refer to those values observed in the presence of drug.

For a worst case analysis we will employ the values used above in the evaluation of Eq. 1, and the following: $\tau_I' < 10^{-4}$ s, $C_I < 1pF$, $\rho_{V_I}' < 10^{-6}\text{V}^2/\text{Hz}$ (DeFelice and Firth, 1971; Sachs and Lecar, unpublished). $\rho_{V_0'}$ appeared to have $1/f$ character at low frequencies, flattening out at about 100 Hz. Its magnitude appeared $< 10^{-11}\text{V}^2/\text{Hz}$ at 1 Hz, and $< 10^{-12}\text{V}^2/\text{Hz}$ at 100 Hz. Although at times the input noise voltage was observed to be significantly greater than these values, such preparations tended not to yield usable data. Thus, after appropriate approximations, the major error term is $\rho_{V_0'}G_m'^2$ with a worst case value of about 10^{-23} A^2/Hz. Since the observed differential noise current was usually significantly greater than the expected value of error at all frequencies of interest, we believe that the observed spectra are a good representation of the true spectra. The possibility of distortion remains, however, since $\rho_{V_0'}$ and τ_I' were not always measured, and were not measured at potentials different from rest.

Increases in τ_I' are to be expected during passage of larger currents and will cause an increase in the observed noise at high frequencies. Force-fitting such data to a Lorentzian

shape would cause an underestimate of τ and an overestimate of γ. This kind of distortion may account for some of the variability of the results (c.f. Fig. 3, lower right, where the data is falling off slightly slower than the theoretical prediction).

RESULTS

The cultures treated with colchicine (10^{-8} M) or vinblastine (10^{-8} M) formed rounded myocytes with diameters of 20–150 μm, as described by Fukuda et al. (1976*a* and *b*). The vinblastine treatment was more reproducible, and became the standard method. The alkaloid treatments do not appear to alter the electrical properties, but they do make the cells isopotential and weaken the force of contraction, two effects that aid voltage clamping.

Mean Currents

Typically, the mean currents used for noise records were in the range of 0.2–10 nA, limited by the resistance of the current electrode and by the degree of desensitization. The currents reached steady state within 15–20 s. Desensitization always occurred, although it was somewhat slower at the low temperatures. The currents could only be explored over a relatively small voltage range, typically −40 to −100 mV. At the most negative potentials, the cells tended to become leaky. At potentials approaching the reversal potential, the ACh noise became too small to be useful, and at positive potentials the outward currents were so large as to saturate the current-driving pipette.

Reversal Potentials

The reversal potential averaged −3 mV, and appeared independent of temperature. This value is essentially identical with the value observed by Ritchie and Fambrough (1975) in tissue cultured rat muscle, and has been used in all subsequent conductance calculations.

Noise Measurements

Fig. 3, upper left, shows the unsmoothed background noise (1 Hz resolution) in a typical control experiment before the application of ACh. The spectrum can be seen to have a rough $1/f$ character at low frequencies, fading into a white-noise background having spectral intensity of 10^{-24} A^2/Hz. The background noise increases again at high frequencies due to instrument-generated noise passed by the membrane capacitance.

The total background noise current in our 500 Hz bandwidth is approximately 30 pA rms (root mean square) (equivalent to the thermal noise current in a 10 kΩ resistance). Spikes can be seen in the power spectrum at the harmonics of 60 Hz (spectra of Fig. 3).

