Desensitization Onset and Recovery at the Potassium-Depolarized Frog Neuromuscular Junction Are Voltage Sensitive

BERTHANN SCUBON-MULIERI and RODNEY L. PARSONS

From the Department of Physiology and Biophysics, University of Vermont College of Medicine, Burlington, Vermont 05401

ABSTRACT The influence of voltage on the time-course of desensitization onset and recovery has been studied at the frog neuromuscular junction. The activation-desensitization sequence was determined from carbachol-induced end-plate currents in potassium-depolarized fibers voltage-clamped either to −40 mV or +40 mV. The time-course of both desensitization onset and recovery developed exponentially, with onset occurring more rapidly than recovery. Desensitization onset was voltage dependent, the onset time constant being 8.3 ± 1.3 s (11 fibers) at −40 mV and 19.3 ± 3.4 s (15 fibers) at +40 mV. Recovery from desensitization was also influenced by voltage. The extent of recovery after 2 min was 80.4 ± 6.3% in those fibers voltage-clamped to −40 mV and 57.4 ± 3.6% in those fibers voltage-clamped to +40 mV. The voltage dependence of desensitization onset and recovery did not result from a difference in ability to control voltage at these two levels of membrane potential. These results demonstrate that in the potassium-depolarized preparation the processes controlling both desensitization onset and recovery of sensitivity from the desensitized state are influenced by membrane voltage.

INTRODUCTION

When acetylcholine or other depolarizing compounds are applied to the end-plate region of skeletal muscle fibers, the postjunctional membrane undergoes a rapid increase in ionic conductance which is slowly reversed by desensitization of the postjunctional membrane if the agonist application is sustained (Thesleff, 1955). Both desensitization onset and the recovery of sensitivity from the desensitized state proceed exponentially with onset being considerably faster than recovery (Scubon-Mulieri and Parsons, 1977*a*). Many factors such as agonist type and concentration, extracellular ionic concentrations, and membrane voltage are known to alter desensitization onset (Thesleff, 1955; Katz and Thesleff, 1957; Manthey, 1966, 1970, 1972; Rang and Ritter, 1970; Nastuk and Parsons, 1970; Magazanik and Vyskocil, 1970, 1975; Parsons et al., 1974; Adams, 1975). In contrast, the recovery from desensitization appears to be relatively independent of these manipulations (Katz and Thesleff, 1957; Magazanik and Vyskocil, 1975; Scubon-Mulieri and Parsons, 1977*a*).

Magazanik and Vyskocil (1970) initially reported that desensitization onset was

voltage dependent. Later these authors reported that the recovery from desensitization was not influenced by membrane voltage (Magazanik and Vyskocil, 1975). In contrast, Steinbach and Stevens (1976) suggested that recovery from desensitization may be voltage sensitive with recovery rate decreasing with hyperpolarization. Preliminary experiments from this laboratory (Scubon-Mulieri and Parsons, 1977*b*) on the onset of and recovery from desensitization in potassium-depolarized fibers also indicated that both processes are voltage sensitive. Consequently, the present study was undertaken to compare the influence of voltage on desensitization onset and recovery at voltage-clamped frog neuromuscular junctions.

These results confirm those of Magazanik and Vyskocil (1970) that the onset of end-plate desensitization is accelerated by hyperpolarization but are in conflict with Magazanik and Vyskocil (1975) who reported that recovery of sensitivity is not voltage dependent. Our results on recovery also differ from those of Steinbach and Stevens (1976) in that we find that recovery is more rapid at −40 than at +40 mV.

METHODS

General Methods

All experiments described in this report were performed in vitro on potassium-depolarized sartorius muscle preparations of the frog (*Rana pipiens*) at temperatures ranging between 13 and 17°C during the period of April to November. The potassium-depolarized preparation was utilized in these studies because (*a*) the contraction normally associated with end-plate activation is eliminated, (*b*) these fibers could be readily voltage-clamped to both negative and positive values of membrane potential (−40 and +40 mV), and (*c*) the inhibition of desensitization onset by Na^+ is avoided (Parsons et al., 1974). In polarized fibers maintained in a sodium Ringer solution, the relative contribution of inward Na^+ current and outward K^+ current to the total carbachol-induced end-plate current is a function of the driving force on the two ions i.e., $(E_m - E_{Na})$ and $(E_m - E_K)$, where E_m is the membrane potential and E_{Na} the equilibrium potential for sodium and E_K the equilibrium potential for potassium. In the potassium-depolarized preparation, only K^+ currents flow (excluding any slight Ca^{2+} contribution) at all potentials thus eliminating any complication which would arise from the voltage-dependent contribution of sodium to the agonist-induced current.

The muscle preparations were equilibrated in the isotonic potassium solution (mM: K propionate, 122.5; $CaCl_2$, 1.29; Ca propionate, 0.51; Tris, 1.0; pH, 6.95–7.05) for 30 min before beginning the experiments, and all experiments were done within 120 min of placing the muscle in the solution. The muscles were kept in a 12-ml bath and continually perfused with fresh bathing solution (50 ml/min flow). Resting membrane potentials ranged from +5 to −5 mV.

Carbachol (1 mM, Sigma Chemical Co., St. Louis, Mo.) dissolved in the isotonic potassium solution was microperfused onto the end-plate region of individual fibers by hydrostatic pressure from a 100-μm diameter pipette placed within 50 μm of the intracellular micropipettes (Manthey, 1966).

