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Depolarization of Electroplax Membrane in Calcium-Free Ringer's Solution

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Summary. Electrical stimulation, either cathodal or anodal, of the monocellular electroplax preparation in Ca-free Ringer's solution results in a sustained depolarization which is determined by the amount of current passed through the cell. The membrane potential recovers only when Ca is added again. These changes take place at the innervated side of the electroplax only. This depolarization of the membrane is pH-dependent; it depolarizes more at pH 6.0 than at pH 9.0. The membrane does not depolarize and the action potential is not blocked within an hour in Ca-free solution unless the cell is stimulated. The sustained depolarization is not prevented or reversed by curare, tetracaine, physostigmine, tetrodotoxin, and tetraethylammonium.

After stimulation, the outward K current remains unchanged regardless of whether Ca is present. In contrast, the inward current is dependent on Ca in the outside solution on the innervated membrane; in the absence of Ca following stimulation, the inward K current is decreased.

The depolarization by carbamylcholine is reduced in Ca-free and increased in Mg-free Ringer's solution. In contrast to the depolarization induced by electrical stimulation, these carbamylcholine depolarizations may be reversed by washing with Ca-free or Ca- and Mg-free Ringer's solution.

Ca has several special physicochemical properties by which it is distinguished from other divalent ions (Katchalsky, 1964). Its osmotic coefficient, for example, is remarkably low compared to other divalent ions such as Mg or Ba. This may have some bearing on the biological role of Ca. During the last decade, its special role in the elementary process of muscular contraction has been elucidated (Weber, Herz & Reiss, 1963; Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962). Another important role of Ca has been found in the permeability control of the membranes of adjacent cells (Loewenstein, 1967).

An essential role of Ca in the excitability of nerve and muscle fibers has long been postulated (Brink, 1954). However, its precise mode of action

remains obscure. In recent years it has been suggested that the acetylcholine (AcCh)-receptor protein may control the release of Ca. When AcCh is released on excitation in the excitable membrane and induces conformational changes of the receptor, Ca, possibly bound by electrostatic forces to carboxyl groups of the protein, may be released by allosteric action and subsequently act on the ionophore of the membrane by producing conformational changes of phospholipids or other polyelectrolytes (Nachmansohn, 1969, 1970).

During the last decade, the monocellular electroplax preparation has provided a large amount of information about the properties and function of the AcCh-receptor in the excitable membrane. It thus appears promising to use this preparation for testing the role of Ca in this membrane and to investigate if some clues may be obtained as to its relationship with the AcCh-receptor. Such studies have been initiated and some of the results obtained so far are described in this paper.

Materials and Methods

Single cells from the electric organ of *Electrophorus electricus* were dissected and mounted in a lucite chamber as described previously (Schoffeniels, 1957). The cells were impaled with a glass microelectrode filled with 3 M KCl. The resting potential was recorded with a Varian Recorder, and the action potentials were monitored on a Tektronix storage oscilloscope. The pH was 7, unless noted otherwise. The cells of the stimulation experiments were stimulated with an anodal current at 10 pulses/sec for 75 sec, each pulse lasting 0.1 msec. The voltage applied varied; it was always slightly above threshold for the action potential. The composition of the eel Ringer's solution was in mM: NaCl 160, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, NaH_2PO_4 0.3, Na_2HPO_4 1.2, and glucose 10. In the solution referred to as "Ca-free", $CaCl_2$ was omitted; otherwise it had the same composition. In experiments where KCl and $CaCl_2$ were increased, NaCl was decreased correspondingly.

