Factors in the Inactivation of Postjunctional Membrane Receptors of Frog Skeletal Muscle

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ABSTRACT Several factors which influence the rate of inactivation of muscle postjunctional membrane (PJM) receptors during the sustained application of carbamylcholine (CARB) have been studied by two methods. The rate of inactivation was increased by elevating the tonicity of the bathing medium, by increasing the CARB concentration, by raising the calcium ion concentration, and by substituting SO₄⁻ for Cl⁻ ions in the extracellular fluid. The relative effectiveness of calcium and other divalent cations in receptor inactivation was compared. In the absence of calcium, other divalent cations such as magnesium, strontium, or manganese were not efficient substitutes for calcium. In the presence of calcium, the addition of strontium or manganese ions accelerated the rate of receptor inactivation, but the addition of magnesium (up to 12 mM) inhibited this process. The inactivation of the membrane receptors in denervated muscle fibers was found to be similar to that in innervated muscle fibers. Various factors in PJM receptor inactivation are discussed. It is suggested that PJM receptor inactivation is influenced by the binding of calcium ions to sites on the internal surface of the PJM.

INTRODUCTION

Many quaternary ammonium compounds including acetylcholine (Ach) are capable of depolarizing the postjunctional membrane (PJM) of skeletal muscle fibers. If the application of such compounds is sustained, their capacity to depolarize the PJM diminishes with time (37). It has been suggested that this reduction in electrogenic activity is caused by some change in the receptor sites located on the PJM and that this change renders the receptors refractory to agents such as Ach. The conversion of receptors into an inactive form has been called “desensitization” (37) but we prefer to describe the process as “receptor inactivation.”

Although many aspects of receptor inactivation and membrane perme-
ability control have been investigated, the molecular mechanisms underlying these processes remain to be clarified. The understanding of receptor activity is limited because the chemical nature of the receptor site is not well-defined. In our laboratory we have investigated the nature of PJM receptor activation-inactivation by studying various factors which influence its rate and extent.

Earlier we have shown (29) that for the population of PJM receptor sites located at a neuromuscular junction, the rate of development of receptor inactivation and the degree to which it occurs depend on the concentration of the quaternary ammonium ions applied to the junctional region. Subsequently Manthey demonstrated (17) that increasing the calcium concentration in the fluid bathing a muscle increases the rate of PJM receptor inactivation which occurs during application of carbamylcholine (CARB). Manthey measured the rate of receptor inactivation by making repeated measurements of the effective membrane resistance (EMR) of single muscle fibers in the postjunctional region before, during, and after local microperfusion of this region with CARB. Manthey's findings have been confirmed in the present study and we have continued to use the EMR measurements in new experiments designed to explore the effect of variables such as ionic composition of the extracellular fluid, concentration of activating quaternary ammonium ion, osmotic pressure, etc. on receptor inactivation.

The mechanism underlying the influence of calcium on receptor inactivation is not known. In an attempt to elucidate the action of calcium in this process, the influence of other divalent cations on the rate of PJM receptor inactivation has been studied. From the results obtained, and from other lines of evidence, it is proposed that PJM receptor inactivation can be influenced by the binding of Ca ions to sites on the internal surface of the PJM.

**METHODS**

**General Methods**

The experiments were performed in vitro on the sartorius muscle of the frog (*Rana pipiens*) at room temperature (18°-23°C). The muscles were dissected and mounted as described previously (24). During these procedures the preparation was bathed in a phosphate-buffered Ringer solution. When the muscle was securely positioned, a test Ringer solution was introduced into the muscle chamber and the preparation was allowed to equilibrate. The test Ringer solutions used in these experiments were buffered with 1.0 mM tris (hydroxymethyl) aminomethane (Tris) to avoid the precipitation of calcium. The pH of these solutions was adjusted to 7.0 by the addition of either HCl or H2SO4. The composition of the test solutions is given in Table I. Modifications of these solutions made during the course of this study are described in the appropriate sections of the results. The chloride-free solutions were made according to the method of Hodgkin and Horowicz (11). In the calcium-free Ringer solutions, the calcium ions were replaced by either magnesium, strontium, or manganese.
ions. To facilitate the removal of calcium, the calcium-free Ringer solution contained 1 mM ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA).

Standard intracellular recording techniques as described in earlier publications from this laboratory were employed to measure resting membrane potentials, action potentials, and effective transmembrane resistance (EMR) of single muscle fibers (17, 24).

In the present study carbamylcholine, an analogue of acetylcholine which is resistant to hydrolysis by acetylcholinesterase was used to activate the PJM receptors and thereby depolarize the postjunctional membrane. The CARB was introduced by adding it to the solution bathing the muscle or by microperfusion through a glass pipette (50–70 μm in diameter) placed at the junctional region of individual fibers. Both these techniques have previously been discussed in detail (17). In a few fibers CARB was applied to the postjunctional membrane iontophoretically using the technique described by Nastuk (25).

The junctional regions of the muscle fibers were located visually under magnification of 150–300 times by following nerve filaments to their termination. Miniature end plate potentials were recorded intracellularly at such junctions (provided that CARB was not present in the bathing fluid) (6).

**Table I**

**COMPOSITION OF RINGER SOLUTIONS**

| Solution | NaCl | KCl | CaCl2 | Na2SO4 | K2SO4 | CaSO4 | Na3HPO4 | Na2HPO4 | Tris | Sucrose |
|----------|------|-----|-------|--------|-------|-------|----------|---------|-----|---------|
|          | mM   | mM  | mM    | mM     | mM    | mM    | mM       | mM       | mM  | mM      |
| A        | 111  | 2.5 | 1.8   | —      | —     | —     | 2.55     | 0.45     | —   | —       |
| B        | 120  | 2.5 | 1.8   | —      | —     | —     | —        | —        | 1   | —       |
| C        | 120  | 2.5 | 1.8   | —      | —     | —     | —        | 300      | 1   | 300     |
| D        | —    | —   | 60    | 1.75   | 8*    | —     | —        | 1        | 57.2| 57.2    |

* Estimated Ca ion concentration = 1 mM.

Measurement of Receptor Inactivation

The methods used to estimate the rate of receptor inactivation depended on the experimental conditions. In some experiments the postjunctional membrane potential was monitored before and after CARB had been added to the solution bathing the muscle. In this instance (with CARB continuously applied) the rate of the PJM repolarization (which followed the initial depolarization) was used as an indicator of the rate of receptor inactivation (28).

In other experiments the effective transmembrane resistance of single muscle fibers was measured at the junctional region prior to, during, and immediately following microperfusion of CARB onto the postjunctional membrane. In this case the rate of return of the EMR toward control values after an initial fall was used as a measure of the rate of receptor inactivation (17).

The method of drug application and the calculation of inactivation half-times are described in detail by Manthey. Two intracellular electrodes were positioned in a single muscle fiber in the region of the neuromuscular junction. One electrode was
used for passing short, hyperpolarizing current pulses of constant amplitude and the resulting changes in membrane potential were recorded with the second electrode. The effective membrane resistance was calculated as a quotient of the transient incremental change in membrane potential and the applied current which produced such change. When 0.27 mM CARB was applied to the neuromuscular junction, action potentials were initiated and vigorous contraction occurred. In Manthey's experiments the recording difficulties caused by muscle contraction were overcome by equilibrating the muscles in Ringer solutions made approximately 2.3 times hypertonic by the addition of sucrose. Under these conditions muscle contraction is minimized (12). This practice was followed in some of the presently reported experiments as indicated in the text (Table I, solution C).

Statistical Methods

Determination that the difference between two means was statistically significant \( P < 0.05 \) was carried out by a standard nonpaired \( t \) test analysis with \( n_1 + n_2 - 2 \) degrees of freedom (\( n_1 \) and \( n_2 \) are the number of measurements in each sample).