End-Plate Localization

Junctional regions of individual fibers were localized visually with a compound microscope (×300) by following nerve fibers to the last node of Ranvier. Close proximity of the microelectrodes and agonist perfusion pipette to the end-plate region was considered a

critical requirement for these studies, because mislocation would be expected to alter the applied dose reaching the end-plate region. Consequently, independent experiments were performed to verify the precision with which the end-plate region could be located with our optical system.

In 10 separate experiments this end-plate localization method was tested by combining it with a cholinesterase stain perfusion. The neuromuscular junction was located visually, and the fiber was impaled in this region. With an electrode implanted, the fiber was perfused through the perfusion-pipette with the potassium propionate solution containing a cholinesterase stain (Koelle and Friedenwald, 1949). In 10 out of 10 such experiments, the microelectrode was inserted into a region which subsequently showed cholinesterase activity by staining. The mean length of the staining pattern in these fibers was 96 ± 42 μm (mean ± SD).

After 2–5 min of perfusion, cholinesterase staining of the impaled end-plate was complete. Continued perfusion for 10 min or longer produced no further staining at this end-plate, but staining of end-plates on other fibers many millimeters away began to appear. This demonstrated that our perfusion adequately covered the end-plate under observation and that nonjunctional regions were not stained. The stain pattern and position of the electrode within the stained region was photographed.

Voltage Clamp Measurement of Agonist-Induced Current

Carbachol-induced end-plate currents (EPC_{carb}) were measured with point voltage clamp techniques (Takeuchi and Takeuchi, 1959) utilizing a two microelectrode voltage clamp system similar to that described previously by Gibbons and Fozzard (1971).

In this system voltage was monitored by a high input impedance, 10-times' magnification electrometer (Industrial Science Associates, Ridgewood, N. Y.), and the signal was further amplified five times using an operational amplifier (model 48K, Analog Devices, Inc., Norwood, Mass.). This signal was compared with the command signal at the summing junction of a control amplifier (Philbrick, model 1022, Teledyne Philbrick, Dedham, Mass.). Any difference between the end-plate membrane potential and the command potential produced an error signal to the control amplifier which supplied current sufficient in sign and magnitude to negate the error signal. The current required to clamp the end-plate membrane was recorded from a current sensing operational amplifier (model 48K, Analog Devices, Inc.) in series with a silver:silver chloride bath electrode. The current amplifier produced a voltage proportional to the current through the end-plate membrane.

Since we have shown that desensitization onset is altered by prior activation of the end-plate membrane (Scubon-Mulieri and Parsons, 1977*a*), only one fiber was studied in an individual preparation. Therefore, the results obtained from different fibers were averaged, and the study was limited to two values of membrane potential (−40 or +40 mV).

The steady-state holding current in the absence of carbachol was greater at the negative voltage than at the positive voltage; the values being 239 ± 57 nA at −40 mV and 64 ± 13 nA at +40 mV. This observation is consistent with that reported previously for potassium-depolarized fibers (Katz, 1949). In most experiments, both the voltage and current electrodes were filled with 3 M KCl and had resistances ranging from 5 to 15 MΩ and 2 to 5 MΩ, respectively. In a few experiments 2 M K citrate-filled current electrodes were used. No difference in results was obtained with either type of current electrode.

Measurement of Postjunctional Membrane Activation, Rate of Desensitization Onset, and Recovery of Sensitivity

The peak value of the carbachol-induced current was taken as an index of the extent of end-plate activation. The time-course of desensitization onset during sustained perfusion

of carbachol was determined from the time constant of the EPC_{carb} decline after the initial peak. Only agonist-induced currents having a time-to-peak value equal to, or less than, 4 s were considered acceptable. In addition, no data were used from fibers in which the holding current was not constant before carbachol perfusion.

The recovery of sensitivity after desensitization was determined by the following procedure. The end-plate activation-desensitization sequence was induced by local application of carbachol. After desensitization was fully developed, i.e., when the EPC_{carb} had plateaued, the perfusion was terminated by raising the perfusion pipette, and the end-plate was allowed to recover for a variable interval, after which time carbachol (at the same concentration) was reapplied, and the test EPC_{carb} was measured. The extent of recovery of sensitivity after desensitization was determined by expressing the test response, EPC_2, as a percent of the magnitude of the initial response, EPC_1, (see Fig. 3).

Statistical Analysis

The time constant of current decay was determined by fitting the raw data using a computerized nonlinear optimization method (Wright, 1977). During carbachol application, the EPC_{carb} initially increases, remains at a peak value which is briefly sustained in some preparations, and then declines as desensitization develops. During the early phase of the response, we believe that agonist concentration is equilibrating so that both activation and desensitization occur; after this the response declines as a single exponential function of time. To determine the decay time constant data, points closest to the peak were successively eliminated until the early nonrandom deviation from the exponential fitted to the whole curve disappeared.

Statistical significance was determined using Student's t test, values of $P \leq 0.05$ being regarded as significant.

RESULTS

Concentration of Carbachol along the End-Plate

The rate of development and equilibrium level of desensitization are dependent on agonist concentration (Katz and Thesleff, 1957; Nastuk and Parsons, 1970; Adams, 1975). Therefore, it was important first to establish the extent to which the concentration of carbachol was uniform along the end-plate membrane with the microperfusion technique.