Results

When the excitable (innervated) membrane of a monocellular electroplax preparation of the electric eel is exposed to Ca-free eel Ringer's solution, the resting and action potentials are not affected up to 1 hr, unless the cell is stimulated frequently. Spontaneous or repetitive firing has never been observed. In the usual Ringer's solution, direct stimulation (cathode at the innervated side) and indirect stimulation (anode at the innervated side) with external silver/silver chloride electrodes at 10 pulses/sec of 0.1 msec duration cause a decrease in the resting potential which is due partly to a summation of the action potentials and partly to the current. The cell repolarizes at a fast rate to its initial resting potential when the stimulation

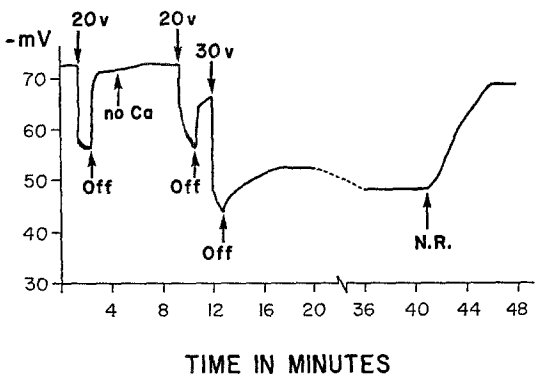

Fig. 1. Stimulation of the electroplax in normal and Ca-free Ringer's solution. Anodal stimulation: 10 pulses/sec of 0.1 msec duration. In Ca-free Ringer's solution, the depolarization persists for 30 min until Ca is added again. The extent of the depolarization depends on the stimulating voltage which is indicated on the Figure. In all figures, the pH was 7.0, temp. 22–25 °C

is discontinued (Bartels, 1968) (Fig. 1). Stimulation at this rate can continue for at least 2 min (it varies from cell to cell) without affecting the action or resting potentials. However, a single, long-lasting pulse will decrease resting and action potentials.

When the cell is stimulated either directly or indirectly in Ca-free Ringer's solution, the cell does not repolarize, or only partially repolarizes, after the stimulation is discontinued. The addition of Ca will repolarize the membrane to its original resting potential (Fig. 1). This sustained depolarization is also observed after conduction is blocked (e.g., by tetracaine or tetrodotoxin); thus, it is not dependent on the action potential. The amount of current passed through the cell determines the extent of the depolarization. In order to observe this sustained depolarization, the current has to be strong enough to depolarize the cell by about 15–20 mV. Otherwise only the recovery rates may be affected. This sustained depolarization occurs only at the excitable membrane; the non-innervated side of the cell maintains the initial resting potential regardless of whether the Ca-free solution is applied to both sides of the cell or to the innervated side alone. Mg cannot replace Ca, as the sustained depolarization is also seen in Ca-free 4 mM Mg-Ringer's solution. However, in the absence of both Ca and Mg, the depolarization owing to stimulation is greater than in Ca-free Ringer's solution; i.e., the absence of Mg increases the effect of Ca-free Ringer's solution. Stimulation in Mg-free Ringer's solution with Ca present does not result in sustained depolarization.

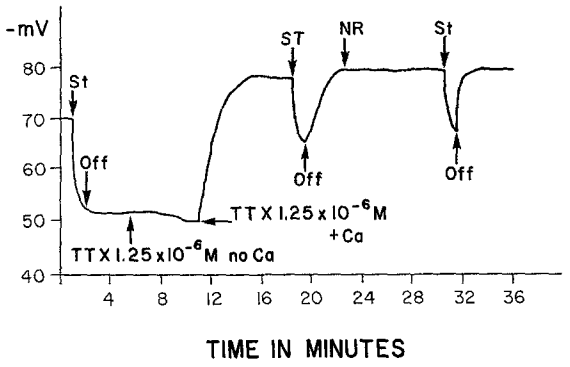

Fig. 2. Stimulation in Ca-free Ringer's solution and recovery on addition of Ca in the presence of tetrodotoxin (TTX). The cell was incubated in Ca-free Ringer's solution for 10 min, prior to the beginning of the experiment. *St.* anodal stimulation, 25 V, 10/sec, 0.1 msec duration. The action potential was blocked after the first stimulation

Inhibitors of AcCh-receptor and of AcCh-esterase, such as curare, tetracaine, eserine and tetraethylammonium at concentrations up to 1×10^{-3} M do not prevent or reverse this depolarization. Tetrodotoxin (TTX) at 10 times the concentration where it blocks the Na conductance of the electroplax membrane (Nakamura, Nakajima & Grundfest, 1965) does not affect the sustained depolarization. As seen in Fig. 2, the addition of 1×10^{-6} M TTX to the Ca-free Ringer's solution does not reverse the depolarization. However, when Ca is added to the TTX solution, the membrane repolarizes immediately. The action potential is blocked after the first stimulation and does not recover. When the sulfhydryl groups of an electroplax cell are reduced by dithiothreitol (DTT) (Karlin & Bartels, 1966) in the absence of Ca and Mg, the sustained depolarization is the same as in the control before reduction; recovery on addition of Ca is also normal.