RESULTS

The Effect of Calcium on the Rate of Inactivation with Bulk Application of CARB

In the first series of experiments we recorded the postjunctional membrane potential of muscle fibers equilibrated for approximately 1 hr in Tris-buffered Ringer solution (Table I, solution B) with the calcium concentration set at 0.18, 1.8, or 10 mM/liter. No attempt was made to maintain constant osmotic pressure, but the maximum deviation was always less than 10% of the control value. Thereafter, Ringer solution containing CARB (0.27 mM) was added to the bath and PJM potentials were recorded as single determinations from individual fibers. Sampling of successive fibers was continued over a period of 1 hr. At the end of this time the preparations were returned to CARB-free Ringer solution and the PJM potentials of an additional series of fibers were recorded successively during the next 30 min.

Immediately after the application of CARB, the muscle fibers twitched vigorously for 2–3 min during which time membrane potentials could not be accurately recorded. In order to obtain PJM potential readings during the earliest moments of CARB application, another technique was employed. Tetrodotoxin (TTX) \( 1 \times 10^{-7} \) g/ml was added to the bath for 20 min before testing with CARB to block the muscle action potential and the twitch resulting from it. However, even when TTX was present muscle contracture still occurred following the application of 0.27 mM CARB although localized to the junctional region. The PJM potential was recorded at individual junctions while CARB was applied by microperfusion (30). Some fibers were impaled twice because the recording micropipette occasionally became dislodged or because the pipette was withdrawn to avoid cell damage during the
early period of vigorous mechanical movement. Minimum values of membrane potential were recorded within 5–30 sec of the start of microperfusion. We assumed from the work of Elmqvist and Feldman (5) and Katz and Miledi (14) that TTX does not affect the response of the PJM to CARB and hence the values obtained for these early readings of membrane potential are included with those obtained at longer times in the absence of TTX.
The results obtained in these experiments are shown in Figs. 1 and 2. An increase in the calcium ion concentration from 1.8 to 10 mM significantly increased the rate and extent of PJM repolarization which occurred (Fig. 1, solid lines) during the sustained application of CARB at 0.27 mM and it also caused a significant change in the minimum membrane potential reached immediately after CARB was applied. The minimum membrane potential reached at 1.8 mM calcium was $-15.6$ mV and at 10 mM calcium it was $-20.7$ mV (Table II).

In additional experiments, during sustained application of 0.27 mM CARB, decreasing the calcium ion concentration from 1.8 to 0.18 mM did not appreciably change the average time course of PJM repolarization produced. However, the repolarization curve representing average results obtained from these experiments conducted on muscles equilibrated in the low Ca++ medium includes two distinct types of responses (Fig. 2). With any individual muscle preparation all the fibers repolarized either slowly (curve A) or rapidly (curve B). Seven of these experiments were carried out and in three of the preparations PJM repolarization occurred much more slowly than was generally observed with muscles equilibrated in 1.8 mM calcium. On the other hand, in the remaining four low Ca++ experiments the time course of PJM repolarization was relatively rapid; i.e., similar to that seen with muscles equilibrated in Ringer solution containing 10 mM calcium. Possible explanations of these results are taken up in the discussion.

When muscles were exposed for 1 hr to 0.27 mM CARB, the postjunctional membrane did not become fully repolarized after this time. Indeed, when the CARB was removed from the bath, the PJM repolarized at an accelerated rate (Fig. 1). This indicated that even after an hour's exposure to 0.27 mM CARB some receptors on the PJM were still being activated and thereby initiated conductance changes which hindered repolarization.

### Table II

| Ringer solution | Ca++ concentration | Control RP | No. fibers | E_min | Control RP | No. fibers |
|-----------------|---------------------|------------|------------|-------|------------|------------|
| B + TTX 1.8     | -91.9 ± 0.9*        | -15.6 ± 0.8| 15         | -91.8 ± 0.7| -14.1 ± 0.8| 14         |
| B + TTX 10      | -92.8 ± 0.5         | -20.7 ± 0.9| 17         | -92.9 ± 1.0| -15.4 ± 1.0| 11         |
| C 1.8           | -95.9 ± 1.4         | -24.1 ± 1.1| 11         | -95.0 ± 1.1| -23.3 ± 1.4| 12         |
| C 10            | -100.4 ± 0.9        | -30.1 ± 2.1| 11         | -99.4 ± 1.2| -24.0 ± 1.3| 11         |

* SEM.
A second series of experiments was undertaken to study the rate of PJM repolarization when the CARB concentration was increased from 0.27 to 5.4 mM. These experiments were done on fibers equilibrated in Ringer solution containing calcium at 1.8 and 10 mM; the results are also illustrated in Fig. 1. In earlier work Gissen and Nastuk (9) demonstrated that the rate of PJM repolarization depends on the concentration of applied CARB. Their finding was confirmed in the present studies. Indeed, when the concentration of CARB was increased from 0.27 to 5.4 mM both the rate and extent of the repolarization were significantly increased. Beyond this, three additional points of information can now be added. First, with the CARB concentration set at 5.4 mM, increasing the calcium concentration did not significantly affect the value of the minimum membrane potential reached immediately after CARB was applied. The minimum values reached were −14.1 mV with Ca++ 1.8 mM and −15.4 mV for calcium 10 mM (Table II). This finding is consistent with the hypothesis that calcium and CARB compete for common PJM receptor sites (30). Second, in fibers treated with 5.4 mM CARB raising the calcium concentration from 1.8 to 10 mM significantly increased the rate of early developed (5 min) PJM repolarization but had no significant influence on the extent of repolarization which had developed at the end of 60 min of this continuous treatment. Finally, in those fibers bathed for 60 min in Ringer solution containing 5.4 mM CARB no change in PJM potential occurred after withdrawal of the CARB. This result can be taken to indicate that practically all the PJM receptors in the fibers were inactivated after exposure to 5.4 mM CARB for 1 hr.

The Effect of Increased Osmotic Pressure on Receptor Inactivation

In order to test the effect of hypertonicity on receptor inactivation we carried out other experiments similar in design to those described in the previous section. Postjunctional membrane potentials were recorded by sampling individual muscle fibers of preparations equilibrated for 1 hr in 2.3 × hypertonic Tris-buffered Ringer solution containing 0.18, 1.8, or 10 mM/liter calcium (Table I, solution C modified as indicated). The hypertonicity of these solutions was maintained at constant level by adjusting the sucrose concentration to compensate for different values of the calcium concentration. After equilibration for 1 hr in one of these solutions, CARB (0.27 mM) was added and PJM potentials were recorded for the next 60 min. At the end of this time, the CARB-containing solution was removed and PJM potentials were sampled for the following 30 min in CARB-free Ringer solution. The minimum level of PJM potentials obtained immediately after CARB application was independently determined by microperfusion techniques.

The results of these experiments, exhibited in Fig. 3, show that elevating the calcium ion concentration from 0.18 to 1.8 mM or from 1.8 to 10 mM
significantly increased the rate and extent of repolarization which occurred in the presence of 0.27 mM CARB. In contrast to the dual response obtained with preparations bathed in isotonic media containing 0.18 mM calcium (Fig. 2), only one pattern of repolarization was evident in fibers equilibrated in hypertonic Ringer solution containing 0.18 mM calcium. Furthermore, with fibers bathed in hypertonic media, the rate of PJM repolarization during CARB application was progressively increased when the Ca++ concentration was raised. Also, very little additional PJM repolarization developed in the recovery period during which time CARB was removed. This result indicated that relatively few receptor sites remained active after an hour's exposure to 0.27 mM CARB in the hypertonic solution.

![Figure 3](image_url)

**Figure 3.** The effect of extracellular Ca++ concentration on the rate of repolarization of the postjunctional membrane of muscle fibers bathed in the hypertonic Ringer solution containing 0.27 mM carbamylcholine. Each point represents results from at least 10 fibers.