A quaternary ion-sensitive electrode (Dionne, 1976) was used to determine the concentration of carbachol as a function of distance from the perfusion pipette. The ion-sensitive electrode was first "calibrated" by perfusing various concentrations of carbachol (dissolved in the potassium propionate solution) onto the electrode at a fixed distance of 80 μm. As reported by Dionne, the response is linear up to ~10 mV (Fig. 1B), a response which was generated by 1 mM carbachol in these experiments. The concentration profile with 1 mM carbachol along a muscle fiber was then determined as follows. The ion-sensitive electrode was initially positioned so that it would be 80 μm away from the agonist perfusion pipette when it was lowered through the fluid interface to the same horizontal plane as the electrode. When the electrode response reached a steady state, the perfusion pipette was raised out of the bathing solution, and the carbachol was allowed to diffuse away. When the ion-sensitive electrode response had returned to the control value, it was moved away in 80 μm increments along a line parallel to the fiber. The fiber was then reperfused. Fig.

1A shows the amplitude of the response of the quaternary ion-sensitive electrode as a function of distance between the electrode and perfusion pipette in one of these experiments. In Fig. 1A is also plotted the time to reach 95% of peak amplitude of this response as a function of distance. Unlike the amplitude which is relatively constant up to 560 μm, the rise time increased precipitously at distances greater than 480 μm. The experiment demonstrates that, with the microperfusion method, agonist concentration equilibrates rapidly and uniformly along a distance greater than that of the average end-plate length which was determined by cholinesterase staining.

This spatial uniformity of concentration depends on the diameter of the perfusion pipette. For instance, in another experiment with the ion-selective electrode, the fiber was first perfused using a perfusion pipette having a tip of 88 μm. With this perfusion pipette the concentration fell sharply after 240 μm.

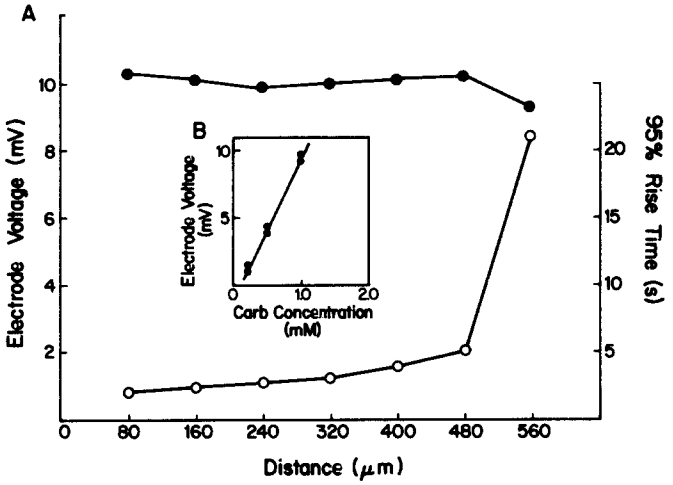


FIGURE 1. Spatial distribution of the carbachol concentration during microperfusion using a quaternary ion-sensitive electrode (QISE). (A) The peak electrode response (●) and 95% rise time (○) plotted as a function of the distance between the QISE and the edge of the perfusion pipette. (B) The dose-response relation of the QISE to carbachol with a constant separation of 80 μm.

However, when the tip was enlarged to 100 μm, the concentration remained uniform for a distance up to 520 μm between the perfusion pipette and quaternary ion electrode. For the present study, the diameter of the perfusion pipettes was always greater than 100 μm.

Comparison of Desensitization Onset and Recovery in Voltage-Clamped Fibers

Scubon-Mulieri and Parsons (1977*a*) have demonstrated in unclamped, potassium-depolarized fibers that both desensitization onset and recovery proceed exponentially with onset being faster than recovery. The first experiments were done to establish that a similar relationship holds for voltage-clamped, potassium-depolarized muscle fibers. For these experiments the fibers were voltage-clamped to +40 mV. The positive potential was chosen because the holding

current required was less at this potential than at −40 mV so that the fibers could be voltage-clamped without any noticeable deterioration for the prolonged duration of these experiments. The results of one of these experiments is summarized in Fig. 2.

Microperfusion of 1 mM carbachol onto the end-plate region of voltage-clamped muscle fibers induced a transient end-plate current (EPC_{carb}) superimposed on the holding current. This current developed with a time-to-peak of 3 s, reached a peak value of 806 nA, and then slowly subsided toward the pre-agonist level even though the agonist application was continued. The rate of

