

Voltage Dependence of Desensitization

Influence of Calcium and Activation Kinetics

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ABSTRACT The voltage dependence of carbachol-induced desensitization has been analyzed in potassium-depolarized frog sartorius muscle preparations with voltage clamp techniques over a wide voltage range (-120 to $+40$ mV). Desensitization developed exponentially at all voltages with τ , the time constant of desensitization onset, varying as a logarithmic function of membrane voltage. The voltage dependence of τ remained in calcium-deficient solutions and was not altered by elevating either the level of extracellular or intracellular calcium. We have analyzed our results according to a simple sequential kinetic scheme in which the rate-limiting step in the development of desensitization is a transition of the receptor channel complex from the activated conducting state to a desensitized, nonconducting state. We conclude (a) that the observed voltage sensitivity of desensitization primarily resides in the voltage dependence of this transition, and (b) the kinetics of activation appear to have a greater influence on the observed rate of desensitization than on its voltage dependence. The magnitude of the voltage dependence suggests that a greater change in free energy is required for the transition to the desensitized state than for the transition between the open and closed states of the receptor channel complex.

INTRODUCTION

When acetylcholine or other agonists are applied to the endplate region of skeletal muscle fibers, the ionic conductance of the postjunctional membrane increases rapidly as endplate receptors are activated. During prolonged application of agonist, the endplate conductance stays elevated for only a short time and then decreases. This gradual decrease of the endplate response to agonist has been described as desensitization (Thesleff, 1955). Factors such as agonist concentration, extracellular ions, and membrane voltage influence desensitization (Katz and Thesleff, 1957; Manthey, 1966; Magazanik and Vyskocil, 1970; Nastuk and Parsons, 1970; Parsons et al. 1974; Adams, 1975; Lambert et al. 1977; Scubon-Mulieri and Parsons, 1977, 1978).

Magazanik and Vyskocil (1970) initially reported that the time-course of development of desensitization is voltage dependent. In their experiments the voltage dependence was studied over a limited range of membrane potentials and without voltage clamp techniques. More recently, Lambert et al. (1977) and Scubon-Mulieri and Parsons (1978) confirmed in voltage-clamped fibers that this process is voltage dependent; desensitization occurs more rapidly at hyperpolarized membrane potentials. Calcium ions similarly increase the rate of desensitization, which suggests that the observed voltage dependence reflects a voltage dependence of calcium action on desensitization. We have investigated the interaction of these two factors on desensitization. Our results suggest that the voltage dependence of desensitization is independent of calcium and represents a direct influence of membrane voltage on the intrinsic rate-determining step of this process.

METHODS

General Methods

All experiments were performed in vitro on voltage-clamped, potassium-depolarized sartorius muscle fibers of the frog (*Rana pipiens*) (Scubon-Mulieri and Parsons, 1978). The potassium-depolarized preparation offers distinct advantages for these studies because (a) the contraction normally associated with end-plate activation is eliminated, (b) these fibers can be voltage-clamped over a wide range of membrane potentials (-120 to $+60$ mV), and (c) the inhibition of desensitization onset by Na^+ is avoided (Parsons et al., 1974).

The muscle preparations were equilibrated in an isotonic potassium solution (millimolar: K propionate, 122.5; CaCl_2 1.29; Ca propionate, 0.51) buffered to pH 7.0–7.25 with either 1 mM Tris or 1 mM HEPES. There was no noticeable difference in results with the two different buffers. All experiments were performed at room temperature (18 – 22°C). In some experiments a calcium-deficient solution was used. In this instance, magnesium (2 mM) was substituted for calcium and 1 mM EGTA was added to the solution to chelate any residual extracellular calcium. When desired, the external calcium concentration was increased by addition of solid calcium chloride to the bathing solution without compensation for the slight increase in solution osmolarity. For the ionophore experiments, X537A (Hoffman-LaRoche, Inc., Nutley, N.J.) was prepared as a solution in dimethylsulfoxide (DMSO, Fisher Scientific Co., Fairlawn, N.J.). An appropriate dilution was made such that the solution bathing the muscle contained $20\ \mu\text{M}$ X537A and 0.5% DMSO. Most experiments were done after at least 15 min of equilibration and within 120 min of placing the muscle in the solution. Exposure to the ionophore, however, was limited to 30 min. Resting membrane potentials ranged from $+5$ to -5 mV.

Endplate Localization and Drug Application

Junctional regions of individual fibers were localized with a compound microscope ($\times 300$) by following nerve fibers to the last node of Ranvier. Scubon-Mulieri and Parsons (1978) have verified the accuracy of this technique.

Carbachol (Sigma Chemical Co., St. Louis, Mo.), dissolved in the isotonic potassium solution, was microperfused onto the endplate region of individual fibers by hydrostatic pressure from a $\sim 100\ \mu\text{M}$ diameter perfusion pipette placed within $50\ \mu\text{M}$ of the intracellular electrodes (Manthey, 1966; Scubon-Mulieri and Parsons, 1978).

Voltage Clamp Measurement of Agonist-Induced Currents and Statistical Analysis

Carbachol-induced endplate currents (EPC_{carb}) were measured with point voltage clamp techniques (Takeuchi and Takeuchi, 1959; Scubon-Mulieri and Parsons, 1978). Because of the large currents recorded under our experimental conditions, membrane voltage was monitored differentially relative to an independent bath reference electrode. In some experiments we measured the currents as the voltage drop across a $1\text{ M}\Omega$ resistor in series with the current-passing electrode rather than by the virtual bath-ground current measuring system. No significant difference in the time-course of the carbachol-induced currents was observed with these two systems.

