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The Antagonistic Effects of Calcium and Potassium on the Time Course of Action of Carbamylcholine at the Neuromuscular Junction

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Summary. The electrogenic action of carbamylcholine on the postjunctional membrane of muscle fibers disappears gradually over a period of several seconds even when this agent is applied by constant perfusion. This waning of drug action, termed "desensitization" by some investigators, occurs also at other cholinergic synapses. It has been found in earlier studies that increased amounts of calcium in the extracellular medium can cause the desensitization process to occur more rapidly and that potassium tends to oppose this action of calcium. In the present investigation, it is found that the relative effects of these ions can be expressed in terms of the quantity $(Ca)/(K)^{1.7}$. A simple ion-exchange mechanism is proposed in which one calcium ion or two potassium ions are able to combine with an anionic site near the cell surface. A general relation between the time course of conductance changes and the extracellular concentration of the ions is derived which appears to be consistent with the conductance measurements over a period of 60 seconds and in the range of calcium concentrations from 0.05 to 20 millimoles/liter and of potassium from 33 to 165 millimoles/liter. Further experiments are presented which suggest that the time course of conductance changes under these conditions is limited primarily by the rate of increase of the calcium concentration at the exchange site.

When quaternary ammonium compounds such as acetylcholine or carbamylcholine are applied for a prolonged time to the postjunctional membrane of skeletal muscle fibers, the rapid initial electrogenic action of these agents gradually disappears over a period of several seconds or minutes even though the substances may continue to be present during this time. This decline in electrogenic effect at the neuromuscular junction has been termed "desensitization" [7] or "inactivation" [12] by some investigators, and it appears to occur at other cholinergic synaptic junctions as well [1, 3, 4, 16]. In an earlier publication [9], it was shown that the rate at which desensitization proceeds is increased greatly when the calcium concentration in the extracellular medium is increased and that this effect of calcium is antagonized by the presence of sodium and potassium. In the

present investigation, the interactions of calcium and potassium with respect to the "desensitization" process were chosen in particular for further study because the use of solutions in which potassium is the major extracellular cation presents several advantages for the measurement of desensitization rate. One of these is that when carbamylcholine is applied to the post-junctional membrane of muscle fibers immersed in low-sodium, high-potassium solutions, little or no change occurs in membrane potential, and this circumstance eliminates the need to consider how changes in membrane potential during drug application might independently affect the time course of drug action. Secondly, muscle fibers in high-potassium solutions are electrically depolarized over their whole length and thus not capable of the vigorous propagated twitch response which under other conditions would make recordings with intracellular electrodes during carbamylcholine action very difficult. Another desirable condition for the quantitative analysis of the calcium-potassium antagonism is the ability to make measurements over a wide range of concentrations of these ions. This was made difficult in the previously cited study [9] because the use of sulfate as the major extracellular anion severely limited the amount of free ionized calcium that could be attained in solution. In the present study, therefore, solutions of the acetate salts of the cations were used thus allowing experiments to be done over a wide range of concentrations of calcium even in the presence of large amounts of potassium.

In the report that follows, measurements of the effective or "input" resistance of single muscle fibers of the frog were used to quantitate the effects of changes in the concentrations of calcium and potassium on the time course of action of carbamylcholine delivered by local perfusion to the junctional region of the fiber. A simple ion-exchange mechanism is suggested which could account for the relative quantitative effects of these ions and which, when investigated further, appears to be consistent with the experimental measurements over at least a 60-sec period of drug action. Additional experiments are described in which an attempt is made to assess the relative contributions of calcium and potassium fluxes in the determination of the rate of desensitization.

Materials and Methods

Muscle Preparation

The sartorius muscle of the frog (*Rana pipiens*) was used in all experiments. Prior to use, animals were kept at room temperature. Muscles were dissected whole from the animal and mounted for microscopic observation in a shallow fluid bath.

Measurement of Carbamylcholine Action

The time course of action of carbamylcholine was estimated by changes in the "input" or effective resistance of single fibers measured in the region of the neuromuscular junction during constant local application of the drug. A solution containing 2.73×10^{-3} M carbamylcholine was delivered to the junctional area of the fiber by local perfusion from a special micropipette with a tip diameter of approximately 50 to 75 μ. The input resistance was estimated by means of two intracellular KCl-filled glass capillary electrodes, one for injecting short (300 to 400 msec), repeated (0.5 per sec) pulses of current and the other for recording the resulting electrotonic potentials. The output from the recording electrode was amplified and led to the vertical amplifier input of an oscilloscope. Vertical deflections of the oscilloscope trace were recorded on slowly moving 35 mm film. Since the test current was delivered to the fiber through a very high series resistance (66×10^6 Ω), the amplitude of the recorded electrotonic potentials is a direct measure of the input resistance. Earlier publications [9, 10] should be consulted for further details about the method of drug application and the measurement and recording of the input resistance.

Solutions

Muscle preparations were dissected and initially mounted in normal Ringer's fluid, the composition of which is shown in the first horizontal row of Table 1. The pH of this and all other solutions was adjusted to approximately 7.3 to 7.4 by the addition of small amounts of HCl. Prior to an experiment, the isolated muscle was equilibrated for 30 to 60 min in one of the high-potassium solutions indicated in Table 1 as A, B, or C. When it was desired to change the concentration of calcium acetate in any of these solutions, adjustments were made in the concentration of sucrose so as to maintain the same osmolarity. Solutions of carbamylcholine for perfusion were made up on the same day of each experiment by dissolving the freshly weighed salt in a medium identical with that bathing the muscle preparation. No more than three or four fibers in widely scattered areas of each muscle preparation were tested and in no case was a fiber perfused more than once. Each muscle preparation was exposed to only one test bath medium. No special provision was made for oxygenation of the bath fluid during the experiments. All experiments were performed at room temperature which varied on different days from 20 to 25 °C.