Additional data concerning the minimum membrane potential reached at the PJM of CARB-activated fibers equilibrated under various conditions are given in Table II. For fibers in isotonic or hypertonic media containing 1.8 mM Ca++, increasing the applied CARB from 0.27 to 5.4 mM caused only a small additional change in the minimum membrane potential. Although this additional depolarization is small, it should not be concluded that the PJM receptors were fully activated by 0.27 mM CARB. From results not included here, we have determined that an increase in CARB concentration from 0.27 to 5.4 mM causes a 2.6-fold decrease in the minimal effective membrane resistance measured at the PJM of fibers equilibrated in the hypertonic medium.
If we return to Table II once again, it can be seen that increasing the Ca\(^{++}\) concentration of the bathing fluid from 1.8 to 10 mM caused the CARB-produced minimum membrane potential to become more negative. Furthermore, under these conditions, increasing the CARB concentration from 0.27 to 5.4 mM produced an appreciable additional depolarization. These results indicate that Ca\(^{++}\) antagonizes CARB in the activation of PJM receptors. The fact that increasing the tonicity of the bathing fluid causes a negative shift in the minimum membrane potential can be interpreted on the basis that loss of intracellular water increased the intracellular Na\(^{+}\) and K\(^{+}\) concentrations and shifted the equilibrium potentials of these ions in the negative direction. Further evidence in support of this argument was provided by the following experiments. Action potentials were recorded over a period of 1 hr from fibers of muscles equilibrated in Tris-buffered isotonic Ringer solution containing 1.8 mM Ca\(^{++}\). Following this the muscles were equilibrated for 1 hr in hypertonic Ringer solution and action potentials were recorded during the next hour of exposure to this medium. The muscles were again immersed in the control solution for 30 min after which action potentials were recorded. Typical recordings are given in Fig. 4 and average results from two muscles are presented in Table III.
For fibers exposed to the hypertonic Ringer solution, the resting potential became more negative and the overshoot diminished. A negative shift in the sodium and potassium equilibrium potentials could explain these changes but in addition there appears to be some influence of hypertonicity on the sodium and potassium conductances of the activated fiber. The rate of rise and fall of the action potentials conducted in these fibers decreased. The changes produced by application of hypertonic Ringer solution appear to be partly reversible. This and other aspects of the problem deserve further study.

Although we concluded that fibers equilibrated in hypertonic solutions and continuously treated with CARB (0.27 mM) repolarize more rapidly than similarly activated fibers equilibrated in isotonic solutions, this conclusion is not readily apparent from curves shown in Figs. 1 and 3. For these two sets of data there are differences both in the control resting potentials and in the minimum values of PJM potential reached early after CARB application. In order to simplify the analysis, the repolarization curves were reconstructed with the PJM repolarization calculated as a percentage of the incremental reduction in membrane potential produced during the initial moments of CARB application. When the values obtained were plotted vs. time for fibers exposed to 1.8 mM Ca++ and CARB 0.27 mM, raising the osmotic pressure of the external fluid significantly increased the rate and the extent of the repolarization of the PJM.

During the recovery period following removal of CARB at 0.27 mM fibers in isotonic Ringer solution (Ca++ 1.8 mM) repolarized by an additional 15 mv as compared with 3 mv additional repolarization achieved by fibers in the hypertonic medium (Fig. 3). The difference may indicate that after an hour’s exposure to CARB (0.27 mM) the extent of postjunctional receptor inactivation is considerably greater for fibers bathed in hypertonic Ringer solution. Parallel conclusions were drawn from the results of similar experiments done with CARB at 0.27 mM and the external Ca++ concentration set at 10 mM.

When it had been demonstrated that PJM repolarization occurs relatively
more rapidly in CARB-activated fibers equilibrated in hypertonic Ringer solution, additional experiments were undertaken to determine the underlying cause of this acceleration. A possible explanation might be that hypertonic Ringer solution causes changes which result in increased reactivity of PJM receptors to CARB. In order to test this we microperfused the PJM with a solution containing CARB at 0.011 mM. The calcium concentration was set at 1.8 mM in this series of experiments. From the data of Table IV, it appears that hypertonic solutions had no appreciable effect on the extent of the depolarization produced by application of 0.011 mM CARB indicating that there is no change in PJM receptor chemosensitivity when fibers are equilibrated in the hypertonic medium.

In order to provide further evidence concerning the effects of tonicity on the rate of inactivation of PJM receptors, measurements of the effective membrane resistance (EMR) were made in individual muscle fibers when CARB was microperfused directly onto the PJM. The half-time of receptor inactivation observed in 12 fibers bathed in hypertonic medium containing 1.8 mM calcium averaged 52 ± 2 sec. When the calcium ion concentration was raised to 10 mM, the average half-time for inactivation in 16 fibers was 20 ± 2 sec. These values are in agreement with those obtained by Manthey (17). Next, to determine the influence of hypertonicity on the rate of recovery of the EMR during the microperfusion of the PJM with 0.27 mM CARB, similar experiments were done on fibers equilibrated in isotonic Ringer solution. In these experiments TTX (1 × 10⁻⁷ g/ml) was applied to prevent initiation of muscle action potentials and the resulting contraction of the muscle fibers. Nonetheless, vigorous contractures developed at the region of the neuromuscular junction and, for fibers bathed in Ringer solution containing 1.8 mM calcium, the mechanical response was strong enough to prevent accurate measurement of both membrane potential and the EMR during the moments immediately following CARB application. However, for fibers equilibrated in Ringer solution containing 10 mM Ca++ plus TTX, the application of CARB caused a shorter lasting contracture which interfered

| Ringer tonicity | Carbamylcholine concentration (mM) | EMR control | EMR perfusion |
|-----------------|----------------------------------|-------------|--------------|
| Isotonic        | 0.011                            | -92.0±0.7   | -70.1±1.5    | 14 |
| Hypertonic 2.3 times | 0.011                        | -96.5±1.5   | -72.7±1.9    | 15 |
less with measurements, and for 12 such fibers, satisfactory experiments were performed. In these experiments the EMR rose from its early developed minimal value to a "plateau" value and the half-time was 30 ± 3 sec. At the plateau, which was reached 5–7 min after CARB was applied, the EMR was still rising slowly and it had attained a value of 72% of the control. For practical reasons the final increments in EMR could not be accurately measured. For 16 fibers bathed in hypertonic Ringer solution containing 10 mM Ca++, the EMR rose to 98.5% of the control value with a half-time of 20 ± 2 sec. Apparently both the rate and the extent of the recovery of the EMR were increased when the tonicity of the Ringer solution was raised. This conclusion parallels that reached from the experiments described above in which the repolarization of the PJM was measured. However, we have reservations about the EMR measurements obtained with fibers bathed in isotonic Ringer solution. In these fibers the intensity of the contractile responses was reduced by raising the extracellular Ca++ concentrations and adding TTX, but it is possible that the residual mechanical responses caused some injury to the muscle fiber which produced leakage pathways around the electrodes at the site of impalement. Such leakage would limit the recovery of the EMR toward control values and thus the figure of 72% given above may be too low.

The Relation between the Concentration of Ca++, the Concentration of the Activating Quaternary Ammonium Ion, and the Production of Receptor Inactivation

The work of previous investigators has shown that the rate of inactivation of PJM receptors by acetylcholine and similar depolarizing agents depends greatly on the method of drug application. This difference in the time course of inactivation ranges from seconds, in the case of iontophoretically applied agents, to many minutes with bath-applied drugs (31).

In their iontophoresis experiments with acetylcholine, Katz and Thesleff (15) obtained a rapid rate of desensitization. They argued that the acetylcholine was applied in a physiological range of concentrations and that the unphysiological part of their experiments was the relatively long time course of application. Work in our laboratory indicates that rapid and extensive PJM receptor desensitization is only obtained if quaternary ammonium compounds are applied in relatively high concentration (31).