The extent of the sustained depolarization in Ca-free Ringer's solution is greater at pH 5.8 than at pH 9.2, as demonstrated in Fig. 3. In this experiment all the solutions were prepared with 1.5 mM phosphate buffer. If the phosphate buffer was increased to 50 mM, the depolarization at pH 6 was further increased and recovery on addition of Ca was slow and often incomplete.

When Na is replaced by an equimolar concentration of choline chloride in Ca-free Ringer's solution, the sustained depolarization after stimulation is also observed and seems to be increased. When the Na is replaced by sucrose, the depolarization after stimulation is only very small and identical with or without Ca, and the membrane depolarizes slowly.

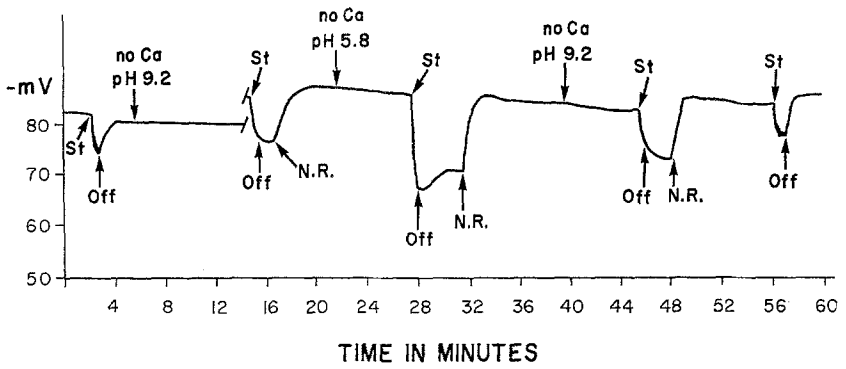

Fig. 3. pH-dependence of stimulation-induced depolarizations in the absence of Ca. The depolarizations at pH 5.8 are larger than those at pH 9.2. *St.* anodal stimulation, 25 V, 10/sec, at 0.1 msec duration. Phosphate buffer 1.5 mM. The interruption of the recording indicates a new impalement

The depolarization caused by an increase of K concentration to 20 mM is the same in the presence and in the absence of Ca (Fig. 4). When the cell is stimulated after equilibration in Ca-free 20 mM KCl solution, a further depolarization occurs only when the voltage applied is strong enough to drive the resting potential below the equilibrium potential of the 20 mM KCl solution (Fig. 4). The same can be seen in the presence of Ca. In the absence of Ca, the resting potential recovers partially when the cell is washed in Ca-free 5 mM KCl solution and recovers completely only when Ca is added again. The last part of the experiment in Fig. 4 shows that the same stimulation in the presence and in the absence of Ca depolarizes the membrane to the same level as in the presence of 20 mM KCl. The experiment seen in Fig. 5 demonstrates that the membrane, after it is depolarized by stimulation in Ca-free Ringer's solution, behaves normally on addition of 50 mM K; in other words, the potential drops to the same level as that observed when the high K solution was added from the initial base-line. It appears as if stimulation in Ca-free Ringer's solution has an effect similar to the raising of the external K concentration. The two depolarizing effects are not additive. It should be noted that only that part of the depolarization which is caused by the addition of 50 mM KCl is completely reversible in Ca-free Ringer's solution. When the KCl concentration is decreased to 1 mM, the membrane [which essentially behaves as a K electrode (Higman, Podleski & Bartels, 1964)] hyperpolarizes in the presence and in the absence of Ca. When the cell is then stimulated, the membrane potential will go to the same level as if it were stimulated in normal Ringer's solution. In other words, this level is the same regardless of whether the cell is hyper-

