The Effect of Calcium on the Desensitization of Membrane Receptors at the Neuromuscular Junction

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ABSTRACT Desensitization, as represented by the progressive decline in the electromotive effects of depolarizing agents at the neuromuscular junction, was studied by observing the time course of changes in effective transmembrane resistance during the prolonged application of 0.27 mM carbamylcholine to the postjunctional region of frog skeletal muscle fibers. The effective transmembrane resistance was measured by means of two intracellular microelectrodes implanted in the junctional region of single muscle fibers. When carbamylcholine was applied to the muscle there was an immediate decrease in the effective membrane resistance followed by a slower return toward control values which was identified as the phase of desensitization. When the calcium concentration was increased from 0 to 10 mM there was an approximately sevenfold increase in the rate of desensitization. On the other hand, an increase in the concentration of sodium from 28 to 120 mM caused a slowing of the rate of desensitization. Even in muscles depolarized by potassium sulfate, calcium increased the rate of desensitization while high concentrations of potassium tended to prolong the process. Some mechanisms by which calcium might exert these effects are discussed.

INTRODUCTION

When acetylcholine is briefly applied to the neuromuscular junction, it causes an increase in the ionic permeability of the postjunctional membrane which results in a local depolarization of the muscle fiber. If, however, the application of acetylcholine is maintained over a period of several minutes, the initial depolarization is followed, despite the continued presence of acetylcholine, by a gradual repolarization. In order to explain this repolarization, Thesleff (1) suggested that during prolonged exposure to acetylcholine the postjunctional membrane became "desensitized," or chemically unreactive toward the drug. Somewhat later Katz and Thesleff (2) further extended this hypothesis.
and proposed that desensitization resulted from the conversion of acetylcholine "receptors" in the postjunctional membrane to an inactive or nondepolarizing form.

Although the Katz-Thesleff proposal is consistent with certain aspects of desensitization, it does not indicate the precise nature of the change which occurs during the process. Research reported in the present paper began with attempts to discover something about the kind of transformation involved in desensitization, and preliminary experiments were conducted to find what factors influenced the time course of the process. It was observed that the concentration of calcium in the extracellular medium had an important effect on the rate at which the desensitized state developed. The following is a report of these results and a discussion of the possible role of calcium during desensitization.

METHODS

In these experiments desensitization was studied by applying a standard concentration of a depolarizing agent to the neuromuscular junction of frog muscle fibers and measuring the rate at which the electromotive effect of the drug on the postjunctional membrane disappeared during the time of application. Instead of using the membrane potential alone as an indication of the degree of drug action, measurements of the effective resistance across the postjunctional membrane were employed for this purpose. It was possible to follow the time course of desensitization by this method because both the potential and effective resistance (with some differences to be noted) returned toward their control values at about the same rate (see Fig. 2).

In order to measure effective membrane resistance two glass capillary electrodes filled with 3 M KCl were placed intracellularly in the postjunctional region of a single sartorius fiber within 50 µ of each other. One electrode was used for passing short repeated pulses of anodal current and the other for recording the resulting changes in membrane potential. The effective membrane resistance was calculated as the ratio of the resulting incremental changes in membrane potential and current. Since the current density delivered across the muscle fiber membrane decreases with distance from the impalement site, this resistance cannot be related in any simple way to a specific area of membrane; therefore the resistance measured in this way will be referred to throughout as an "effective membrane resistance." The electrode for recording membrane potential was mounted in a Lucite cylinder filled with Ringer's solution and containing a silver-silver chloride electrode coupled directly to the input of an electrometer. The output of the electrometer was led directly to one channel of a double-beam oscilloscope. The current-passing electrode was connected via a silver-silver chloride electrode to a stimulus isolation unit and square wave pulse generator. The potential difference across a 49K resistor in the ground connection of the isolation unit was applied directly to the second channel of the oscilloscope and from it the amount of current delivered to the muscle fiber could be calculated. The amount of this current varied in different experiments from 5 to 60 X 10⁻⁶ amp. In order to mark the onset and end of drug perfusion, a 0.4 mv dc potential was in-
introduced into the current delivery circuit by a manual switch producing a small
deflection of the corresponding oscilloscope trace. The operation of this switch caused
a negligible change in the total resistance of the current delivery circuit. A large
silver-silver chloride electrode in contact with the bath fluid acted as an indifferent
lead. Photographic records were obtained on the slowly moving film of a camera
mounted directly in front of the oscilloscope face.