After the ACh pipette had been brought close to the surface of the cell and the drug was allowed to diffuse out, the spectrum of Fig. 3, lower left, was obtained (smoothed 5 Hz resolution). The spectral density in this case was 6×10^{-23} A^2/Hz at low frequency and fell off as frequency squared at high frequency. The upper right panel of

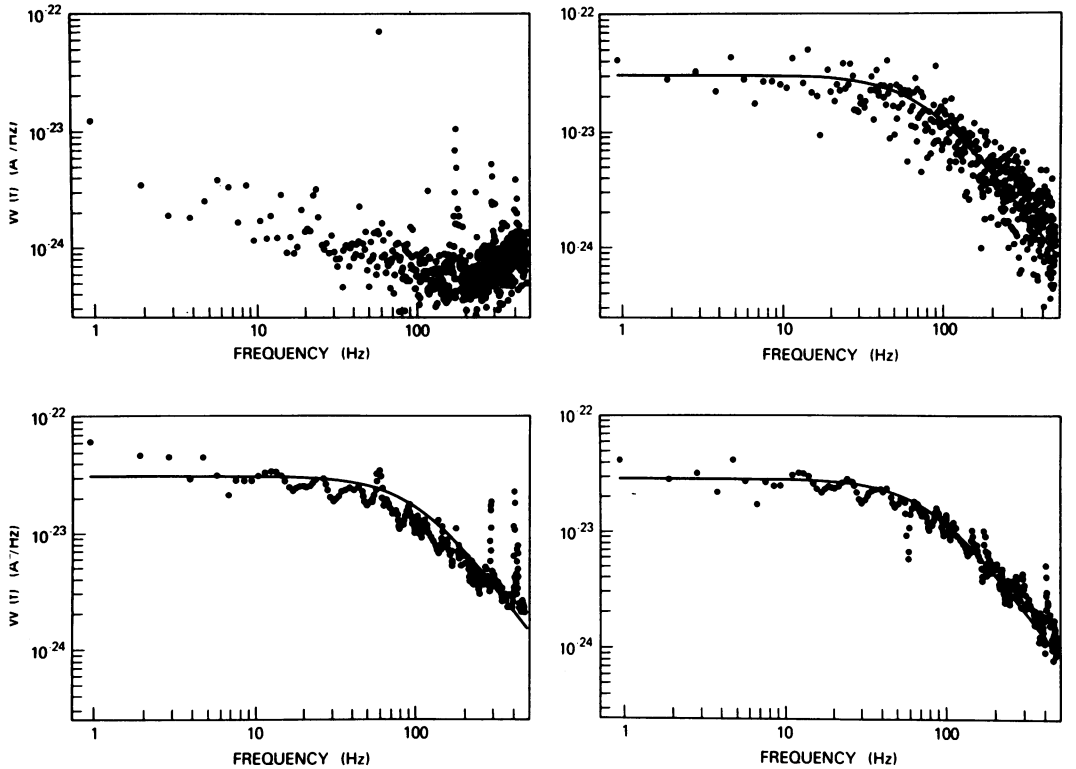

FIGURE 3 Current fluctuation spectra for one cell. Temperature 30°C, membrane potential −100 mV, average of 10 spectra. *Upper left:* Background current noise spectrum, before ACh application (unsmoothed, 1 Hz resolution). *Lower left:* Current noise after application of ACh. Mean depolarizing current, 0.93 nA. Curve is fit to equation of form $W(f) = W(0)/[1 + (2\pi f\tau)^2]$, (smoothed 5 Hz resolution). *Upper right:* Difference spectrum of ACh current fluctuations, with curve fit, (unsmoothed, 1 Hz resolution). *Lower right:* Smoothed difference spectrum (5 Hz resolution), with curve fit. τ = 3 ms based on curve fit.

Fig. 2 shows an unsmoothed difference spectrum (1 Hz resolution), in which the harmonics of 60 Hz are not completely cancelled, possible due to slow drifts in the interfering signals over the course of the measurements. This incomplete cancellation of 60 Hz spikes points out the limitations of the difference spectra for small ACh perturbations. The total rms noise current in that spectrum is of the order of 56 pA, or about twice the background noise current. However, the ACh-induced noise is concentrated into approximately 100 Hz of bandwidth, so that it has a much higher spectral density than the background at low frequency.