Although Manthey (17) demonstrated that elevating the calcium ion concentration increases the rate at which CARB-treated PJM receptors become inactivated, Katz and Thesleff (15) did not observe any significant effect of calcium on the rate of inactivation when CARB and similar drugs were applied. As the concentration of applied CARB is increased, the effect of Ca++ on the rate of receptor inactivation diminishes, which suggests that
in Katz and Thesleff's iontophoresis experiments, the depolarizing agents used were locally applied in high concentration.

In order to investigate the importance of drug concentration for receptor inactivation, the rate of recovery of the EMR was determined with the concentration of applied CARB set at 0.27, 5.4, and 100 mM. These experiments were performed on fibers equilibrated in hypertonic Ringer solution containing Ca++ at either 1.8 or 10 mM and the results are summarized in Table V. As the CARB concentration was increased from 0.27 to 100 mM the rate of inactivation increased significantly for fibers exposed to 1.8 or 10 mM calcium. In addition, the influence of calcium on the rate of inactivation diminished as the CARB concentration was elevated; calcium had a statistically significant effect only in the experiments done with 0.27 mM CARB.

**Table V**

**Effect of Carbamylcholine and Calcium on Rate of Receptor Inactivation**

| Carbamylcholine concentration (mM) | Calcium concentration (mM) | Receptor inactivation half-time (sec) | No. of fibers |
|---------------------------------|---------------------------|------------------------------------|--------------|
| 0.27                            | 1.8                       | 51.9±10.0*                         | 12           |
| 0.27                            | 10                        | 19.9±1.8                           | 16           |
| 5.4                             | 1.8                       | 21.6±4.4                           | 11           |
| 5.4                             | 10                        | 13.4±1.7                           | 11           |
| 100                             | 1.8                       | 10.5±2.5                           | 6            |
| 100                             | 10                        | 8.2±1.3                            | 6            |

*SEM.

**The Effect of Replacing Calcium with Strontium or Magnesium on the Rate of PJM Repolarization**

In the next experiments, we studied the rate of PJM repolarization in the presence of bath-applied CARB when magnesium or strontium replaced calcium in the Ringer solution. Postjunctional membrane potentials were measured in muscle fibers equilibrated for approximately 1 hr in a calcium-free Tris-buffered Ringer solution (solution B) which contained either strontium or magnesium as substitutes for calcium. After a 60 min equilibration period, Ringer solution of similar composition but containing 0.27 mM CARB was added to the bath and over a period of 1 hr, postjunctional membrane potentials were measured from a succession of fibers. At the end of this time the preparations were returned to CARB-free Ringer solution and PJM potentials were recorded over the next 30 min. In order to facilitate calcium removal when Mg++, was substituted for Ca++, 1 mM EGTA was added to all bathing solutions. However, when calcium was replaced with
strontium, EGTA was added to bathing solutions only during the equilibration period prior to CARB application. No attempt was made to maintain the osmotic pressure constant in these solutions to compensate for changes in the magnesium or strontium ion concentrations. In agreement with Jenden and Reger (13), resting potentials were maintained in the control range when strontium or magnesium was substituted for calcium.

The influence of strontium and magnesium on the rate at which the PJM repolarized in the presence of 0.27 mM CARB is shown in Fig. 5. These experiments were done on muscles equilibrated in Ringer solution which contained either 12 mM strontium or 12 mM magnesium. CARB-activated muscle fibers equilibrated in the calcium-free Ringer solutions containing either Sr++ or Mg++ repolarized significantly more slowly than fibers equilibrated in the control Ringer solution which contained 1.8 mM calcium. The difference becomes more marked after 5 min of CARB treatment. In order to determine PJM potential at a time immediately following CARB application, 0.27 mM CARB was microperfused onto individual fibers in other muscles equilibrated in either the strontium- or magnesium-containing Ringer solution. In muscles equilibrated in Ringer solution containing 12 mM strontium, the application of 0.27 mM CARB caused vigorous twitching, consequently TTX ($1 \times 10^{-7}$ g/ml) was added. In contrast, muscles equilibrated in Ringer solution containing 12 mM magnesium did not twitch when 0.27 mM CARB was applied.

![Figure 5](image-url)
Because muscles equilibrated in the calcium-free Ringer solution containing 12 mM magnesium did not twitch when 0.27 mM CARB was added, experiments were undertaken to determine why the PJM depolarization was insufficient to initiate an action potential in these muscle fibers. Four muscles which had been equilibrated in the calcium-free Ringer solution containing 12 mM magnesium and 1 mM EGTA were used. In three of these muscles, eight junctions were microperfused with 0.27 mM CARB and the PJM potential was driven from -88.1 to -18.1 mV without the initiation of an action potential. Furthermore, in four fibers no action potentials were initiated when nonjunctonal regions were microperfused with 3 M KCl thereby reducing the membrane potential to -32 mV. At this stage the micropipette was dislodged by the strong locally produced contracture.

In contrast, when muscle fibers of preparations equilibrated in Mg++-substituted solution were electrically stimulated at junctional or nonfunctional regions through a second intracellular micropipette, action potentials and accompanying twitches were produced without exception. In 11 fibers sampled from such muscles, the resting potential at nonjunctonal regions was -86 mV after placement of two intracellular pipettes. The critical membrane potential ($E_{crit}$) for the initiation of an action potential was determined to be -42 mV (this value is a few millivolts less than the $E_{crit}$ value of -49 mV which was obtained in 20 fibers of muscles equilibrated in solution B). Apparently in these Mg++-treated fibers, perfusing the PJM with 0.27 mM CARB or perfusing the nonjunctonal regions with 3 M KCl produced depolarization exceeding the $E_{crit}$ value.

We speculated that action potentials might not have been initiated during the microperfusion of CARB onto the PJM because the rate of depolarization might be relatively slow. In order to test this possibility, CARB was iontophoretically applied to the PJM (25). By this method of CARB application, the membrane potential could readily be reduced to the critical value within 30-50 msec and in all the Mg++-treated fibers tested with suprathreshold CARB pulses, action potentials were initiated. However, if the bias voltage on the CARB-containing iontophoretic pipette was reduced, thereby allowing CARB to diffuse from its tip, PJM depolarization developed more gradually and under these circumstances, no action potentials were initiated.

The failure of a more slowly developing depolarization to initiate action potentials appears to be specifically related to the high extracellular magnesium concentration, rather than to the lack of calcium in the Mg++-substituted Ringer solution, because this behavior was not observed in muscle fibers equilibrated in either the Mg++ (2 mM) or Sr++ (3 or 12 mM) substituted Ca++-free Ringer solutions. Furthermore, from experimental results not given here we found that nonjunctonal action potentials recorded from muscle fibers equilibrated in Ca++-free, Mg++-substituted (12 mM) Ringer
solution show significant reductions in overshoot and in maximum rates of rise and fall. One interpretation of this result is that the ionic conductance changes associated with these action potentials are reduced in magnitude.

The Effect of Replacing Calcium with Strontium or Magnesium on the Rate of Recovery of the EMR

Activation-inactivation of PJM receptors produced during prolonged local microperfusion of 0.27 mM CARB onto individual junctions was estimated from the rate of return of the effective transmembrane resistance (EMR) toward the control value after its initial decline. The hypertonic Ringer solutions used contained either 10 mM calcium, 12 mM strontium, or 12 mM magnesium. The muscles were equilibrated for approximately 30 min first in an approximately isotonic Ringer solution (B) which contained either 10 mM calcium, 12 mM strontium, or 12 mM magnesium, then for 30 min in the hypertonic medium of similar ionic composition, before starting the experiment. When calcium was replaced by strontium, 1 mM EGTA was added to solutions applied during the equilibration period to facilitate calcium removal, but in this case the EGTA was removed from bathing solutions applied during the test period. Throughout the experiments in which magnesium was substituted for calcium, 1 mM EGTA was present.