The test depolarizing agent used throughout these experiments was carbamyl-
choline, an analogue of acetylcholine which is resistant to hydrolysis by cholinesterase.
A solution of 0.27 mM carbamylcholine was delivered by local perfusion to the junc-
tional region of the impaled fiber through a special micropipette having a tip diam-
eter of about 40 to 50 μ. When, at the desired moment of drug application, the tip of
this pipette was lowered into the bath fluid, the drug solution was expelled by an
hydrostatic force of approximately 17 cm of water. With the tip of the pipette with-

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\begin{array}{cccccccc}
\text{Na}^+ & \text{K}^+ & \text{Ca}^{2+} & \text{Cl}^- & \text{SO}_4^{2-} & \text{HPO}_4^{2-} & \text{H}_2\text{PO}_4^- & \text{Sucrose} \\
\text{mM/liter} & \text{mM/liter} & \text{mM/liter} & \text{mM/liter} & \text{mM/liter} & \text{mM/liter} & \text{mM/liter} & \text{mM/liter} \\
\hline
\text{Normal Ringer's solution} & 116 & 2.5 & 1.8 & 117 & - & 2.55 & 0.45 & - \\
A & 120 & 2.5 & 20.0 & 162 & - & 0.22 & 0.06 & 245 \\
B & 120 & 2.5 & - & 122 & - & 0.22 & 0.06 & 306 \\
C & 28 & 2.5 & 20.0 & 70 & - & 0.22 & 0.06 & 429 \\
D & 28 & 2.5 & - & 30 & - & 0.22 & 0.06 & 489 \\
E & 0.5 & 38 & 1.56* & - & 20 & 0.22 & 0.06 & 188 \\
F & 0.5 & 38 & - & - & 19 & 0.22 & 0.06 & 195 \\
G & 0.5 & 76 & 1.56* & - & 40 & 0.22 & 0.06 & 142 \\
H & 0.5 & 76 & - & - & 38 & 0.22 & 0.06 & 142 \\
\end{array}
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* Estimated value of free calcium ion in solution.

drawn above the level of the fluid, however, this pressure was insufficient to force
fluid from the delivery apparatus. In all cases carbamylcholine was delivered in a solu-
tion of the same composition as that of the bathing medium. Several control experi-
ments in which impaled fibers were perfused in this way with solutions containing no
carbamylcholine showed that the force of the expelled fluid did not affect the meas-
urement of effective membrane resistance.

Solutions  Table I presents a summary of the composition of the various bath
solutions used in these studies. In solutions A, B, C, and D sucrose was added to bring
the total osmolarity to about 2.3 times that of normal Ringer's solution. This was
done in order to eliminate the repetitive twitching of the muscle fibers which normally
occurs when carbamylcholine is applied to the neuromuscular junction. Hodgkin
and Horowicz (3) have shown in this regard that muscle fibers equilibrated in hyper-
tonic solutions will continue to conduct action potentials at a time when the twitch
response has disappeared. To obtain solutions with calcium concentrations between
0 and 20 mM, solutions A and B were mixed in proportionate amounts. In a series of
experiments performed in sodium-depleted media, solutions C and D were mixed to obtain intermediate calcium concentrations. When immersed in the potassium sulfate solutions E to H, muscle fibers are depolarized over their entire length and thus incapable of any twitch response. The calcium ion concentrations noted for solutions E to H were calculated on the basis of a dissociation constant for CaSO$_4$ of $5.3 \times 10^{-4}$ M/liter (4).