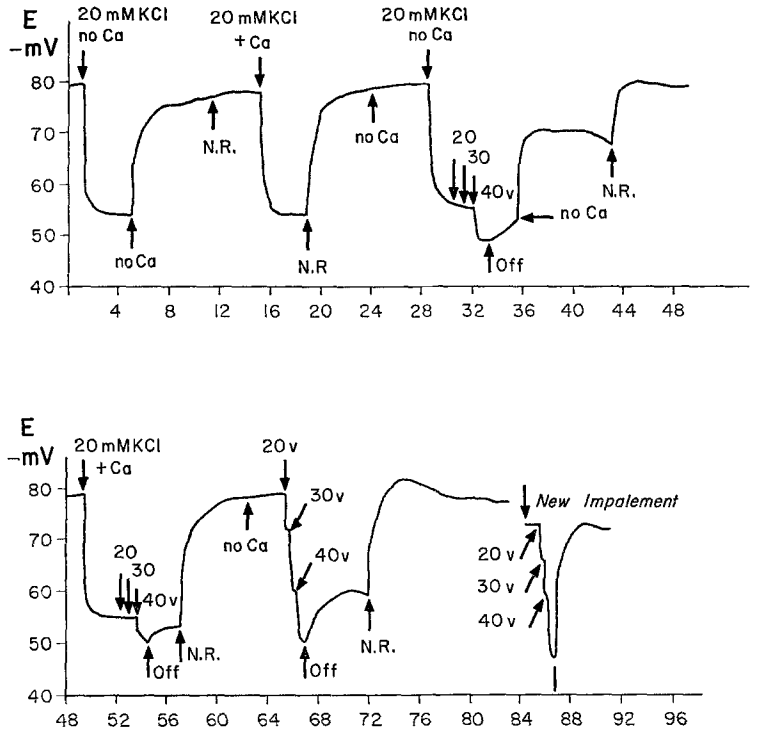

Fig. 4. Anodal stimulation of a cell in Ca-free Ringer's solution exposed to 20 mM KCl. A further depolarization caused by stimulation occurs only if the stimulating voltage is strong enough to drive the potential below the equilibrium potential for 20 mM KCl

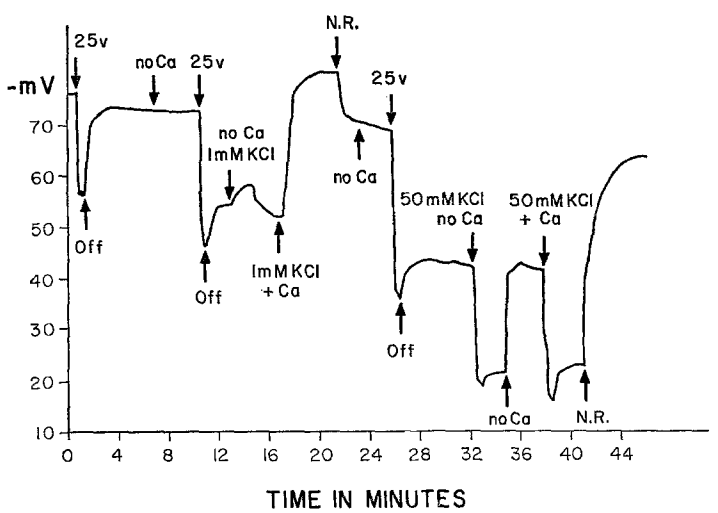

Fig. 5. Effect on the resting potential of a stimulation-depolarized cell by a decreased and increased concentration of KCl in Ca-free solution. Addition of 1 mM KCl causes only a transient 3 mV repolarization, whereas addition of 50 mM KCl drives the potential to the same level as in the presence of Ca (*see* text)