Typical results obtained in three muscle fibers which were equilibrated in 10 mM calcium, 12 mM strontium, or 12 mM magnesium Ringer solution are shown in Fig. 6 A–C. In the fiber, equilibrated in 10 mM calcium Ringer solution (Fig. 6 A), the half-time of recovery of the EMR was 18 sec. In contrast, the half-times of EMR return observed in two fibers one of which was equilibrated in the 12 mM strontium (Fig. 6 B) and the other in 12 mM magnesium Ringer solution (Fig. 6 C) were 138 and 216 sec, respectively. Thus we concluded that Mg++ and Sr++ are not as effective as Ca++ in accelerating the inactivation of PJM receptors.

One additional fact is evident from Fig. 6. In the fibers equilibrated in the 10 mM calcium or 12 mM strontium Ringer solution, only a very small increase in the EMR occurred when the CARB was removed. However, in the fibers which were equilibrated in the 12 mM magnesium Ringer solution, the EMR markedly increased when the CARB perfusion ended. This result indicates that when magnesium was present, activation of the PJM receptors continued to occur during almost 800 sec of CARB perfusion and receptor inactivation was not complete even at the end of this time.

Manthey (17) demonstrated in CARB-perfused fibers that the rate of recovery of the EMR decreases as the calcium ion concentration is lowered. Additional experiments were undertaken to determine whether in the absence of calcium ions, the rate of recovery of the EMR would be diminished if the magnesium concentration was lowered from 12 to 2 mM.
In the absence of calcium ions, lowering the magnesium concentration from 12 to 2 mM had little effect on the rate of recovery of the EMR. The half-time of EMR return was found to be 201 sec (average) in four fibers equilibrated in calcium-free Ringer solution containing 2 mM Mg$^{2+}$ and it averaged 208 sec in five fibers equilibrated in calcium-free Ringer solution containing 12 mM Mg$^{2+}$.

![Graph A](image1)

**Figure 6.** Time course of changes in effective membrane resistance measured at the region of the postjunctional membrane. Records A, B, and C represent measurements from experiments performed on muscle fibers equilibrated in hypertonic (2.3 times) Ringer-sucrose solutions containing 10 mM Ca$^{2+}$, 12 mM Sr$^{2+}$, or 12 mM Mg$^{2+}$, respectively. Vertical arrows indicate the onset and termination of the microperfusion of the neuromuscular junction with 0.27 mM carbachol.

In contrast with results obtained by varying the Mg$^{2+}$ concentration, when the strontium ion concentration was reduced from 12 to 3 mM, there was a slight decrease in the rate of EMR recovery. In nine fibers equilibrated in a hypertonic Ringer solution containing 3 mM Sr$^{2+}$ the average half-time for return of the EMR was 175 sec whereas in eight fibers equilibrated in the 12 mM strontium Ringer solution the half-time of EMR recovery was 145 sec.
The Influence of Strontium and Magnesium on the Rate of Recovery of the EMR in the Presence of Calcium

In the previous experiments, using muscles which had been equilibrated in a calcium-free magnesium- or strontium-containing Ringer solution, neither magnesium nor strontium was an effective substitute for calcium in the receptor inactivation process. Because these experiments might have been complicated by changes in membrane behavior often produced in muscle and nerve fibers bathed in calcium-free solutions (7), experiments were done to study the effects of added strontium or magnesium on the rate of receptor inactivation when calcium was present. As in the preceding section the rate of recovery of the EMR during sustained perfusion of the PJM with 0.27 mM CARB was taken as an indication of the rate of receptor inactivation. The tonicity of the Ringer solution was maintained approximately 2.3 times normal and changes in the sucrose concentration were made to compensate for variations in the calcium, strontium, or magnesium ion concentration. A summary of the results obtained from muscles equilibrated in hypertonic Ringer solution containing various concentrations of calcium, magnesium, or strontium is presented in Table VI.

In the presence of calcium, addition of strontium accelerated the rate of receptor inactivation. In six fibers from two preparations equilibrated in hypertonic Ringer solution containing 1.8 mM calcium and 12 mM strontium, the average half-time of recovery of the EMR was 23 sec. In contrast, the

| Calcium concentration (mM) | Strontium concentration (mM) | Magnesium concentration (mM) | EMR recovery half-time (sec) | No. of fibers |
|---------------------------|----------------------------|-----------------------------|-----------------------------|---------------|
| 1.8                       | 0                          | 0                           | 52±10*                     | 12            |
| 10.0                      | 0                          | 0                           | 20±2                       | 16            |
| 0                         | 3.0                        | 0                           | 175±17                     | 9             |
| 0                         | 12.0                       | 0                           | 148±16                     | 8             |
| 0                         | 0                          | 2.0                         | 201±34                     | 4             |
| 0                         | 0                          | 12.0                        | 206±21                     | 5             |
| 1.8                       | 0                          | 6.0                         | 75±19                      | 8             |
| 1.8                       | 0                          | 12.0                        | 105±17                     | 8             |
| 1.8                       | 0                          | 18.0                        | 70±13                      | 10            |
| 5.0                       | 0                          | 12.0                        | 37±12                      | 7             |
| 10.0                      | 0                          | 12.0                        | 25±5                       | 7             |
| 1.8                       | 12.0                       | 0                           | 25±6                       | 6             |

* SEM.
average half-time of EMR return was 52 sec in 12 fibers which were equili-
brated in the control hypertonic Ringer solution containing 1.8 mM calcium (Table VI).

Similar experiments were done to determine the influence of magnesium on the rate of receptor inactivation in the presence of calcium. Initially, the rate of recovery of the EMR was determined in fibers equilibrated in a hyper-
tonic Ringer solution in which the calcium ion concentration was main-

Figure 7. The effect of increasing the extracellular Mg\(^{2+}\) concentration on the half-
time of inactivation exhibited by fibers microperfused with 0.27 mM carbamylcholine. The extracellular Ca\(^{2+}\) concentration was 1.8 mM in all experiments. Each point repre-
sents results from at least seven fibers.

Figure 8. The effect of increasing the extracellular Ca\(^{2+}\) concentration on the half-time of inactiva-
tion exhibited by fibers microperfused with 0.27 mM carbamylcholine. The extracellular Mg\(^{2+}\) concen-
tration was 12 mM in all experiments. Each point represents the results from at least five fibers.

tained at 1.8 mM and the magnesium ion concentration was increased up to 18.0 mM. The average half-time of return of the EMR obtained in seven or more fibers, plotted as a function of the extracellular magnesium ion concentra-
tion, is presented in Fig. 7. As the magnesium ion concentration was increased to 12 mM the half-time of the recovery of the EMR increased. This inhibitory influence of magnesium on the inactivation process appears to be limited; on increasing the magnesium ion concentration from 12 to 18 mM, the inhibitory influence of this ion diminishes. Experiments were done to determine whether the inhibitory influence exhibited by 12 mM magnesium
can be reversed by increasing the calcium ion concentration. Consequently, the magnesium ion concentration was maintained at 12 mM while the calcium ion concentration was varied from 0 to 10 mM. The average half-time of EMR return obtained in five or more fibers has been plotted as a function of the calcium ion concentration (Fig. 8). In the presence of 12 mM magnesium raising the calcium ion concentration reduced the mean half-time of EMR recovery. However, even in the presence of 10 mM calcium, 12 mM magnesium ions decreased the rate of EMR recovery. In 16 fibers which were equilibrated in the magnesium-free Ringer solution containing 10 mM calcium, the half-time of EMR recovery was 20 sec whereas in 7 fibers equilibrated in the 10 mM calcium, 12 mM magnesium Ringer the half-time of EMR return was 29 sec.

The Effect of Manganese Ions on the Rate of EMR Recovery

The results from the previous sections indicate that the action of calcium ions in accelerating the process of receptor inactivation is not shared by magnesium ions and that strontium is a poor substitute for calcium in this process. Additional experiments were done to study the influence of manganese ions on the inactivation process. Other workers have demonstrated that manganese ions are potent inhibitors of certain membrane responses which are calcium-dependent. For instance, the action potentials in barnacle muscle fibers and in some crustacean muscle fibers depend upon Ca\(^{++}\) ion influx and the addition of manganese ions blocks these action potentials (10). Further, in guinea pig smooth muscle, manganese ions abolish the small spontaneous calcium-dependent action potentials which occur randomly in the preparation (32).