During sustained drug perfusion, carbachol-induced currents developed with a time-to-peak equal to or less than 4 s and then declined towards the preagonist level. The time constant of current decay was determined using a computerized least squares exponential fitting program (Wright, 1977). In the initial experiments the raw data were measured from photographic records and then analyzed. In later experiments the currents were recorded on a Hewlett-Packard (Palo Alto, Calif.) FM tape recorder and then digitized by a DEC PDP 8e (Digital Equipment Corp., Maynard, Mass.) computer. No difference was obtained with these two different methods of analysis. To determine the decay time constant, data points closest to the peak were eliminated and the remainder of current was fitted as a single exponential function of time (Scubon-Mulieri and Parsons, 1978).

All values are expressed as mean \pm standard error of the mean (SEM). Semilogarithmic plots of the decay time constant as a function of membrane voltage were analyzed by a least squares linear regression.

Estimate of Mean Channel Lifetime in Isotonic Potassium Propionate Solution

Miniature endplate current (MEPC) decay rates (α MEPC) were analyzed in the isotonic potassium solution to estimate the voltage dependence of mean channel lifetime. MEPCs could be recorded from individual fibers for variable lengths of time after placement in the isotonic potassium solution, but after 30–60 minutes, the MEPCs of most preparations were either absent or too small to be accurately measured (see Gennaro et al., 1978). MEPCs were recorded at different membrane potentials ranging between -80 and $+60$ mV. At each potential, 10–20 MEPCs were collected, averaged, and analyzed with the aid of a digital computer. At all voltages, MEPCs decayed with a single exponential time-course. The decay rate constant was determined by confining the analyzed portion to between 20 and 80% of the peak value.

RESULTS

Peak Carbachol-Induced Current and Time-Course of Current Decay Depend on Membrane Voltage

Microperfusion of carbachol onto the endplate region of voltage-clamped muscle fibers induced a transient EPC_{carb} superimposed on the steady-state holding current. This current developed with a time-to-peak of a few seconds and then slowly decayed towards the base line even though agonist application was continued. A typical response, obtained with $250\ \mu\text{M}$ carbachol in a fiber voltage clamped to -100 mV, is illustrated in Fig. 1 A. The decay phase of the carbachol-induced EPC was exponential over most of its time-course and could be adequately described by the following equation:

$$EPC(t) = Ae^{-t/\tau} + B, \quad (1)$$

where A is a constant, B is a constant representing the value of the final "plateau" current reached in the presence of carbachol, and τ is the time constant of current decay. The exponential nature of the decay time-course is shown in Fig. 1 B. The carbachol-induced currents did not always return to the original value of the holding current when desensitization reached its equilibrium level. The level of the plateau current was generally less than 10% of the peak current value.

Large holding currents are required to voltage clamp potassium-depolarized fibers to negative membrane potentials (Katz, 1949; Scubon-Mulieri and Parsons, 1978). Furthermore, the voltage level is not constant but falls with distance from the site of current injection, the extent of the decay depending

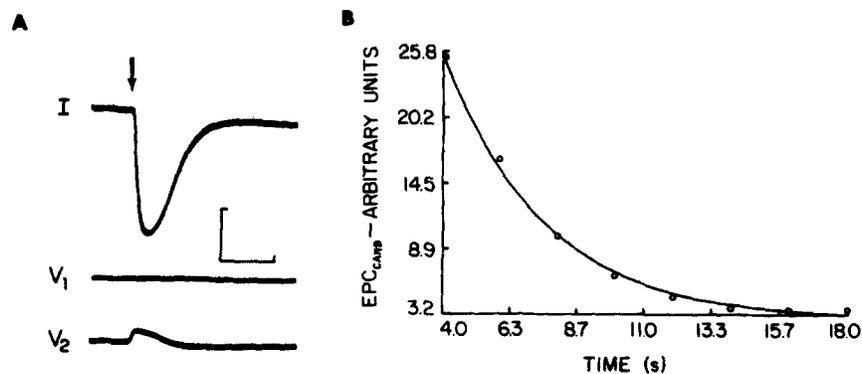


FIGURE 1. A typical EPC_{carb} during microperfusion of 250 μM carbachol in a fiber voltage clamped at -100 mV. (A) Estimate of the voltage deviation during the peak EPC_{carb} at -100 mV. Trace I is the EPC_{carb} response. Traces V_1 and V_2 show the membrane voltage recorded from the primary voltage electrode and 104 μM away, respectively. The vertical arrow indicates the onset of carbachol perfusion. (B) The decline of the EPC_{carb} expressed in arbitrary units is plotted as a function of time. The circles represent the data points (taken from trace I in A), and the solid line is drawn as a single exponential with a decay time constant of 3.5 s. Vertical calibration bar: 0.5 μA for trace I and 20 mV for traces V_1 and V_2 . Horizontal calibration bar: 15 s.