Table 1. Composition of some of the solutions used to bathe isolated muscle preparations[a]

Solution	Na^+	K^+	Ca^{++}	Cl^-[b]	CH_3COO^-	THAM[c]	Sucrose
Normal Ringer's	120	2.5	1.8	126.1	—	1.0	—
A	3.0	165	1.8	—	171.6	1.0	55
B	3.0	100	1.8	—	106.6	1.0	185
C	3.0	33	1.8	—	39.6	1.0	319

[a] All concentrations are in units of mmoles/liter.
[b] Entries in this column do not include the small amounts of chloride ion which are present because of the neutralization of THAM with HCl.
[c] Tris[hydroxymethyl]aminomethane.

Measurement of Small Local Contractions in the Postjunctional Region of Fibers

In one series of experiments, an attempt was made to measure the time courses and approximate relative amplitudes of small local contractions which appear under some conditions in the junctional region of fibers during perfusion with carbamylcholine. The procedure for this was as follows. Two glass capillary micropipettes, identical with those used to measure the input resistance, were positioned under microscopic observation (150 × magnification) near the junctional region of a muscle fiber. One of the pipettes was inserted into the fiber while the other was positioned above and outside the fiber but within a few microns of the first. Perfusion of the area with a solution of carbamylcholine was then begun using the special perfusion pipette described above, and during this time motion pictures of the microscope image were made on 16 mm film at a rate of 12 frames per sec. Any local contraction in response to drug perfusion produced a movement of the intracellular pipette relative to the position of the extracellular pipette, and this could be measured as a displacement of the pipette images on the filmed

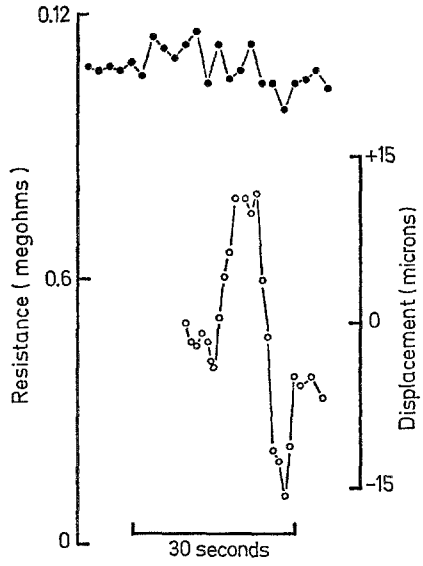

Fig. 1. Simultaneous measurements of the input resistance and mechanical displacement of implanted electrodes during artificial movement of the muscle preparation. The input resistance (filled circles) was measured by means of two intracellular capillary electrodes as described in the text. Artificial movement of the muscle fiber was produced by manipulation of the microscope stage (*see* text) and the displacement of the electrode tips was measured on 16 mm motion pictures of the microscope image. The position of the electrodes before the onset of the movement is arbitrarily designated as zero on the right-hand ordinate. The bath medium in this experiment contained 33 mM potassium acetate and 0.5 mM calcium acetate. Results similar to those shown here were obtained as well in solutions A, B, and C of Table 1. The microelectrodes used in this and other experiments were pulled from glass tubing having an outside diameter of 3 mm and a wall thickness of 0.6 mm. The resistance of these electrodes was usually between 5 and 10 MΩ and the distance from shoulder to tip was always about 12 to 13 mm. The diameter of the fiber in this experiment was estimated to be about 40 μ

record. Displacements as small as 1 to 2 μ could be measured by this means. Control experiments showed that the force of the perfusion fluid by itself produced almost no displacement of the pipettes unless the large perfusion pipette was moved from its normal delivery position 0.1 mm away to a point within a few microns of the impalement site.

It is easy to imagine that the occurrence of these "microcontractions" in experiments where the input resistance is being measured might disturb the impalement site sufficiently to cause electrolytic "leaks" around the implanted electrodes and thereby affect the measurement of the input resistance. In practice, however, it is found that the input resistance is fairly insensitive to small mechanical disturbances such as those produced by the microcontractions. A clear demonstration of this is provided by the experiment illustrated in Fig. 1. During measurement of the input resistance with two microelectrodes as described above, a small movement of the muscle fiber along its longitudinal axis was produced by external manipulation of the microscope stage causing a displacement of the tips of the implanted electrodes which resembled that produced in the most extreme cases by the microcontractions. This displacement was meanwhile recorded as described above on motion picture film. The results in Fig. 1 show that, whereas this maneuver caused a maximum displacement of 28 μ, the input resistance measured simultaneously remained within about 10% of its average value during this time. For comparison, it should be noted that microcontractions produced by carbamylcholine caused a maximum displacement of the implanted micropipette of only about 11 μ (*see* Fig. 7) concomitant with a 75 to 90% change in input resistance.

Data Analysis

The values of b in Eqs. (1) and (11) were obtained with the aid of the IBM 1620 computer. The computer was also employed in fitting the regression lines in Fig. 4*A* and in the comparisons of their slopes. Further details about the analysis in each case are described in the text.

Results

The Antagonistic Effects of Calcium and Potassium with Respect to the Time Course of Carbamylcholine Action in Acetate Solutions

The filmed records shown in Fig. 2*A–D* illustrate the effect of calcium on the time course of the desensitization process and how this effect is altered by changes in the concentration of potassium. In the first example (Fig. 2*A*), the muscle preparation had been soaked prior to the experiment in a solution containing 1.8 mM calcium and 165 mM potassium acetate. The first small downward deflection of the oscilloscope trace (vertical arrow marked 1) represents the measurement of the initial membrane potential when the recording electrode is first implanted in the fiber. This value of potential is close to zero because of the high concentration of potassium present in the bath fluid. A few seconds later the current-passing electrode is implanted (vertical arrow marked 2) and repeated pulses of negative current delivered to the inside of the fiber causing deflections of the membrane potential which appear as a series of dots in the filmed record. Since