In a study of 43 cells, the range of ACh-induced depolarizing current was 0.1–29 nA. Over this range we measured the ratio of noise current (area of spectrum) to mean current to infer a value of the unitary conductance. A variety of arguments can be used to show that the current through a single channel can be estimated from the ratio of

variance-to-mean of the current (Hagins, 1965; Katz and Miledi, 1972; Anderson and Stevens, 1973; Sachs and Lecar, 1973).

For the present purposes, we can give a simple argument not dependent on the details of the statistics of channel activation. Consider a conducting channel with only one open state. Let Θ be the fraction of time that the channel stays open. The mean current would be:

$$\bar{I} = \Theta\gamma(V - \epsilon), \tag{3}$$

where γ is the channel conductance, V the membrane potential, and ϵ the reversal potential, and ϵ the reversal potential. For rectangular pulses, the mean value of the current squared is the duty cycle times the square of the current:

$$\overline{I^2} = \Theta\gamma^2(V - \epsilon)^2. \tag{4}$$

From Eqs. 3 and 4 we can write the ratio of variance to mean as

$$(\overline{I^2} - \bar{I}^2)/\bar{I} = (1 - \Theta)\gamma(V - \epsilon). \tag{5}$$

Eq. 5 thus enables one to estimate γ once the membrane potentials are measured. Generally experiments are done for doses of Ach sufficiently small so that $\Theta \ll 1$ (Anderson and Stevens, 1973). If Θ is in fact small, the calculated γ should be independent of $\bar{I}$. A linear regression of γ and $\bar{I}$ at 25°C yielded a line with a slope of 0.54 pS/nA. However, the coefficient of determination, r^2, was 0.03, which indicated a slope not significantly different from 0. Additional evidence for Θ being small is treated in the Discussion section.

The mean value of γ for 15 cells measured at 25°C was $\gamma = 39 \pm 5$ pS(SEM). The standard error here reflects the variability from cell to cell. The variability between successive measurements within a given cell was much less than the variability between cells.

Fig. 4 shows the ACh conductance values as a function of temperature. Although there is considerable scatter in the data, an increase of conductance with temperature is apparent. The line in the figure is a least squares regression, with a slope corresponding to an activation enthalpy of 9.3 ± 1.9 kcal/M($Q_{10} \approx 1.7$). (This is not to suggest that a single barrier model is appropriate for ion permeation. The functional form was simply chosen for compatibility with Fig. 4.)

The differential power spectra of Fig. 3 were fitted to a spectral density function of the Lorentz form, characteristic of single time constant processes:

$$\rho_I(\omega) = \frac{\rho_I(0)}{1 + \omega^2\tau^2} \tag{6}$$

$\rho_I(0)$ can be expressed in terms of the two-state model parameters by integrating $\rho_I(\omega)$ over positive frequencies to get the variance and substituting back into Eq. 5 to get: $\rho_I(0) = 4\tau\bar{I}(V - \epsilon)$. $\bar{I}$ is the mean drug-induced current, τ is the mean channel open time (for low channel duty cycles), and the other quantities have been pre-

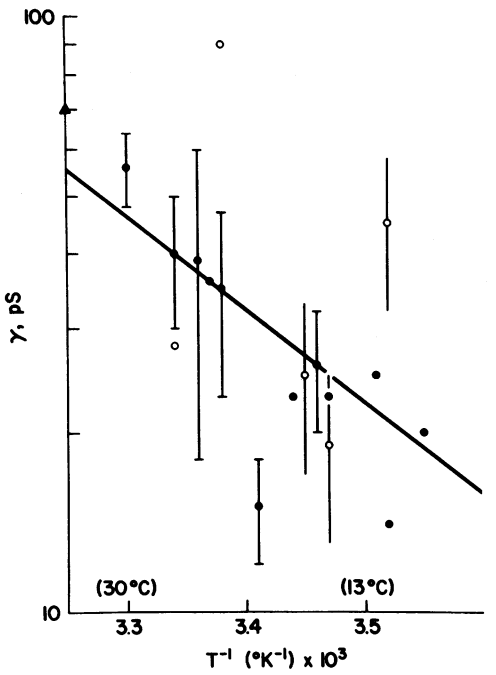

FIGURE 4 Temperature dependence of the average conductance of single channel as a function of reciprocal temperature derived from drug-induced noise. Filled circles are for acetylcholine and the open circles are for carbachol. Error bars represent ± SEM. Points without error bars represent the mean from a single cell. The line is a least squares regression with a slope equivalent to a $Q_{10} \approx 1.7$. The triangle represents the mean from the data of Sachs and Lecar (1973).

