The Interaction between Caffeine and Calcium in the Desensitization of Muscle Postjunctional Membrane Receptors

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ABSTRACT The interaction between caffeine and calcium on the rate of desensitization of muscle postjunctional membrane (PJM) receptors during the sustained application of 0.27 mM carbamylcholine (CARB) has been studied in vitro on the sartorius muscle of the frog. The rate of PJM repolarization with CARB added to the solution bathing the muscle or the recovery of the effective transmembrane resistance (EMR) during the microperfusion of CARB directly onto the end-plate region of individual fibers was used as an index of the rate of desensitization. Caffeine (1.5 mM) increased the rate of PJM repolarization with bulk application of CARB in a 1.8 or 10 mM calcium Ringer solution but had no effect on PJM repolarization in a calcium-deficient, 4 mM magnesium Ringer solution. For EMR measurements the preparation was rendered mechanically quiescent by repeated challenges with isotonic KCl during an exposure of several hours to a calcium-free, 4 mM magnesium–1 mM EGTA Ringer solution. In these fibers, the microperfusion of 0.27 mM CARB together with 1.8 mM calcium plus 1.5 mM caffeine significantly increased the rate of EMR recovery above that observed in the absence of caffeine. Raising the calcium concentration to 10 mM had a similar effect; however, no additional increase was noted by the inclusion of 1.5 mM caffeine. It is suggested that the major role of caffeine in PJM desensitization is to increase the calcium permeability of the surface membrane. The transmembrane movement of calcium and the consequent intracellular accumulation of calcium is seen as a critical factor in controlling the rate of PJM desensitization.

INTRODUCTION

The application of many quaternary ammonium compounds such as acetylcholine (ACh) or carbamylcholine (CARB) to isolated skeletal muscle produces a rapid depolarization of the postjunctional membrane (PJM). If the

presence of such depolarizing agents is maintained there is a gradual repolarization of the PJM accompanying a general reduction in chemosensitivity (Thesleff, 1955). This loss of chemosensitivity of the end-plate receptors has been termed desensitization (Thesleff, 1955) or inactivation (Nastuk and Parsons, 1970). While the actual molecular mechanisms underlying the process of desensitization are unknown, evidence has accumulated which suggests that there is a relationship between an increase in calcium permeability at the end plate and the rate of PJM desensitization (Magazanik, 1968; Parsons, 1969 *b*; Magazanik and Vyskočil, 1970; Manthey, 1970). If the above relation is correct, then factors which raise the permeability to calcium may be expected to increase the rate of PJM desensitization. Consistent with this view, Nastuk and Parsons (1970) and Manthey (1970) have shown that elevating the CARB concentration, which is thought to increase the permeability to calcium at the end plate (Takeuchi, 1963; Parsons and Nastuk, 1969), accelerates the rate of PJM desensitization.

Caffeine has been used in many studies concerning excitation-contraction coupling in muscle (see Sandow, 1965; Chiarandini et al., 1970 *a*, *b*). The results of earlier work have demonstrated that many of the effects of caffeine can be explained by its ability to change the distribution of ionized calcium within muscle fibers and to enhance the calcium fluxes across the muscle fiber membrane (Bianchi, 1968; Chiarandini et al., 1970 *a*, *b*). If calcium permeability of the surface membrane is a critical factor in PJM desensitization, then treatment of the muscle with caffeine might be expected to alter the desensitization process. The present study of the effects of caffeine on PJM desensitization was undertaken to obtain additional information on the importance of transmembrane calcium movements in this process. It was found that caffeine accelerates desensitization of the PJM and our results suggest that this action of caffeine is related to its ability to increase calcium influx at the end plate during CARB activation.

METHODS

General Methods

The experiments were performed in vitro on the sartorius muscle of the frog (*Rana pipiens*) at room temperature (16°–22°C). The muscles were dissected and mounted as described previously (Parsons, 1969 *a*, *b*; Nastuk and Parsons, 1970). All experimental Ringer solutions used in this study were buffered with 1.0 mM tris(hydroxymethyl)aminomethane (Tris) to avoid the precipitation of calcium. The composition of the test solutions is given in Table I except when indicated. Note that, in the calcium-free Ringer solutions, the calcium ions were replaced by magnesium ions and, to facilitate the removal of calcium, 1 mM ethylene glycol bis(β-aminoethyl ether)*N*, *N*,*N*′,*N*′-tetraacetic acid (EGTA) was added. The pH of all solutions was adjusted to 7.2–7.3 by the addition of HCl.

Standard intracellular recording techniques were employed to measure the resting membrane potentials and the effective transmembrane resistance (EMR) of single muscle fibers (Parsons, 1969 *a*, *b*).

As in earlier investigations, carbamylcholine chloride (CARB) (K & K Laboratories Inc., Plainview, N.Y.), an analogue of acetylcholine which is resistant to hydrolysis by acetylcholinesterase, was used to activate the end-plate receptors. The CARB was introduced either by incorporation into the solution bathing the muscle or by local microperfusion through a glass micropipette (50–100 μm tip diameter) placed at the end-plate region of individual fibers. Both procedures have been discussed in detail (Manthey, 1966; Parsons, 1969 *a*, *b*; Nastuk and Parsons, 1970). The maximum depolarization observed during the continuous perfusion of CARB was assumed to be an index of the extent of end-plate receptor activation. In some experiments caffeine (Eastman Organic Chemicals, Rochester, N.Y.) was included with

TABLE I

COMPOSITION OF EXPERIMENTAL RINGER SOLUTIONS

Solution	NaCl	KCl	$CaCl_2$	$MgCl_2$	Tris	EGTA
A	120	2.5	1.8	—	1.0	—
B	—	122.5	—	—	1.0	—
C	120	2.5	—	4.0	1.0	1.0

CARB in the solution bathing the muscle or was microperfused onto the end-plate region of individual fibers together with CARB.

The junctional regions of individual fibers were visually located by following nerve filaments to their termination under magnification 300. Miniature end-plate potentials were recorded intracellularly at such junctions (provided that CARB was not present in the bathing fluid) (Fatt and Katz, 1952).

Measurement of Receptor Desensitization

The two methods used to estimate the rate of receptor desensitization were similar to those previously described in detail (Manthey, 1966; Nastuk and Parsons, 1970). In some experiments the postjunctional membrane potential was monitored before and after CARB had been added to the solution bathing the muscle. Under these conditions (with CARB continuously present), the rate of PJM repolarization was used to indicate the rate of receptor desensitization.

In other experiments, the effective transmembrane resistance of single muscle fibers was measured at the end-plate region before, during, and immediately after microperfusion of CARB onto the PJM. In this instance, the rate of return of the EMR toward control values after an initial fall was assumed to be an indication of the rate of receptor desensitization (Manthey, 1966; Parsons, 1969 *b*; Nastuk and Parsons, 1970). The method used to calculate the degree of recovery of the EMR was similar to that described by Manthey (1966).

Normally, when 0.27 mM CARB is perfused onto the end plate, muscle fibers are depolarized and vigorous contraction occurs. To minimize the recording difficulties

caused by muscle contraction, previous studies were performed using muscles equilibrated in sodium Ringer solutions made hypertonic by the addition of sucrose (Manthey, 1966; Parsons, 1969 *b*; Nastuk and Parsons, 1970). Unfortunately, hypertonicity increases the rate of receptor desensitization (Nastuk and Parsons, 1970), perhaps due to an elevated intracellular calcium ion concentration evident under these conditions (Isaacson, 1969; Caputo, 1966). In the current study another method was used to minimize muscle movements enabling CARB-produced changes in EMR to be estimated. Muscles were maintained in a calcium-free, 4 mM magnesium solution (solution C) for 4–7 hr. During this time they were challenged at least four times (but not more than six times) with a KCl Ringer solution (solution B). Tetrodotoxin (TTX) (1×10^{-7} g/ml) was then added to the bathing solution approximately 30 min before the perfusion of 0.27 mM CARB to eliminate muscle spiking (Kao, 1966). It was assumed from the work of Elmqvist and Feldman (1965) and Katz and Miledi (1967 *a*) that TTX does not affect the sensitivity of the PJM receptors to CARB. After exposure of the muscle to the calcium-free environment as described above, it was possible to maintain two intracellular micropipettes in an individual fiber during the initial moments of CARB application in 114 of 126 fibers (38 muscles) studied as CARB-induced contractures at the end-plate regions were greatly diminished.

In a few experiments, a sodium Ringer solution made approximately 2.3 times hypertonic by the addition of sucrose was utilized to determine the influence of caffeine on PJM receptor activation (Nastuk and Parsons, 1970). This solution was chosen for these experiments because it was essential that muscle movement not interfere with an accurate estimate of the extent of the CARB depolarization.