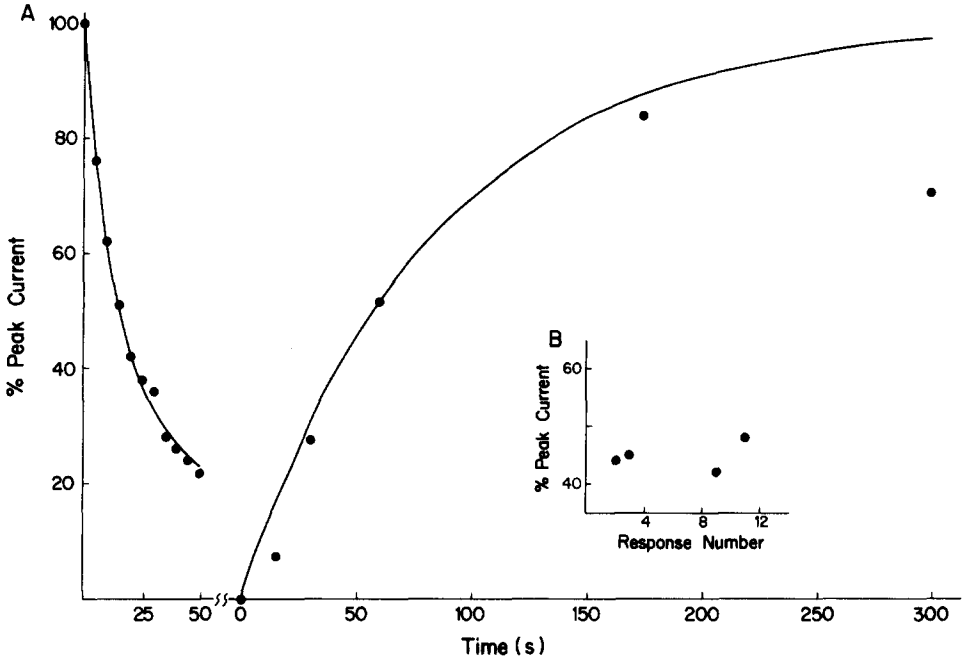


FIGURE 2. (A) Time-course of desensitization onset (curve on left) during the initial carbachol exposure and the time-course of recovery of sensitivity (curve on right). The time scale is identical for desensitization onset and recovery. The time constant for desensitization onset was 12 s whereas that for recovery of sensitivity was 84 s. (B) Peak value of EPC_{carb} (expressed as percent of initial value) produced by application of 1 mM carbachol after 1 min of recovery plotted as a function of the perfusion number.

decline of the EPC_{carb}, which is an index of desensitization onset time-course, was exponential. In this example, the time constant of current decay was 12 s (Fig. 2A). To estimate the time-course of recovery from desensitization the end-plate was activated by 1 mM carbachol application a total of 10 times. The time-course of recovery of sensitivity was determined by plotting activation (in percent of the EPC_{carb} obtained during the initial carbachol perfusion) as a function of the recovery interval. In this example the time constant of recovery of sensitivity was 84 s. The data points have been fitted on the assumption of a single exponential approach to 100% recovery. The assumption of 100%

recovery is based on preliminary experiments where we achieved 100% recovery after prolonged periods (15 min) using our constant flow system. In other experiments done without flow, recovery appeared to be incomplete even after 15 min. This observation corroborates the report by Katz and Thesleff (1957) that desensitization appeared to be partially irreversible in some instances.

To insure that the response to carbachol was not changing with time, the EPC_{carb} after a 1-min recovery interval was determined at four different times (the 2nd, 3rd, 9th, and 11th perfusions) during this experiment. These observations are presented in Fig. 2B and demonstrate that no progressive decline occurred with time or repeated activations.

In three similar experiments using voltage-clamped fibers the time-course of both desensitization onset and recovery was exponential. The fact that recovery appeared to develop progressively made us feel that we could investigate the influence of membrane voltage on recovery without having to generate a complete recovery curve for each fiber. This was important because of difficulties in voltage-clamping fibers for long periods. Consequently, the extent of recovery was determined after either a 1- or 2-min wash interval in the remainder of the experiments.

Desensitization Onset and Recovery are Both Voltage Sensitive.

The time-course of desensitization onset and the extent of recovery of sensitivity were compared in potassium-depolarized muscle fibers voltage-clamped to either −40 mV or +40 mV. The extent of recovery of sensitivity after desensitization was determined after a 1- or 2-min wash interval. These results indicate that both desensitization onset and recovery are voltage-sensitive processes.

The protocol for comparing the influence of membrane voltage on desensitization onset and recovery is summarized in the example presented in Fig. 3. This tracing of a record shows the activation and desensitization of a fiber which was voltage-clamped to +40 mV and perfused twice with 1 mM carbachol. In Fig. 3A and B the top trace represents membrane voltage (+40 mV), the middle trace is the current, and the bottom trace is an event marker which denotes the beginning and end of the carbachol perfusion. During each perfusion the time-course of development of desensitization was determined from the decline of the EPC_{carb} from the peak value towards the pre-agonist level. The extent of recovery (in this example after 2 min of wash), estimated from the ratio of the peak EPC_{carb} value during the second carbachol exposure to the peak EPC_{carb} value during the initial perfusion was 59%. No correction was made for non-zero end-plate current at equilibrium desensitization (see below). In all fibers voltage-clamped to either −40 or +40 mV, the desensitization produced by 1 mM carbachol during the first and second perfusion developed exponentially.

Desensitization during the initial carbachol perfusion developed more rapidly in those fibers voltage-clamped at −40 mV than at +40 mV. The average time constant of desensitization for 11 fibers voltage-clamped at −40 mV was 8.3 ± 1.3 s (mean ± SEM) whereas the average time constant of desensitization for 15 other fibers voltage-clamped to +40 mV was 19.3 ± 3.4 s (mean ± SEM). The means are significantly different ($P \leq 0.005$). These observations are consistent

with the results presented previously by Magazanik and Vyskocil (1970) that desensitization onset is accelerated by hyperpolarization.

The peak EPC_{carb} value during the first carbachol application was also significantly different in those fibers voltage-clamped at these two voltages. In the 11 fibers voltage-clamped at −40 mV, the peak inward current in the presence of 1 mM carbachol had an average value of 338 ± 53 nA (mean ± SEM). The peak outward current in the presence of 1 mM carbachol in the 15 fibers voltage-clamped to +40 mV was 559 ± 47 nA (mean ± SEM). The reversal potential for carbachol is −8 mV in the potassium-depolarized preparation.[1] The different values of the EPC_{carb} are consistent with the difference in the