viously defined. From the lower right panel of Fig. 3 it can be seen that the mean open duration is about 3 ms at 30°C. Fig. 5 shows the relaxation time, τ, as a function of temperature. The temperature dependence is marked. An activation enthalpy, ΔH, of 30 ± 2 kcal/M ($Q_{10} \approx 5$), was calculated from the least squares regression.

15 cells were studied with carbachol as agonist. Fig. 4 shows the carbachol-induced conductance (open circles) plotted on the same scale as the ACh results. The data are too scattered to observe a reliable trend. However, taking the value for γ at 17°C, for which there was the greatest number of measurements (eight cells), $\gamma = 25 \pm 3$ pS (SEM), and assuming a Q_{10} of 1.7 for Carb just as for ACh, this extrapolates at 25°C to $\gamma \simeq 35$ pmho, consistent with the value found for ACh. The relaxation times for carbachol are plotted against temperature in Fig. 5. The straight line in the figure is the best-fit line for the ACh data. The carbachol points (open circles) all below the line, indicating that the open duration is shorter for this agonist at all temperatures. A best-fit line to the τ values for carbachol indicates an activation enthalpy of about 42 ± 4 kcal/M.

The half-power frequencies were weakly dependent on membrane potential, suggestive of longer open time for more negative potentials. Generally, however, the po-

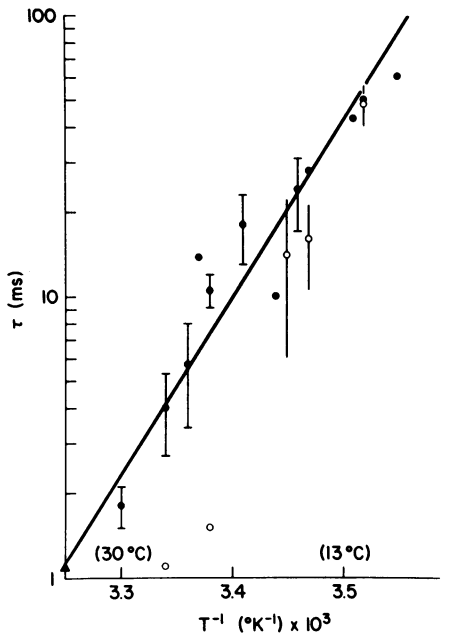

FIGURE 5 Temperature dependence of conductance relaxation time derived from drug-induced noise. Data has been averaged over all potentials (usually −40 to −100 mV). Notation as in Fig. 3. The line represents a least squares regression on the acetylcholine data (filled circles). The calculated enthalpy of activation is 30 ± 2 kcal/mol.

tential dependence was too variable to quantify. One exceptionally precise experiment done on one cell at 25°C showed a clear exponential dependence of τ on voltage with a proportionality of 70 mV/e-fold change in τ.

Extraneous ACh-Induced Noise

A major consideration in this study was to rule out extraneous noise sources that might be caused by the ACh application. One source of noise that can be troublesome in voltage-noise experiments, electrically excitable-channels opened by ACh-induced depolarization, is eliminated in voltage-clamp. However, the iontophoretic currents themselves are not eliminated in a difference experiment. Noise produced by current fluctuations in the iontophoretic pipette could be particularly insidious, since such noise would correlated with the presence of drug release.