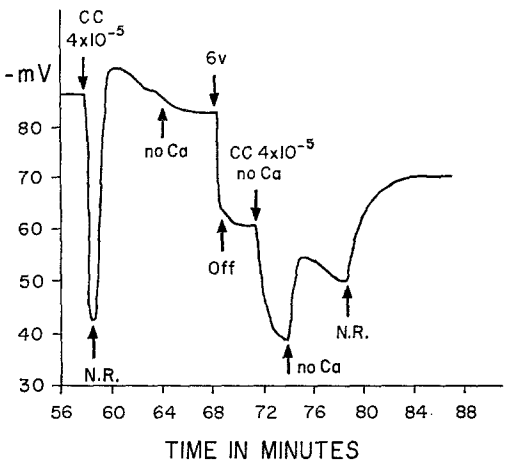

Fig. 6. Same experiment as Fig. 5, except 4×10^{-5} M carbamylcholine was used instead of KCl. After stimulation in Ca-free Ringer's solution, the response to carbamylcholine is reduced to give the same total depolarization as the control in normal Ringer's solution before stimulation

polarized or depolarized by low or high external K concentrations. On the other hand, as can be seen in the first part of the experiment shown in Fig. 5, when the K concentration is decreased to 1 mM after stimulation in the absence of Ca, only a small transient repolarization of 3 mV occurs and the addition of Ca is required to hyperpolarize the cell as expected. When 1 mM KCl in Ca-free solution is changed to 5 mM KCl without Ca after stimulation in Ca-free Ringer's solution, the membrane remains at the same potential.

An experiment similar to that in Fig. 5 is demonstrated in Fig. 6, except that the cell is exposed to 4×10^{-5} M carbamylcholine instead of 50 mM K after stimulation in Ca-free Ringer's solution. The cell also depolarized further, only at a slower rate, which is due to the inhibitory effect which the absence of Ca has on the carbamylcholine depolarizations, as will be described below. When the cell is stimulated in Ca-free Ringer's solution after exposure to carbamylcholine, there is always a small decrease in potential, regardless of how much the membrane would depolarize on stimulation before the addition of carbamylcholine. This is different than with KCl (Fig. 4). The effects of stimulation in high-Ca Ringer's solution can be seen in Fig. 7. An increase in Ca concentration up to 10 mM decreases the recovery rates only. The recovery of the resting potential in 15 and 20 mM $CaCl_2$ is incomplete. After changing to normal Ringer's solution, the membrane depolarizes completely at a fast rate and irreversibly.

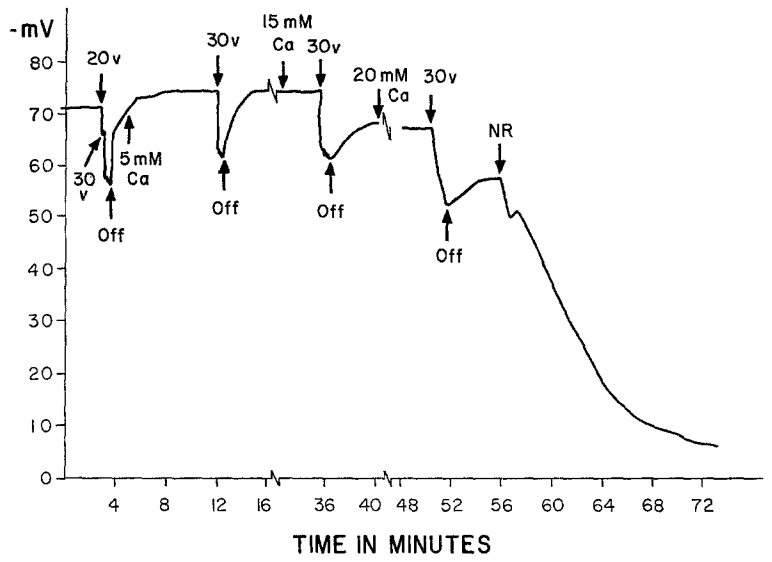

Fig. 7. Stimulation (10/sec, 0.1 msec duration) in increasing concentrations of Ca. In 5 and 10 mM Ca (not shown), the rates of recovery are increased; in 15 and 20 mM Ca, the recovery of the resting potential is only partial. On returning the cell to normal Ringer's solution, the cell depolarizes irreversibly

When the log of the recovery rates after stimulation in low concentrations of Ca (less than 2 mM) is plotted against time, a straight line is consistently obtained. This indicates that the recovery is limited by a pseudo first-order reaction. However, it was not possible to establish an unequivocal dependence of the recovery rates on the Ca concentration because of the wide variation of these rates in different cells (unpublished observations in collaboration with Dr. T. Rosenberry).