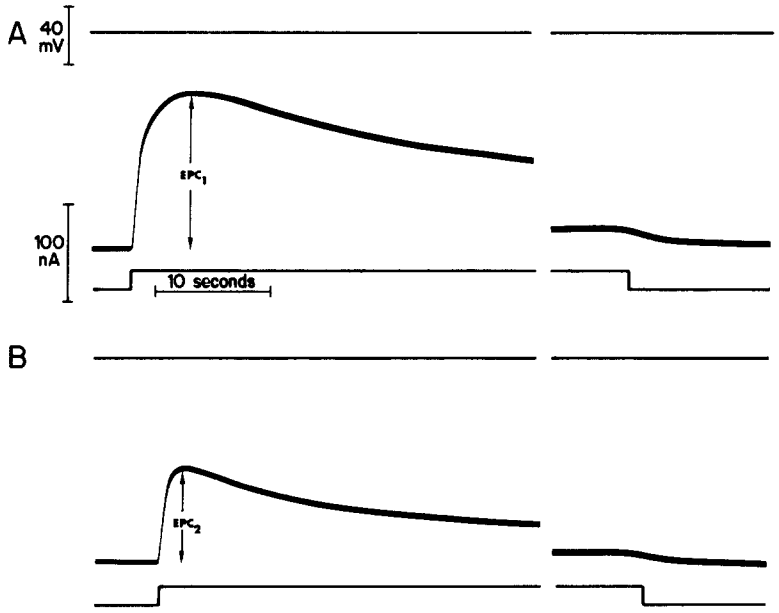


FIGURE 3. End-plate currents recorded in a fiber voltage clamped to +40 mV (A) during an initial carbachol application (EPC_1) and (B) during a second perfusion done after a 2-min recovery interval (EPC_2). In both (A) and (B), the top trace is membrane voltage, the middle trace is current, and the bottom trace is the onset and termination of 1 mM carbachol application. The portion of the record to the right of the break shows the equilibrium level of desensitization. Recovery of sensitivity was calculated as the amplitude of EPC_2 in percent of EPC_1.

driving force at the two voltages. This observation differs from that reported by Dionne and Stevens (1975). They demonstrated that agonist effectiveness diminished as membrane potential was made more positive. This effect was attributed to a voltage dependence of the rate constants of channel opening and closing. In contrast, agonist effectiveness was not different at −40 mV or +40 mV in the present experiments. The discrepancy between the results of Dionne and Stevens and those reported here can be attributed to differences in the agonist concentration employed and (or) in the buffers used. First of all, Dionne

[1] Parsons, R. L., B. Scubon-Mulieri, and P. M. Spannbauer. Unpublished observation.

and Stevens (1975) used low concentration of agonist whereas we have employed much higher concentrations. Recently, Adams (1976*a*) has reported that the voltage dependence of agonist-induced conductance was less marked as concentration was elevated. Secondly, Dionne's and Stevens' experiments were done in a phosphate-buffered Ringer's solution whereas we employed a Tris-buffered solution. Kordas (1977) has reported that in Tris-buffered solution the rate of decay of neurally-evoked end-plate currents becomes independent of voltage at membrane potentials more positive than approximately −40 mV, whereas in phosphate-buffered solutions the end-plate current decay becomes progressively faster as membrane potential is made more positive.

The results summarized in Table I indicate that the extent of recovery of sensitivity after desensitization is also influenced by membrane voltage. The extent of recovery after a 1-min interval at −40 mV and +40 mV was not significantly different ($0.05 \leq P \leq 0.1$) but the means are representative of the trend. After a 2-min wash period the extent of recovery of sensitivity was significantly greater in those fibers voltage-clamped at −40 mV than at +40 mV.

TABLE I

INFLUENCE OF VOLTAGE ON RECOVERY OF SENSITIVITY AND ALTERATION IN τ_{ONSET}

Recovery interval	Membrane potential	Extent of sensitivity recovery	Alteration in desensitization onset	Fibers
min	*mV*	*% initial* EPC_{carb}	$\tau_{onset\,1}/\tau_{onset\,2}$*	n
1	−40	62.8 ± 7.9‡	2.29±0.41	6
	+40	48.6±6.7‡	2.81±0.40	5
2	−40	80.4±6.3§	1.87±0.12	5
	+40	57.4±3.6§	4.09±1.00	8

* $\tau_{onset\,1}$ is the desensitization onset time constant for the first perfusion, and $\tau_{onset\,2}$ is the desensitization onset time constant for the second perfusion.
‡ Significance $P \leq 0.10$.
§ Significance $P \leq 0.005$.

Evidence That 1 mM Carbachol Produces Complete Desensitization

Often the carbachol-induced current did not return exactly to the original value of the holding current when desensitization appeared to reach its equilibrium level (see Fig. 3). At +40 mV the residual current at equilibrium desensitization was 8.06 ± 1.38% of the peak response (n = 13), and at −40 mV the residual current was 11.81 ± 3.28% of the initial response (n = 8). To test whether this represents a pool of potentially activable or non-desensitizable receptors remaining when desensitization has reached equilibrium, the following experiments were done. The end-plate region was perfused with 1 mM carbachol to induce the activation-desensitization sequence. At equilibrium densensitization (5 min of exposure to 1 mM carbachol), a 10 mM carbachol perfusion was superimposed on the 1 mM carbachol response. As demonstrated in Fig. 4, this application of the higher carbachol concentration produced no alteration in the current. This suggests that no additional receptor activation or change in the level of desensitization occurs when the agonist concentration was increased. In

five fibers voltage-clamped to +40 mV, the response to 10 mM carbachol was 0.59 ± 0.88% of the first response to 1 mM carbachol, and in two fibers voltage-clamped to −40 mV, the response, if any, was within the noise. From this we conclude that no free receptors exist at equilibrium desensitization to 1 mM carbachol, and that desensitization is equally complete at both −40 and +40 mV. Consequently, we did not correct for any residual current when desensitization had reached its equilibrium level.