There are several lines of evidence to suggest that in fact the observed spectra are not caused by fluctuations in the ACh iontophoretic current. The best control for the noninterference of iontophoretic current came from bringing a large drug electrode (20 Mohm) near the cell, depending only on drug leakage to stimulate the cell. The results from these experiments were consistent with the results from experiments using iontophoretic drug ejection. Moving the drug electrode 30 μm away from the cell abolished the mean current and reduced the power spectra to background levels. Re-

versing the ACh current, i.e., increasing the holding current, during the test yielded only background fluctuations. In terms of the experimental results themselves, the very steep temperature dependence of the half-power frequency strongly suggests that the differential power spectra are not the result of instrumental errors, and finally, the difference in the half-power frequencies with different transmitters points to the fluctuations arising at membrane-receptors.

DISCUSSION

The value we have obtained for the conductance of ACh channels in tissue-cultured striated muscle is 39 pS, measured at 25°C. This value is consistent with values of 25–32 pS obtained in the frog (Neher and Sakman, 1975; Colquhoun et al., 1975; Anderson and Stevens, 1973). Using our earlier data (Sachs and Lecar, 1973) and the new value of -3 mV for the reversal potential, we calculate $\gamma = 70$ pS at 36°C. This value of conductance is consistent with the extrapolated value of 63 pS from the present work. Since the total noise power and the probable density of receptors is about 100-fold less for the cultured muscle cells than for the frog endplate, the agreement of the conductance values suggests the noise does indeed arise from the transitions of independent conducting channels rather than from some domain mechanism dependent on site density.

The estimated channel conductance at 25°C, 39 pS, is between the large-pore values of the order of 100–1,000 pS found for EIM channels in lipid bilayers (Ehrenstein et al., 1970; Lecar et al., 1975), and the smaller values of the order of a few picoSiemens for the electrically excitable channels of axon membranes inferred from noise measurements (Conti et al., 1975).

The temperature dependence of the unit channel conductance is consistent with the value obtained for membrane pores in both natural (Huxley, 1959) and synthetic membranes (Latorre et al., 1974). Although the channel conductance data are scattered, the temperature dependance of the channel conductance, or more precisely, the difference between the open and closed channel conductance, is clear. This differs from the results of Anderson and Stevens (1973), where the conductance was not visibly temperature dependent. Part of the difference may lie merely in the scatter of the data, since Anderson and Stevens did not explore as wide a temperature range as we have. However, the difference may be real, and should be further investigated. Recently Anderson et al. (1976) found a Q_{10} of 2.1 for the conductance of glutamate-activated channels in locust muscle.

A striking feature of the data is the steep temperature dependence of τ. The enthalpy of activation, ΔH, with ACh as the agonist, is 30 kcal/M, corresponding to a Q_{10} of 5.3. Anderson and Stevens' (1973) work on the frog endplate indicated a Q_{10} between 2.7 and 3.6 depending on the voltage, equivalent to $\Delta H \approx 20$ kcal/mol. The difference in ΔH between the two preparations appears to be real, and may reflect differences in the membrane structure between poikliotherms and homeotherms, or the fact that one preparation is denervated and the other is not. The rate constants for

denervated muscle are slower than for innervated (Neher and Sakman, 1975; Katz and Miledi, 1972).

The difference in ΔH for channel closing between ACh and Carb also appears significant, and would not be surprising in view of the difference in rate constants with the different agonists. This temperature dependence is particularly significant in view of Catterall's (1975) result that under saturating concentrations of ACh and Carb, the drug-induced Na flux in these cells is essentially independent of temperature.

Since flux is a steady-state property, related to the number of open channels, the flux rate is related to the equilibrium constant of the channel activation process: $k = \beta/\alpha$ where β is the opening rate, and α the closing rate. For k to be independent of temperature, β must have approximately the same temperature dependence as α. This leads to a picture in which the closed and open states are of approximately the same energy, and separated by a large barrier. Further evidence from Catterall's work shows that under saturating drug doses, before desensitization, the observed flux per channel (2×10^7 ions/min at 2°C, based on determinations of the net flux and the number of α-bungarotoxin-binding sites), is only a small fraction of the 10^9/min expected from a channel conductance of 12 pS (scaled to 2°C). This implies that the channel duty cycle is always much less than one, even under saturating drug concentrations. The energy of the open state appears somewhat higher than that of the closed state, although either is much less than the free energy barrier between them. This result lends credence to the low duty cycle approximation used to drive the channel conductance from the power spectrum (see also Anderson and Stevens, 1973).