on the space constant of the fiber (1.0–1.5 mm in potassium-depolarized fibers) (Jack et al., 1975). Loss of voltage control along the fiber may be accentuated during agonist application when the endplate membrane conductance is increased, especially if the chemosensitive membrane extends a considerable distance along the fiber (Rang, 1975). In fact, Scubon-Mulieri and Parsons (1978) have shown that some additional loss of voltage control along the fiber did occur when 1,000 μM carbachol was applied to voltage-clamped endplates in potassium-depolarized fibers. However, the voltage deviation was similar at -40 and $+40$ mV even though the holding current values were very different at the positive and negative values of membrane voltage. In the present study we wanted to use a concentration of agonist which desensitized the endplate membrane to equilibrium levels rapidly

enough so that stable voltage clamp recordings could be obtained over a wide range of membrane voltages. Also, we felt it important that the voltage deviation during endplate activation be maintained at an acceptable level. Consequently, experiments were done to estimate the voltage deviation during application of 250 μM carbachol. The spatial uniformity of voltage control was estimated with a second voltage electrode inserted approximately 100 μm from the primary voltage and current electrodes (Scubon-Mulieri and Parsons, 1978). These experiments were done in fibers voltage-clamped to -100 mV. An example of the voltage deviation during carbachol application is shown in the lower portion of Fig. 1 A. The middle and lower traces indicate membrane voltage measured adjacent to the current-passing electrode (V_1) and 104 μm (V_2) from the current-passing electrode, respectively. The peak inward carbachol-induced EPC was 1.2 μA . No discernible deviation occurred at the primary voltage electrode, but a measurable depolarization, ~ 4 mV, occurred at the second electrode, V_2 . The results of four similar experiments indicated that the voltage deviation 100 μm from the current-passing electrode during carbachol activation was $<5\%$ of the driving force ($E_{\text{clamp}} - E_R$). Because desensitization produced by 250 μM carbachol reached its equilibrium level rapidly enough to maintain stable voltage clamp conditions in the range of voltages desired (Lambert et al., 1977), this concentration of carbachol was used.

PEAK CARBACHOL-INDUCED CURRENT-VOLTAGE RELATIONSHIP IS NON-LINEAR The magnitude of the peak EPC_{carb} was not a linear function of membrane potential in the range of $+40$ to -120 mV (Fig. 2). In this figure, each point represents the average value obtained from at least five different fibers voltage-clamped at the designated voltage.

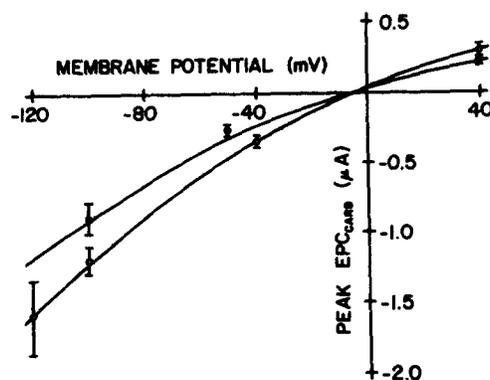


FIGURE 2. Peak EPC_{carb} plotted as a function of membrane potential. Each point represents the mean peak current \pm SEM of at least five fibers perfused with 250 μM carbachol in isotonic potassium solution. The initial holding current has been subtracted in each case. (○) Responses obtained in 1.8 mM Ca^{++} ; (●) responses obtained in the Ca-deficient solution containing 2 mM Mg^{++} and 1 mM EGTA. Lines through the data points were drawn by eye.

An estimate of the voltage dependence of peak EPC_{carb} , A , was determined from the slope of a plot of $\ln [EPC_{carb}/(V - V_r)]$ vs. V . The value of A was 0.0049 mV^{-1} . This value is similar to that reported for carbachol by others using muscles in sodium Ringer solution (Dionne and Stevens, 1975; Neher and Sakmann, 1975; Adams, 1976). However, because the rate of desensitization increases with hyperpolarization, the amount of curvature in the current-voltage relationship in our experiments may be decreased. Adams and Sakmann (1978) demonstrated that the voltage dependence is decreased with increasing concentrations of agonist. Hence we believe that the curvature in the peak EPC_{carb} -voltage relation may be underestimated. Nevertheless, the nonlinearity suggests that the influence of voltage-dependent channel gating observed in normal sodium Ringer solution (Dionne and Stevens, 1975) is also present in the isotonic potassium solution. The value of E_R , determined by interpolation, was $\sim -5 \text{ mV}$ in the isotonic potassium solution.

TIME-COURSE OF CARBACHOL-INDUCED CURRENT DECAY IS VOLTAGE SENSITIVE Scubon-Mulieri and Parsons (1978) observed that the time constant of EPC_{carb} decay (τ) of voltage-clamped potassium-depolarized fibers was smaller at -40 mV than at $+40 \text{ mV}$. We have confirmed this voltage dependence for $250 \mu\text{M}$ carbachol over a much wider voltage range, -120 to $+40 \text{ mV}$. Results that demonstrate the exponential decay and the voltage dependence of τ are presented in Fig. 3. This figure shows the time-course of carbachol-induced currents in three different muscle fibers voltage-clamped to $+40$, -40 , and -100 mV , respectively. The time constants of decay determined from the exponential fits shown in Fig. 3 B were 38 s for the fiber voltage clamped at $+40 \text{ mV}$, 13 s at -40 mV , and 5 s at -100 mV . The voltage dependence of τ is adequately described by an exponential relationship. This is illustrated by the semilogarithmic plot of τ as a function of membrane potential in Fig. 4 (●). Each data point on this graph is the average value of τ from at least three fibers and the line is a least squares regression line drawn using all the individual values at each voltage. We conclude that at least over the voltage range of -120 to $+40 \text{ mV}$ the time constant $\tau(V)$ may be described by

$$\tau(V) = \tau(0)e^{DV}, \quad (2)$$

where D is the coefficient of voltage sensitivity of τ and $\tau(0)$ is the value of τ at zero voltage. For these experiments in potassium propionate solution containing 1.8 mM calcium the estimated value of D was -0.012 mV^{-1} .