The action of Mn\(^{++}\) ions on the rate of recovery of the EMR during microperfusion of single muscle fibers with 0.27 mM CARB was used to estimate PJM receptor inactivation. All these experiments were done in muscle fibers equilibrated in modified hypertonic Ringer solution to minimize muscle contraction. In the first experiments, the hypertonic Ringer solution contained 1.8 mM calcium ions and 10 mM manganese ions. A shortened equilibration procedure was used because the resting potential declined in fibers bathed in the Mn\(^{++}\) hypertonic media. For 4 fibers from 2 muscles equilibrated in hypertonic Ringer solution containing 1.8 mM calcium and 10 mM manganese, the average half-time of recovery of the EMR was 26 ± 2 sec, whereas for 16 fibers which were equilibrated in the hypertonic Ringer solution containing 1.8 mM calcium and no manganese, the half-time of EMR recovery was 52 ± 10 sec.

When it had been demonstrated that in the presence of calcium, the addition of manganese ions accelerates the process of receptor inactivation, experiments were undertaken to determine the capacity of manganese ions to
replace calcium in the inactivation process. Three muscles were bathed for approximately 30 min in a calcium-free hypertonic Ringer solution containing 1.8 mM Mn\textsuperscript{++} ions. In order to facilitate calcium removal 1 mM EGTA was added during the pretest equilibration period. In seven fibers the half-time of recovery of the EMR was 131 ± 28 sec. In three additional experiments, the concentration of Mn\textsuperscript{++} was raised to 12 mM. In these experiments, the half-time of recovery of the EMR was 21, 40, and 96 sec. These results suggest that manganese is more effective than strontium as a substitute for calcium in the inactivation process.

Receptor Inactivation in Muscle Fibers Equilibrated in Chloride-Free Ringer Solution

Manthey (17) demonstrated that Na\textsuperscript{+} and K\textsuperscript{+} ions antagonize the acceleratory action of calcium on receptor inactivation. There has been no attempt to study the influence of anions, particularly chloride, on the process of receptor inactivation.

Consequently, we next studied the development of receptor inactivation for fibers equilibrated in chloride-free Ringer solution (Table I, solution D). The calcium ion concentration was estimated to be 1.0 mM as indicated by Hodgkin and Horowicz (11). When the muscle was first placed in the chloride-free medium, twitching and contracture of the fibers often occurred. This activity quickly subsided and at the end of a 1 hr equilibration period, the membrane potential was in the normal range.

First, the influence of chloride ions on the rate of PJM repolarization was investigated during bulk application of 0.27 mM CARB. The design of these experiments was similar to that described previously. In reducing the external Cl\textsuperscript{−} ion concentration, NaCl was removed and Na\textsubscript{2}SO\textsubscript{4} was substituted keeping the Na\textsuperscript{+} concentration unchanged and sucrose was added to maintain constant osmotic pressure. The results obtained are shown in Fig. 9. In experiments done with the chloride-free Ringer solution, the rate and extent of repolarization were greater than those observed for fibers bathed in control Ringer solution containing chloride at 126.2 mM and Ca\textsuperscript{++} at 1.8 mM. For fibers equilibrated in Cl\textsuperscript{−}-free Ringer solution only a small additional PJM repolarization occurred when the CARB was removed. In contrast, a more pronounced PJM repolarization occurred following removal of CARB from fibers bathed in the control Ringer solution containing chloride. This difference is taken to indicate that in the absence of chloride, a more complete inactivation of PJM receptors occurs during CARB application.

Takeuchi's results (35) show that reduction of the chloride ion concentration has no influence on the membrane potential at which end plate current reverses. Thus it might be expected that changes in extracellular Cl\textsuperscript{−} concentration would not affect the minimum level of membrane potential reached
during intensive activation of the PJM with CARB. In two muscles this expectation was checked using the microperfusion techniques discussed previously. For fibers equilibrated in control Ringer solution (1.8 mM Ca++) or chloride-free Ringer solution (1.0 mM Ca++) the minimum value of the PJM potential reached during CARB perfusion was identical (-15.6 mV). In these experiments TTX (1 × 10⁻⁷ g/ml) was used to block the production of muscle action potentials.

When it had been demonstrated that the rate of repolarization (and presumably receptor inactivation) was increased in fibers equilibrated in chloride-free Ringer solution when CARB was bath-applied, additional experiments utilizing EMR measurements were done to determine the rate of inactivation in individual muscle fibers when CARB (0.27 mM) was microperfused directly onto the PJM. During this perfusion, the EMR was repeatedly determined and the rate of recovery of the EMR was used as an index of receptor inactivation. The muscle equilibrated in Cl–free Ringer solution (Table I, solution D, made hypertonic by the addition of extra sucrose [300 mM]). The mean half-time of recovery of the EMR was found to be 21 ± 2 sec in the hypertonic chloride-free Ringer solution (1 mM Ca++). This average value representing data from five junctions in two muscles may be compared with the 52 sec half-time for fibers in hypertonic Ringer solution containing Ca++ 1.8 mM, Cl–126.2 mM, and tested with 0.27 mM CARB (Table VI).
Reduction of the calcium ion to 0.2 mM slowed the development of receptor inactivation. The mean half-time of recovery of the EMR obtained in five fibers (two muscles) immersed in the hypertonic chloride-free Ringer solution containing 0.2 mM calcium was 130 ± 18 sec.

Receptor Inactivation in Denervated Muscle Fibers

In normally innervated muscle fibers the membrane region sensitive to acetylcholine and related drugs is localized primarily at the PJM of the neuromuscular junction. However, many workers have demonstrated that after denervation, muscle fibers become sensitive to acetylcholine along their entire length (21, 22). At the present time, few studies concerning the process of receptor inactivation have been done on denervated muscle. Katz and Thesleff (15) applied acetylcholine iontophoretically to muscles which had been denervated for 3–4 wk and demonstrated that receptors located in the PJM (the region previously innervated) exhibit desensitization and recovery which parallel the behavior of receptors in normal muscle. Nastuk et al. (31) showed, using chronically denervated muscle fibers, that membrane depolarization produced by application of CARB in moderate concentration (0.05 mM) was well-sustained over a 90 min period. The following is an extension of our earlier studies of receptor inactivation in denervated muscle.

These experiments were performed using muscles which had been denervated for 12–20 wk. The muscle fibers were extremely sensitive to mechanical disturbance and consequently all the experiments were performed on preparations equilibrated in the hypertonic Ringer solution. Even though the bathing medium was hypertonic, these fibers often developed strong contractures immediately following application of 0.27 mM CARB. No attempt was made to distinguish between the formerly innervated regions and nonjunctional areas of the muscle fibers.

Experiments were carried out to determine whether denervated muscle fibers would repolarize when continuously exposed to 0.27 mM bath-applied CARB, and the results obtained are shown in Fig. 10. In 2 denervated muscles, resting potentials were obtained from 10 fibers, then Ringer solution containing CARB (0.27 mM) was added and resting potentials from other muscle fibers were sampled over the next 50 min. Denervated fibers equilibrated in the hypertonic medium repolarized during continuous application of CARB and the repolarization curve is similar to that seen with normal muscle (Fig. 3). The mean value of the minimum membrane potential reached immediately following CARB application was determined by microperfusion techniques and found to be −21.9 ± 1.4 mv (seven fibers). The value is comparable to the −24.1 ± 1.1 mv value obtained in normal muscle fibers equilibrated in a hypertonic medium (Table II). We concluded from the foregoing
demonstration that the chemoreceptors in denervated muscles become inactivated during prolonged exposure to CARB.