The relation giving the unit conductance as derived in the simple argument leading to Eq. 5 presupposes that a channel conductance has only two values, zero and γ. This assumption has received strong support from the experiments of Neher and Kakman (1976), in which they were able to observe the currents from individual channels in denervated frog muscle. The elementary events did, in fact, appear to have only one value of conductance in the open state.

While this paper was in press, Lass and Fischbach (1976) presented data suggesting that, in a preparation very similar to ours, the temperature dependence of γ is discontinuous. We have no clear answer for the discrepancy. However, it should be pointed out that the ratio of variance to mean (Eq. 5) is not really γ, but $\gamma(1 - \Theta)$. Denoting the ratio of variance to mean by γ', the temperature dependence of γ' is given by: $d\gamma'/dT = (1 - \Theta)\, d\gamma/dT - \gamma d\Theta/dT$ where T is temperature. Thus, changes in duty cycle, produced by temperature dependence of the rate constants, can affect the apparent channel conductance.

Smooth changes in the rate constants may lead to abrupt changes in $d\gamma'/dT$, since as shown above, $d\gamma'/dT$ depends on the difference of two temperature-dependent factors. It should also be pointed out that even when the low duty cycle approximation is valid ($\Theta \ll 1$), $d\Theta/dT$ may be large. Thus the slope of an Arrhenius plot does not necessarily signify the activation energy for ion permeation through an open channel, even if the permeation itself were described by a single barrier model.

Experimentally, the one major difference between the experiments of Lass and

Fischbach and ours are the much larger currents that they were able to use. It is possible that with the presumed higher agonist concentrations, a measurable degree of receptor saturation was taking place, i.e. an increase in Θ. Such changes might also affect $d\Theta/dT$.

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APPENDIX

Spectral distortion produced by the voltage clamp system.

In this analysis, the response times of the voltage recording system and the clamp amplifier have been ignored. This simplification is useful in that the algebra is greatly simplified, and the origin of distortion produced by stray capacity of the current electrode is more clearly demonstrated.

A schematic diagram of the simplified voltage clamp is shown in Fig. 2. In the figure we note that: V_m is the membrane potential relative to rest; R_I, C_I, and δV_I are, respectively, the resistance, stray capacitance, and rms noise voltage of the current electrode; ΔV_V is the rms noise voltage of the voltage electrode; ϵ is the reversal potential for the ACh channel; δG is the ACh-induced fluctuating conductance (the major quantity we wish to record); $Y_m = G_m + j\omega C_m$ is the membrane admittance; K is the gain of the control amplifier, and δV_c is any extraneous noise that may appear on the command voltage. (Stray capacity between the current and voltage electrodes has been ignored for simplicity.) To simplify notation, the following analysis has been done in the frequency domain. The "δ's" are the Fourier amplitudes of random time functions, and are assumed to be uncorrelated. The output voltage of the feedback system is

$$V_0 = K(V_c + \delta V_c - V_m - \delta V_v). \tag{1A}$$

But this is equal to the drop across membrane and current microelectrode,

$$V_0 = V_m + R_I I_m + \delta V_I. \tag{2A}$$

The membrane current is

$$I_m = (V_m - \epsilon)\delta G + V_m Y_m. \tag{3A}$$

Equating 1 A and 2 A, we can solve for V_m in terms of V_c and various noise sources (letting $\delta V_0 = \delta V_v + \delta V_c$).

$$V_m = \frac{K}{K+1}(V_c + \delta V_0) - \frac{1}{K+1}[R_I I_m + \delta V_I]. \tag{4A}$$