250 μM CARBACHOL PRODUCES COMPLETE DESENSITIZATION Scubon-Mulieri and Parsons (1978) reported that the equilibrium level of desensitization was similar in fibers microperfused with $1,000 \mu\text{M}$ carbachol either at -40 or $+40 \text{ mV}$. A similar result was obtained in the present study with $250 \mu\text{M}$ carbachol. We used a double perfusion procedure similar to that described previously (Scubon-Mulieri and Parsons, 1978). The $250 \mu\text{M}$ carbachol application was maintained until the current reached a plateau (after $\sim 2 \text{ min}$). Application of $1,000 \mu\text{M}$ carbachol at this time produced no obvious alteration in the current level in fibers voltage clamped either at -40 or $+40 \text{ mV}$. We concluded, therefore, that no activable receptors existed when desensitization

had reached its equilibrium level with 250 μM carbachol, and that desensitization is equally complete at both -40 and $+40$ mV.

Voltage Dependence of Desensitization Onset Does Not Require External Calcium

Calcium accelerates the rate of carbachol-induced desensitization (Manthey, 1966; Parsons, 1978). Further, Nastuk and Parsons (1970) suggested that the

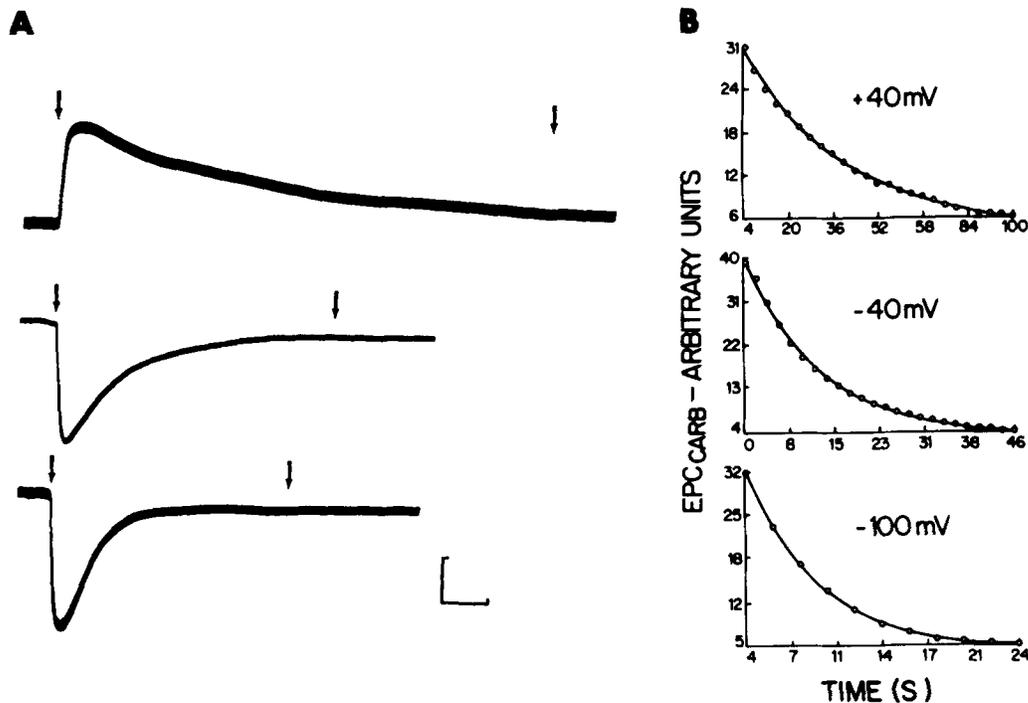


FIGURE 3. (A) Examples of EPC_{carb} measured in three voltage-clamped potassium depolarized muscle fibers during microperfusion of 250 μM carbachol. Holding voltage of individual records was $+40$ mV (upper), -40 mV (middle), and -100 mV (lower). Vertical arrows indicate the onset and termination of carbachol perfusion. Outwardly directed current is indicated by an upward deflection and inward current by a downward deflection. (B) The decline of the respective EPC_{carb} records shown in A expressed in arbitrary units and plotted as a function of time. The time constants of decay are $+40$ mV, 38 s; -40 mV, 13 s; and -100 mV, 5 s. Vertical calibration bar: $0.05 \mu\text{A}$ for the upper, $0.1 \mu\text{A}$ for the middle, and $0.5 \mu\text{A}$ for the lower traces, respectively. Horizontal calibration bar: 15 s.

site of calcium action is located on the internal surface of the postjunctional membrane. Normally the level of intracellular ionized calcium is low so that there is a large inward electrochemical gradient for calcium. The electrochemical driving force ($E_{\text{Ca}} - E_M$) should increase with hyperpolarization. Therefore, the acceleration of desensitization with hyperpolarization may reflect an acceleration of the process caused by increased calcium entry through agonist-

activated endplate channels as the membrane potential is made more negative (Parsons, 1978). We have addressed this possibility in a series of experiments in which activation-desensitization was studied using muscles maintained in a calcium-deficient, isotonic potassium solution containing 1 mM EGTA and 2 mM magnesium. Different fibers were voltage clamped to -100 , -50 , or $+40$ mV. Endplate currents produced by $250 \mu\text{M}$ carbachol in the calcium-deficient solution were similar to those observed in the presence of calcium. The EPC_{carb} rose rapidly to a peak and then decayed exponentially to a

