Further Studies of the Effect of Calcium on the Time Course of Action of Carbamylcholine at the Neuromuscular Junction

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ABSTRACT The rate at which the postjunctional membrane of muscle fibers becomes desensitized to the action of carbamylcholine is increased after the muscle has been soaked in solutions containing increased concentrations of calcium. Some further aspects of this effect of calcium were investigated by measuring changes in the input resistance of single fibers of the frog sartorius during local perfusion of the neuromuscular junction with 2.73×10^{-3} M carbamylcholine in isolated muscles immersed in 165 mM potassium acetate. It was found that (*a*) sudden changes in the local concentration of calcium brought about by perfusing fibers with carbamylcholine solutions containing 20 mM calcium, 40 mM oxalate, or 40 mM EDTA were followed within 20 sec by marked changes in the rate of desensitization; (*b*) prior to 13 sec after the introduction of carbamylcholine, however, no effect on the input resistance could be detected even though the muscle had been presoaked in 10 mM calcium; (*c*) the ability of high concentrations of calcium to bring about rapid desensitization disappears when a lower concentration of carbamylcholine (0.137×10^{-3} M) is applied to the muscle fiber. These findings suggest that calcium present in the extracellular fluid can act directly on the postjunctional membrane to promote the desensitization process and that an increased permeability of the membrane to calcium brought about by the presence of carbamylcholine is a factor which contributes to this action.

INTRODUCTION

The application of acetylcholine to isolated skeletal muscle causes an immediate and rapid depolarization of the postjunctional membrane of the muscle fibers. If the muscle remains in contact with this agent for more than a few seconds, however, repolarization of the postjunctional membrane begins

to occur at a slow rate even though the concentration of acetylcholine is maintained at a constant level. This gradual disappearance of the depolarizing action of acetylcholine is one aspect of a general decrease in chemosensitivity which occurs when cholinoceptive tissues are exposed to this agent for long periods. The phenomenon has been referred to by different authors as "desensitization" (1), "inactivation" (2), or "fade" (3), and it may also be related to the "second phase block" produced by decamethonium at the neuromuscular junction (2,4) and nicotine in autonomic ganglia (5). Since desensitization occurs during the action of several different cholinergic compounds (6) and in different tissues (3, 7–10), it may be due to some general and fundamental feature of their action at cholinoceptive sites. Several mechanisms based on this assumption have been proposed (2–4, 10).

Desensitization in frog skeletal muscle has been studied in this laboratory by seeing what are the important factors which influence its rate. Two factors which are known to cause an increase in the rate of desensitization are (*a*) an increase in the concentration of acetylcholine applied to the muscle (2) and (*b*) an increase in the concentration of calcium present in the extracellular fluid (11). In the present investigation the action of calcium on the rate of desensitization was examined in greater detail in order to further specify its mode of action and its relationship to the desensitization process.

METHODS

Because it is resistant to hydrolysis by tissue cholinesterase, carbamylcholine was used throughout these experiments as a test agent for producing desensitization in muscle fibers. In order to prevent twitches and contractures during drug application and to allow constant recording with intracellular capillary electrodes, muscle preparations were immersed in solutions containing 165 mM potassium acetate, a procedure which causes the fibers to be almost completely depolarized and therefore electrically inexcitable. Since the application of carbamylcholine to the postjunctional membrane under these conditions produces little or no change in the membrane potential, it was necessary to use some measurement other than membrane potential for the detection of drug action. Therefore, the input or "effective" resistance of the fiber was chosen for this purpose.

Muscle Preparation

Whole isolated sartorius muscles of the frog (*Rana pipiens*) mounted for direct microscopic observation were used throughout these experiments. Prior to use, the animals were stored at room temperature.