Procedure The same general procedure was followed in all experiments. The right sartorius of the frog (*Rana pipiens*) was dissected and mounted in normal Ringer's solution. The mounting dish was attached to the microscope stage, and the two electrodes and perfusion pipette were brought into position above the viewing field by means of micromanipulators. The test solution, in which appropriate alterations had been made in the concentrations of sodium, potassium, and calcium, was introduced into the bath chamber. After a 1 hr equilibration period, the neuromuscular junction of a fiber to be tested was identified visually and the current-passing and recording electrodes were inserted intracellularly in the postjunctional region of the fiber within 50 $\mu$m of each other. Rectangular pulses of anodal current were delivered to the fiber at 2 sec intervals. The amplitude and duration of these pulses were adjusted so as to produce 10 to 20 mv hyperpolarizations of the postjunctional membrane of about 200 to 400 msec duration. After a 1 min control period, local perfusion of the junctional area with 0.27 mM carbamylcholine was begun by rapidly lowering the perfusion pipette (previously above the level of the bath fluid) to a position about 0.1 mm above the impaled fiber. Recordings and perfusion were continued until no further changes were noted in the amplitude of the membrane potential pulses. In most cases drug perfusion lasted from 5 to 10 min. At the conclusion, the electrodes and perfusion pipette were withdrawn and the bath fluid exchanged. After about 15 to 30 min another junctional region was identified in an area of the preparation some 2 to 3 cm away, and the impalement and perfusion procedure were repeated.

RESULTS

Experiments in Hypertonic Solutions In the first experiments the effects of calcium on the desensitization process were studied in solutions made hypertonic by the addition of sucrose (solutions A–D of Table I). The muscle fibers in this altered medium showed no mechanical response to the marked depolarization produced by 0.27 mM carbamylcholine, their resting potentials remained greater than 80 mv for several hours, and throughout this treatment there was little decrease in the sensitivity to applied carbamylcholine. It was possible, therefore, to impale a fiber with electrodes prior to drug perfusion and to measure changes in potential and effective resistance throughout the total period of drug perfusion without mechanical movements of the fiber dislodging the electrodes. On the other hand, the use of hypertonic solutions caused shrinkage of the fibers and probably some changes in the activity of intracellular ions.

The record in Fig. 1 $a$ is from an experiment performed in a "hypertonic sodium-sucrose" medium containing 1.8 mM calcium (the amount present in
normal Ringer's solution). The traces denoting membrane potential and current are marked $E$ and $I$ respectively. A series of dots above the $E$ and $I$ traces are momentary displacements produced by short pulses of anodal current delivered at 2 sec intervals. At the beginning of the period of drug perfusion, the duration of which is indicated by the upward step in the $I$ trace, there is a rapid depolarization of the postjunctonal membrane accompanied by a sharp decrease in the amplitude of the membrane potential pulses ($E$
trace). This is followed by a more gradual repolarization and recovery of the original pulse amplitude. The slow repolarization during the continued application of carbamylcholine indicates that desensitization is occurring throughout this time.

Measurements of the effective membrane resistance, calculated from Fig. 1a as the ratio of the amplitudes of the membrane potential and current pulses, appear on a somewhat compressed time scale in Fig. 2a. The time course of changes in membrane potential is also shown for comparison. At the onset of drug perfusion, indicated by the first vertical arrow, there is a rapid decrease of membrane potential and effective resistance to low values. As perfusion continues, both potential and effective resistance gradually return toward control values until after about 300 sec there is little further change and the perfusion is stopped (indicated by the second arrow). In this and in most other experiments the effective membrane resistance at this time has recovered more completely than has the membrane potential. Nevertheless, the similarity in the time rate of change of both curves shows that the desensitization process affects the underlying ionic permeability of the postjunctional membrane as well as the membrane potential itself. This fact makes it possible to redefine desensitization in terms of changes in effective membrane resistance rather than membrane potential. In these experiments, therefore, the phase of desensitization was identified as the time during the application of carbamylcholine when the effective resistance of the muscle fiber membrane measured in the region of the neuromuscular junction increases toward its original control value. In order to compare results from different experiments, the half-period of desensitization (τ₁/₂ in Fig. 2a) was defined as the time taken for the effective membrane resistance to return toward control values by an amount equal to one-half the total increase in resistance which occurred during the desensitization phase (ΔR, in Fig. 2a). The final "plateau" value of effective resistance at the end of drug perfusion was used to calculate the desensitization half-period whether or not this value was the same as the control resistance prior to drug application. In the experiment of Fig. 2a the half-period measured in this way was about 35 sec.