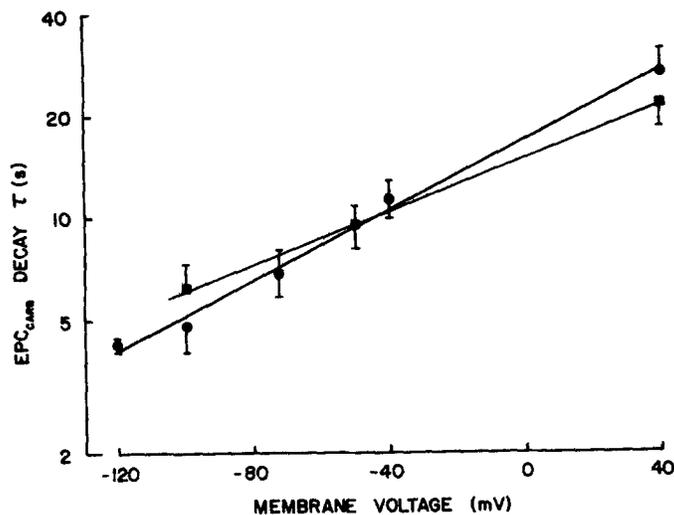


FIGURE 4. The decay time constant for EPC_{carb} (τ) is plotted semilogarithmically as a function of membrane voltage for calcium-containing (1.8 mM) (●) and calcium-deficient (▲) isotonic potassium solution. Each point represents the mean \pm SEM of at least three fibers voltage clamped at the designated potentials. The data were fit using Eq. 2 with values indicated in the text.

plateau level. The peak EPC_{carb} values obtained in the calcium-deficient solution were slightly smaller at comparable voltages than those observed in the presence of calcium; however, the curvature of the EPC_{carb} -voltage relationship was similar (Fig. 2). At -100 mV we observed a progressive increase in the amount of current required to voltage clamp some fibers maintained in the calcium-deficient solution. Consequently, the final plateau current during carbachol application did not always return to the initial base-line and plateau currents between 10 and 15% of the peak current value were frequently observed. However, in spite of this difficulty, it was readily apparent that desensitization remained voltage dependent in the calcium-deficient solution. The coefficient of voltage dependence, D , for the fibers maintained in the calcium-deficient solution was -0.0092 mV^{-1} ; a value not markedly different from that obtained in the presence of calcium (-0.012 mV^{-1} , Fig. 4, ●). Our

results indicate that the voltage dependence of desensitization does not require external calcium and does not result from an enhanced calcium influx during endplate activation at hyperpolarized values of membrane potential.

Elevation of the External or Internal Calcium Concentration Does Not Modify the Voltage Dependence of Desensitization

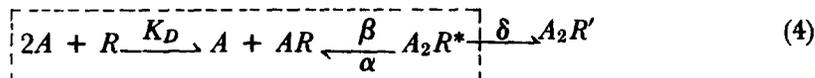
Calcium and voltage each independently change the intrinsic rate of desensitization. Consequently, we have investigated the interaction of these two factors on desensitization. Two series of experiments were done. In the first, the external calcium concentration was raised to 9.0 mM and the voltage dependence of EPC_{carb} decay was estimated over a voltage range of -80 to $+40$ mV. In the second group of experiments, muscles were pretreated with the calcium ionophore X537A (Pressman, 1972; Scarpa et al., 1972). DeBassio et al. (1976) demonstrated that X537A accelerates desensitization, presumably because the ionophore raises internal calcium (Mobley, 1977). We determined the voltage dependence of EPC_{carb} decay in the ionophore-treated muscles over a voltage range of -80 to $+40$ mV. In preliminary experiments we determined that DMSO, the solvent used for the ionophore, had no influence on the rate of desensitization (see also DeBassio et al., 1976). The results with elevated external calcium and ionophore treatment were compared to those obtained in muscles maintained in 1.8 mM calcium.

Raising external calcium and treatment with X537A each increased the rate of desensitization over that observed in the presence of 1.8 mM calcium. In all three solutions, the time-course of EPC_{carb} decay was adequately described by a single exponential function of time at all voltages. The average values of τ obtained at each voltage in elevated calcium and in the ionophore solution were consistently smaller than values in the control 1.8 mM calcium group. However, the differences were small and variability such that only the values at -40 mV in the ionophore and elevated calcium solutions were significantly different ($P < 0.05$) from the control values (1.8 mM calcium). In all three cases, τ was an exponential function of voltage. The slope of the $\ln \tau$ vs. voltage relationship (Fig. 5) was determined by a least squares regression line drawn through each set of data points. The coefficient of voltage dependence of desensitization, D (Eq. 2), was -0.0109 mV $^{-1}$ for muscles maintained in 1.8 mM calcium, -0.0109 mV $^{-1}$ for fibers maintained in 9.0 mM calcium, and -0.0098 mV $^{-1}$ for those preparations pretreated with the ionophore. These results demonstrate that elevating external calcium or treatment with X537 increased the rate of desensitization without changing the voltage dependence of this process.