Measurement of Input Resistance

The input resistance of single muscle fibers on the exposed inner surface of the preparation was measured by the "electrotonic pulse" method. Two glass capillary electrodes filled with 3 M KCl were inserted into a muscle fiber within 50 μ of each other

in the region of the neuromuscular junction under microscopic observation at a magnification of 150 times. One electrode was used to deliver repeated pulses of current from a square wave stimulator. The amplitude of this "test" current was always between 9 and 11 $\times$ 10^{-8} amp and the duration of each pulse was 300 msec. The deflections of membrane potential produced by the test current were recorded by the other electrode, amplified, and then displayed on an oscilloscope. Records of the oscilloscope trace were obtained on moving 35 mm film by means of an oscilloscope recording camera. Since the total resistance of the current delivery circuit was always much greater than the input resistance of the muscle fiber membrane, the current delivered to the membrane was virtually independent of changes in the input resistance produced by carbamylcholine. Therefore, the amplitudes of the pulsatile changes in membrane potential caused by the injected test currents (such as appear in Fig. 1) are a direct measure of the input resistance. No corrections were made for the variations in internal resistance of individual fibers.

Drug Application

Solutions of carbamylcholine were applied to the junctional region of surface muscle fibers by means of a special perfusion pipette. This pipette, which measured approximately 30–50 μ at the tip, was connected to a length of glass tubing and both were filled with a solution identical in composition to the fluid bathing the muscle except for the addition of carbamylcholine and in some cases a change in the concentration of calcium. At the time of drug application this pipette, initially above and outside the bath fluid, was rapidly brought into position about 0.1 mm above the impaled fiber. When in contact with the bath fluid, the solution within the pipette was expelled at a constant rate by gravity. It was determined in control experiments that this method of local perfusion did not alter the measurement of input resistance to any significant degree (11). It is not known how closely the concentration of carbamylcholine produced at the surface of the fiber approximates that present in the perfusion pipette. Nevertheless, a certain degree of reproducibility in electrical response was attained by using the same pipette in many experiments and maintaining as nearly as possible the same geometrical relation of the pipette tip and the impalement site. Another variable which was difficult to control was the rate of onset of drug action. In most cases the time taken for the input resistance to decline by one-half of the maximum decrease of the onset phase varied from 0.5 to 1.0 sec although extreme values of 0.2 and 1.5 sec were also encountered.

Solutions

Muscle preparations were dissected and initially mounted in normal Ringer fluid which contained the following amounts of dissolved salts in mM/liter: NaCl, 120, KCl, 2.5, $CaCl_2$, 1.8, THAM (tris[hydroxymethyl]aminomethane), 1.0. The pH was adjusted to approximately 7.3 by the addition of HCl. Prior to an experiment, the isolated muscle was equilibrated for 30–60 min in a bath medium containing the following concentrations in mM/liter of dissolved solids: potassium acetate, 165, calcium acetate, 1.8, sodium acetate, 3.0, sucrose, 55, THAM, 1.0. The pH was adjusted to approximately 7.3 by the addition of HCl. In experiments in which increased

amounts of calcium were added to the bath or perfusion fluids, corresponding adjustments were made in the concentration of sucrose so as to maintain the same osmolarity. Solutions of carbamylcholine chloride were made up from the freshly weighed salt on the same day of each experiment. It was determined in control experiments that the amount of chloride in the most concentrated solution of carbamylcholine used (2.73×10^{-3} M) did not affect the input resistance of the fiber when applied to the impalement site. Furthermore, this concentration of carbamylcholine did not produce any mechanical response that could be seen under microscopic observation at a magnification of 150 times. All experiments were performed at room temperature which varied on different days from 22 to 25°C.

Statistical Analysis

Student's t test was used in comparing means of groups of data. A valid difference between two means was assumed to exist if the calculated P value was less than 0.05. In some cases the correlation coefficient (r) was used to establish a relationship between variables. The 95% confidence interval for r was found by consulting Table A7 on page 175 of reference 12.

RESULTS

A. *Effect on the Rate of Desensitization of Rapid Changes in the Local Concentration of Calcium in the Extracellular Fluid*

It was shown in an earlier study (11) that the rate at which muscle fibers become insensitive to the action of carbamylcholine is greatly increased when the muscle is presoaked in a solution containing five times the normal amount of calcium. In the further investigation of this action of calcium, one important question concerns its time of onset. If, for example, the accumulation of a large amount of calcium in the intracellular phase were necessary for this effect, changes in the time course of drug action would occur gradually over a period of many minutes, whereas a very rapid onset might indicate a simple action of the calcium ion at the cell surface. Experiments were undertaken, therefore, to find out how soon the rate of desensitization reaches a maximum after a sudden and rapid increase is made in the concentration of calcium in the extracellular fluid.

