Changes in the Membrane Permeability of Frog's Sartorius Muscle Fibers in Ca-Free EDTA Solution

H. KIMIZUKA and K. KOKETSU

From the Research Laboratories, Department of Psychiatry, University of Illinois College of Medicine, Chicago

ABSTRACT The changes in the membrane permeability to sodium, potassium, and chloride ions as well as the changes in the intracellular concentration of these ions were studied on frog sartorius muscles in Ca-free EDTA solution. It was found that the rate constants for potassium and chloride efflux became almost constant within 10 minutes in the absence of external calcium ions, that for potassium increasing to 1.5 to 2 times normal and that for chloride decreasing about one-half. The sodium influx in Ca-free EDTA solution, between 30 and 40 minutes, was about 4 times that in Ringer's solution. The intracellular sodium and potassium contents did not change appreciably but the intracellular chloride content had increased to about 4 times normal after 40 minutes. By applying the constant field theory to these results, it was concluded that (*a*) P_{Cl} did not change appreciably whereas P_K decreased to a level that, in the interval between 10 and 40 minutes, was about one-half normal, (*b*) P_{Na} increased until between 30 and 40 minutes it was about 8 times normal. The low value of the membrane potential between 30 and 40 minutes was explained in terms of the changes in the membrane permeability and the intracellular ion concentrations. The mechanism for membrane depolarization in this solution was briefly discussed.

INTRODUCTION

The membrane potential of frog's skeletal muscle fibers starts to drop within a few seconds after the external calcium ions are removed (Koketsu *et al.*, 1962), and the rate of drop of the potential is enhanced by adding ethylenediaminetetraacetic acid disodium salt (EDTA) to the Ca-free Ringer's solution (*cf.* Koketsu and Noda, 1962). From the standpoint of the ionic theory (Hodgkin, 1951; Frankenhaeuser and Hodgkin, 1957), such a depolarization would be explained in terms of the membrane permeability to ions as well as the intracellular concentration of ions. The present experiment was designed to study the changes in the relative membrane permeability to sodium, potassium,

and chloride ions, and also the changes in the intracellular concentration of these ions. To determine the membrane permeability to these ions the efflux of potassium and chloride ions and the influx of sodium ions were tentatively regarded as passive movements driven by an electrochemical force in accordance with the recent concept of the ionic theory.

METHODS

Pairs of sartorius muscles of *Rana pipiens* were isolated with extreme care. In all experiments one of a pair was used as a control muscle and the other as a test muscle.

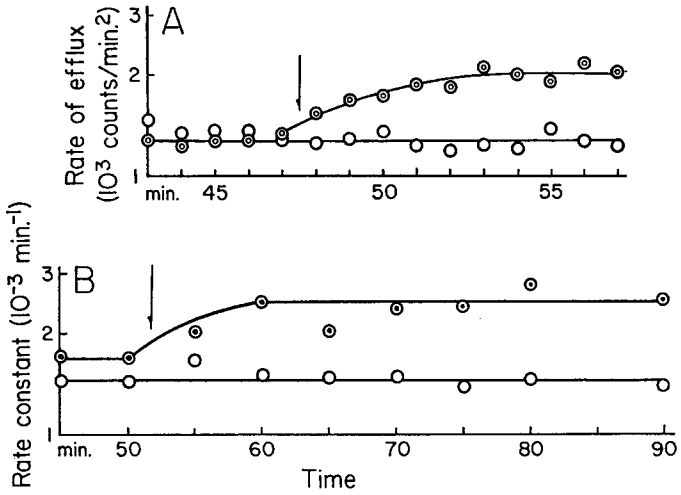

FIGURE 1. Effect of Ca-free EDTA solution on the K^{42} efflux from sartorius muscle. (A) Rate of K^{42} efflux from a pair of muscles. Abscissa indicates the minutes after the washing-out period was started in Ringer's solution. Solution was changed to Ca-free EDTA solution (arrow) for the test muscle (double circles). (B) Rate constant of K^{42} efflux from a pair of muscles. Abscissa indicates the minutes after the washing-out period was started in Ringer's solution. Solution was changed to the test solution (arrow) for the test muscle (dotted circles).

The control and test solutions used were Ringer's solution (112 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, and 2.4 mM $NaHCO_3$; pH, 8.1 ± 0.1) and Ca-free EDTA solution (112 mM NaCl, 2 mM KCl, 4 mM Na_2–EDTA, and 2.4 mM $NaHCO_3$; pH, 6.4 ± 0.1), respectively, unless otherwise stated. Experiments were performed at room temperature (24 ± 1°C) between April and September. Since results were compared between paired muscles of nearly equal weight, no attempt was made to calculate the absolute fluxes nor to apply diffusion corrections.