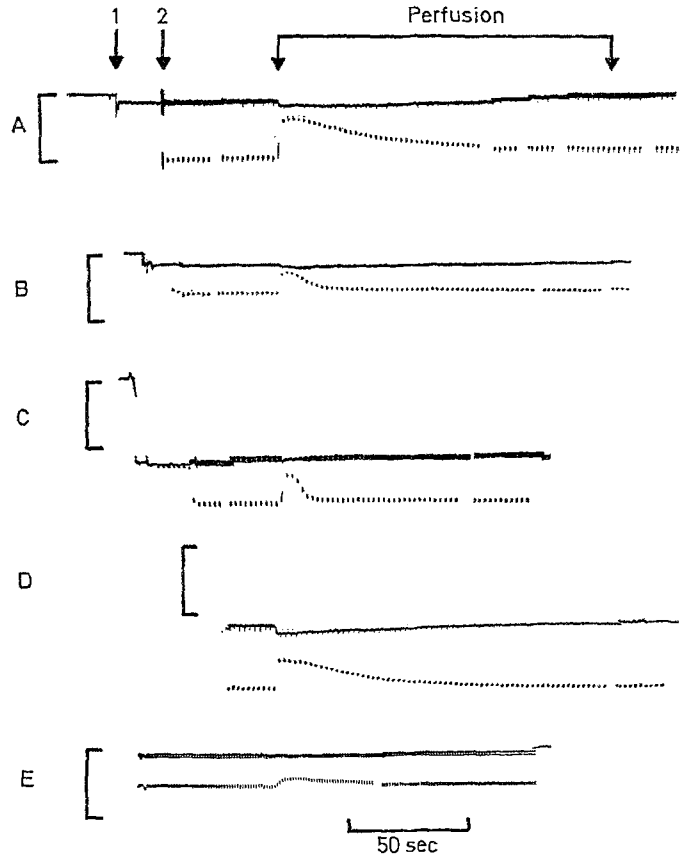

Fig. 2. Records showing the effects of calcium and potassium on the time course of changes in input resistance of single fibers during perfusion of the junctional region with 2.7×10^{-3} M carbamylcholine. (*A*) Perfusion experiment performed in a solution containing 165 mM potassium and 1.8 mM calcium acetate. The upper and lower extremes of the calibration mark indicate, respectively, 0 and -30 mV potential relative to the potential of the extracellular medium. *See* text for a more detailed description of this record and an explanation of the deflections marked "1" and "2". The period of perfusion with carbamylcholine in this experiment is indicated above the record. The blank portions in the dot sequence in the control period before perfusion and in the latter moments of the perfusion indicate a lapse of 60 sec during which the recording camera was turned off. The bath fluid in the other examples shown contained the following: (*B*) 165 mM potassium, 20 mM calcium acetate; (*C*) 33 mM potassium, 1.8 mM calcium acetate; (*D*) 33 mM potassium, 0.1 mM calcium acetate. In (*D*) the initial deflection of the potential trace is not shown. In (*E*) the bath and perfusion fluids contained 2.5×10^{-6} M d-tubocurarine in addition to 165 mM potassium and 1.8 mM calcium acetate; also the current pulses were delivered at 1-sec intervals instead of every 2 sec as in (*A*)–(*D*)

this current was delivered through a high resistance, the amplitude of these deflections is a measure of the input resistance of the fiber. After a control period, local perfusion with carbamylcholine was begun, causing the abrupt

decrease in input resistance seen in the record. Following this initial decline, however, the resistance begins to increase slowly toward the control value even though drug perfusion was continued. This gradual recovery of the membrane resistance in the presence of carbamylcholine is considered to be a direct manifestation of the desensitization process. The ability of increased amounts of calcium to cause a more rapid onset of the desensitization process is shown by the next example in Fig. 2*B*. The muscle in this case has been equilibrated previously in a solution containing 20 mM calcium and 165 mM potassium acetate and the return of the input resistance to control levels during the desensitization phase is here very much more rapid.

In addition to this primary effect of calcium, an increase in desensitization rate can also be brought about by lowering the concentration of the potassium salt in the bath fluid. This is shown by a comparison of the result in Fig. 2*C*, which was obtained in a solution of 33 mM potassium and 1.8 mM calcium acetate, with the earlier example in Fig. 2*A* where the same amount of calcium is present in a solution of 165 mM potassium. The desensitization rate in the low-potassium medium (Fig. 2*C*) is much faster and resembles the rapid sequence in Fig. 2*B* which was produced by increasing the calcium concentration in the high-potassium medium to 20 mM. On the other hand, the slower pattern of desensitization can also be obtained in the low-potassium medium by reducing the calcium concentration to 0.10 mM as shown by the next result in Fig. 2*D*. It appears, then, that the reduction of the potassium concentration in *C* and *D* has reinforced the desensitizing action of calcium in some manner so that the effect of this ion is now evident at a much lower concentration. A similar antagonism between the effects of calcium and potassium on desensitization rate was demonstrated over a more limited range of concentrations in an earlier study [9] where the sulfate salts of these ions were used. In addition, therefore, to extending these earlier results, the present findings also show that the interaction of calcium and potassium in this regard is not dependent on the chemical nature of the anion present.

Some differences in the electrical characteristics of fibers in the high-potassium and low-potassium solutions are evident in the records of Fig. 2, although these are not thought to be primary factors causing changes in the desensitization rate. One obvious difference is the higher membrane potential of fibers exposed to solutions of 33 mM potassium. The mean initial potential for all experiments in this medium was −38.3 mV whereas that for fibers in 165 mM potassium was only −6.6 mV. It is difficult to design experiments which will test the effect on desensitization rate of this factor

alone since deflection of the membrane potential by whatever means for many seconds or minutes may also cause changes in the concentrations of ions in the immediate intracellular and extracellular environment. Nevertheless, if differences in membrane potential consequent to changes in the extracellular concentration of potassium exert any effect, such is not evident as a change in the desensitizing action of calcium since, as shown somewhat later (Fig. 4*A*), no differences can be demonstrated in the relationship between desensitization rate and per cent change in calcium concentration among three series of measurements in the presence of 165, 100, and 33 mM potassium. Another difference in electrical behavior of the muscle fibers exposed to low-potassium solutions is a general increase in input resistance both before and during carbamylcholine application. The mean control resistance in 33 mM potassium was 0.27 MΩ and that in 165 mM potassium was 0.16 MΩ. The values at the peak of drug action in the initial moments of perfusion were 0.10 MΩ and 0.043 MΩ, respectively, for the low- and high-potassium media. The possible effect of this factor on desensitization rate is also difficult to test in isolation. The result in Fig. 2*E* provides one indication, however, that an increased resistance during drug action does not of itself cause an increase in desensitization rate. In this experiment, the bath and perfusion fluids contained 2.5×10^{-6} M d-tubocurarine in addition to 165 mM potassium and 1.8 mM calcium. It can be seen that although this degree of curarization reduced the maximum change of conductance during perfusion compared to that in Fig. 2*A*, the rate of desensitization was nevertheless not greatly increased.