The next experiment, the results of which are shown in Figs. 1b and 2b, illustrates the effect on the time course of desensitization of raising the calcium concentration of the bathing medium to 10 mM (solutions A and B of Table I were mixed in proportionate amounts). After the initial fall in resistance on exposure to carbamylcholine (Fig. 2b), the return toward control levels is here more rapid than in the presence of 1.8 mM calcium (Fig. 2a) and displays a half-period of about 12 sec. The time course of changes in potential shows a similar acceleration in the recovery phase. In this experiment the increased calcium concentration has evidently accelerated the desensitization process.
The effect of decreasing the calcium concentration to very low values was next examined. Measurements from an experiment performed in a calcium-free medium (solution B, Table I) are shown in Fig. 2 c. Compared with the previous results (Fig. 2 a, b) the return of effective membrane resistance is here very much slower and occurs with a half-period of nearly 100 sec. The membrane potential also remains at a low value with only a limited repolarization.
zation during the perfusion with carbamylcholine. From this result it is clear that the removal of calcium has greatly prolonged the time course of desensitization.

Considered together, the observations in Figs. 1 and 2 show that increasing the concentration of calcium up to 10 mM can greatly accelerate the desensitization process. The degree of this effect is displayed in the upper curve of Fig. 3 which shows the average half-period of desensitization from several experiments performed at different concentrations of calcium. There is an approximately sevenfold decrease in the desensitization half-period as the calcium concentration is increased from 0 to 10 mM. Raising the calcium concentration to 20 mM did not result in any further decrease in the half-period below that seen at 10 mM.

The lower curve in Fig. 3, which summarizes the results of a series of similar experiments performed in sodium-depleted media, illustrates another important finding. These experiments, which were undertaken to see what effect the removal of sodium might have on the time course of desensitization, were performed in media in which 77% of the sodium chloride was substituted by an osmotically equivalent amount of sucrose. Variations in the calcium concentration were obtained by mixing proportionate amounts of solutions C and D of Table I. The average half-periods of desensitization from several such ex-

![Figure 3. The effect of calcium on the half-period of desensitization. Each point represents the mean of 3 to 8 determinations of the desensitization half-period (calculated as in Fig. 2 a). The mean half-periods for experiments in 120 and 28 mM sodium are significantly different at the 5% level of probability for calcium concentrations of 0.5, 1.0, 1.8, and 5.0 mM. The Student t test was used as the test of significance.](image-url)
Experiments at different concentrations of calcium are the values plotted in the lower curve of Fig. 3. It is evident from a comparison of the results in 120 and 28 mM sodium that at every level of calcium below 10 mM, the removal of sodium has potentiated the effect of calcium and caused a further reduction in the half-period of desensitization. Therefore, in this range of calcium concentrations, the presence of sodium antagonizes the ability of calcium to bring about rapid desensitizations.

Experiments in Potassium Sulfate Solutions  Since the application of carbamylcholine in the previous experiments always caused substantial depolarizations of the postjunctional membrane, it was of interest to see what the time course of desensitization would be in the absence of any changes in membrane potential. This was undertaken by performing perfusion experiments with the muscle immersed in solutions of potassium sulfate. Under these conditions the muscle fibers are depolarized over their whole length, and the application of carbamylcholine causes little or no change in membrane potential. Nevertheless, the action of the drug results in a substantial decrease in the effective membrane resistance which can be easily measured by the same method adopted in the previous experiments.