Efflux of K^{42}

Paired muscles were immersed in Ringer's solution containing K^{42} (prepared from $K^{42}Cl$ obtained from the Oak Ridge National Laboratory) for 5 to 6 hours. The extracellular radioactivity was first washed out in Ringer's solution for 40 minutes.

The K^{42} efflux into the control solution was then observed for a given period during which muscles were transferred every 1 or 5 minutes into a series of beakers containing 2 ml of Ringer's solution, and the same procedure was continued using beakers containing Ca-free EDTA solution for the test series. At the end of the experiment, the muscle was digested in 2 ml of 10 per cent sodium hydroxide solution, which was diluted to 200 ml with distilled water. The radioactivity of each solution and of 2 ml of the digested muscle solution was counted by means of a GM counter. After corrections for radioactive decay were made, the rate of efflux, the desaturation, and the rate constant curves were constructed.

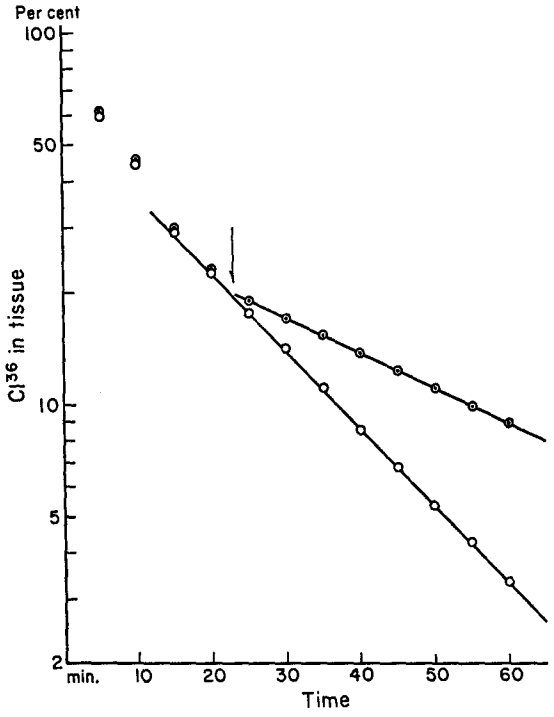

FIGURE 2. Time course of loss of tissue Cl^{36} from a pair of muscles which were soaked in hot K-rich Ringer's solution. Control (open circles) and test (dotted circles) muscles were washed out in Ringer's solution for 20 minutes after which the solution was changed to Ca-free EDTA solution (arrow) for test muscle (20 to 60 minutes).

Efflux of Cl^{36}

HCl^{36} (obtained from the Oak Ridge National Laboratory) was neutralized with sodium hydroxide. To avoid possible contamination from radioactive PO_4 and SO_4 ions, non-radioactive sodium salts of sulfate and phosphate were dissolved in the radioactive chloride solution to which barium chloride solution was added, and subsequently filtered. The excess barium in the filtrate was removed by passing over a column of cation exchange resin.

Muscles were immersed in Ringer's solution or K-rich (30 mM) Ringer's solution

(*cf.* Harris, 1958) containing Cl^{36} for 4 hours after which they were transferred every 5 minutes into a series of beakers (1 ml). In this case, the initial washing-out period in Ringer's solution lasted for 20 or 30 minutes. At the end of each run, muscles were cut into pieces and extracted in Ringer's solution (2 ml) overnight to determine the radioactivity remaining in the muscle. The muscle extract and 0.5 ml of each collection solution were dried on planchets, and the radioactivity was measured with a gas-flow counter.

Influx of Na^{24}

$Na^{24}Cl$ was obtained from the Oak Ridge National Laboratory. Muscles were first exposed to Na^{24}-labeled Ca-free EDTA solution (test muscle) and Ringer's solution

TABLE I

UPTAKE OF Na^{24} IN PAIRS OF MUSCLES

Soaking period	Experiment No.	Intracellular radioactivity of Na^{24} (in test solution/in control solution)
min.		
0–5	1	0.6_8
	2	0.8_4
0–10	1	1.2_1
	2	0.9_9
	3	1.0_6
30–40	1	4.1_2
	2	4.1_3
	3	4.1_2
	Mean	$4.1_2 \pm 0.0_0$

±SE of mean.