Although the input resistance is the quantity obtained by direct measurement of the pulse amplitude in records such as those in Fig. 2, it was thought useful to express the time course of carbamylcholine action in terms of the reciprocal of this value, the input conductance (G), for the following reason. The initial decrease in resistance brought about by carbamylcholine is regarded in the first place as the result of the "opening" to increased ion flow of parallel pathways in the postjunctional membrane and the desensitization process the subsequent "reclosing" of these pathways. Since the input conductance is equal to the algebraic sum of all the parallel current pathways across the membrane, it is considered that the number of pathways remaining open at any time and hence the portion of the membrane which has not yet undergone desensitization is a direct linear function of the input conductance. This may be only approximately true, however, since the current pathways to all parts of the postjunctional membrane from the current electrode are not exactly equal, and the current through the membrane is not strictly uniform. Nevertheless, the input conductance would

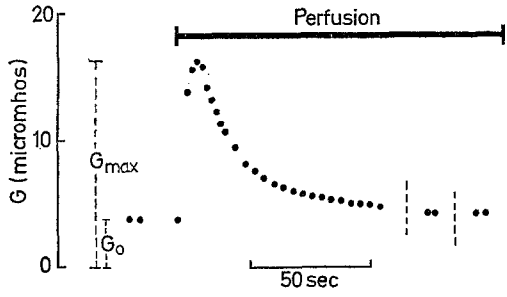

Fig. 3. The time course of changes in input conductance G during perfusion of a single fiber with 2.7×10^{-3} M carbamylcholine. The measurements shown are derived from the record in Fig. 2*A*. *See* text for explanation of G, G_0, and G_{max}. The vertical dashed lines in the right-hand part of the figure indicate lapses of 60 sec during which no measurements were made

seem to be more nearly a linear function of the number of open pathways than the input resistance.

Fig. 3 shows the time course of changes in input conductance derived from the record in Fig. 2*A*. The conductance in the control period prior to drug perfusion (G_0) is low but upon introduction of carbamylcholine rapidly increases to a maximum value (G_{max}). The desensitization process in this representation is defined as the decrease in input conductance during the application of carbamylcholine which follows the attainment of G_{max}. The time of half-decline of the input conductance ($T_{1/2}$) was used as a rough quantitative indication of the rate of desensitization. This was measured as the time taken for the conductance to decrease from G_{max} to a value halfway between G_{max} and G_0.

The relative effects of calcium and potassium on the mean $T_{1/2}$ of desensitization over a wide range of calcium concentrations in the presence of 33, 100, and 165 mM K are shown on logarithmic coordinates in Fig. 4*A*. In all three cases, the desensitizing effect of calcium is shown by a decrease in the $T_{1/2}$ as the calcium concentration increases, and the antagonism of this action by potassium appears as a shift to the right on the concentration axis when the amount of potassium is increased from 33 mM to 165 mM. In addition to the results shown, experiments were also performed in potassium acetate solutions to which no calcium had been added, and it was found that the mean $T_{1/2}$ in these cases was very nearly the same as the highest values of $T_{1/2}$ shown in Fig. 4*A*. This apparent upper limit to the Ca-$T_{1/2}$ relationship is indicated by the horizontal dashed lines in the figure.

An interesting aspect of the results in Fig. 4*A* is the similarity of slope of all the regression lines. This finding suggests that the relative effects of

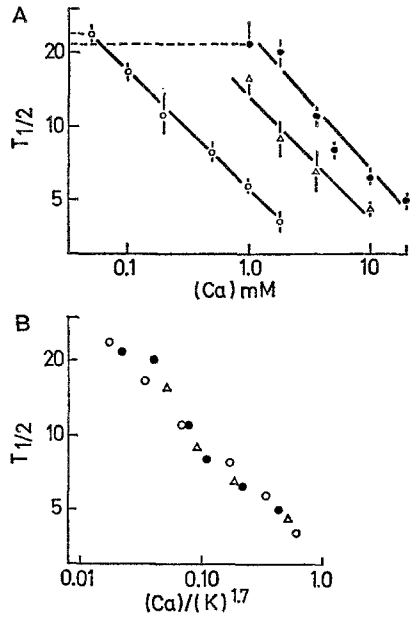

Fig. 4. Effects of changes in the calcium and potassium concentrations of the extracellular fluid on the time of half-decline of input conductance ($T_{1/2}$). $T_{1/2}$ was calculated as the time taken for the input conductance G to decline from G_{max} to a value halfway between G_{max} and G_0 (*see* Fig. 3). (*A*) The effect of calcium on $T_{1/2}$ in the presence of 165 mM potassium (filled circles), 100 mM potassium (open triangles), and 33 mM potassium (open circles). Each point represents the mean $T_{1/2}$ determined in 7 to 21 single fibers. The vertical lines about the points indicate the value of 1 SEM. The least squares line for each series is shown. No differences among the slopes of the lines could be demonstrated at the 95% level of significance. The meaning of the horizontal broken lines near the top of the figure are explained in the text. (*B*) The same data as in (*A*) plotted as a function of the quantity $(Ca)/(K)^{1.7}$ where the symbols in parentheses represent concentrations in the extracellular fluid of the respective ions in moles/liter

calcium and potassium on desensitization rate can be expressed in terms of a single quantity, $(Ca)^a/(K)^b$, where the exponents a and b are constants and the chemical symbols in parentheses represent the concentration of ions in the extracellular medium. To find what relative values of a and b might give this result, the following procedure was employed. All the $T_{1/2}$ measurements were considered collectively and a best least squares fit was determined for the following function:

$$\ln T_{1/2} = m[a \ln(Ca) - b \ln(K)] + c \tag{1}$$

where m is the slope on log coordinates, c the intercept, $a = 1$, and b was considered a variable. It was found that the best fit was obtained when

$b = 1.7$. Accordingly, the $T_{1/2}$ measurements are replotted in Fig. 4*B* as a function of the quantity $(Ca)/(K)^{1.7}$, and it appears that the results from all three series of experiments fall more nearly on a single line.