The Voltage Dependence of Desensitization Onset and Recovery Is Not Due to Inadequate Voltage Control

The possibility that the voltage dependence of desensitization onset and recovery resulted from a difference in voltage control at these two levels of membrane

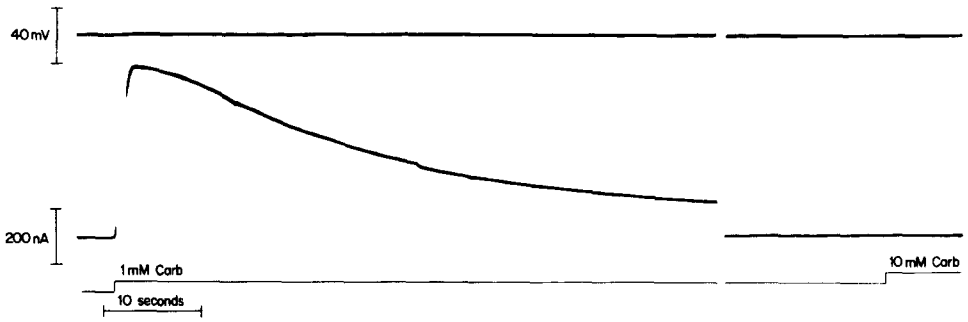


FIGURE 4. A double-perfusion experiment demonstrating that increasing the carbachol concentration during equilibrium desensitization by 1 mM carbachol produces no additional activation or desensitization. The first deflection of the bottom trace indicates the onset of 1 mM carbachol perfusion which is maintained for the duration of the experiment. After a 5-min exposure to 1 mM carbachol, a 10 mM carbachol perfusion was superimposed. The beginning of the second perfusion is indicated by the second deflection of the bottom trace. The break in the record is a 240-s interval. The upper trace is membrane voltage clamped to +40 mV, and the middle trace is the carbachol-induced current.

potential was tested in a separate series of experiments. The extent of the voltage deviation in the end-plate region was measured during carbachol perfusion.

In these experiments a second voltage electrode was inserted in the same fiber approximately 100 μm distant from the primary voltage and current micropipettes to estimate the spatial extent of voltage control during 1 mM carbachol perfusion in fibers voltage-clamped either to +40 or −40 mV. Sample records illustrating the extent of the deviation in two different fibers, one voltage-clamped at +40, the other at −40 mV, can be seen in Fig. 5. No discernible deviation occurred at the primary voltage electrode, but a measurable voltage deviation occurred at the second voltage electrode. At −40 mV, the deviation was 4.3 ± 1.2 mV (mean ± SD, four fibers) at an average distance of 108 μm from the command electrode whereas at +40 the deviation was 5.3 ± 2.3 mV (three fibers) at an average distance of 104 μm. Considering that the carbachol

reversal potential in these potassium-depolarized fibers is −8 mV, the deviation in membrane potential during carbachol action was similar at the two voltages, being ~13% at −40 mV and ~11% at +40 mV. Therefore, the difference in desensitization onset rate and extent of recovery at −40 mV and +40 mV cannot be attributed to a difference in voltage control at these two values of membrane potential.

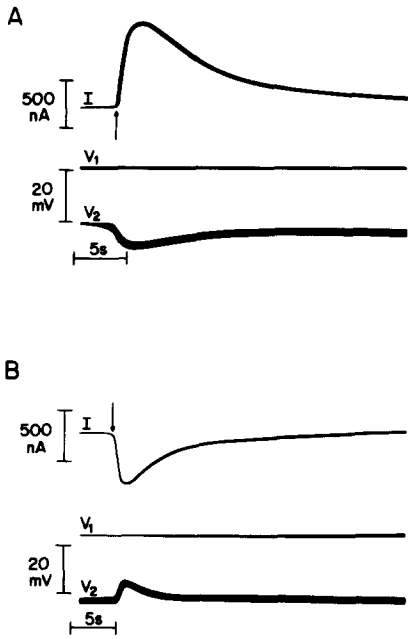


FIGURE 5. Records showing the voltage deviation at a distance of 112 μm from the command electrode during 1 mM carbachol perfusion. (A) Fiber voltage clamped to +40 mV. (B) Fiber voltage clamped to −40 mV. For both (A) and (B), I is the current trace, V_1 is the command voltage, and V_2 shows the membrane potential recorded with a second electrode 112 μm away from the command and current passing electrodes. The onset of carbachol perfusion is shown by the arrows. No discernible voltage deviation occurred at the command electrode during carbachol application whereas a small voltage change was seen at the distant electrode.

Desensitization Onset Is Faster during Recovery from Desensitization at Both Holding Voltages

Previously, Scubon-Mulieri and Parsons (1977*a*) observed that the time constant of desensitization onset was not the same with the initial carbachol exposure and with subsequent exposures during the recovery period. A similar finding was obtained in the present experiments using fibers voltage-clamped at either −40 or +40 mV. An example of this change in desensitization onset time constant for one fiber voltage-clamped at +40 mV can be seen in Fig. 6. In this example the time constant of desensitization onset during the initial carbachol exposure