Fig. 8 shows the response of 3×10^{-5} M carbamylcholine in the presence of various concentrations of Ca. The depolarization effect of carbamylcholine as a function of Ca concentration (0–20 mM) passes through a maximum at 2 mM.

Fig. 9 shows the tracing of an experiment with depolarizations of the membrane by carbamylcholine 2×10^{-5} M in the absence of Ca, Mg or both. It can be seen that the absence of Ca reduces whereas the absence of Mg increases the carbamylcholine depolarization. In the absence of both Ca and Mg, the effect of the lack of Ca prevails. It should be noted that the membrane potential recovers completely in the absence of Ca and Mg or of Ca alone.

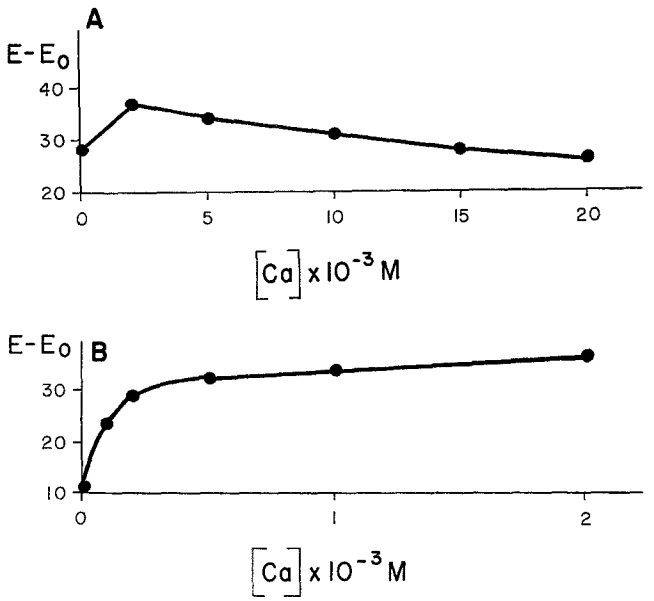

Fig. 8A and B. Response of 3×10^{-5} M carbamylcholine as a function of Ca concentrations. A, Ca-concentrations from 0 to 20 mM: B, from 0 to 2 mM. E_0 initial resting potential, E steady state potential in the presence of 3×10^{-5} M carbamylcholine. The records were obtained on two different cells

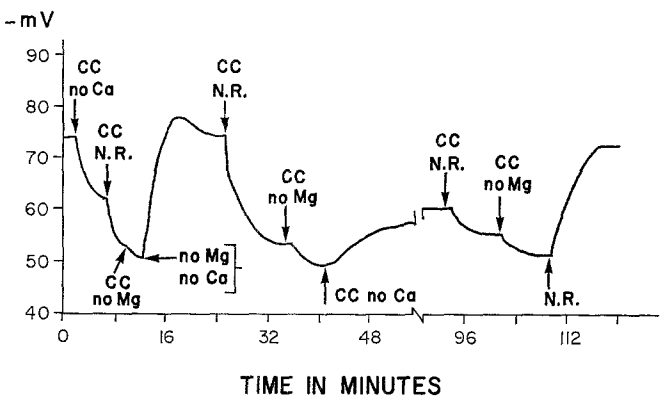

Fig. 9. Changes in carbamylcholine-induced depolarizations in Mg- and Ca-free Ringer's solution. *CC*, 2×10^{-5} M carbamylcholine. There is no difference in the recovery in Ca- and Mg-free as compared to normal eel Ringer's solution