A Simple Ion-Exchange Model for the Potassium-Calcium Interaction

Results similar in some respects to those presented above were found by Lüttgau and Niedergerke [8] in a study of the antagonistic effects of calcium and sodium on contractions of cardiac muscle. In this case, the effects on tension output produced by reducing the sodium concentration of the extracellular medium could be expressed as a single function of the ratio $(Ca)/(Na)^2$. A model consistent with these findings was suggested in which either one calcium ion or two sodium ions combined with a divalent anionic site near the surface of the cell. Since the effects of calcium and potassium on desensitization rate in the present study are related in the exponent by nearly a 1:2 ratio, it may be that a model similar to that proposed by Lüttgau and Niedergerke can be applied to the present results. The following are some simple postulates suggested as a possible basis for such a model.

(a) Anionic sites ($A^=$) are present in the muscle fiber, which, when combined with calcium, cause a decrease in membrane conductance by some unknown mechanism but which do not do so when in combination with potassium. No independent evidence is offered for this assertion which, in any case, simply states that the antagonistic actions of calcium and potassium occur at some site.

(b) The action of carbamylcholine increases the permeability of the postjunctional membrane to several ions present in the extracellular medium (including potassium and calcium) and allows access of the anionic sites to these ions which, in the absence of carbamylcholine, is denied. No assumptions are made about the mechanism by which carbamylcholine causes increased ion permeability, but evidence for the participation of potassium and calcium in these permeability changes is readily available especially in the work of A. and N. Takeuchi [14, 15]. Evidence that the desensitization site prior to the action of carbamylcholine is not immediately available to combination with extracellular ions is presented in an earlier publication [10] and consists principally of the finding that calcium added to the extracellular medium does not exhibit a desensitizing action until several seconds after the onset of action of carbamylcholine. Since the cholinergic receptor itself can react within a few milliseconds with quaternary ammonium compounds applied to the external surface of the cell, this delay in the desensitizing action of calcium suggests that the desensitization site is distinct from the

cholinergic receptor and that cations in the extracellular medium do not have as ready access to this site as to the acetylcholine receptor group. It is not intended to specify beyond this a precise locus for the desensitization site although it may be reasonable to suppose that it lies somewhere within the membrane but somehow "shielded" from the external medium or perhaps, as suggested by Nastuk and Parsons [13], at the internal surface of the membrane.

(c) During the desensitization phase of carbamylcholine action, calcium and potassium ions combine with $A^=$, the desensitization site, in an ion exchange such as the following:

$$Ca^{++} + K_2 A \rightleftharpoons Ca\,A + 2K^+. \tag{2}$$

Since potassium is the most abundant species in these experiments and since the membrane permeability for this ion is usually much greater than that for calcium, it is suggested that by the time the membrane conductance has reached its maximum, about 2 to 6 sec after the onset of carbamylcholine action (*see* Fig. 3), all the sites are combined with potassium and that the amount of decline in conductance at any moment in the following desensitization phase depends on the fraction of sites which subsequently combine with calcium to form Ca A. No assumptions are made, however, about whether or not other ions can combine with the sites under appropriate conditions or about what ions may be associated with the sites prior to carbamylcholine action. Since the desensitization sites appear to be somewhat removed from direct contact with ions of the ambient external medium (*see above*), the activities or concentrations of calcium and potassium in the immediate vicinity of the sites during desensitization may not always be the same as in the bulk extracellular fluid and for this reason are given the special symbols $(Ca)_s$ and $(K)_s$. An "equilibrium constant" can be written for the ion exchange in Eq. (2) as follows:

$$\Psi = \frac{(Ca\,A)(K)_s^2}{(K_2 A)(Ca)_s}. \tag{3}$$

Since the activities of the various species in the right-hand expression are not known, Ψ is not a true equilibrium constant and should perhaps be termed instead a "selectivity coefficient" [5].

The occurrence of the ratio $(K)_s^2/(Ca)_s$ in Eq. (3) suggests a possible relationship to the findings presented earlier in Fig. 4 where the $T_{1/2}$ measurements were shown to be related to the quantity $(Ca)/(K)^{1.7}$. Some further

development of the ion exchange model is described now to show how this relation might be made more explicit and also to provide a means of further testing the applicability of the model to the conductance measurements.

In most ion-exchange reactions the exchange step itself is very rapid, and the overall rate is usually controlled by diffusion or penetration of the exchanging ions to and from the exchange site. On this basis, it is assumed that the rate-limiting step is not chemical exchange represented in Eq. (2) but rather some other process which controls the rate at which $(Ca)_s$ and $(K)_s$, the concentrations of the ions near the anionic site, approach the values for the respective concentrations of these ions in the ambient extracellular medium subsequent to the onset of carbamylcholine action. In general, therefore, $(Ca)_s$ and $(K)_s$ are related to the respective extracellular concentrations by some functions of time, $f_1(t)$ and $f_2(t)$, which are not specified:

$$(Ca)_s=(Ca)f_1(t), \tag{4}$$

$$(K)_s=(K)f_2(t). \tag{5}$$

Since the degree of desensitization depends on the amount of calcium combined with the anionic site, it is supposed that in the early moments of "drug perfusion" when the membrane conductance is maximum, $(Ca)_s$ is much less than (Ca) and that in the subsequent desensitization phase, $(Ca)_s$ gradually increases as more calcium from the extracellular phase penetrates to the site. Although it is postulated that $(K)_s$ depends on (K), no assumptions are made for the present about the relative magnitudes of these quantities. For the purposes of notation the following definitions are made using the symbols in Fig. 3:

$$F=\frac{\Delta G}{\Delta G_{max}}=\frac{G-G_0}{G_{max}-G_0}. \tag{6}$$

The symbol F denotes the fraction of the total carbamylcholine-induced conductance change remaining at any time. To relate chemical events at the desensitization site with observed conductance changes, the approximation is made that the fraction of sites occupied by calcium is directly related to the fractional decline in conductance following the attainment of G_{max}:

$$(Ca\,A)=1-F. \tag{7}$$

If the total number of desensitization sites is taken as unity:

$$(Ca\,A)+(K_2\,A)=1 \tag{8}$$

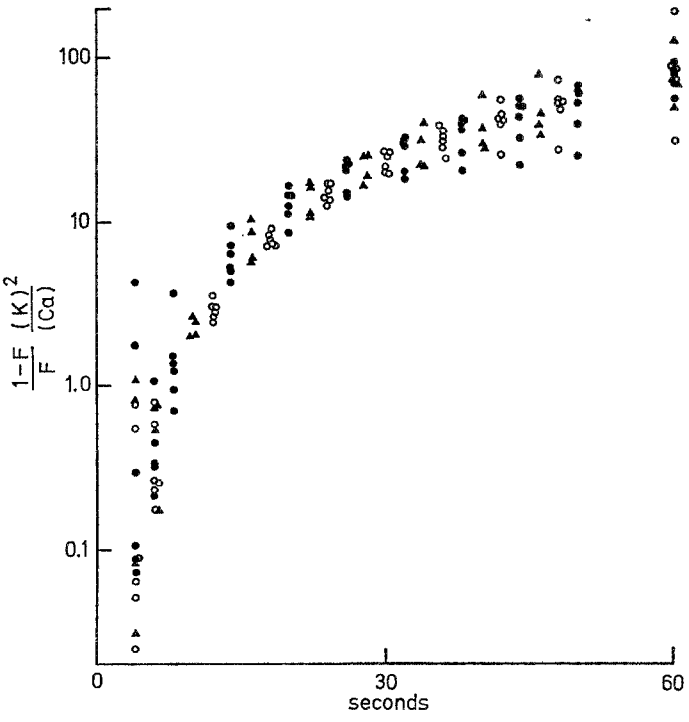

Fig. 5. Time plot of the quantity on the left of the equals sign in Eq. (10). The symbol F is defined in the text. The chemical symbols in parentheses on the ordinate represent the concentrations of the respective ions in the extracellular medium expressed in moles/liter. Each point represents data derived from experiments on 7 to 21 fibers. The data are grouped according to the concentration of potassium in the extracellular medium: filled circles, 165 mM; filled triangles, 100 mM; open circles, 33 mM. The concentration of calcium varies overall in the three series from 0.05 to 20 mM. For convenience in plotting, the results from only one series are shown in each of the 2-sec intervals

and Eq. (8) combined with Eq. (3) yields:

$$\frac{(\mathrm{Ca}\,A)}{1-(\mathrm{Ca}\,A)} = \Psi \frac{(\mathrm{Ca})_s}{(\mathrm{K})_s^2}. \tag{9}$$

Substituting Eq. (7) and the time-dependent functions, Eqs. (4) and (5), in Eq. (9) and rearranging yields Eq. (10):

$$\frac{1-F}{F}\frac{(\mathrm{K})^2}{(\mathrm{Ca})} = \Psi \frac{f_1(t)}{[f_2(t)]^2}. \tag{10}$$

According to Eq. (10), the quantity to the left of the equals sign when expressed as a function of time will describe a single complex time course which represents a combination of the time functions $f_1(t)$ and $f_2(t)$. Fig. 5

shows time plots of this quantity over a period of 60 sec obtained from experiments performed in 33, 100, and 165 mM potassium. As predicted, the results of all three series of experiments appear to fall nearly on a single curve. To find more exactly the relative values of the exponents for potassium and calcium which would produce optimal coincidence of the experimental points, the following procedure was used. The conductance measurements in all three series of experiments were considered collectively at every fourth second during the 60-sec period shown in Fig. 5. For each of these moments, in turn, the best least squares fit was determined for the following function:

$$\ln\left(\frac{1-F}{F}\right) = a\ln(\mathrm{Ca}) - b\ln(\mathrm{K}) + c \tag{11}$$

where the quantity F is defined in Eq. (6), $a=1$, c is the intercept, and b is considered a variable. The value of b which best fit the data varied from 1.45 to 2.05 at the selected times between 6 and 60 sec. The average value for all intervals so tested was 1.9. It should be noted that Eq. (10) was derived specifically for the conditions where there is a demonstrable relation between desensitization rate and the concentration of calcium in the extracellular medium and cannot be applied, therefore, in circumstances where this relation is not evident.

Experiments to Determine if Net Flux of Potassium and/or Calcium Occurs during Carbamylcholine Action

The relationship in Eq. (10) was derived for the general case in which the concentrations near the desensitization site of both potassium and calcium are changing during the period of desensitization. Some indication of whether or not this is true might be obtained from an examination of the transmembrane fluxes of these ions, since the absence of a net flux in the case of either Ca or K would predispose against the possibility of a change in the concentration of that species near the surface of the cell where it is presumed the desensitization site is located. Although measurement of these fluxes with radioactive tracers was not attempted, it appears already from evidence in the records of Fig. 2 that in the case of potassium, little or no net flux occurs during sustained application of carbamylcholine. If passive transmembrane diffusion of potassium ion is considered the major determinant of the membrane potential in these experiments (assuming relative impermeability of the acetate and calcium ions), any appreciable net flux of potassium should be accompanied by a major change in this potential. As all the examples in Fig. 2 show, however, very little change in the baseline

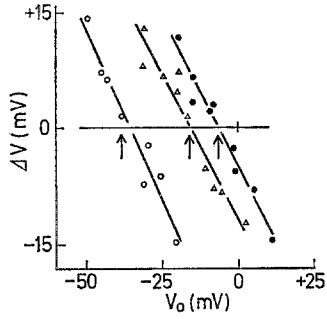

Fig. 6. Changes in the maximum deflection of the membrane potential upon carbamylcholine perfusion when various amounts of constant current are applied to the postjunctional membrane. The symbols V_0 and ΔV are defined in the text. Three sets of experiments are shown in which the following concentrations of potassium are present: 165 mM, filled circles; 100 mM, open triangles; 33 mM, open circles. The concentration of calcium in all cases was 1.8 mM. Each point represents a single determination on a different fiber. The least squares line is shown for each set of experiments. The vertical arrows indicate on the axis corresponding to $\Delta V=0$ the mean values of membrane potential recorded in each medium in the absence of carbamylcholine and applied current. From left to right these correspond to the experiments in 33 mM, 100 mM, and 165 mM potassium