Statistical Methods

In all cases, results are reported as a mean value ($\bar{Y}$) ± the standard error ($s\bar{Y}$). No distinction was made between end plates obtained from the same or different muscles. Tests of significance between different treatment groups were made either by student's *t* test (nonpaired) or by conventional analysis of variance (ANOV). Differences in sample means whose estimated probability of chance occurrence corresponded to the 5% level were taken as significant.

RESULTS

The Effect of Caffeine on the Rate of Desensitization with Bulk Application of CARB

Initially it was determined that caffeine accelerated the rate of PJM repolarization with bulk application of 0.27 mM CARB. For these experiments the PJM potential was recorded from muscle fibers equilibrated for approximately 45 min in the Tris-buffered 1.8 mM calcium Ringer solution (solution A). The muscles were then exposed for 60 min to solution A plus 0.27 mM CARB (in the presence or absence of caffeine at the concentrations indicated). During this period, the PJM potential of individual fibers was sampled at regular intervals. The preparations were then returned to the CARB-

free, caffeine-free Ringer solution (solution A) and the PJM potential was recorded after 15 min.

The results obtained are summarized in Fig. 1. We assumed that 0.5–1.5 mM caffeine did not significantly alter the minimum values of membrane potentials during the initial 0.27 mM CARB application. The evidence for this view is described in the following sections.

We found that 1.5 mM caffeine increased the rate of repolarization in the 0.27 mM CARB–10 mM calcium solution (Fig. 2), although the effect of caf-

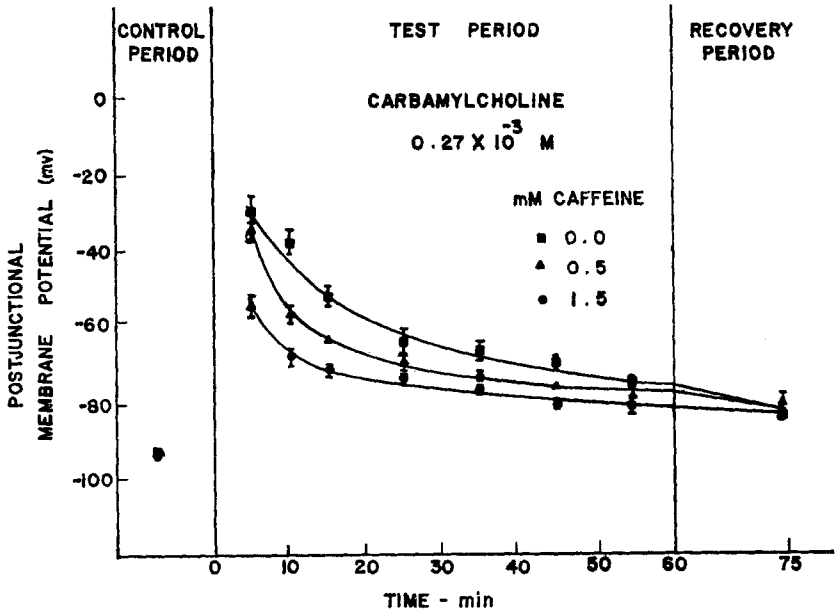


FIGURE 1. The effect of caffeine on the rate of repolarization of the postjunctional membrane of muscle fibers bathed in 1.8 mM Ca^{2+} Ringer solution containing 0.27 $\times$ 10^{-3} M carbamylcholine. Each point represents results from at least five fibers from at least five muscles. Vertical lines in Figs. 1, 2, and 3 represent the standard error about the mean. When omitted, the standard error was encompassed by the solid symbols. Recovery in CARB-free, caffeine-free solution.

feine was less striking in the presence of the elevated calcium concentration. These experiments were performed in a manner identical to that described above except the calcium concentration of solution A was raised from 1.8 to 10 mM (no attempt was made to compensate for the increased tonicity as the change was less than 10%).

For those experiments done in the 10 mM calcium Ringer solution, we determined the influence of 1.5 mM caffeine on the depolarization produced by the microperfusion of 0.27 mM CARB onto the end-plate region of individual muscle fibers by utilizing other muscles treated with TTX (Nastuk and Parsons, 1970). The depolarization produced by 0.27 mM CARB alone

(70 ± 2 mv in eight fibers) was not significantly different from that produced by 0.27 mM CARB and 1.5 mM caffeine (67 ± 2 mv in six fibers).

The Influence of Caffeine on the Rate of Desensitization with Bulk Application of CARB in a Calcium-Free Solution

The importance of extracellular calcium in the acceleration of PJM repolarization by caffeine was next determined using experiments similar in design

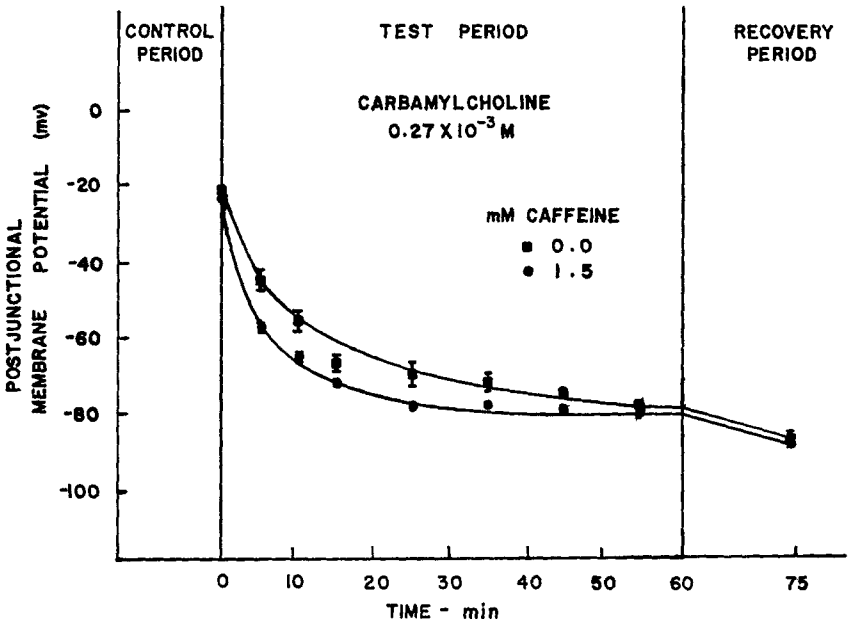


FIGURE 2. The effect of caffeine on the rate of repolarization of the postjunctional membrane of muscle fiber bathed in 10 mM Ca^{2+} Ringer solution containing 0.27 × 10^{-3} M carbamylcholine. Each point represents results from at least five fibers from at least five muscles. Recovery in CARB-free, caffeine-free solution.

to those described in the previous section. Postjunctional membrane potentials were sampled from muscles equilibrated for 45 min in a calcium-free, 4 mM magnesium solution (solution C). Carbamylcholine (0.27 mM) was then added (in the presence or absence of 1.5 mM caffeine) and PJM potentials were recorded for 60 min. At the end of this time, the muscles were returned to the calcium-free, 4 mM magnesium solution and the PJM potentials were measured after 15 min in the CARB-free (caffeine-free) solution. The results of these experiments (Fig. 3) demonstrate that in the absence of extracellular calcium, 1.5 mM caffeine did not increase the rate of PJM repolarization.

Influence of Caffeine on PJM Receptor Sensitivity

Mambrini and Benoit (1963) previously indicated that caffeine augmented the response of end-plate receptors to acetylcholine. A possible explanation for the acceleration of PJM repolarization by caffeine with the bulk application of CARB might be that caffeine causes an increased sensitivity of the end-plate receptors to CARB. To investigate this possibility, the influence of 1.5 mM caffeine on the sensitivity of the PJM to CARB was determined in