Fig. 4 shows the results of perfusion experiments performed in solutions containing 19 mM K₂SO₄ and various concentrations of calcium up to 1.3 mM. Carbamylcholine was applied as before in a concentration of 0.27 mM. Variations in the calcium concentration of the bathing medium were obtained by mixing solutions E and F of Table I. A low concentration of K₂SO₄ was used in order to minimize the common ion effect of the sulfate ion on the dissociation of calcium sulfate. The resting potential of fibers equilibrated in this medium was about 20 to 30 mv (inside negative) and did not change more than 5 to 10 mv upon application of 0.27 mM carbamylcholine to the neuromuscular junction. Comparison of the results in Fig. 4 with those of the previous experiments (Fig. 2) shows that during perfusions with carbamylcholine in potassium sulfate solutions the effective membrane resistance goes through a cycle of changes similar to that seen in hypertonic sodium-sucrose. At the onset of drug application there is a rapid decrease of the effective membrane resistance to low values, and this is followed by a slower return toward control values. As before, the phase of desensitization was identified as the time during the application of carbamylcholine when the effective membrane resistance increases toward its control value. In some experiments (Fig. 4, 1.0 and 1.3 mM Ca) a small secondary decrease in effective resistance was seen during the period of desensitization. This was in contrast to the less complex time course usually observed in hypertonic sodium-sucrose solutions (Fig. 2). Nevertheless, by comparing the time courses observed in 0.34 and 1.35 mM calcium (Fig. 4) it is again evident that calcium causes a marked accelera-
tion of the desensitization process. These results demonstrate, therefore, that desensitization can occur in depolarized fibers and independently of any change in membrane potential. They also show that calcium can bring about an acceleration of desensitization under the same conditions.

By performing similar experiments in potassium sulfate solutions containing twice the concentration of potassium ion that was used in Fig. 4a, it was possible to show that the addition of potassium antagonizes the effect of calcium on desensitization. The results of two such experiments performed in solutions containing 38 mM potassium sulfate are seen in Fig. 4b (solutions G and H of Table I were mixed to obtain intermediate calcium concentrations). The results obtained in these experiments over the range of calcium ion concentrations from 0.8 to 1.3 mM resemble closely desensitizations observed in 19 mM
potassium sulfate (Fig. 4 a) at 0.3 to 0.7 mM calcium ion. Since the addition of potassium in Fig. 4 b has the same effect as the removal of calcium in Fig. 4 a, potassium may be said to antagonize the ability of calcium to bring about rapid desensitizations.

DISCUSSION

The term “desensitization,” as applied by Thesleff (1), was used in part to refer to the repolarization of the postjunctional muscle fiber membrane during prolonged exposure to various depolarizing agents. In the present study, desensitization has been defined as the process which causes the effective resistance of the postjunctional membrane to return toward control values during this time. This redefinition of desensitization is possible because, as seen in Fig. 2, the membrane potential and effective resistance show similar variations in time course. However, one notable difference between the two measurements is that at the end of the period of drug application the membrane potential frequently remains at a low value of around 60 to 70 mV whereas the effective resistance has returned to control values (Fig. 2 a, c). This residual depolarization may indicate that the desensitization process is not yet complete and that some part of the postjunctional membrane is still sensitive to the action of carbamylcholine. Nevertheless, despite the possibility that desensitization does not proceed to full completion in these experiments, it is clear from Fig. 2 that a major part of the process is characterized by parallel changes in both membrane potential and effective membrane resistance.

Although in the early moments of carbamylcholine application the effective membrane resistance decreased by as much as 90% of its control value, it should not be inferred that the resistance measured by the method used in these experiments was solely that of the chemosensitive postjunctional membrane. If the length constant (λ) of the muscle fibers is assumed to have an approximately normal value of 2.5 mm and if the chemosensitive postjunctional membrane extends roughly 100 μ along the fibers, then the area of membrane traversed by the current pulses used in these experiments to measure effective resistance must include a substantial portion of the adjacent extrajunctional membrane. This consideration may indicate that the properties of the nearby electrically excitable membrane exert some influence on the time course of the desensitization process. Because of the efficient electric coupling between the junctional and extrajunctional regions of the muscle fiber, it would be difficult in the present experiments to estimate what influence changes in the surrounding electrically excitable membrane might have.