potential occurs throughout the time of drug perfusion. To investigate this point further, a new series of experiments was conducted in which the membrane potential prior to carbamylcholine application was preset at various values above and below the "resting" level by introduction of a constant d–c potential in the current delivery circuit. Application of carbamylcholine under these altered conditions caused a rapid and marked change in membrane potential (ΔV) which was measured and then plotted in each case as a function of the absolute value of the "preset" membrane potential just prior to drug action (V_0). The results of several such experiments in solutions containing 33, 100, and 165 mM potassium are shown in Fig. 6. The vertical arrows indicate on the V_0 axis the mean value of membrane potential in each series measured in the absence of carbamylcholine and any applied current, and in all cases the regression line relating ΔV and V_0 intersects $\Delta V=0$ at very near this value. These results show, therefore, that carbamylcholine action can produce a net flux of potassium when an extrinsic potential is applied but that this net flux approaches zero when the extrinsic potential is removed.

In the absence of any net change in the potassium concentration at the desensitization site, an increase in the degree of occupancy of these sites by calcium during desensitization could result from a gradually increasing concentration of calcium near the sites. If these sites, furthermore, are located

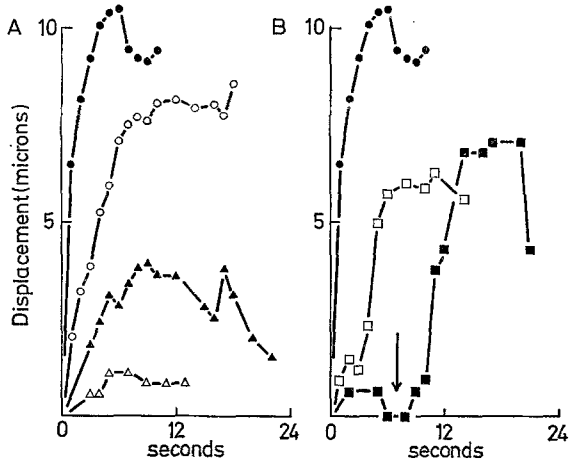

Fig. 7. Mechanical deflections of intracellular capillary micropipettes produced by local contractions in the junctional region of single fibers during carbamylcholine perfusion. The vertical axis shows displacement of the intracellular pipette relative to a marker pipette immediately above and outside the fiber. The initial distance between these pipettes has been subtracted from subsequent displacements to obtain the values shown in the figure. (*A*) The effect of changes in calcium concentration of the extracellular medium on the amplitude of the displacements. The extracellular fluid contains 33 mM potassium and the following amounts of calcium: 10 mM (filled circles, 4 fibers); 1.8 mM (open circles, 4 fibers); 1.0 mM (filled triangles, 2 fibers); and 0.5 mM (open triangles, 1 fiber). (*B*) Contraction responses under other conditions. The response in 10 mM calcium (filled circles) is repeated from (*A*). The open squares show the result obtained in the same medium when the perfusion fluid contains 16.5 mM K_2EDTA in addition to carbamylcholine. The filled squares show the result of a double perfusion experiment which is explained in the text

in the surface region of the fiber or within the membrane itself, an increase in calcium concentration at this locus could occur in association with a net transmembrane movement of calcium from the extracellular medium. One feature of the present experiments which suggests such a net influx of calcium is the occurrence of minute local contractions which are visible under certain conditions in the junctional region of fibers upon perfusion with carbamylcholine. These local "microcontractions" appear within 1 to 2 sec of the onset of perfusion and continue for many seconds thereafter. They are especially evident when the bath solution contains increased amounts of calcium and/or decreased amounts of potassium. A crude method was developed to measure the time course and relative amplitude of these contractions employing cinemicrophotographic measurements as described earlier (*see* Materials and Methods). The results of several such measurements from muscles in 33 mM potassium acetate and various amounts of calcium are shown in Fig. 7*A*. It is evident that the amplitude of the drug-

induced contractions decreases as the concentration of calcium in the extracellular medium is lowered. One possible explanation of this finding is that calcium from the extracellular fluid traverses the postjunctional membrane during carbamylcholine action and initiates contraction of the myofibrils in the immediate junctional region of the fiber. On this basis, the appearance of local contractions during drug action implies a net inward flux of calcium from the external medium and would be evidence suggesting that the concentration of calcium in the surface phase of the cell near the desensitization site is increasing with time. Another possibility, which has been suggested by Marco, Mikiten and Nastuk [11], is that local contractions during carbamylcholine action are caused by calcium already present in the intracellular compartment which is in some way "mobilized" or released by drug action. In this case, the dependence of contraction amplitude on extracellular calcium shown in Fig. 7*A* could be explained on the basis of various degrees of "precalcification" of the intracellular compartment during the control period prior to carbamylcholine application. While not denying this last possibility, the experiments in Fig. 7*B* suggest that some part at least of the local contraction is probably caused by a rapid net influx of calcium from the extracellular medium during the time of drug action. The upper curve (filled circles) in this figure shows the mean amplitude and time course of contractions observed when both the bath and perfusion fluids contain 33 mM potassium acetate and 10 mM calcium. The curve below this (open squares) was obtained from experiments in the same bath medium but in which the perfusion fluid contained 16.5 mM K_2EDTA. The decline in contraction amplitude in the latter case would seem to be a consequence of a decrease in extracellular free calcium ion since in the short time of onset of the contraction it is unlikely that EDTA could penetrate the intracellular compartment or cause depletion of calcium sequestered there. A further indication of the participation of extracellular calcium in the local contraction is provided by the last curve in Fig. 7*B* (filled squares). In this experiment, two perfusion pipettes were used, one containing carbamylcholine in a calcium-free solution and the second containing 10 mM calcium in addition to carbamylcholine. The bath medium as well as the solutions in both pipettes contained 33 mM potassium acetate, but no calcium was present in the bath medium. At zero time in Fig. 7*B*, perfusion was initiated with the Ca-free carbamylcholine pipette; little or no response was observed. At the moment marked by the vertical arrow (about 7 sec after onset of the first perfusion) perfusion with the other pipette containing 10 mM calcium was begun, and at this time a contraction was observed. These results suggest that "precalcification" of the intracellular compartment is

not necessary for the contraction response and that calcium introduced into the extracellular medium can penetrate rapidly in the presence of carbamylcholine to the intracellular sites of contraction. It is possible, therefore, that the local contractions result from a net inward flux of calcium from the external medium during carbamylcholine action and, in this event, it may be reasonable to suppose that the concentration of calcium near or within the membrane phase is increasing during this time.