We next studied the effect of calcium on receptor inactivation in denervated muscle. The rate of recovery of the EMR was considered to be a measure of the rate of receptor inactivation. Very strong contractures developed in these denervated muscle fibers (denervated 15–17 wk) during perfusion with 0.27 mM CARB, and the mechanical movement was a severe experimental problem, especially when the calcium concentration was 1.8 mM. In 13 out of 16 fibers perfused with CARB both intracellular microelectrodes remained inside the fiber under these conditions. Of these 13 fibers, the results from only 7 were considered to be acceptable. In the remaining six fibers, there was evidence of injury caused by movement of the muscle fibers. By this we mean that in the latter fibers there was no repolarization and the EMR recovered slowly (mean half-time was approximately 202 sec with a range of 170 to 276 sec). In contrast, the EMR recovered much more rapidly in the remaining seven fibers and the mean half-time was 74 sec (range 22 to 142 sec), which is comparable with the value of 52 sec obtained in innervated fibers (Table VI). In the satisfactory experiments on seven denervated fibers, membrane repolarization and EMR recovery showed parallel time courses as is apparent in normal muscle (17).

When the calcium ion concentration was raised from 1.8 to 10 mM the halftime of recovery of the EMR was decreased to 35 sec (eight fibers). In these experiments, the increased Ca++ concentrations were compensated for by appropriate reduction in the Na+ concentration leaving the sucrose concentration unchanged. Strong contractures appeared despite the fact that the calcium ion concentration had been increased to 10 mM and as a result, of
the 13 fibers perfused with CARB only the data from 8 were acceptable. Because the denervated muscles were mechanically active and their membranes appeared to be more susceptible to damage we feel that our present results should be regarded as only qualitative.

**DISCUSSION**

When an electrogenic quaternary ammonium compound such as acetylcholine or carbamylcholine is placed on the external surface of the postjunctional membrane (PJM) of a muscle fiber, this membrane undergoes a rapid increase in conductance. The resultant ionic fluxes of small diameter cations, principally Na\(^+\) and K\(^+\), quickly drive the potential difference across the PJM to a value near \(-15\) mV (35). In normal neuromuscular transmission these events are speedily terminated because the neurally released acetylcholine is hydrolyzed and also because the transmitter rapidly diffuses away from the PJM. However, if electrogenic quaternary ammonium ions are topically applied to the PJM and are allowed to remain there for a relatively long time, the initially produced increase in membrane conductance and the decrease in membrane potential are slowly reversed and both return toward control values at roughly parallel rates (17).

We have used the term receptor activation to characterize the initial molecular events which make possible an increase in PJM conductance when electrogenic quaternary ammonium ions are applied. Further, we have used the term receptor inactivation to indicate the steps following receptor activation which bring about a more slowly developed reduction in PJM conductance. At present there is no direct evidence which reveals the nature of the molecular changes which are presumed to occur during activation and inactivation of membrane receptors. Therefore we have investigated these processes in an indirect manner on the assumption that determinations of the time course of the PJM potential and PJM conductance provide a useful measure of the molecular events occurring at membrane receptor sites.

One limitation of the techniques used in the present study is that conductance changes produced in the PJM by applied CARB cannot be measured directly. Such a measurement is presently not possible because the chemosensitive membrane is electrically coupled to the adjacent conductile membrane of the muscle fiber. Thus the "input resistance" measurements reported in the present study represent the conductance of the PJM in parallel with that of the adjacent conductile membranes. An additional complication is introduced by the fact that the conductance of this extrajunctional membrane is membrane potential dependent. Despite these limitations it has been possible in this and earlier work to obtain useful information relative to PJM conductance changes. In future studies of this kind it may be preferable to make conductance measurements on voltage-clamped fibers during application of
CARB and similar agents. Another approach is to use fibers totally depolarized by application of isotonic potassium Ringer solutions.

During the sustained application of CARB it was generally found that the initial increase in PJM conductance subsided more rapidly than the repolarization of the PJM membrane, the two being simultaneously measured in such experiments (17). This result is to be expected largely because changes in PJM conductance are non-linearly related to changes in PJM potential (20). However, additional subsidiary factors may cause the PJM repolarization to lag behind the return of PJM conductance toward control values. Suppose that after sustained application of CARB in high concentration, the PJM conductance has returned to its control value. During the preceding period when the membrane conductance is elevated, appreciable ionic influx and efflux occur at the junctional and extrajunctional regions producing alteration of the ionic composition of the intracellular phase. It is assumed that these ionic alterations persist for some time after the PJM conductance increase has subsided. One such expected alteration is an increased intracellular Cl\(^{-}\) concentration as a result of a preceding increase in Cl\(^{-}\) influx. An ionic shift of this kind would keep the membrane potential reduced below its control value for a time after full inactivation of PJM receptors had been achieved.

In our present experiments, the contribution of Cl\(^{-}\) influx to the time course of the change in PJM potential produced during CARB application was tested by measurements on muscle fibers equilibrated in Cl\(^{-}\)-free Ringer solution. The results show that substitution of SO\(_4^{2-}\) for extracellular Cl\(^{-}\) increases the rate of PJM repolarization (Fig. 9) which provides support for the above line of reasoning. However, the argument is complicated by the fact that substitution of SO\(_4^{2-}\) for Cl\(^{-}\), also produces an unexpected further increase in the rate of recovery of the PJM conductance which itself would speed PJM repolarization. In these SO\(_4^{2-}\) substitution experiments we cannot estimate the comparative effectiveness of Cl\(^{-}\) removal vs. the additional acceleration of receptor inactivation on the rate of PJM repolarization produced by this ionic substitution. For this reason it now appears better to make direct chemical determination of any intracellular Cl\(^{-}\) concentration changes in order to evaluate the contribution of such shifts to the rate of repolarization in CARB- depolarized fibers.

In earlier publications from our laboratory we have presented evidence concerning various factors which influence the rate and extent of receptor inactivation (27). The previously discussed criteria for inactivation have been used and it has been shown that this process is speeded as the concentration of the applied quaternary ammonium ions is increased (27). The range of our earlier results has now been extended by the presently reported experiments which indicate that inactivation of a large fraction of PJM receptors can be
produced in a few seconds during topical application of very high concentrations of quaternary ammonium ions. Katz and Thesleff (15) reported a rapid rate of receptor inactivation from experiments in which quaternary ammonium ions were applied to the PJM iptonophoretically. They contrasted these rapid changes with the relatively slow rate of inactivation produced by adding various quaternary ammonium ions in low concentrations to solutions bathing whole muscles. We suggest that in Katz and Thesleff's iontophoresis experiments, quaternary ammonium ions were applied in high concentration to a small area of the PJM and thereby receptor inactivation was rapidly produced in this discreet region. This argument is further supported by the fact that in Katz and Thesleff's experiments they found receptor inactivation to be insensitive to increase in the extracellular Ca++ concentration and to the tonicity of the bathing fluid. As we have shown in the present study, such comparative independence becomes evident when CARB is applied to the PJM in high concentrations.

The fact that CARB must be applied in very high concentrations to inactivate a large fraction of the PJM receptors in a short time is an interesting and important point which requires interpretation. One possible explanation is that some of the PJM receptors lie in tortuous channels whose openings are guarded by barriers having low permeability to ions such as CARB. Thus one action of hypertonic solutions in accelerating receptor inactivation might stem from the fact that such solutions shrink various extramuscular cellular elements at the neuromuscular junction thereby allowing easier access of CARB to postjunctional receptor sites. Dr. Phillip Brandt of this institution kindly prepared electron micrographs of frog sartorius muscles after they had been equilibrated with the hypertonic solutions we used. The muscle fibers showed a marked increase in the diameter of the transverse tubules (1, 8). Unfortunately no sections were made at the neuromuscular junction but from our preliminary results it appears that the problem is worthy of further investigation.