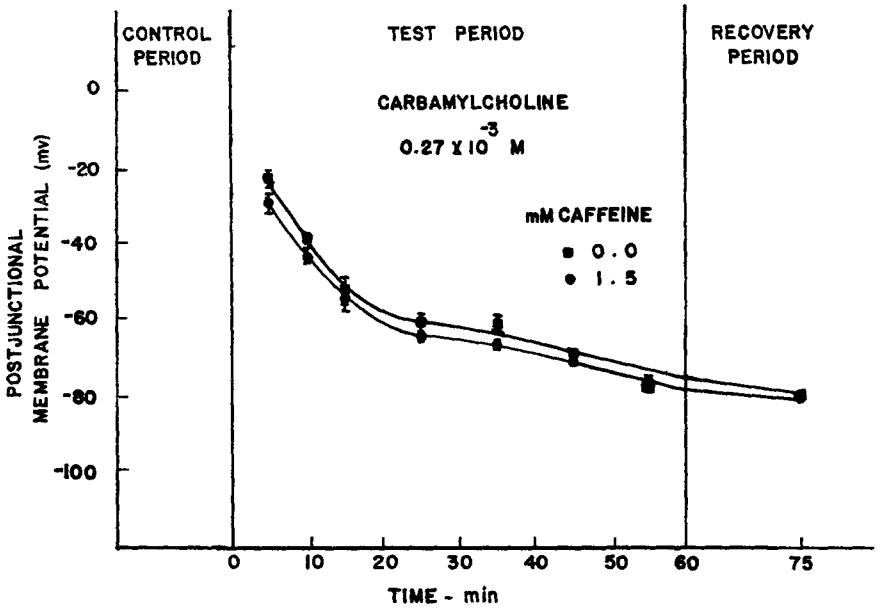


FIGURE 3. The effect of caffeine on the rate of repolarization at the postjunctional membrane of muscle fibers bathed in 4 mM Mg^{2+}–1 mM EGTA Ringer solution containing 0.27 × 10^{-3} M carbamylcholine. Each point represents results from at least five fibers from at least five muscles. Recovery in CARB-free, caffeine-free solution.

muscles equilibrated in either the 1.8 mM calcium, 10 mM calcium, or calcium-free, 4.0 mM magnesium Ringer solutions. Postjunctional membrane sensitivity was estimated by the depolarization of the PJM produced by the microperfusion of CARB alone as compared to that produced by CARB plus 1.5 mM caffeine (Nastuk and Parsons, 1970; Lambert and Parsons, 1970). In many of these experiments TTX (1 × 10^{-7} g/ml) was present to eliminate muscle action potentials (Kao, 1966). From Table II, it is evident that 1.5 mM caffeine significantly reduced the depolarization produced by 0.011 or 0.022 mM CARB in muscles equilibrated in the 1.8 mM calcium or calcium-free, 4.0 mM magnesium Ringer solutions, respectively. In contrast, the caf-

feine had no appreciable effect on the extent of depolarization produced by 0.022 mM CARB in muscles equilibrated in the 10 mM calcium solution.

Although caffeine reduced the end-plate response to 0.011 and 0.022 mM CARB, the depolarization produced by microperfusion of 0.27 mM CARB was not decreased (Table III). For these experiments, muscles were equilibrated in either the 10 mM calcium, 1.8 mM calcium, or calcium-free, 4.0 mM magnesium Ringer solution each made 2.3 times hypertonic by the addition of sucrose to eliminate muscle movements. Nastuk and Parsons (1970) have shown that there is no alteration in PJM receptor sensitivity when muscles fibers are bathed in a hypertonic environment.

TABLE II

INFLUENCE OF CAFFEINE ON PJM RECEPTOR SENSITIVITY

Ca^{2+}	Mg^{2+}	CARB	Caffeine	Control RP*	E_{min}‡	ΔV (voltage)	No. of fiber
mM	*mM*	*mM*	*mM*	*mv*	*mv*		
—	4.0	0.022	—	−84.7±0.9	−62.4±2.2	22.3±1.9§	7
—	4.0	0.022	1.5	−84.6±1.1	−68.7±1.4	15.9±1.6§	7
1.8	—	0.011	—	−91.1±1.7	−68.9±2.0	22.0±1.7‖	9
1.8	—	0.011	1.5	−91.5±1.4	−76.7±2.2	15.0±1.6‖	12
10.0	—	0.022	—	−92.0±1.3	−69.0±1.4	23.0±0.5¶	6
10.0	—	0.022	1.5	−92.0±0.6	−70.4±1.9	21.6±2.0¶	5

* Control PJM potential before CARB perfusion.

‡ Minimum PJM potential during CARB perfusion.

§ The mean depolarizations with and without caffeine were significantly different at $P < 0.05$.

‖ The mean depolarizations with and without caffeine were significantly different at $P < 0.01$.

¶ The mean depolarizations with and without caffeine were not significantly different.

The Interaction Between Calcium and Caffeine on the Rate of Recovery of the EMR

To provide further evidence concerning the influence of caffeine on the rate of PJM receptor desensitization, measurements of the effective transmembrane resistance were made in individual muscle fibers before, during, and after 0.27 mM CARB was microperfused directly onto the PJM. The half-time of return of the EMR after an initial decline in the presence of CARB was used as an index of the rate of desensitization (Manthey, 1966). The half-time of EMR recovery represents the time taken for the input resistance to increase after an initial decline with activation to a value equal to one-half the total change measured during desensitization (Manthey, 1966, 1970; Nastuk and Parsons, 1970). All muscles in these experiments were equilibrated in a calcium-free, 4.0 mM magnesium Ringer solution (solution C) for 4-7 hr, during which time the preparations were exposed at least four times to an isotonic potassium chloride Ringer solution for 3-5 min (solution B). Tetrodotoxin (1×10^{-7} g/ml) was added to the bathing medium during the last 30 min of equilibration to eliminate muscle action potentials (Kao,

1966). In a total of 126 fibers from 38 muscles, the average resting membrane potential after insertion of two micropipettes within 50 μm of each other was −74.8 ± 0.5 mv (range −60 to −92 mv) and the mean EMR was 0.40 ± 0.01 MΩ (range, 0.26-0.75 MΩ).

A. SINGLE PERFUSION STUDIES

It is well established that calcium accelerates the desensitization of PJM receptors (Manthey, 1966, 1970; Magazanik, 1968; Parsons, 1969 *b*; Nastuk and Parsons, 1970; Magazanik and Vyskocil, 1970). A similar result was obtained in the present study (Table IV). In these experiments the muscles were not pretreated with either calcium or caffeine, calcium or caffeine being present only along with CARB during the perfusion. Examples of the

TABLE III

EFFECT OF CAFFEINE ON MAXIMUM DEPOLARIZATION PRODUCED BY 0.27 mM CARB IN HYPERTONIC RINGER SOLUTION

Ca^{2+}	Mg^{2+}	Caffeine	Control RP	E_{min}*	ΔV	No. of Fibers
mM	*mM*	*mM*	*mv*	*mv*		
—	4.0	—	−84.4±0.7	−24.6±3.2	58.9±2.8‡	7
—	4.0	1.5	−82.3±0.6	−24.1±1.1	58.2±1.0‡	6
1.8	—	—	−92.1±1.7	−27.7±1.9	64.4±2.2‡	7
1.8	—	1.5	−90.7±1.5	−27.4±0.9	63.3±2.0‡	10
10.0	—	—	−94.0±1.5	−26.9±2.4	67.1±1.9‡	8
10.0	—	1.5	−93.7±1.9	−26.3±0.9	67.4±1.3‡	7

* Minimum PJM potential during CARB perfusion.

‡ The mean depolarizations with and without caffeine were not significantly different at the 5% level.

response obtained in the presence of the calcium-free, 4.0 mM magnesium, 5.4 mM calcium, and 10 mM calcium solutions are shown in Fig. 4 and the calculated resistance changes are presented in Fig. 5. As the concentration of calcium in the microperfusate increased, the rate of recovery of the EMR increased. The half-time of EMR return for the fibers presented in Fig. 4 was 160, 90, and 25 sec in the presence of calcium-free, 4.0 mM magnesium, 5.4 mM calcium, and 10 mM calcium, respectively. The average values of EMR recovery from numerous fibers are presented in Table IV.

The rate of PJM receptor desensitization was not significantly different in the calcium-free, 4.0 mM magnesium Ringer solution (solution C) or the 1.8 mM calcium Ringer solution (solution A). The average half-time of EMR recovery was 152.7 ± 23.3 sec in 12 fibers perfused with 0.27 mM CARB in solution C and 173.3 ± 28.9 sec in 6 fibers exposed to 0.27 mM CARB in solution A. However, the addition of 1.5 mM caffeine to solution A significantly increased the rate of PJM receptor desensitization produced by 0.27

mM CARB. In contrast, 1.5 mM caffeine did not significantly change the rate of EMR recovery produced by 0.27 mM CARB in the calcium-free, 4.0 mM magnesium solution (Table IV). Sample records which demonstrate the acceleration of PJM receptor desensitization by caffeine in the 1.8 mM calcium Ringer solution are presented in Fig. 6 and the calculated resistance changes as a per cent of control in Fig. 7. In Fig. 6 A the half-time of PJM desensitization in the presence of 0.27 mM CARB in solution A was 150 sec and in Fig. 6 B the half-time of desensitization during the perfusion of 0.27 mM CARB in solution A plus 1.5 mM caffeine was 70 sec.