The major findings in this study with respect to desensitization are that calcium can cause a several fold increase in the rate of desensitization and that this effect of calcium is inhibited by both sodium and potassium. Of the possible explanations for this action of calcium two may be mentioned. One possi-
bility is to adopt the Katz-Thesleff scheme of desensitization (2). According to this proposal, the reaction of carbamylcholine with postjunctional membrane receptors occurs in a sequence of steps in which an active or depolarizing form of the drug-receptor combination is gradually converted to an inactive or nondepolarizing form. The action of calcium could be adapted to this kind of mechanism by postulating that calcium promotes the conversion of active receptors to their inactive form such as in the following:

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\text{Carb + receptor} \rightleftharpoons \text{Carb} \cdot R \xrightarrow{\text{Ca}} \frac{\text{Carb} \cdot R'}{\text{Na}, \text{K}}
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In order to account for the antagonistic action of sodium and potassium, it would be necessary to postulate in addition that the monovalent ions compete with calcium or acted in some way to promote the reverse reaction; i.e., the reversion of inactive receptors to their active form.

Another way in which calcium might act to bring about rapid desensitization is through its well known property as a membrane “stabilizing agent.” It has been frequently observed in many different natural membranes that calcium acts to limit the permeability of the membrane to other ions, especially the monovalent cations sodium and potassium. Of the familiar examples of this that may be cited, it is especially interesting to note that this effect of calcium can be observed in membranes not ordinarily sensitive to the action of depolarizing agents such as acetylcholine and carbamylcholine. Thus in the squid axon (5), human erythrocytes (6), the frog skin (7), as well as in other systems, calcium has been shown to cause a decrease in the transmembrane movement of sodium and potassium. Since desensitization in the present experiments is characterized by a gradually increasing membrane resistance (or decreasing ion permeability) and since calcium accelerates this change, it may not be unreasonable to think that calcium is acting during desensitization by a similar nonspecific “membrane-stabilizing” mechanism. A more recent contribution by Takeuchi (8) agrees in some respects with this interpretation. Using a modified voltage clamp technique, Takeuchi has shown that an increased concentration of calcium causes a shift in the reversal point of the acetylcholine potential toward more negative values and a decrease in the acetylcholine-produced end plate current. Analysis of these results indicated that during the action of acetylcholine added calcium caused a reduction in the membrane current associated with sodium ions, but that it did not much affect the potassium conductance.

No clear explanation is available for the delayed secondary decline in effective resistance seen in some of the experiments performed in potassium sulfate solutions (Fig. 4 a, 1.0 and 1.3 mm Ca). Such oscillatory behavior was not observed in hypertonic sodium-sucrose media. Further experiments would
be necessary to determine whether this more complex time course is related to the absence of changes in membrane potential, the presence of potassium sulfate itself, or to some other factor.

A final comment may be made concerning the difference between the rates of desensitization measured in the present experiments and those found by Katz and Thesleff (2). In the latter study acetylcholine was applied electrophoretically to the postjunctional membrane of frog sartorius fibers from two separate barrels of a double micropipette. Small amounts of acetylcholine were delivered from one barrel so as to produce repeated 10 to 20 mv depolarizations; the other barrel was used to deliver a long sustained concentration of drug to the same area. The degree of desensitization was measured as the decrease in amplitude of the repeated "test" depolarizations after a steady concentration of acetylcholine was applied from the second or "conditioning" barrel. Desensitization half-times measured in this way ranged from 0.7 to 7 sec whereas the half-periods found in the present study from measurements in effective membrane resistance varied from 10 to 120 sec. The reason for this difference is not known. Although Katz and Thesleff used a high concentration of calcium (9 mm) in most of their experiments, this probably does not explain the shorter time course since the same results were obtained in solutions containing normal amounts of calcium. A second possibility is that the high concentration of acetylcholine (up to several molar) in the electrophoretic micropipettes caused a significant increase in the rate of desensitization over that seen in the present study where a concentration of 0.27 mm was regularly used (see Nastuk and Gissen, 9). Finally, it may be that the desensitization measured by the technique of Katz and Thesleff is not the same process as that defined in this study in terms of changes in effective resistance and potential and hence may occur with an entirely different time course.

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