In order to serve as a basis for later comparison, the records in Fig. 1 a and b show first the time course of carbamylcholine action in muscle fibers that have been equilibrated for long periods in solutions containing 1.8 mM calcium (Fig. 1 a) and 20 mM calcium (Fig. 1 b). Since the bath medium in these experiments also contains 165 mM potassium acetate, the resting potential of the postjunctional membrane (denoted by the thick continuous line in the record) is always close to zero mv. The series of elongated dots below the resting potential level are momentary deflections of the membrane potential produced by repeated pulses of test current. Because this current is delivered to the fiber from a "constant current" source, the amplitude of

these deflections is a measure of the input resistance of the muscle fiber. When, as shown in Fig. 1 a, perfusion of the postjunctional region with carbamylcholine is begun (heavy vertical line), there is at first a rapid decrease in the input resistance. As the perfusion continues, however, the input resistance gradually returns toward control values at a slow rate. Since the latter period of increasing membrane resistance occurs in the continued presence of carbamylcholine, it signifies a gradual waning of the drug effect and there-

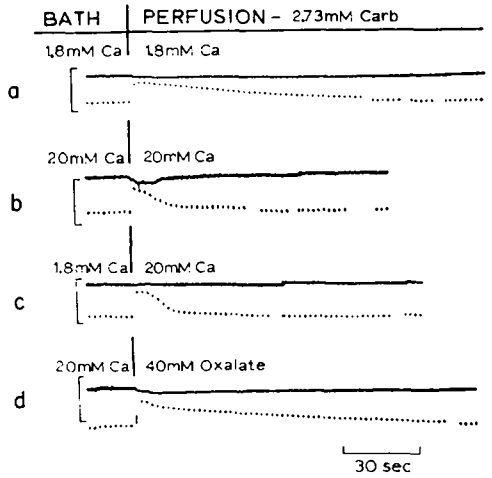

FIGURE 1. The time course of changes in input resistance of single muscle fibers during perfusion with 2.73×10^{-3} M carbamylcholine under various conditions. The bath and perfusion fluids both contained 165 mM potassium acetate. The muscle preparations were soaked in the bath solution for at least 60 min prior to perfusion. The upper and lower limits of the calibration marks to the left of each record indicate respectively 0 and −20 mv potential with respect to the bath medium. The series of dots are momentary deflections of the membrane potential produced by pulses of test current delivered to the fiber every 2 sec. The blank areas in the dot sequences of each record indicate periods of 60 sec during which the recording camera was stopped. Perfusion was begun at the moment indicated by the heavy vertical lines and continued at a constant rate thereafter.

fore the onset of the desensitization process. In this and subsequent experiments, the "time of half-desensitization" is used as a measure of the rate of desensitization. This is calculated as the time taken for the input resistance to increase toward control values by an amount equal to one-half the total change in resistance during the phase of desensitization. In Fig. 1 a, where the membrane resistance increases rather slowly, the time of half-desensitization is about 44 sec, whereas in Fig. 1 b it is only 11 sec, indicating a much more rapid rate of desensitization. The mean times of half-desensitization from several experiments in 1.8 and 20 mM calcium are shown in the first two horizontal rows of Table I. These measurements confirm the observation (11) that soaking muscle fibers in high calcium solutions produces an increase in

the rate of desensitization and they show further that the same action of calcium is evident in fibers depolarized by solutions of potassium acetate.

An attempt was now made to find out how fast the time of half-desensitization approaches a low value when the concentration of calcium in the bath fluid is suddenly increased from 1.8 to 20 mM. Initial experiments in which the bath medium was simply exchanged with one containing 20 mM calcium showed that the rate of desensitization had already reached a maximum within 2–3 min after exposure to the high concentration of calcium. Therefore, in the next experiment (Fig. 1 c) the conditions were changed so as to

TABLE I

THE TIME OF HALF-DESENSITIZATION IN MUSCLE FIBERS PERFUSED WITH 2.73×10^{-3} M CARBAMYLCHOLINE WHEN DIFFERENT AMOUNTS OF CALCIUM ARE PRESENT IN THE BATH AND PERFUSION FLUIDS