TABLE IV

EFFECT OF CALCIUM AND CAFFEINE ON EMR RECOVERY*

Ca^{2+}	Mg^{2+}	Caffeine	EGTA	EMR recovery half-time	No. of Fibers
mM	*mM*	*mM*	*mM*	*sec*	
—	4.0	—	1.0	152.7±23.3‡	12
—	4.0	1.5	1.0	138.0±11.4§	5
1.8	—	—	—	173.3±28.9‖	6
1.8	—	1.5	—	65.6±14.5¶	5
5.4	—	—	—	75.0± 5.3*	6
10.0	—	—	—	26.4± 4.0**	9
10.0	—	1.5	—	26.6± 4.9‡‡	7

* In all cases, the perfusion contained 0.27 mM CARB and the indicated calcium, magnesium, and/or caffeine concentrations.

‡ *versus* §, the mean EMR half-times were not significantly different.

§ See ‡.

‖ *versus* ¶, the mean EMR half-times were significantly different ($P < 0.01$).

¶ See ‖.

** *versus* ‡‡, the mean EMR half-times were not significantly different.

‡‡ See **.

In addition, we found that caffeine did not significantly increase the rate of EMR recovery in those fibers exposed to CARB in the presence of the 10 mM calcium solution (Table IV).

B. DOUBLE PERFUSION STUDIES

In a second series of experiments the influence of calcium and caffeine on PJM desensitization was determined following CARB activation of the end-plate receptors in the calcium-free solution (solution C). A double perfusion technique similar to that described previously by Parsons (1969 *b*) was used. Table V summarizes the results of these experiments. In all cases the initial perfusate contained 0.27 mM CARB in the calcium-deficient, 4.0 mM magnesium solution (solution C). After an initial perfusion period of 30–70 sec the first perfusion pipette was rapidly replaced with a second perfusion pipette containing 0.27 mM CARB plus calcium and caffeine. Desensitization of

PJM receptors was estimated in these experiments from the rate of EMR recovery during the second CARB perfusion. Following the initial exposure to CARB in the calcium-free medium, the rate of PJM desensitization was slow (Fig. 8). The addition of 1.8 mM calcium (Fig. 8 B) or 10 mM calcium

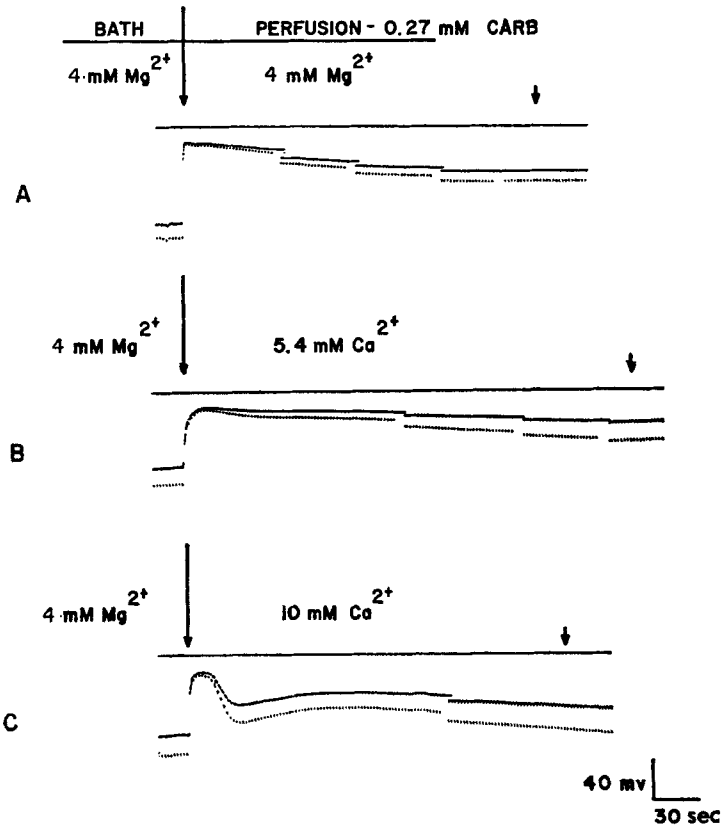


FIGURE 4. The influence of calcium on the time-course of changes in effective transmembrane resistance measured at the region of the postjunctional membrane. Records A, B, and C represent measurements from experiments performed on muscle fibers perfused with 0.27 mM CARB containing 4 mM Mg^{2+}, 5.4 mM calcium, or 10 mM Ca^{2+}, respectively. The upper trace represents zero potential and the lower trace the membrane potential in millivolts. The dots below the potential trace denote the hyperpolarizations produced by constant amplitude anodal current pulses delivered at 2-sec intervals. The breaks in the records indicate periods of 60 sec during which the recording camera was stopped. Vertical arrows indicate the onset and termination of the microperfusion.

(Fig. 8 D) in the second CARB perfusion dramatically increased the rate of recovery of the EMR. Caffeine (1.5 mM) significantly potentiated the action of 1.8 mM calcium (Fig. 8 C), but did not affect the rate of EMR recovery occurring in the presence of 10 mM calcium. No change in the rate of the EMR recovery was apparent when the second perfusion contained CARB

in the calcium-deficient, 4.0 mM magnesium Ringer solution. If, however, the second perfusion contained CARB plus 1.5 mM caffeine in the calcium-deficient, 4.0 mM magnesium Ringer solution, the rate of EMR recovery was increased. The calculated change in resistance as a per cent of control for those records presented in Fig. 8 is shown in Fig. 9.

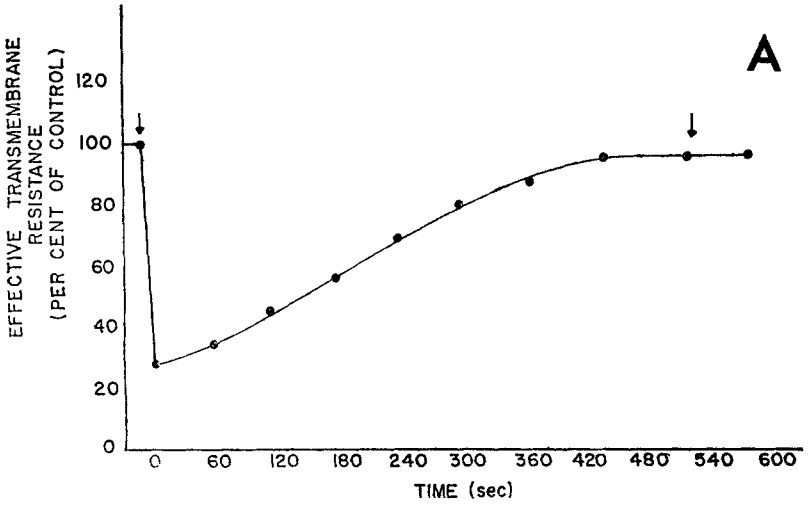
The Time-Course of Calcium Effect

Manthey (1970) demonstrated in depolarized muscle fibers that the influence of calcium on PJM desensitization occurs approximately 13 sec after the onset of CARB application. We have also determined the time-course of calcium action in the present study. The time-course of EMR recovery in the calcium-free, 4.0 mM magnesium solution was compared to that observed in the 10 mM calcium Ringer solution. In order to average the results obtained from individual fibers, the point of maximum receptor activation (minimum EMR) was taken to be "zero time" and the results presented as a per cent change in EMR. The composite time-course of the per cent change in EMR in the 4.0 mM magnesium solution (circles in Fig. 10) and 10 mM calcium solution (triangles in Fig. 10) from the single perfusion experiments is presented in Fig. 10. A student's t test was used to determine when calcium significantly increased the EMR. From Fig. 10 it can be seen that calcium significantly ($P < 0.05$) increased the rate of EMR recovery after approximately 6 sec of CARB perfusion in the single perfusion experiments. The composite time-course of the per cent change in EMR in the calcium-free, 4.0 mM magneisum solution (circles in Fig. 11) and the 10 mM calcium solution (triangles in Fig. 11) during the double perfusion experiments is presented in Fig. 11. Zero time was taken at the start of the second perfusion. A student's t test was used to determine when calcium significantly increased the EMR above the control, calcium-free solution. Calcium significantly ($P < 0.05$) increased the rate of EMR recovery within 2 sec (Fig. 11).

The Influence of Calcium on Membrane Resistance in the Absence of CARB

One additional fact should be mentioned concerning the changes in EMR obtained in the present study. Note from Figs. 5, 7, and 9 that, in the 1.8 mM calcium solution and in the 10 mM calcium Ringer solution, the EMR

FIGURE 5. Time-course of changes in effective transmembrane resistance produced by 0.27 mM CARB. (A) EMR change recorded from a fiber perfused with 0.27 mM CARB in the Ca^{2+}-free, 4 mM Mg^{2+} solution equilibrated in the Ca^{2+}-free, 4 mM Mg^{2+}–1 mM EGTA Ringer solution. (B) EMR change measured from a fiber perfused with 0.27 mM CARB plus 5.4 mM Ca^{2+} equilibrated in the Ca^{2+}-free, 4 mM Mg^{2+}–1 mM EGTA Ringer solution. (C) EMR change observed in a fiber perfused with 0.27 mM CARB plus 10 mM Ca^{2+} equilibrated in the Ca^{2+}-free, 4 mM Mg^{2+}–1 mM EGTA Ringer solution. The vertical arrows indicate the onset and termination of the CARB microperfusion.