Discussion

The observations reported above may indicate that membrane-bound Ca is released in the excitable membrane by passing a cathodal or anodal current through the electroplax cell. Support for this hypothesis is provided

by the experiment illustrated in Fig. 3. The negatively charged groups in the membrane, to which Ca may be bound, may become neutral at low pH, and this effect may facilitate the release of Ca. This might explain the greater depolarization observed at low pH as compared to high pH. The sustained depolarization cannot be caused by a Ca flux through the cell, since in that case stimulation leading to inward flow of current should have different effects from that leading to outward current. Additional support is the fact that the sustained depolarization is observed only when Ca-free Ringer's solution is applied at the innervated side; the normal Ca concentrations have been maintained at the non-innervated side of the electroplax throughout most of the experiments. The non-innervated side maintains its normal resting potential when the cell is stimulated in the absence of Ca.

Changes in Na permeability do not seem to be the cause of the sustained depolarization, since tetrodotoxin which blocks the inward current produced by Na influx (Nakamura *et al.*, 1965) has no effect. Also, substituting choline chloride for Na does not prevent the sustained depolarization.

Observations on the electroplax by Nakamura *et al.* (1965) and Ruiz *et al.* (1970) suggest that there is a high K permeability at rest and a decrease in K conductance during the action current. The extent of this decrease is determined by the amount of current passed through the membrane and occurs only when the cell is about 30 mV depolarized, at which point a threshold is reached. The authors did not find any effect with tetraethylammonium, tetrodotoxin, or with an increase in external Ca or K concentrations. These observations are similar to the ones reported here. Therefore, one possible explanation for the effect of stimulation in Ca-free Ringer's solution is that the sustained depolarization is a prolonged state of decrease in K conductance, and Ca is apparently involved in the reversal to the resting state. The experiments illustrated in Figs. 4 and 5 with different K concentrations may indicate that the outward flow of K is independent of external Ca concentration, in contrast to the inward flow produced by low K concentrations. This appears to be a kind of rectification.

According to Nachmansohn (1969), excitation releases AcCh from its storage form which then reacts with the receptor protein inducing a conformational change that leads to molecular rearrangements in the membrane. One possible effect may be the release of Ca ions. Once the membrane is depolarized following stimulation in the absence of Ca in the external fluid, the permeability to K is apparently decreased in one direction only, namely for inward current; outward current remains unchanged or may be even increased. Apparently, Ca has to be rebound to some component in the

membrane before repolarization or hyperpolarization may occur, i.e., an increase in inward current and thus the restoration of the resting state of the membrane. Additional evidence is the observation that the depolarizing rates after stimulation are always the same, independent of the external Ca, whereas the repolarization rates in low Ca are slower than in the controls. The fact that the recovery rates follow first-order kinetics may be a further indication of the direct Ca dependence of the recovery process.

The absence of Ca in the outside solution does not have a direct effect on AcCh-binding sites, since potent and specific inhibitors of the AcCh-receptor and -esterase do not influence the sustained depolarization and its recovery. The amount of Ca released seems to determine the extent of depolarization and depends within the limits tested on the amount of current applied. Thus, with subthreshold stimulation in the absence of Ca, the resting potential recovers completely. This may be the explanation why the membrane recovers normally after depolarization by carbamylcholine in the absence of Ca. Carbamylcholine acts at the synaptic junction only, and the amount of Ca released may be smaller than the one released by a strong non-physiological current.

The depolarization induced by carbamylcholine appears to be significantly smaller in the absence of Ca, but stronger in the absence of Mg. No explanation can be offered at the present for these effects, but they further support the assumption of a close interaction between receptor and Ca and a possible regulatory function of the protein and an interaction between these two components of the membrane. This effect of Ca may have a different basis from that regulating the change in K permeability when the cell is electrically stimulated.

The electroplax does not depolarize and conduction is normal in Ca-free Ringer's solution at rest. This absence of any direct action is in contrast to other preparations (Brink, 1954; Davis & Dettbarn, 1962). The excitable membrane may be better protected in this preparation against outside variation of Ca concentration or the molecular organization may have some different characteristics. In this connection, it appears noteworthy that the membrane did depolarize in Ca-free Ringer's solution after the cell underwent several depolarizations or at the end of a long experiment, when some organizational changes may have taken place.

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