Bath fluid	Perfusion fluid (2.73×10^{-3} M carbamylcholine)	Mean time of half-desensitization
1. 1.8×10^{-3} M Ca	1.8×10^{-3} M Ca	47.7* sec ± 4.2‡ (15)§
2. 20×10^{-3} M Ca	20×10^{-3} M Ca	9.8 ± 0.7 (10)
3. 1.8×10^{-3} M Ca	20×10^{-3} M Ca	12.8 ± 1.4 (9)
4. 20×10^{-3} M Ca	40×10^{-3} M oxalate	43.0 ± 9.4 (7)
5. 20×10^{-3} M Ca	40×10^{-3} M EDTA	40.8 ± 3.2 (5)

* One value for the time of half-desensitization was more than 9 SD from the mean and was not included in this calculation.

‡ The value of 1 SEM.

§ Figures in parentheses are no. of fibers tested.

reduce as far as possible the time of contact of the muscle fiber with the high calcium solution. In order to accomplish this, the muscle was soaked first in a bath medium containing 1.8 mM calcium, and then at the time of drug application perfusion of a fiber was begun with a solution which contained 20 mM calcium in addition to the usual amount of carbamylcholine. It is assumed that at the onset of perfusion under these conditions there is a rapid rise in the local concentration of calcium at the fiber surface which coincides very nearly with the delivery of carbamylcholine itself. The result in Fig. 1 c shows that, despite this short exposure to the high calcium solution, the membrane resistance again increases at a rapid rate within 20 sec after the beginning of the perfusion. The mean time of half-desensitization from several such experiments shown in the third row of Table I is not different at the 5% level of probability from the value in the second row ($P > 0.05$) obtained from experiments in which the bath and perfusion fluids both contained 20 mM calcium. In control experiments perfusion with a solution containing 20 mM calcium in the absence of carbamylcholine did not affect the input

resistance of the fibers. These findings demonstrate that, when carbamylcholine is present, the full accelerating effect of calcium on the rate of desensitization appears within a few seconds of introduction of the ion into the extracellular medium.

Further experiments were done in order to find whether a rapid decrease in the extracellular concentration of calcium will also cause a rapid disappearance of its effect. In the example shown in Fig. 1 d the muscle was first soaked in a solution containing 20 mM calcium and then perfusion of a single fiber was carried out with a solution of carbamylcholine which contained in addition 40 mM potassium oxalate. It is assumed that the sudden delivery of oxalate by this method causes a rapid decrease in the local concentration of ionized calcium at nearly the same time as the onset of the action of carbamylcholine. The result in Fig. 1 d shows that, even in the early moments of the desensitization phase, the rate of increase of resistance is much slower than in Fig. 1 b where a high concentration of calcium was present in both the bath and perfusion fluids. The mean time of half-desensitization from several experiments of this kind appears in the fourth row of Table I and this value is different at the 5% level of probability from that in the second row which was obtained from experiments in which 20 mM calcium was present in both the bath and perfusion fluids. Similar experiments were performed in which the perfusion fluid contained 40 mM EDTA, and these also showed prolonged phases of desensitization (fifth row of Table I). When, therefore, the concentration of free ionized calcium in the extracellular fluid is rapidly decreased from a high value, there is a corresponding immediate slowing of the desensitization process.

B. *The Effect of Calcium in the Early Moments of Carbamylcholine Action*

Although calcium applied in the external medium can act rapidly to promote the desensitization process, it is not clear from the previous results whether or not this effect is also present in the very early moments of carbamylcholine action. This was tested in a separate group of experiments in which a higher frequency of test pulses and a more rapid film speed were used in order to measure the early events of drug action more accurately. Fig. 2 presents composite time courses of drug action from several experiments performed in 1.8 mM calcium (open circles) and 10 mM calcium (closed circles). Each point was obtained by first superimposing the records from many experiments so that the initial moments of onset of drug action coincided (zero time) and then calculating a mean value of input resistance at subsequent intervals of 1 sec. The muscles used in these experiments were presoaked in the respective solutions for 30–60 min prior to testing, and the perfusion fluid always contained the same concentration of calcium as that of the general bath medium.