A
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40
20
0
0
60
120
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360
420
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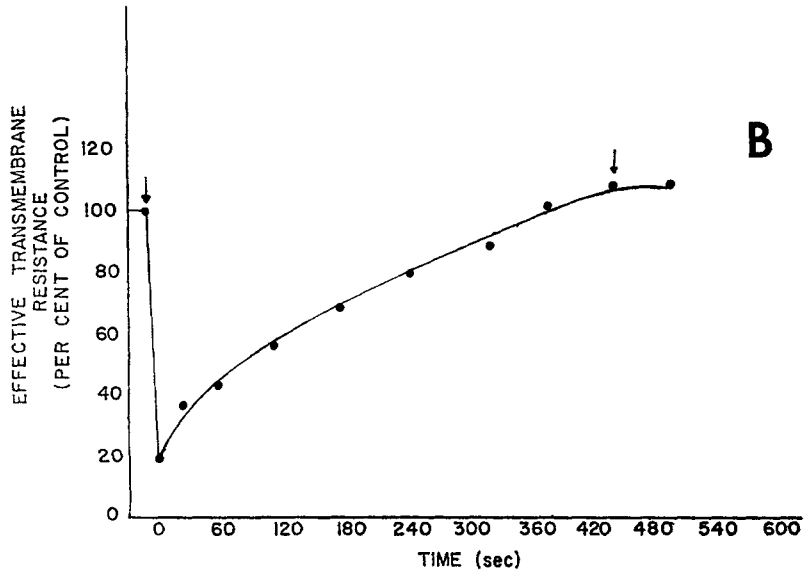
TIME (sec)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EFFECTIVE TRANSMEMBRANE RESISTANCE (PER CENT OF CONTROL)
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100
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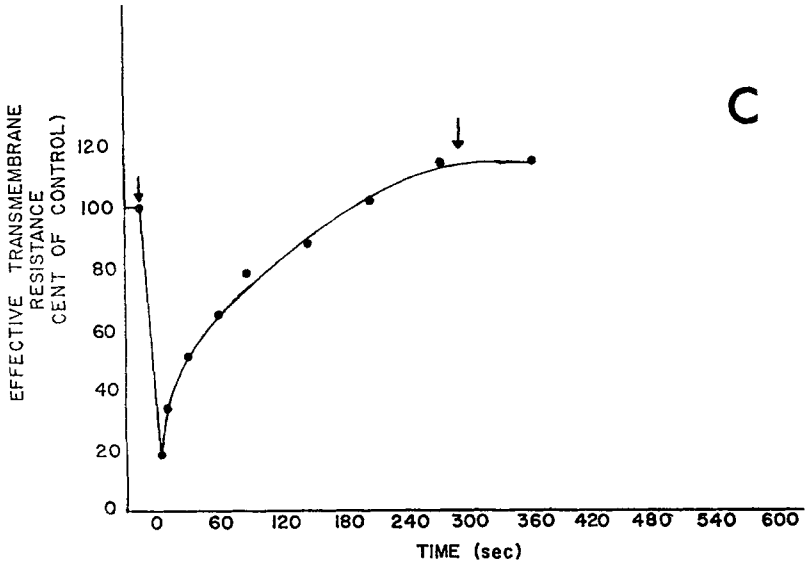
600
TIME (sec)


C
EFFECTIVE TRANSMEMBRANE RESISTANCE CENT OF CONTROL)
120
100
80
60
40
20
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120
180
240
300
360
420
480
540
600
TIME (sec)

after an initial decline returned to a value greater than the preperfusion level. This was a consistent finding in these experiments. These results suggested to us that calcium exerted a stabilizing influence which was independent of the inactivation process. This view was substantiated in additional experiments. Muscles were maintained in the calcium-free, 4.0 mM magnesium–1 mM EGTA Ringer solution and treated as described in the previous section. Muscle fibers were perfused both at junctional and nonjunc-

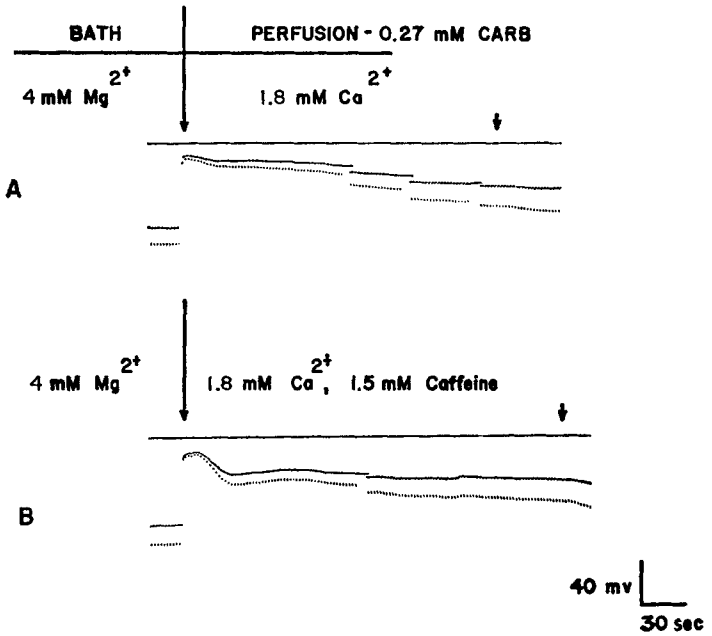

FIGURE 6. The influence of caffeine on the time-course of changes in effective transmembrane resistance measured at the region of the postjunctional membrane. Records A and B represent measurements from experiments performed on muscle fibers perfused with 0.27 mM CARB Ringer containing either 1.8 mM calcium or 1.8 mM calcium plus 1.5 mM caffeine.

tional regions with 1.8 or 10 mM calcium (in the presence and absence of 1.5 mM caffeine) to determine if the EMR was increased by the addition of calcium. We have combined the results obtained from junctional and nonjunctional areas of the fibers as no difference between areas was noted. It can be seen from Table VI that, after the application of 1.8 mM calcium, the EMR increased by an average of 28.6%. No additional significant increase in EMR was noted when 1.8 mM calcium and 1.5 mM caffeine were applied simultaneously. The change in EMR noted when 10 mM calcium and 1.5 mM caffeine were applied together is significantly ($P < 0.01$) greater than