Discussion

The results of the present experiments as well as those in an earlier publication [9] show that in the presence of a constant amount of calcium, the rate at which the postjunctional membrane becomes desensitized to carbamylcholine increases when the potassium concentration of the extracellular medium is decreased in the range from 165 to 33 mM/liter. The simple view is adopted that this action of potassium is directly related to changes in its activity with respect to that of calcium, and a test of the proposed ion-exchange model is derived on the basis of a mass-action effect of these ions at the desensitization site. However, the fact that changes in the potassium concentration are also accompanied inevitably by changes in transmembrane potential suggests the additional possibility that acceleration of the desensitization process in low-potassium solutions can result in some part from an increase in the transmembrane electric field. One way, for example, in which the transmembrane potential could participate is by causing an increased movement of calcium into or across the postjunctional membrane and permitting thereby a more rapid occupation of desensitization sites by this ion. Some findings in the present study suggest, however, that if a synergistic effect of the membrane electric field such as described above is present, it does not alter greatly the characteristic dependence of desensitization rate on calcium concentration. Since the average value of membrane potential of fibers in 165 mM potassium is only about -7 mV, the slope of the line relating the logarithms of $T_{1/2}$ and calcium concentration in this medium (Fig. 4) provides a measure of the effect of calcium on desensitization rate in the presence of a relatively weak membrane electric field. On the assumption that an increase in the membrane field promotes the movement of calcium to the desensitization site, this slope may be expected to increase somewhat in the experiments performed in 100 and 33 mM potassium, since the average values of potential in these cases are -16 and -38 mV, respectively. Contrary to this, however, no differences among the

slopes of the three lines could be demonstrated at the 95% level of significance. When considered in this way, therefore, any contributions to the determination of desensitization rate of a mechanism of this kind are apparently not detectable under the present conditions. It is possible, nevertheless, that transmembrane potentials greater than those encountered in this study might alter the effect of calcium on desensitization rate.

A simple ion-exchange mechanism is proposed for the antagonistic actions of calcium and potassium on desensitization rate in which both ions combine with a fixed number of anionic sites in a mass-action relationship. An equation relating the fractional change in input conductance and the "mass-action ratio" of the potassium and calcium concentrations in the extracellular fluid was derived with the assumption that an unspecified rate-limiting process controls the appearance of ions from the extracellular phase at the active site (Eq. (10)). Since this relationship was obtained for cases in which the desensitization rate depends on the extracellular ion concentrations, its applicability is limited by the circumstance, indicated in Fig. 4 by the horizontal dashed lines, that in certain concentration ranges the rate of desensitization is not affected by changes in the amount of calcium in the extracellular medium. It is not known what factors control the desensitization process under these conditions. Nastuk and Parsons [13] have suggested recently that a desensitization site is located on the internal surface of the membrane and that the desensitization process can be affected, therefore, by ionized calcium released from bound or sequestered sources in the intracellular compartment. Other intracellular divalent ions such as magnesium may also be able to act on the desensitization site instead of calcium. With these possibilities in mind, therefore, it may not be necessary to abandon the idea of an ion exchange at the desensitization site even though quantitative predictions based on ion concentrations in the extracellular medium may fail at certain low values of calcium.

Since time-dependent changes in ion concentration at the desensitization site are postulated as rate-limiting steps in the ion-exchange mechanism, further experiments were done in an attempt to demonstrate whether, in support of such a possibility, a net flux of either calcium or potassium occurred during the desensitization process. From measurements of changes in membrane potential (Fig. 6), it appears that there is very little net flux of potassium during carbamylcholine action, whereas observations of small local contractions (Fig. 7) show that some net flux of calcium probably does occur. Simple calculations were done now in an effort to estimate upper and lower limits of the relative magnitudes of these fluxes. The greatest amount of change in membrane potential during carbamylcholine action

was found to occur in solutions containing 33 mM potassium, and the average change for all experiments in this medium was 2.7 mV. If this change in potential is attributed entirely to potassium flux and if the capacitance of the muscle fiber membrane is taken as 6×10^{-6} farads cm^{-2} [6], the maximum total net transfer of potassium throughout the desensitization process would be approximately 1.7×10^{-13} moles cm^{-2}. The true net flux of calcium in the contraction experiments (Fig. 7) cannot be determined from the present data, but it may be possible to calculate an approximate lower limit for net calcium influx from available measurements of radioactive calcium exchange in muscle. Bianchi and Shanes [2] have estimated the influx for resting frog muscle to be about 0.1×10^{-12} moles cm^{-2} sec^{-1} in the presence of 1.0 mM calcium. Assuming that this value is near that for muscle fibers in the potassium-rich solutions used in the present study, contractions induced by carbamylcholine must entail a calcium influx greater than this even if the calcium outflux were not to increase during drug action. If a minimum time of 10 sec is allowed for the calcium influx (the contractions last much longer in most cases), the minimum net influx could not be less than 1.0×10^{-12} moles cm^{-2}, a value nearly 6 times greater than that calculated for the maximum net potassium flux. Such calculations provide some basis then for believing that the concentration of calcium near the surface of the fiber in the present experiments changes to a much greater degree during the desensitization process than does that of potassium. Furthermore, if these changes are related to the occupation of active sites, the time course of desensitization would appear to be dictated primarily by the rate of increase of the calcium concentration at these sites. This possibility can be incorporated in the ion-exchange model simply by specifying that $f_2(t)$, the time function which relates the potassium concentration of the extracellular fluid and that present at the desensitization site, is constant. In this event, the time course of the quantity on the ordinate of Fig. 5 would be directly related only to $f_1(t)$, the time course of change in calcium concentration at the desensitization site.

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