The two sets of data were first examined to find at what stages a true difference due to the increased calcium concentration can be detected. This was done by using Student's t test to compare the mean input resistance in each of the 1 sec intervals. The intervals of time in which P values smaller than 0.05 were obtained are indicated by the horizontal line in the upper

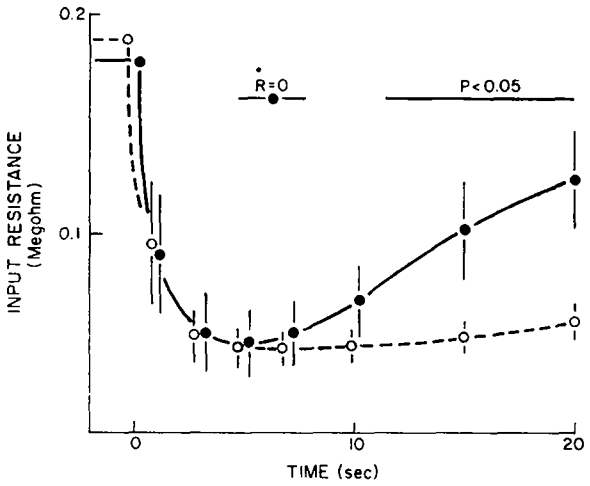

FIGURE 2. The effect of calcium on the time course of changes in input resistance of single fibers during the first 20 sec of perfusion with 2.73×10^{-3} M carbamylcholine. The open circles denote results obtained in solutions containing 1.8 mM calcium and the closed circles in 10 mM calcium. Perfusion with carbamylcholine was begun at time zero. Each point represents the mean of 9 or 10 experiments performed on different fibers from several muscle preparations. The vertical lines about each point represent 2 SEM. The horizontal line at the upper right indicates the interval of time in which a valid difference ($P < 0.05$) was found between the results in 1.8 mM and 10 mM calcium. The mean time of minimum resistance, determined as the moment when the time rate of change of resistance ($\dot{R}$) was zero, is indicated at the upper left as well as the interval which includes 2 SEM.

right section of Fig. 2. The analysis shows that at the 5% level of probability the increased calcium concentration does not have any effect on the input resistance until about 13 sec after the onset of drug action.

From these calculations it also appears that the action of calcium occurs somewhat later than the moment of maximum drug action when the membrane resistance is at its lowest value. In order to verify this the time of minimum resistance was determined for each experiment by plotting the time rate of change of resistance ($\dot{R}$) in each 1 sec interval and estimating when this value was zero. The mean time of minimum resistance obtained in this way from both sets of data is also shown in the upper portion of Fig. 2. It can be seen that within an interval containing 2 SEM the time of minimum resistance occurs sooner than the onset of action of calcium.

Although calcium does not seem to have much effect on the input resistance in the early moments of perfusion with 2.73×10^{-3} M carbamylcholine, it may be that some effect is present but not evident at this high concentration of the drug. Therefore, further experiments were done using one-tenth and one-twentieth of the concentration of carbamylcholine in the perfusion fluid, and the mean values of minimum input resistance obtained under these conditions are summarized in Table II. No difference between the results with 1.8 and 10 mM calcium could be demonstrated at any of the concentrations of carbamylcholine tested ($P > 0.05$).

In summary, these experiments show that the influence of calcium on the time course of action of carbamylcholine appears only after the drug has

TABLE II

THE EFFECT OF CALCIUM ON THE MAXIMUM CHANGE IN INPUT RESISTANCE PRODUCED BY VARIOUS CONCENTRATIONS OF CARBAMYLCHOLINE

Concentration of carbamylcholine in the perfusion fluid	Mean maximum fractional decrease in input resistance*	
	1.8 mM Ca	10 mM Ca
2.73×10^{-3} M	0.753 ± 0.016‡	0.736 ± 0.018
0.273×10^{-3} M	0.442 ± 0.092	0.431 ± 0.024
0.137×10^{-3} M	0.329 ± 0.056	0.282 ± 0.028

* Calculated as the maximum change in input resistance attained in the initial moments of drug perfusion divided by the control resistance immediately prior to perfusion.
‡ Value of 1 SEM.

been present for about 13 sec, and that prior to this, when the action of carbamylcholine is maximal, calcium does not produce any significant degree of desensitization as measured by changes in the input resistance.