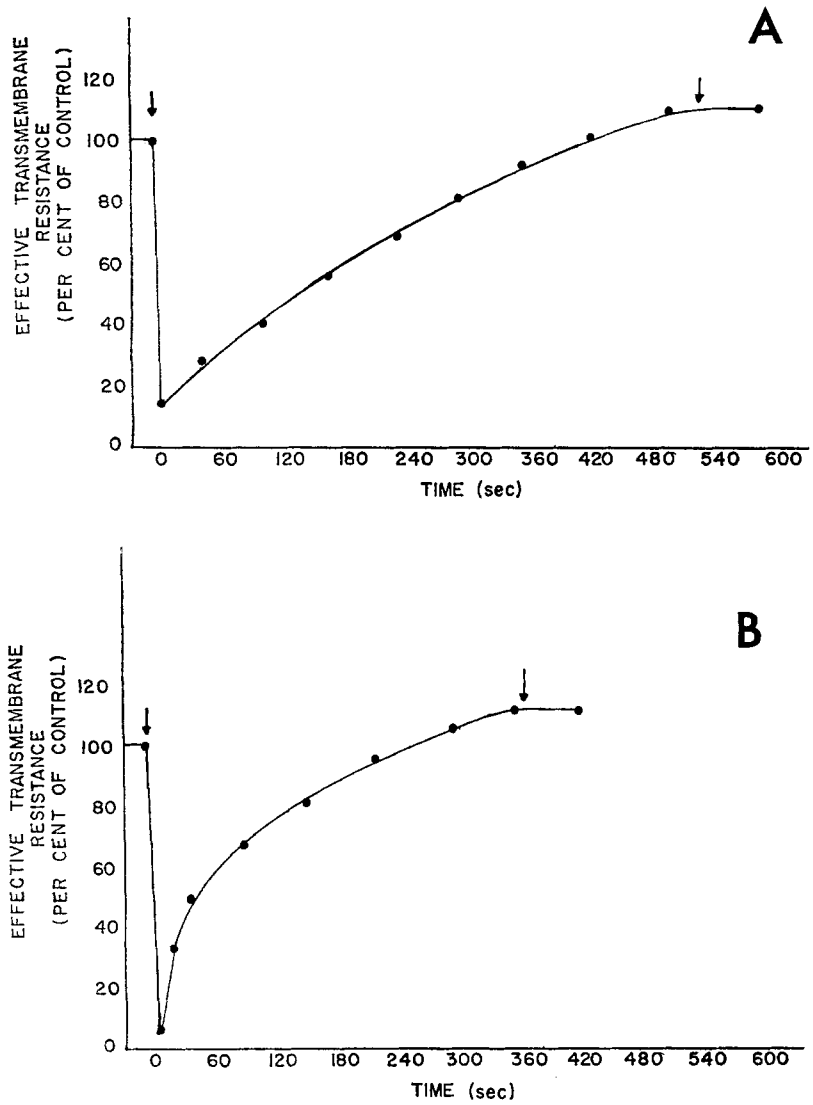

FIGURE 7. Time-course of changes in effective transmembrane resistance produced by 0.27 mM CARB. (A) EMR changes observed in a fiber perfused with 0.27 mM CARB plus 1.8 mM Ca^{2+} equilibrated in the Ca^{2+}-free, 4 mM Mg^{2+}–1 mM EGTA Ringer solution. (B) EMR change measured in a fiber perfused with 0.27 mM CARB plus 1.8 mM Ca^{2+} and 1.5 mM caffeine. The vertical arrows denote the onset and termination of the CARB microperfusion.

that produced by 1.8 mM calcium, 1.8 mM calcium plus 1.5 mM caffeine, or 10 mM calcium alone. Caffeine in the absence of calcium had no effect on the EMR.

DISCUSSION

Manthey (1966) initially reported that calcium accelerated PJM receptor desensitization. Many workers have since demonstrated a relationship between an increase in extracellular calcium and the rate of PJM desensitization (Magazanik, 1968; Parsons, 1969 *b*; Nastuk and Parsons, 1970; Magazanik and Vyskočil, 1970; Manthey, 1970). Normally, in muscle there is an inward gradient for calcium of some four to five orders of magnitude (Bianchi, 1968). Consequently, an influx of calcium would be expected if membrane permeability to calcium increases. Previously, evidence had been

TABLE V

EFFECT OF CALCIUM AND CAFFEINE ON EMR RECOVERY IN DOUBLE PERFUSION EXPERIMENTS*

Ca^{2+}	Mg^{2+}	Caffeine	EGTA	EMR recovery half-time‡	No. of fibers
mM	*mM*	*mM*	*mM*	*sec*	
—	4.0	—	1.0	140.2± 9.7§	10
—	4.0	1.5	1.0	89.2±10.2‖	5
1.8	—	—	—	66.5±18.1¶	5
1.8	—	1.5	—	27.0±12.0**	4
10.0	—	—	—	34.6±10.3‡‡	5
10.0	—	1.5	—	27.0± 9.6§§	3

* In all cases, the first perfusion contained 0.27 mM CARB in the 4 mM Mg^{2+}–1 mM EGTA Ringer solution. The second perfusion contained 0.27 mM CARB and the indicated calcium and/or caffeine concentrations.

‡ EMR half-time calculated from the time of the second perfusion.

§ *versus* ‖, the mean EMR half-times were significantly different ($P < 0.001$).

‖ See §.

¶ *versus* **, the mean EMR half-times were significantly different ($P < 0.01$).

** See ¶.

‡‡ *versus* §§, the mean EMR half-times were not significantly different.

§§ See ‡‡.

presented to support the view that calcium permeability increased at the end plate in the presence of CARB. For example, Parsons and Nastuk (1969) suggested that the CARB contractures which occur in potassium depolarized muscles (only in the presence of calcium) were due to an influx of calcium locally at the end plate during receptor activation. It has also been shown that CARB-induced contractures can be produced in depolarized, denervated muscle and that extracellular calcium is required (Jenkinson and Nicholls, 1961; Parsons et al., 1971). Direct evidence that the permeability to calcium is increased by ACh is derived from the work of Takeuchi (1963) and Katz and Miledi (1969). The latter authors demonstrated that end-plate potentials and miniature end-plate potentials could be recorded from muscles bathed in an isotonic calcium chloride Ringer solution. Two sites

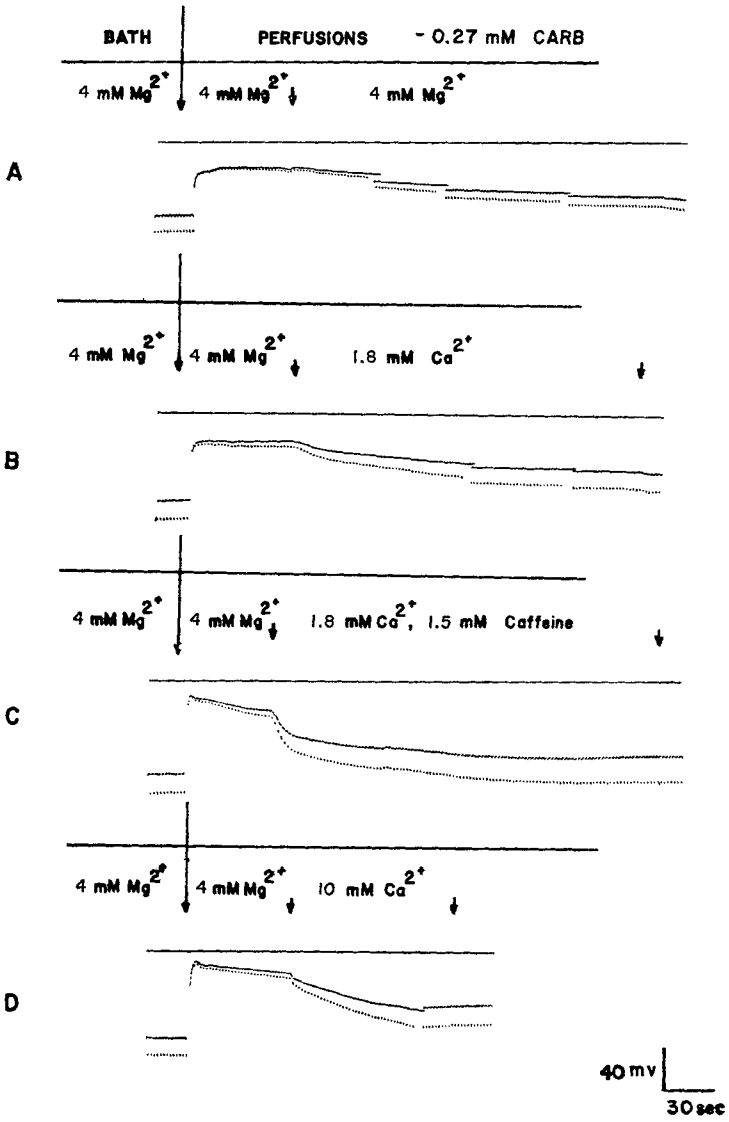

FIGURE 8. Examples of double perfusion experiments showing the effect of calcium and caffeine on the rate of return of the effective transmembrane resistance. The first vertical arrow denotes the initial microperfusion of 0.27 mM carbamylcholine in 4 mM Mg^{2+}–1 mM EGTA Ringer solution onto the neuromuscular junction. The second vertical arrow indicates the application of 0.27 mM carbamylcholine containing either 4 mM Mg^{2+}, 1 mM EGTA (record A), 1.8 mM Ca^{2+} (record B), 1.8 mM Ca^{2+} plus 1.5 mM caffeine (record C), or 10 mM Ca^{2+} (record D). The microperfusion was terminated at the third vertical arrow.