In addition to the morphological changes produced by application of a hypertonic solution, we thought that this treatment might influence the kinetics of receptor site inactivation by CARB. For example, a hypertonic solution could directly affect the receptor site structure or its state of hydration. We suppose that such changes might be revealed as a change in the sensitivity of the PJM to CARB but as the data show (Table IV) no such alteration was detected in fibers treated with hypertonic solution. Finally, we wondered whether the accelerating action of hypertonic solutions on receptor inactivation might not result from an increase in intracellular Ca++ in CARB depolarized fibers produced both as a result of enhanced Ca++ influx and by facilitated release of Ca++ from the intracellular stores. According to the work of A. Isaacson (personal communication) application of Ringer solution...
made hypertonic by addition of sucrose increases the calcium content of frog sartorius muscle. We presume that the calcium loading produced by hypertonic solutions is further promoted by the depolarization produced during CARB application.

In the model which we have been considering (see below) receptor inactivation is thought to be accelerated by a rise in intracellular Ca++ concentration which promotes the reaction of Ca++ with anionic sites on the inner surface of the PJM. We suppose that in CARB-depolarized fibers the [Ca++]r increase is produced both by increased Ca++ influx (33) and by increased mobilization of Ca++ sequestered in the sarcoplasmic reticulum (20). On this basis reduction in extracellular Ca++ concentration would be expected to slow receptor inactivation and this result is obtained in fibers bathed in hypertonic solution. It shows up in the form of slowed PJM repolarization and slow recovery of the EMR during sustained CARB application (Fig. 3, Table V).

Many experiments conducted on muscles bathed in isotonic Ringer solution gave results parallel with those discussed above, that is reduction of extracellular Ca++ to 0.18 mM slows the rate of repolarization developed during CARB application (Fig. 2, curve A). However, some of the muscles treated in this manner behaved differently in that reduction of (Ca++) to 0.18 mM accelerated membrane repolarization (Fig. 2, curve B). All the fibers of a particular muscle behaved either as shown in curve A or in curve B. The result seems to be dependent on some unspecified preconditioning factors operating in the various individual muscle preparations used. Possibly in these so-called anomalous experiments a larger than usual amount of Ca++ was liberated intracellularly because the relationship between membrane potential and Ca++ release shifted favorably and also because some fibers have unusually large amounts of Ca++ stored in their sequestration depots. Our only further comment on these paradoxical results is that possibly the rapid repolarization seen in the curve B experiments may not simply represent PJM receptor inactivation. For example, the extrajunctional membranes of the fibers of these muscles might have shown unusually large increases in potassium conductance. For instance, Frankenhaeuser and Hodgkin (7) showed, from experiments on squid axons, that reducing (Ca++) caused large increases in potassium current at moderate depolarization. However, it is hard to imagine why this behavior should not also be exhibited by fibers bathed in low calcium hypertonic Ringer solutions.

Our data indicate that Ca++, among the ions thus far studied, is the most effective in accelerating PJM receptor inactivation. Based on the rate of return of effective membrane resistance and the rate of repolarization of CARB-exposed fibers, Sr++ or Mn++ is a very weak substitute for Ca++ in receptor inactivation. However, if some Ca++ is already present in the bathing
fluid, addition of Sr$^{++}$ or Mn$^{++}$ accelerates receptor inactivation to an appreciable extent.

Magnesium ions are unique among the cations studied in that they appear to inhibit receptor inactivation as shown in Figs. 6-8. The antagonism between Ca$^{++}$ and Mg$^{++}$ in receptor inactivation brings to mind the fact that these cations exert opposing action on the release of acetylcholine from motor nerve terminals (4). We suppose that Mg$^{++}$ acts to displace membrane-bound Ca$^{++}$ or that it competes with Ca$^{++}$ for common mechanisms of influx operative during membrane depolarization. This competitive relation between Mg$^{++}$ and Ca$^{++}$ in receptor inactivation deserves further study.

The molecular processes involved in postjunctional membrane permeability control and the role of postjunctional receptors in these processes have been the subject of many reviews and speculations (38). The nature of receptor inactivation has also been speculated about. Models for receptor inactivation have been suggested in a paper by Katz and Thesleff (15) and in publications from our own laboratory (27, 31). From earlier and more recent work carried out in our laboratory we are now led to suggest that receptor inactivation involves the reaction of free intracellular Ca$^{++}$ ions with anionic sites available on the inner surface of the PJM. These anionic sites might be negatively charged groups of membrane carrier molecules. Thus combination of such sites with intracellular Ca$^{++}$ ions could reduce the mobility of carriers and limit their capacity to transport univalent ions such as Na$^{+}$ and K$^{+}$.

In order to obtain the required increase in the intracellular Ca$^{++}$ concentration one can promote Ca$^{++}$ influx and also reduce or reverse intracellular Ca$^{++}$ sequestration.

Several lines of evidence and argument make the above ideas attractive to us. (a) Ca$^{++}$ influx is increased when the PJM is activated by depolarizing quaternary ammonium compounds (33, 36). A transient increase in (Ca$^{++}$)$_{i}$ requires an increase in Ca$^{++}$ influx. Such influx could be achieved by sudden widespread activation of PJM receptor sites such as is produced by application of a concentrated solution of an active quaternary N$^{+}$ compound to the external surface of the PJM. (b) The rate and extent of receptor inactivation are dependent on (Ca$^{++}$)$_{o}$ (17). (c) The PJM depolarizing power of an agent such as CARB depends on the external Ca$^{++}$ concentration. Increase in Ca$^{++}$ concentration above 2 mM limits PJM depolarization by CARB apparently as a result of competition of these two ions for PJM receptor sites (30). (d) The rate of receptor inactivation by CARB and the rate of reversal of inactivation are relatively slow processes which fact could conceivably be explained by the appreciable time required to increase and decrease intracellular (Ca$^{++}$)$_{i}$.

(e) Sustained application of various quaternary ammonium compounds increases the amount of Ca$^{++}$ localized in the region of the PJM (18) and
such treatment can cause sarcomeric oscillations in that region (19). The controlling factor in such Ca$$^{++}$$ accumulation appears to be PJM depolarization. Although quaternary ions can penetrate the PJM (26) there is no evidence that they directly cause release of Ca$$^{++}$$ from the sarcoplasmic reticulum (34). (f) Caffeine which causes sarcomeric oscillations (19) increases the rate of PJM repolarization during sustained application of CARB (Mikiten and Nastuk, unpublished data). Caffeine is commonly believed to act by limiting Ca$$^{++}$$ sequestration at the sarcoplasmic reticulum. (g) Receptor inactivation can be produced by applying CARB to muscle fibers completely depolarized by increased (K$$^{+}$$)o. Activation of the contractile system can also occur under these conditions (33).

The action of Mg$$^{++}$$ in inhibiting receptor inactivation represents one of the important findings in our present study. Magnesium and calcium ions exhibit parallel behavior in that both are competitors of CARB in receptor activation (3, 27), but these ions have opposing action in receptor inactivation. We suppose that the inhibitory action of Mg$$^{++}$$ depends on its competition with Ca$$^{++}$$ in transmembrane movement and possibly for binding to anionic sites on the internal surface of the PJM.

In order to provide more direct evidence for the validity of the above proposal, we are attempting to elevate (Ca$$^{++}$$)i by various means such as iontophoresis and by the use of chemical agents such as caffeine. Powerful intracellular Ca$$^{++}$$ sequestration mechanisms operating in muscle fibers and axons keep the (Ca$$^{++}$$)i at very low values and make experimental maneuvers of the above kind difficult. Thus, under normal physiological conditions, one can reasonably assume that the Ca$$^{++}$$ influx which accompanies neuromuscular transmission and excitation-contraction coupling produces only a short enduring rise in (Ca$$^{++}$$)i.

The action of Ca$$^{++}$$ at extracellular sites on conductile and postsynaptic membranes has long been appreciated and carefully studied (2, 16, 23, 30, 36). We now suggest that Ca$$^{++}$$ also has an important action at internal membrane sites in controlling the permeability of postsynaptic and perhaps also the conductile membranes as well. It may be that (Ca$$^{++}$$)i is an important regulator of the coupling between motor neuron and muscle fiber.

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