Voltage-Dependent Channel Gating May Contribute to the Observed Voltage Dependence of Desensitization Onset

The cyclic model of desensitization, proposed by Katz and Thesleff (1957) adequately describes the desensitization onset-recovery process (Rang and Ritter, 1970; Gage, 1976; Steinbach and Stevens, 1976; Scubon-Mulieri and

Further, these authors observed that at equilibrium with sustained agonist exposure no activable receptors remained. We have used procedures similar to those of Scubon-Mulieri and Parsons (1978) in the present study and no appreciable recovery was evident when desensitization had developed to its equilibrium level. Since no significant recovery occurred under our experimental conditions the kinetics of development of desensitization can be described by the following sequential scheme.



We have modified Eq. 4 to account for recent evidence which suggests that the channel opens as the receptor binds the second in a sequence of two agonist molecules with each binding step having equal equilibrium affinities (Dionne et al., 1978). This model assumes that (a) the desensitized nonconducting state, A_2R' , develops only from the activated conducting state, A_2R^* ; (b) additional agonist binding steps are not required for this transition; and (c) the enclosed portions of the kinetic scheme occur rapidly relative to the onset of desensitization. We have chosen to analyze this simple sequential scheme because, to our knowledge, there is no experimental evidence to suggest that alternative, more complex, schemes are more appropriate. Consequently, under the conditions of our experiments, the transition from the activated to the desensitized state represents the rate-limiting step for the development of desensitization. The voltage dependence of desensitization would reflect a voltage dependence of δ as described by $\delta(V) = \delta(0)e^{D'V}$, where D' represents the coefficient of voltage dependence for desensitization.

The sequential model described by Eq. 4 predicts that the experimentally observed onset rate of desensitization, k_{obs} , and its voltage dependence reflect a contribution from both activation and desensitization kinetic parameters according to the following relationship (Pallotta, 1978):

$$k_{\text{obs}}(V) = \frac{\delta(0)e^{-D'V}}{1 + \frac{\alpha(0)e^{AV}}{\beta} \left[\frac{1}{[c]} + \frac{K_D}{[c]^2} \right]}, \quad (5)$$

where $\delta(0)e^{-D'V}$ represents the voltage-dependent rate constant of desensitization onset; $\alpha(0)e^{AV}$ describes the voltage-dependent channel open time; β is the rate constant of channel opening; $[c]$ is the agonist concentration; and K_D is the dissociation constant for the agonist-receptor complex.

In the present study the time constant of agonist-induced current decay has been measured experimentally (Fig. 1). With sustained agonist application, the magnitude of the EPC_{carb} is some function of the number of conducting A_nR^* complexes at any defined time. Anderson and Stevens (1973) have reported previously that the kinetic properties of individual channels are not altered as desensitization develops. Therefore, the gradual decline of the EPC_{carb} after the peak reflects the time-dependent transition of conducting receptor channel complexes, A_nR^* , into the nonconducting desensitized state,

$A_n R'$. Given that recovery from the desensitized state proceeds considerably slower than its onset, then the operationally defined K_{obs} for desensitization should be approximated by τ^{-1} , the reciprocal of the time constant of decay of agonist-induced currents.

Numerical analysis of Eq. 5 was used to estimate the possible contribution of voltage-dependent channel gating of K_{obs} and its observed voltage dependence.

Each of the single-channel parameters, K_D , α , and β may be voltage sensitive and could contribute to the observed voltage dependence of desensitization. Since β has been reported to have little voltage sensitivity relative to α at the frog endplate (Magleby and Stevens, 1972 *a, b*; Dionne and Stevens, 1975) and in the electroplaque (Sheridan and Lester, 1977), we would not expect β to contribute significantly to our observed voltage dependence. The voltage dependence of K_D is unknown and we have assumed that the voltage dependence of gating is primarily reflected in the voltage dependence of α (Anderson and Stevens, 1973).

We felt it necessary to estimate the voltage dependence of mean channel lifetime under the conditions of our experiments, i.e., in the isotonic potassium propionate solution, because Van Helden et al. (1977) and Gage and Van Helden (1979) observed quantitative differences in endplate channel lifetime and conductance when external sodium was replaced by other monovalent cations. Therefore, MEPC decay rates (α MEPCs) were analyzed to estimate mean channel lifetime (Anderson and Stevens, 1973). The relationship between α MEPCs and membrane potential was exponential and yielded a value for the coefficient of voltage dependence, A , of $0.0041 \pm 0.0005 \text{ mV}^{-1}$ (mean \pm SE) at temperatures ranging between 18 and 21.5°C. This value is slightly less than that reported previously for muscles in normal sodium solution at 18°C (Anderson and Stevens, 1973; Dionne and Stevens, 1975; Adams and Sakmann, 1978). The MEPC decay at identical voltages was faster in the potassium solution than in sodium solution. Similar results in potassium solutions have been reported (Linder and Quastel, 1978; Gage and Van Helden, 1979). The value of $\alpha(0)$ obtained from MEPC decay in muscles maintained in the isotonic potassium solution was 1.57 ms^{-1} .

Mean channel lifetime differs with different agonists but its coefficient of voltage dependence does not (Katz and Miledi, 1973). The value of τ for carbachol is ~ 0.75 that for acetylcholine in the normal sodium containing solution. We assumed that the duration of channel lifetime with carbachol also is 0.75 of that for acetylcholine in the isotonic potassium solution. Therefore, the value of $\alpha_{carb(0)}$ used in our analysis was 2.1 ms^{-1} with the coefficient of voltage dependence, A , equal to 0.0041 mV^{-1} .