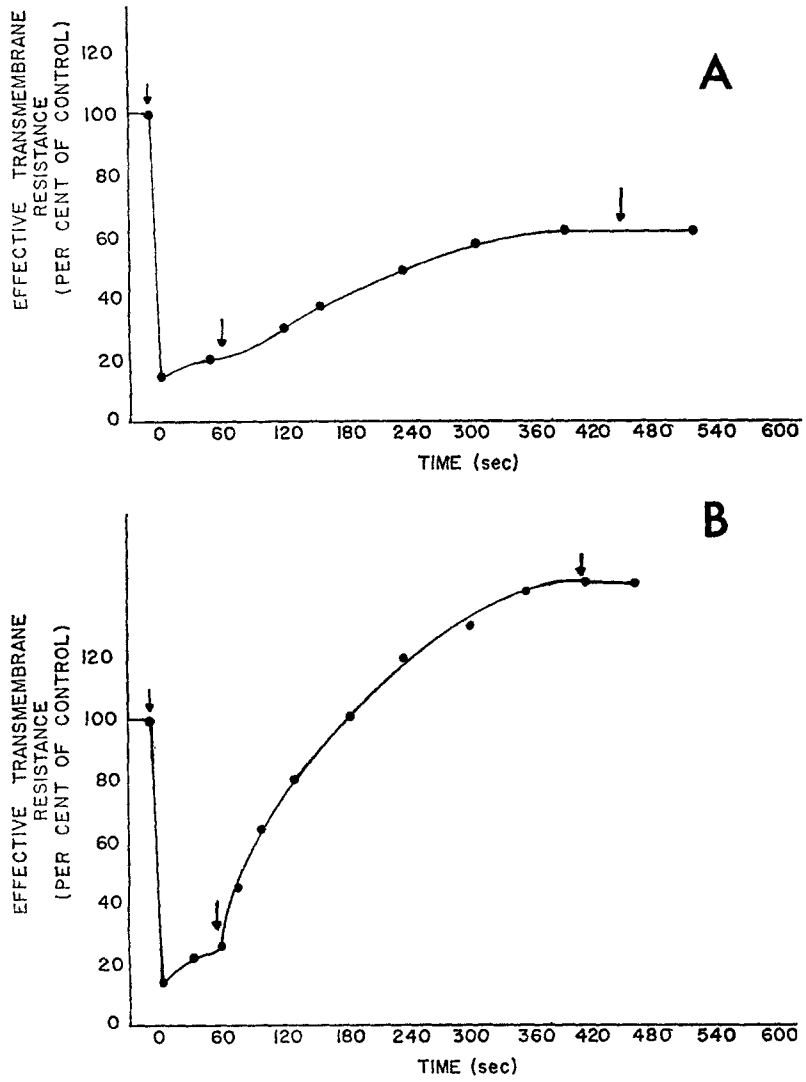

FIGURE 9. Time-course of changes in effective transmembrane resistance produced by 0.27 mM CARB in the double perfusion experiments. In all cases the initial perfusion contained 0.27 mM CARB in the Ca^{2+}-free, 4 mM Mg^{2+}–1 mM EGTA Ringer solution. The first vertical arrow denotes the onset of the initial perfusion; the second vertical arrows represents the termination of the initial perfusion and the onset of the second perfusion. The third vertical arrow indicates the termination of the second microperfusion. (A) EMR change observed in a fiber when second perfusion contained 0.27 mM CARB in the Ca^{2+}-free, 4 mM Mg^{2+}–1 mM EGTA solution. (B) EMR change recorded in a fiber when the second perfusion contained 0.27 mM CARB plus 1.8 mM Ca^{2+}. (C) EMR change measured in a fiber when the second perfusion contained 0.27 mM CARB plus 1.8 mM Ca^{2+} and 1.5 mM caffeine. (D) EMR change observed in a fiber when the second perfusion contained 0.27 mM CARB plus 10 mM Ca^{2+}.

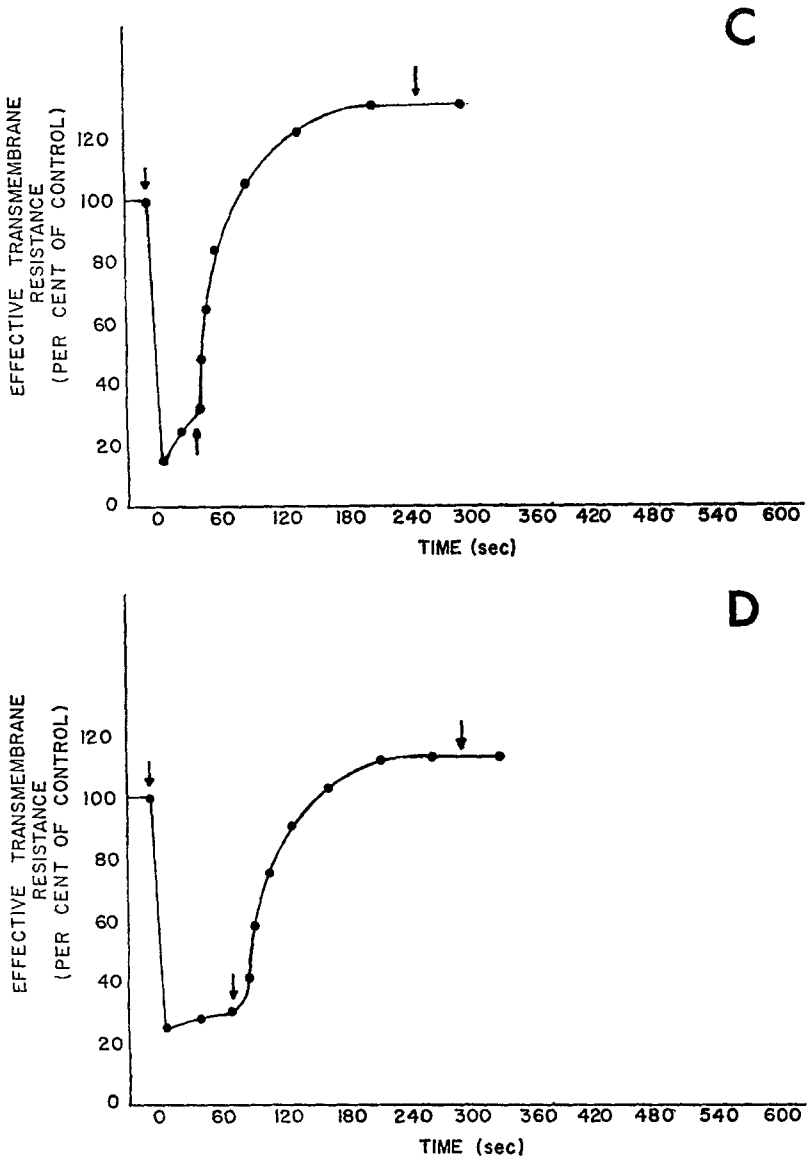

FIGURE 9

of calcium action, both of which are dependent on calcium influx, have been proposed as a possible scheme by which calcium accelerates desensitization. Parsons (1969 *b*) suggested that the reaction of calcium with anionic sites along the ionic conductance pathways accelerated PJM desensitization. Similarly, Magazanik and Vyskočil (1970) have proposed that during the prolonged application of ACh at the end plate, free calcium ions accumulate in the membrane and form complexes which control the pathways for ionic

permeability. In another recent report (Nastuk and Parsons, 1970) the intracellular accumulation of calcium has been implicated as an essential step in the desensitization process. These authors (Nastuk and Parsons, 1970) proposed that PJM desensitization may be accelerated by the binding of calcium to anionic sites on the internal surface of the PJM and that the reaction between these sites and calcium controls the ionic permeability of the membrane.

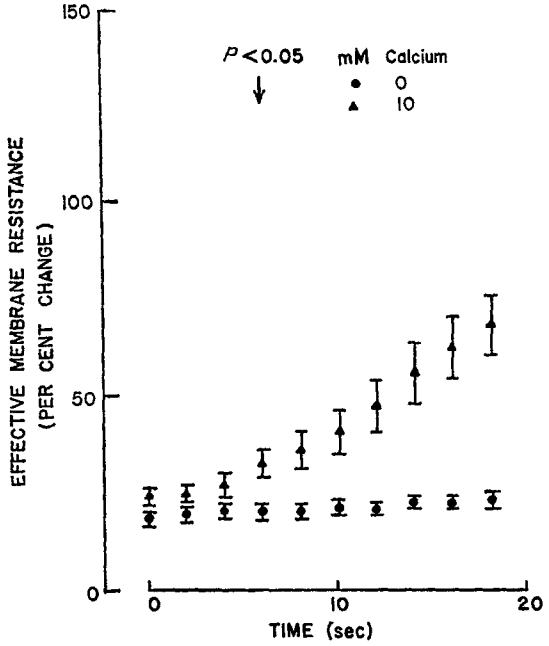

FIGURE 10. The effect of calcium on the time-course of the per cent change in effective membrane resistance of single muscle fibers during the first 20 sec of perfusion with 0.27 mM carbamylcholine. The closed circles denote results obtained with 4 mM Mg^{2+}–1 mM EGTA and the closed triangles results obtained with 10 mM calcium in the perfusion solution. The vertical line about each point represents the standard error of the mean of five different fibers from several muscles. The vertical arrow indicates the time at which a significant ($P < 0.05$) difference was found between the results in 4 mM Mg^{2+}–1 mM EGTA and 10 mM Ca^{2+}.