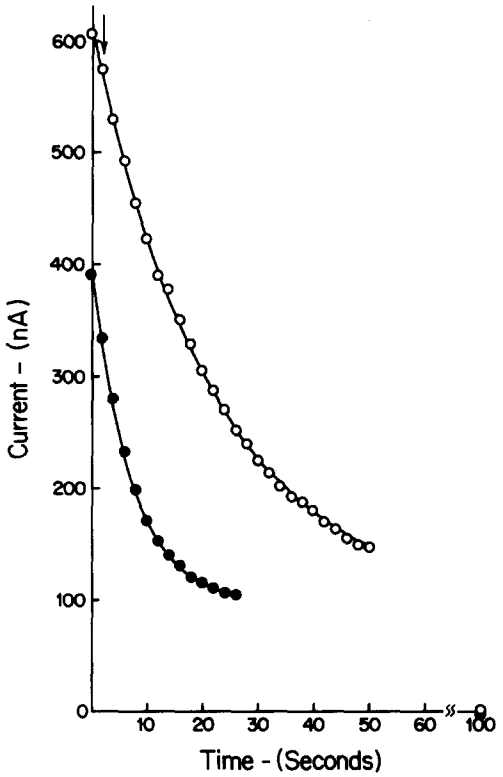


FIGURE 6. The exponential fit to the decline of the EPC_{carb} during the first and second application of 1 mM carbachol in a fiber voltage clamped to +40 mV. (○) Data points and fitted curve for the first carbachol perfusion (τ = 22 s); (●) data points and fitted curve for the second perfusion done after a 2-min wash interval (τ = 6.9 s). Only the data points after the arrow in the upper curve were used to generate the exponential. Final equilibrium values are shown in single symbols after the break.

was 22 s, whereas during the second carbachol perfusion (after 2 min of recovery) the time constant of desensitization onset was 6.9 s.

The data for the change in τ_{onset} in those fibers voltage-clamped at either −40 or +40 mV are summarized in Table I. In these experiments the recovery of τ_{onset} appears to be slower than the recovery of sensitivity which confirms our earlier observation that these two processes recover with different time-courses (Scubon-Mulieri and Parsons, 1977*a*). In the present study we examined sensitivity at times when τ_{onset} showed little recovery, and no significant amount of recovery of τ_{onset} was evident after 1 or 2 min of wash at either −40 or +40 mV. Consequently, the results of the present study do not indicate whether the recovery of τ_{onset} is also voltage dependent.

DISCUSSION

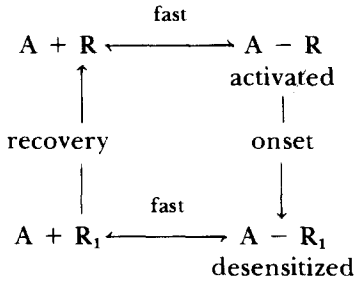
Scubon-Mulieri and Parsons (1977*a*) have recently shown that desensitization onset and recovery proceed exponentially with onset occurring faster than

recovery in the potassium-depolarized preparation at about −10 mV. A qualitatively similar relationship between the time-courses of desensitization onset and recovery was obtained in the present study using potassium-depolarized muscle fibers voltage-clamped at +40 mV and −40 mV (Fig. 3).

In the potassium-depolarized preparation, both desensitization onset and recovery are influenced by membrane voltage. The voltage dependence of desensitization onset obtained in the present study is in agreement with the prior observations by Magazanik and Vyskocil (1970) using muscles maintained in sodium Ringer's solution and by Lambert et al. (1977) using potassium-depolarized fibers. However, Magazanik and Vyskocil (1975) did not observe an influence of voltage on the time-course of recovery from desensitization in polarized fibers. In contrast, recovery proceeded more rapidly at −40 mV than at +40 mV in the present study with the potassium-depolarized muscle fibers. Differences in experimental conditions between those of Magazanik and Vyskocil (1975) and those used in the present study may account for the different results; the most obvious difference was a difference in bathing solutions. Magazanik and Vyskocil (1975) used a sodium Ringer's solution whereas the muscles were bathed in an isotonic potassium solution in the present study. A second important difference in the experimental conditions of these two studies is that a much larger voltage range was utilized in the present experiments. Magazanik and Vyskocil (1975) compared recovery at −60 mV and −90 mV, (a 30 mV difference) whereas in the present work recovery was compared at −40 mV and +40 mV (an 80-mV difference). A third difference between these two studies is that Magazanik and Vyskocil (1975) did the majority of their experiments in the presence of drugs such as SKF 525 (Smith, Kline & French Laboratories, Philadelphia) or chlorpromazine which accelerate desensitization onset. If these agents also increase the rate of recovery, then the drug effect may have masked an influence of voltage on recovery. Lastly, the agonist and method of drug application were different in the two studies. Magazanik and Vyskocil (1975) applied acetylcholine iontophoretically to the postjunctional membrane whereas in the present study desensitization was produced by hydrostatically controlled microperfusion of carbachol. With the former method a considerable degree of spatial nonuniformity of agonist concentration occurs over the postjunctional membrane (Katz and Thesleff, 1957; Adams, 1976*b*) whereas these experiments show that the agonist concentration is uniform over the end-plate region with microperfusion of 1 mM carbachol.

Recently a voltage dependence of recovery from desensitization has been suggested by Steinbach and Stevens (1976) who reported a voltage dependence opposite to that observed in the present study. As no detailed information concerning the methods employed has been published, we cannot speculate on the basis of this difference in results. However, both studies demonstrate that future work on the kinetics of desensitization onset and recovery must be done under conditions where membrane voltage is controlled.

The weight of evidence (Rang and Ritter, 1970; Scubon-Mulieri and Parsons, 1977*a*) presently support the cyclic model of desensitization onset and recovery first proposed by Katz and Thesleff (1957). In such a model, schematically presented below,



the activated A − R complex is slowly converted to a desensitized state A − R_1 with sustained exposure to agonist. The recovery of sensitivity after desensitization would occur as A − R_1 dissociated and R_1 reverts to R and is complete when all receptor has returned to the free R configuration. The results of the present study indicate that the rates of both desensitization onset and recovery are influenced by membrane voltage. The mechanisms by which membrane voltage influences onset and recovery rates is unknown. Because onset and recovery are influenced in a similar direction by membrane voltage in the potassium-depolarized preparation, it might be suggested that the more rapid recovery at the negative potential is a consequence of the more rapid onset rate at this voltage. However the previous observation that onset rate can be increased by raising external Ca^{2+} or carbachol concentration without influencing recovery argues against this simple correlation (Scubon-Mulieri and Parsons, 1977*a*). Rather it appears that the molecular processes responsible for both desensitization onset and recovery are influenced by membrane voltage.