Fig. 6 illustrates the contribution of voltage-dependent activation to the voltage dependence of desensitization. Predicted values of δ (●) are plotted as a function of voltage according to Eq. 5, which includes a term for the voltage dependence of activation. For comparison values of δ are plotted at comparable voltages assuming that all of the voltage dependence resides in the transition to the desensitized state: $\delta(v) = k_{obs}(0)e^{-DV}$. In this analysis the

following experimentally obtained values were substituted into Eq. 5; $\delta(0) = 0.067 \text{ s}^{-1}$; $D = 0.0109 \text{ mV}^{-1}$; $\alpha(0) = 2.1 \text{ ms}^{-1}$; $A = 0.0041 \text{ mV}^{-1}$; $c = 250 \text{ } \mu\text{M}$. The values of K_D ($250 \text{ } \mu\text{M}$, Dionne et al. 1978) and β ($2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) were obtained from the literature with the assumption that they are not altered in the isotonic potassium solution. The value used for the opening rate constant was 5.0 ms^{-1} . This was obtained from Sakmann and Adams (1978) assuming a Q_{10} for β of 3.0.

This analysis (Fig. 6) demonstrates that under our experimental conditions the observed rate of desensitization but not its voltage dependence is markedly

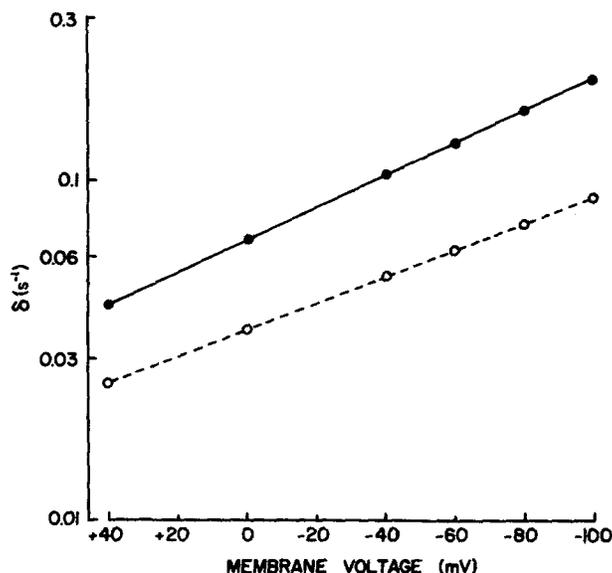


FIGURE 6. An estimate of the contribution of the voltage dependence of activation to the voltage dependence of desensitization. Solid line is drawn through data points calculated based on the assumption that $\delta(V) = k_{\text{obs}}$, which assumes that all the voltage dependence resides in the transition to the desensitized state. The line through the open circles is drawn according to Eq. 2 and is a theoretical plot describing the simple sequential scheme discussed in the text. See text for specific values.

altered by the influence of activation kinetics. Our k_{obs} overestimated δ by $\sim 46\%$ at 0 mV membrane potential. This difference increased with hyperpolarization such that at -100 mV k_{obs} differed from the predicted value by 56%. Values of the coefficient of voltage dependence obtained from the slope were -0.010 mV^{-1} for k_{obs} and -0.009 mV^{-1} obtained from the kinetic analysis. These values indicate that the voltage dependence of activation contributes $\sim 10\%$ to the observed voltage dependence of desensitization.

The channel opening rate constant, β , has been reported to have concentration dependence, but little voltage dependence (Dionne and Stevens, 1975). If β is concentration dependent, then according to Eq. 5, the contribution of

$\alpha(0)e^{AV}/\beta$ to k_{obs} would be a function of agonist concentration. We tested this prediction experimentally by comparing the voltage dependence of τ at two carbachol concentrations (250 and 1,000 μM). These experiments were done at membrane voltages of -40 and $+40$ mV and are depicted in Fig. 7. Although the τ was significantly decreased with 1,000 μM carbachol, the voltage dependence of τ was not significantly different at the two concentrations of carbachol. These results suggest that a concentration dependence of channel gating does not contribute to the observed voltage dependence of desensitization onset.

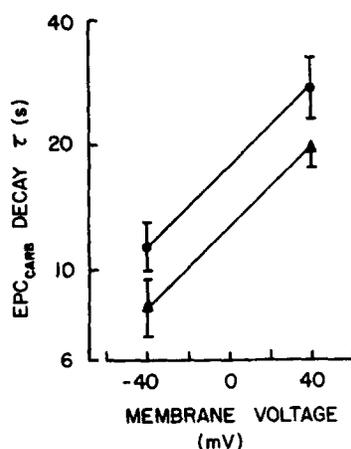


FIGURE 7. The affect of agonist concentration on the time constant of EPC_{carb} decay (τ) and its relation to the observed voltage sensitivity. The decay time constant of EPC_{carb} is plotted semilogarithmically as a function of membrane potential for fibers voltage clamped at $+40$ and -40 mV in isotonic potassium solution. EPC_{carb} responses were obtained from fibers microperfused with either 250 μM (●) or 1,000 μM (▲) carbachol. Each point represents the mean \pm SEM of at least seven fibers.