Previously, Mambrini and Benoit (1963) found that caffeine augmented the response of end-plate receptors to iontophoretically applied acetylcholine. In contrast, we found that caffeine was a weak inhibitor of the PJM receptors. No explanation of this discrepancy is readily apparent. The present results demonstrated that caffeine decreases PJM receptor chemosensitivity in the 1.8 mM calcium or calcium-free, 4.0 mM magnesium solutions, but not in the 10 mM calcium solution. Formerly, Jenkinson (1960) demonstrated that calcium reduces the inhibition produced by curare and our results indicate a

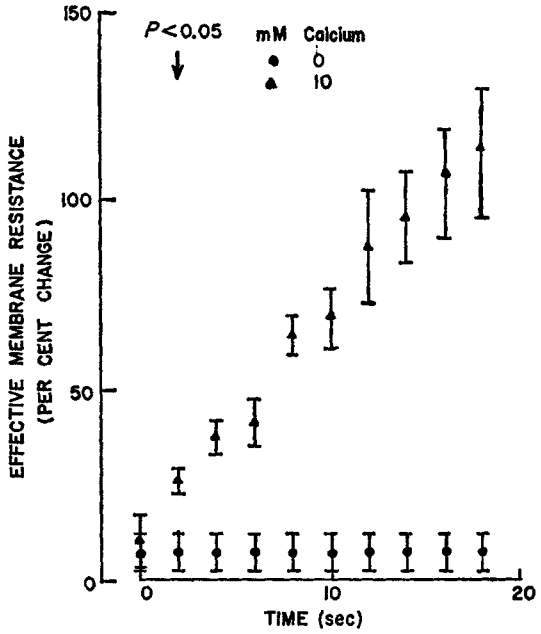

FIGURE 11. The effect of calcium on the time-course of the per cent change in effective membrane resistance of single muscle fibers during the first 20 sec of the second perfusion with 0.27 mM carbamylcholine during the double perfusion experiments. Time zero is the start of the second perfusion. The closed circles denote results obtained with 4 mM Mg^{2+}–1 mM EGTA and the closed triangles results obtained with 10 mM calcium in the perfusion solution. The vertical line about each point represents the standard error of the mean of five different fibers from several muscles. The vertical arrow indicates the time at which a significant ($P < 0.05$) difference was found between the results in 4 mM Mg^{2+}–1 mM EGTA and 10 mM Ca^{2+}.

TABLE VI

THE EFFECT OF CALCIUM AND CAFFEINE ON THE EMR IN THE ABSENCE OF CARB*

Ca^{2+}	Caffeine	% increase in EMR	No. of fibers
mM	*mM*		
1.8	—	28.6±2.9‡	7
1.8	1.5	34.7±3.2§	6
10.0	—	30.4±9.0‖	5
10.0	1.5	52.5±9.3¶	8

* The response includes results obtained from both junctional and extrajunctional areas of muscle fibers.

‡ ANOV comparison *versus P* value (where NS = not significant): (‡ + § + ‖) vs. ¶, $P < 0.01$; (‡ + §) vs. ‖, NS; ‡ vs. §, NS.

§ See ‡.

‖ See ‡.

¶ See ‡.

similar action of calcium on the inhibition of receptors by caffeine. Our results also confirm earlier work demonstrating that an elevation of external calcium inhibits the response of the end-plate receptors to CARB (Nastuk and Liu, 1966; Parsons and Nastuk, 1969).

In the present study, caffeine accelerated the rate of PJM receptor desensitization that occurs with the normal, 1.8 mM calcium concentration. This result is opposite to that reported by Magazanik and Vyskočil (1970) who suggested that caffeine did not influence this process. The discrepancy between the results of Magazanik and Vyskočil (1970) and our findings is most likely due to differences in the method of drug application used in the two studies. In the former study, Magazanik and Vyskočil (1970) applied acetylcholine iontophoretically. With this method, the quaternary ammonium ion concentration is high over a small area of the PJM and receptor desensitization is rapidly produced in this discreet region. Unfortunately, the influence of other factors (e.g., calcium, hypertonicity) which accelerate the rate of PJM desensitization is not evident when the activator concentration is high (Nastuk and Parsons, 1970).

Caffeine did not accelerate PJM desensitization as measured by repolarization in those muscles maintained in a calcium-free Ringer solution (Fig. 3). This suggests to us that the release of calcium by caffeine (1.5 mM) from internal sequestering sites (Isaacson and Sandow, 1967) is not an essential effect of caffeine during PJM desensitization. It also has been shown that caffeine, in concentrations similar to those employed in this study, enhances the influx and efflux of calcium in frog muscle fibers (Bianchi, 1961, 1962; Isaacson and Sandow, 1967). We suggest therefore that the acceleration of PJM desensitization by caffeine is related primarily to an increase in cell membrane permeability to calcium. When calcium influx is increased, the intracellular concentration of calcium would be raised allowing calcium to bind at sites on the internal surface of the PJM, and Ca^{2+} binding at these sites increases the rate of desensitization. Previously Manthey (1970) has indicated that the onset of calcium activity in PJM desensitization was too rapid for intracellular accumulation of calcium to be a factor in this process. However, seconds represent a reasonably long time interval if one compares the time-course of action of calcium in PJM desensitization and in excitation-secretion coupling at nerve terminals (Katz and Miledi, 1967 *b*). Although we favor the view that the acceleration of desensitization by caffeine is related to an increased calcium permeability, the possibility that the effect of caffeine in this process is related to a change in calcium binding at the external surface membrane cannot be eliminated.

In the double perfusion experiments, when the second perfusion pipette contained 0.27 mM CARB plus 1.5 mM caffeine in the absence of calcium, an increase in the rate of desensitization above the control (0.27 mM CARB

alone) value was noted (Table V). This effect of caffeine was not observed when caffeine and CARB were applied together in the single perfusion experiments (Table IV). These results may indicate that, in partially depolarized fibers, the ability of caffeine to release calcium from internal stores is enhanced and the increased intracellular calcium may be sufficient to accelerate desensitization. Previously, Lüttgau and Oetliker (1968) noted that caffeine contractures were affected in a similar fashion by partial depolarization of the muscle fiber. In their study, the addition of 2 mM caffeine to fibers depolarized by an elevated potassium solution yielded nearly a maximal tension response, whereas in fibers maintained at the normal potassium level 2 mM caffeine produced minimal tension changes.

It is interesting that the effectiveness of caffeine was more apparent when the calcium concentration was 1.8 mM than at 10 mM (compare Figs. 1 and 3, and Table IV). This may indicate that there is a critical concentration of calcium in this process and that, once this is exceeded, no further acceleration of desensitization occurs. When the external calcium concentration is raised approximately fivefold, the inward driving force must be sufficient to move calcium to some critical site and the increased calcium permeability produced by caffeine is not necessary. It has been demonstrated by Bianchi (1968) that an increase in calcium influx similar to that obtained with caffeine occurred when the external calcium concentration was tripled. Support for our view is also derived from an earlier study (Nastuk and Parsons, 1970) of the interaction between calcium concentration and CARB concentration in PJM desensitization. These authors demonstrated that, at high CARB concentrations, elevating the calcium concentration fivefold had very little additional effect on the rate of desensitization.

The importance of calcium as a membrane stabilizer of excitable cells has been recognized for some time (Shanes, 1958; Bianchi, 1968). However, the role of membrane stabilization by calcium in PJM desensitization remains in question. It is felt that this particular action of calcium is involved in the desensitization process but is not the major role of calcium. Membrane stabilization by calcium may involve a reaction of calcium with sites on the external membrane surface which may not be of primary importance in PJM desensitization. This view is based on the following lines of evidence.

(*a*) During desensitization the initial rate of EMR recovery was identical in the calcium-free, 4.0 mM magnesium solution and the 1.8 mM calcium Ringer solution, although the final EMR value was consistently greater in the latter situation.

(*b*) Calcium in the absence of CARB increased the EMR at both junctional and nonjunctional regions.

(*c*) Caffeine accelerated the rate of PJM desensitization but did not potentiate the increase in EMR produced by 1.8 mM calcium in the absence

of CARB (Table VI). Only when the calcium concentration was increased approximately fivefold, to 10 mM, did caffeine have an effect on the EMR in the absence of CARB.

In conclusion, a major factor controlling the rate of desensitization of skeletal muscle PJM receptors produced by the sustained application of carbamylcholine is proposed to be a rise in intracellular calcium concentration and a subsequent binding of calcium ions at sites on the internal surface of the PJM. It is proposed that the binding of calcium at these sites limits the ionic permeability of the PJM. The major influence of caffeine noted in the present investigation is suggested to be the result of an increased intracellular calcium level which results from an increased membrane permeability to calcium produced by this alkaloid.

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