We thank Dr. William A. DeBassio for assistance with the cholinesterase staining technique. Also we wish to thank Drs. V. E. Dionne and L. A. Mulieri for their helpful comments on this manuscript. This study was supported by U.S. Public Health Service grant NS-07740.

Received for publication 26 August 1977.

REFERENCES

ADAMS, P. R. 1975. A study of desensitization using voltage clamp. *Pfluegers Arch. Eur. J. Physiol.* **360:**135–144.

ADAMS, P. R. 1976*a*. Voltage dependence of agonist responses at voltage-clamped frog endplates. *Pfluegers Archiv. Gesamte Physiol. Menschen Tiere.* **361:**145–151.

ADAMS, P. R. 1976*b*. A comparison of the time course of excitation and inhibition by iontophoretic decamethonium in frog endplate. *Br. J. Pharmacol.* **57:**59–65.

DIONNE, V. E., and C. F. STEVENS. 1975. Voltage dependence of agonist effectiveness at frog neuromuscular junction: resolution of a paradox. *J. Physiol. (Lond.).* **251:**245–270.

DIONNE, V. E. 1976. Characterization of drug iontophoresis with a fast microassay technique. *Biophys. J.* **16:**705–717.

GIBBONS, W. R., and H. A. FOZZARD. 1971. Voltage dependence and time dependence of contraction in sheep cardiac Purkinje fibers. *Circ. Res.* **28:**446–460.

KATZ, B. 1949. Les constances électriques de la membrane du muscle. *Arch. Sci. Physiol.* **3:**285–300.

KATZ, B., and S. THESLEFF. 1957. A study of the 'desensitization' produced by acetylcholine at the motor end-plate. *J. Physiol. (Lond.).* **138:**63–80.

KOELLE, G. B., and J. S. FRIEDENWALD. 1949. A histochemical method for localizing cholinesterase activity. *Proc. Soc. Exp. Biol. Med.* **70**:617–622.

KORDAS, M. 1977. On the role of junctional cholinesterase in determining the time course of the end-plate current. With an appendix by P. Jakopin and M. Kordas, *J. Physiol. (Lond.)*. **270**:133–150.

LAMBERT, D. H., P. M. SPANNBAUER, and R. L. PARSONS. 1977. Desensitization does not selectively alter sodium channels. *Nature (Lond.)*. **268**:533–555.

MAGAZANIK, L. G., and F. VYSKOCIL. 1970. Dependence of acetylcholine desensitization on the membrane potential of frog muscle fibre and on the ionic changes in the medium. *J. Physiol. (Lond.)*. **210**:507–518.

MAGAZANIK, L. G., and F. VYSKOCIL. 1975. The effect of temperature on desensitization kinetics at the post-synaptic membrane of the frog muscle fibre. *J. Physiol. (Lond.)*. **249**:285–300.

MANTHEY, A. A. 1966. The effect of calcium on the desensitization of membrane receptors at the neuromuscular junction. *J. Gen. Physiol.* **49**:963–976.

MANTHEY, A. A. 1970. Further studies of the effect of calcium on the time course of action of carbamylcholine at the neuromuscular junction. *J. Gen. Physiol.* **56**:407–419.

MANTHEY, A. A. 1972. The antagonistic effects of calcium and potassium on the time course of action of carbamylcholine at the neuromuscular junction. *J. Membr. Biol.* **9**:319–340.

NASTUK, W. L., and R. L. PARSONS. 1970. Factors in the inactivation of postjunctional membrane receptors of frog skeletal muscle. *J. Gen. Physiol.* **56**:218–249.

PARSONS, R. L., R. M. SCHNITZLER, and D. E. COCHRANE. 1974. Inhibition of end-plate desensitization by sodium. *Am. J. Physiol.* **227**:96–100.

RANG, H. P., and J. M. RITTER. 1970. On the mechanism of desensitization at cholinergic receptors. *Mol. Pharmacol.* **6**:357–382.

SCUBON-MULIERI, B., and R. L. PARSONS. 1977*a*. Desensitization and recovery at the frog neuromuscular junction. *J. Gen. Physiol.* **69**:431–477.

SCUBON-MULIERI, B., and R. L. PARSONS. 1977*b*. Voltage dependence of desensitization onset and recovery at the neuromuscular junction. *Biophys. J.* **17**:190a.

STEINBACH, J. H., and C. F. STEVENS. 1976. Neuromuscular transmission in frog. *In* Neurobiology: a Handbook. R. Llinas and W. Precht, editors. Springer-Verlag New York, Inc. 1046 pp.

TAKEUCHI, A., and N. TAKEUCHI. 1959. Active phase of frog's end-plate potential. *J. Neurophysiol.* **22**:395–411.

THESLEFF, S. 1955. The mode of neuromuscular block caused by acetylcholine, nicotine, decamethonium, and succinylcholine. *Acta Physiol. Scand.* **34**:218–231.

WRIGHT, R. 1977. A separable variables method for least squares exponential approximation. *SIAM J. Numer. Anal.* Accepted for publication.