C. *Dependence of the Action of Calcium on the Concentration of Carbamylcholine in the Perfusion Fluid*

Since the main effect of calcium appears only some time after the moment of maximal drug action, it may be that activation of the postjunctional membrane by carbamylcholine limits or controls in some way the action of calcium. In experiments designed to examine further this possibility, the time of half-desensitization was measured during perfusion with high (2.73×10^{-3} M) and low (0.14×10^{-3} M) concentrations of carbamylcholine while the concentration of calcium in the bath and perfusion fluids was varied between 1.8 and 10 mM. The results are summarized in Fig. 3. The lower curve (closed circles) shows that when fibers are perfused with 2.73×10^{-3} M carbamylcholine an increase in the calcium concentration from 1.8 to 10 mM causes a fourfold decrease in the time of half-desensitization. The

correlation coefficient calculated for these data is −0.61 and the 95% confidence interval of this value lies between −0.32 and −0.80. On the other hand, when the perfusion fluid contains only 0.14×10^{-3} M carbamylcholine (open circles) little or no change occurs over the same range of calcium concentrations. The correlation coefficient in this case is −0.073 and the 95% confidence interval from +0.37 to −0.5. The ability of calcium to bring about a rapid rate of desensitization therefore depends on the presence of a sufficient concentration of carbamylcholine acting on the postjunctional membrane.

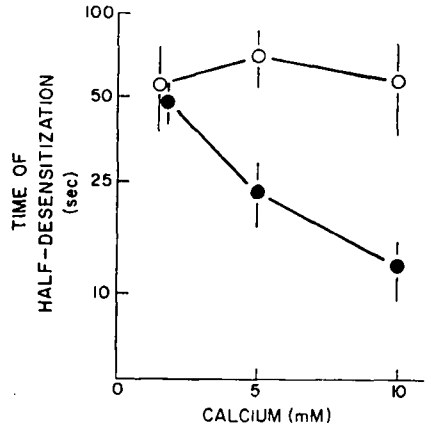

FIGURE 3. The effect of calcium on the time of half-desensitization during perfusion with different concentrations of carbamylcholine. The closed circles denote results obtained during perfusions with 2.73×10^{-3} M carbamylcholine and the open circles with 0.137×10^{-3} M carbamylcholine. Each point represents the mean time of half-desensitization in 6 to 15 different fibers from several different muscle preparations. In no case was the same fiber perfused with more than one concentration of carbamylcholine. The vertical lines represent 2 SEM.

DISCUSSION

As shown in this and a previous communication (11), there is a marked increase in the rate of desensitization to applied carbamylcholine when isolated muscle fibers are soaked in solutions containing increased amounts of calcium ion. In the first group of experiments some indication was sought whether calcium introduced rapidly into the extracellular fluid acted immediately to produce this result or whether a very slow process was involved, such as the accumulation in the intracellular compartment of a high concentration of calcium. If the latter were true, changes in the rate of desensitization would be expected to take place gradually over a period of several minutes or perhaps hours (13) because of the very low membrane permeability of this ion. The results in Fig. 1 c and Table I, row 3, show, on the contrary,

that the effect of calcium is apparent within 20 sec of its rapid introduction by means of a perfusion pipette. Furthermore, even after long exposure to a high concentration of calcium, the desensitizing action of the ion can be rapidly removed by perfusion with oxalate or EDTA (Fig. 1 d; Table I, rows 4 and 5). Since these agents probably penetrate cell membranes slowly (14), it is likely that in this short time they are reacting primarily with calcium present in the extracellular fluid rather than some intracellular fraction of calcium. The rapidity with which these sudden changes in the local extracellular concentration of calcium are followed by changes in the time course of carbamylcholine action suggests that calcium in the extracellular fluid can act directly on the chemosensitive surface membrane and that a large accumulation or depletion of the ion in the intracellular phase is not an essential step.