DISCUSSION

Our results demonstrate that desensitization onset exhibits a marked voltage dependence when analyzed over a wide range of membrane potentials. The voltage dependence of desensitization does not require external calcium and appears independent of elevated calcium levels or agonist concentration, both of which affect the rate of development of desensitization. Our kinetic analysis, however, suggests that voltage-dependent receptor channel activation contributes to the experimentally observed rate of desensitization and its estimated voltage sensitivity. According to the sequential model this contribution to the voltage dependence is small and the major component of the voltage dependence resides in the voltage sensitive transition step from the activated A_nR^* state to the desensitized, A_nR' state.

These experiments were done using potassium-depolarized frog sartorius muscle fibers. Kuno et al. (1971) and McMahon et al. (1972) reported that the endplate region of this preparation extends hundreds of micrometers along the fibers. Our estimates were less (Scubon-Mulieri and Parsons, 1978). If the endplate regions are long, some degree of nonuniformity of agonist concentrations as well as some additional loss of voltage control may have occurred, and this could introduce errors in our estimates of the time-course of desensitization. However, we doubt that such problems markedly influenced our results. We have observed a similar voltage dependence of desensitization when carbachol is applied to snake fast muscle fibers maintained in an isotonic potassium propionate solution.¹ Neuromuscular junctions in the snake preparation are 50–70 μM long—much shorter than the fiber space constant (~ 1 mm) (Ridge, 1971). Agonist concentration during microperfusion is therefore uniform over the entire endplate region and effective voltage control is maintained.

Elevation of external calcium increased the rate of desensitization without altering the voltage dependence (Fig. 5). We did not observe as marked an acceleration of desensitization by calcium as reported previously for the potassium-depolarized preparation (Manthey, 1972; Scubon-Mulieri and Parsons, 1977). Several major differences in experimental conditions may account for this discrepancy. In the previous work the agonist concentration was considerably higher (1–3 mM) than the concentration used in our experiments. In addition, the previous studies were not done on voltage-clamped muscle fibers. Manthey (1972) observed that the slope of the relationship between calcium concentration and desensitization half-time was similar at three concentrations of external potassium. Rearranging Manthey's data (1972; see Fig. 4) and plotting half-time vs. membrane potential (using values of membrane potential stated in the paper for the three external potassium concentrations), we obtained a relationship similar to that seen in (Fig. 5). Elevation of external calcium accelerated desensitization without changing its voltage dependence. Our results do not rule out the possibility of a calcium-potassium competition as suggested by Manthey (1972); however, we suggest that these previous studies may be better interpreted in terms of the marked influence of membrane potential on the rate of desensitization.

The receptor-channel complex resides within the membrane and is therefore sensitive to the existing membrane field. Consequently, different conformational states would be expected to appear and disappear with rates dependent on the value of the membrane potential. We propose that the transition of the receptor-channel complex from the activated, conducting state to the desensitized state, $A_nR^* \xrightarrow{\Delta} A_nR'$, reflects the kinetics of a conformational change in the receptor channel complex, and that this conversion is governed by the orientation of voltage-sensitive macromolecules in the membrane. Our treatment of the transition rates between states is therefore formulated in terms of protein-membrane field interaction as used previously to describe the kinetics of receptor channel activation (Magleby and Stevens, 1972 *b*, Anderson and

¹ Fiekers, J. F., and R. L. Parsons. Unpublished observations.

Stevens, 1973; Stevens, 1978). A basic assumption is that each individual complex is inactivated in an "all or none" fashion as desensitization develops so that current decay reflects the progressive decrease in the number of activated complexes rather than a continuum of change in α or γ of individual complexes. Evidence in support of this assumption is derived from the observation that acetylcholine-induced current fluctuations do not change in form as desensitization progresses (Anderson and Stevens, 1973). In addition, ion selectivity is not altered at partially desensitized endplates (Koester, 1971; Lambert et al., 1977; Katz and Miledi, 1977).

With the assumption of distinct conformational states, it is reasonable to suggest that an energy barrier must be overcome to complete the transition between states. Further, if each state has a dipole moment, then a voltage-sensitive component of the free energy is expected. The change in dipole moment between the receptor molecule in its open and transition state will result in unequal changes in the difference in free energy, μ , with voltage. This would lead to a voltage-sensitive reaction rate (Magleby and Stevens, 1972 *b*; Stevens, 1978). With the assumptions outlined by Magleby and Stevens (1972 *b*) and Stevens (1978), we estimate the value of μ , the difference between the two states in dipole moment normal to the field strength, for the transition of $A_nR^* \xrightarrow{\Delta} A_nR'$ to be 72.7 *D*. Since this value is ~ 1.5 times that estimated for the transition between the open and closed states of the receptor channel complex, (Magleby and Stevens, 1972 *b*), it suggests that a greater difference in free energy exists for the transition of this complex to a desensitized state.

Our results predict that a small component of the experimentally derived voltage dependence of desensitization is contributed by voltage-dependent activation. This conclusion is based on an analysis of a sequential kinetic scheme. We realize that the accuracy of this analysis and further quantitation depend on the validity of the assumptions made in the development of this scheme as well as the substituted values. This analysis indicates, however, that the voltage dependence of activation could contribute to the overall voltage dependence of desensitization and must be considered in any appropriate scheme.

Even at the most hyperpolarized values of membrane potential used in this study, the molecular transition to the desensitized state occurred very slowly relative to channel gating. Therefore, we do not consider desensitization to be a major factor in normal synaptic functioning at the neuromuscular junction and expect that it can only be observed under conditions of prolonged agonist application.

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