For $K \gg 1$, this becomes approximately

$$V_m \cong V_c + \delta V_0. \tag{5A}$$

There is capacitative shunt current across the walls of the current microelectrode,

$$I_s = j\omega C_I V_0. \tag{6A}$$

Thus the total current passing through the measuring device is $I_{tot} = I_m + I_s$. Substituting from Eq. 3 A, 4 A, and 6 A, we have,

$$I_{tot} = V_m Y_m + (V_m - \epsilon)\delta G + j\omega C_I V_0. \tag{7 A}$$

but from Eq. 2 A,

$$V_0 = V_m + R_I I_m + \delta V_I = V_m + R_I V_m Y_m + R_I(V_m - \epsilon)\delta G + \delta V_I.$$

Rearranging terms,

$$V_0 = V_m(1 + R_I Y_m + R_I \delta G) - R_I \epsilon \delta G + \delta V_I. \tag{8 A}$$

Substituting Eq. 8 A into Eq. 7 A,

$$I_{tot} = V_m Y_m + (Vm - \epsilon)\delta G + j\omega C_I[V_m(1 + R_I Y_m + R_1 \Delta G) - R_I \epsilon \delta G + \delta V_I]$$

Let $\tau_I = R_I C_I$ and $V_m = V_c + \delta V_0$. Dropping terms greater than first order in fluctuations and rearranging,

$$\begin{aligned} I_{tot} \approx\ & \delta G(V_m - \epsilon)(1 + j\omega\tau_I) \\ & + \delta V_I(j\omega C_I) \\ & + \delta V_0[Y_m + j\omega C_I(1 + R_I Y_m)] \\ & + V_c[Y_m + j\omega C_I(1 + R_1 Y_m)] \end{aligned} \tag{9 A}$$

The first term in Eq. 9 A represents fluctuations in the current caused by drug-induced conductance fluctuations (with a distortion factor, $j\omega\tau_I$). The second term represents current fluctuations induced by noise in the current electrode coupled through C_I. The third term represents the fluctuations in the current due to noisy inputs to the clamp, and the last term, the average total current.

To calculate the power spectrum, we square I_{tot} and calculate the expectation value of each fluctuating term. Cross products of "δ" quantities cancel since they are uncorrelated. The spectral density, ρ, of a "δ" quantity is $<\delta^2>$. Letting $\tau_m = C_m/G_m$, $\rho_{Itot} = \rho_G(V_m - \epsilon)^2(1 + \omega^2\tau_I^2) + \rho_{V_I}(\omega^2 C_I^2) + \rho_{V_0} G_m^2(1 + \omega^2\tau_m^2 + \omega^2\tau_m^2\omega^2\tau_I^2)$.

For a differential noise measurement some terms will cancel so that the power spectrum will contain the following:

$$\begin{aligned} \Delta\rho_{I_{tot}} = \rho_G(V_m - \epsilon)^2(1 + \omega^2\tau_I'^2) + (\rho_{V_I'} - \rho_{V_I})(\omega^2 C_I^2) + \rho_{V_0}[(G_m'^2 - G_m^2) \\ + \omega^4 C_m^2(\tau_I'^2 - \tau_I^2)]. \end{aligned} \tag{10 A}$$

The primed quantities refer to values during drug application, and the unprimed to control values. We have assumed that the cell's conductance and the resistance and noise of the current electrode are the only parameters likely to change significantly upon application of the drug. The worst errors will occur when the primed quantities are much larger than the unprimed, in which case:

$$\Delta\rho_{I_{tot}} < \rho_G(V_m - \epsilon)^2(1 + \omega^2\tau_I'^2) + \rho_{V_I}\omega^2 C_I^2 + \rho_{V_0} G_m'^2(1 + \omega^4\tau_m'^2\tau_I'^2) \tag{11 A}$$

It must be remembered that the ρ's may have frequency dependencies of different form.

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