Although these results demonstrate a fairly rapid action of calcium, further measurements were made in order to determine more accurately when during the action of carbamylcholine the desensitizing effect of calcium is first evident (Fig. 2). This was done in particular to examine the possibility that the calcium ion can bring about a desensitized state within a very short time of the activation of membrane receptors by carbamylcholine. An onset time of only a few milliseconds, for example, would probably be the case if calcium acted only by simple adsorption to a site on the outer surface of the postjunctional membrane since the rates of such reactions are usually limited only by free diffusion in the fluid near the reactive site. An example of this type of reaction is perhaps the antagonism of the depolarizing action of acetylcholine by *d*-tubocurarine, which can occur with apparently little or no delay in experiments in which both drugs are applied by microelectrophoresis and diffusion time is only a few milliseconds (15). The method of microperfusion used in the present study, however, would not provide an accurate measure of such a rapid time course of action since, even in the most favorable cases, full activation of the postjunctional membrane by applied carbamylcholine was not achieved until at least one or more seconds after the onset of perfusion. Nevertheless, a desensitizing action of calcium which is faster than this would be evident in these experiments as an increase in the minimum input resistance in the initial moments of drug application, since in the 1 or 2 sec required to reach full postjunctional activation some degree of desensitization would already have been complete. When the early events of drug perfusion were measured carefully, however, it was found (Fig. 2) that an increased concentration of calcium (10 mM) which produces an almost maximal increase in the rate of desensitization has no demonstrable effect on the minimum input resistance in the initial moments or at times thereafter until about 13 sec following the onset of perfusion. Moreover, this lack of effect on the initial input resistance was found to be the case over a

wide range of concentrations of carbamylcholine (Table II). Therefore, on the basis of these results, it appears that the desensitizing action of calcium, although rapid, does not occur immediately upon the activation of the membrane receptors by carbamylcholine (as might happen if the ion acted by adsorption to an exposed surface membrane site) but only after a delay of several seconds.

There may be several possible explanations for this delay. The calcium ion, for example, could be the initiating factor in a slow change or series of changes in membrane structure which ultimately lead to a desensitized state but which take several seconds to reach completion. A mechanism of this type, however, would be difficult to explore further at present because of general lack of detailed knowledge of membrane structure. A simpler hypothesis is that the site at which the calcium ion acts, although perhaps part of the membrane structure, nevertheless, is not immediately available to ions of the extracellular medium and that the time needed for the calcium ion to penetrate to this site results in the observed delay in its effect. One implication of this proposal which is susceptible to further test is that the membrane permeability of calcium is a rate-limiting factor in its effect on the desensitization process. On this basis some relation would be expected between the ability of calcium to bring about rapid desensitization and the degree of activation of the postjunctional membrane, a factor which Takeuchi (16) has shown can increase the membrane permeability of calcium. Such a relationship is demonstrated by the results in Fig. 3 which show that the action of calcium in accelerating the desensitization process disappears when the concentration of carbamylcholine in the perfusion fluid (and hence the degree of postjunctional activation) is reduced. Although it is possible that some other aspect of the action of carbamylcholine is responsible for this result, the simplest explanation at present seems to be that the more potent desensitizing effect of calcium seen with higher concentrations of carbamylcholine depends on the ability of the latter to increase the permeability of the membrane to calcium.

A final word is added concerning the apparent discrepancy between some of the results presented here and those of Nastuk and Liu (17). In the latter study calcium was shown to reduce the maximal response of the postjunctional membrane to carbamylcholine while in the present investigation such an effect was not observed. No explanation for this is offered at this time but two important differences in the experimental conditions should be noted: (*a*) Change in membrane potential was used by Nastuk and Liu as a measure of drug action, whereas in the present work change in input resistance was employed for this purpose. These two measurements may differ with respect to their sensitivity to drug action and also their susceptibility to the effects of the intracellular accumulation of ions. (*b*) Most of the concentrations of

carbamylcholine reported by Nastuk and Liu were below 10^{-4} M, while the lowest concentration used here was above this level. It is possible that a competitive interaction between carbamylcholine and calcium at the receptor site would be more evident at low concentrations of the drug.

The author expresses his sincere thanks to Dr. Charles Sheppard of the Department of Physiology and Biophysics for his helpful suggestions during the preparation of the manuscript, and also to Miss Sherry K. Diggs and Mr. Herbert Simkin for their valuable technical assistance in the performance of the experiments.

This work was supported by NSF Grant GB-8495 from the National Science Foundation.

Received for publication 19 February 1970.

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