(control muscle) for 5 or 10 minutes. The collection into a series of beakers (2 ml) was then continued for 90 minutes, each collection period lasting for 5 minutes. The total Na^{24} entering the cellular phase was roughly determined by extrapolation from the linear part of the desaturation curve (between 40 and 90 minutes) to zero time. To estimate the Na influx after 30 to 40 minutes in Ca-free EDTA solution, muscles were soaked in non-radioactive test and control solutions for 30 minutes before exposure to the radioactive ones. At the end of each collecting period the muscle was digested in 2 ml of sodium hydroxide solution, which was diluted to 50 ml with distilled water. The radioactivity of each solution and that of the muscle solution (2 ml) were measured by means of a GM counter. Corrections for radioactive decay and coincidence loss were made before the desaturation curves were plotted.

Intracellular Na and K

Pairs of muscles were soaked in Ca-free EDTA solution and Ringer's solution for 40 minutes. Muscles were then ashed and the amounts of intracellular sodium and potassium were determined with a flame photometer.

Intracellular Cl

Control and test muscles were first soaked for 40 minutes in Ringer's and Ca-free EDTA solutions containing Cl^{36} of a given specific activity, respectively, and then transferred every 5 minutes into a series of beakers (1 ml) for 40 minutes. During this washing-out procedure, nitrate Ringer (112 mM $NaNO_3$, 2 mM KNO_3, 1.8 mM $CaCl_2$, and 2.4 mM $NaHCO_3$) was used instead of Ringer's solution (*cf.* Adrian, 1961). The

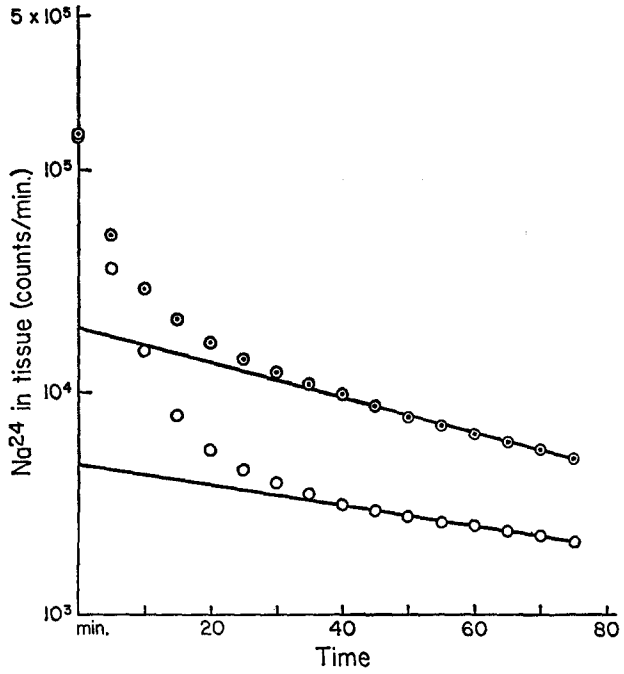

FIGURE 3. Time course of loss of tissue Na^{24} from a pair of muscles. Test (dotted circles) and control (open circles) muscles were first soaked in non-radioactive Ca-free EDTA solution and Ringer's solution for 30 minutes, and then soaked in radioactive Ca-free EDTA solution and Ringer's solution, respectively, for 10 minutes.

total Cl^{36} entering the cellular phase was estimated by extrapolating the linear part (between 20 and 40 minutes) of the desaturation curve to zero time. The ratio of the intercepts at zero time was taken as the ratio of the intracellular chloride concentration of a control to test muscle. This ratio remains unchanged even with allowances made for the correction factor of 25 per cent (Adrian, 1961) used for the estimation of the intracellular Cl^{36}. In the course of the present experiment, it was confirmed that there was no difference between the total radioactivity of Cl^{36} entering the slow phase of the muscle pool during 40 minutes of exposure and that entering during 2 hours of exposure to Cl^{36}-labeled Ringer's solution. This indicates that the isotopic exchange between the intracellular and external chloride ions is almost equilibrated within 40 minutes. Thus, it can be considered that the specific activities of the intracellular chloride ions in a control and in a test muscle exposed to Cl^{36}-labeled Ringer's and Ca-free EDTA solutions, respectively, for 40 minutes are nearly equal. The intra-

cellular chloride concentration was estimated by assuming that the specific activity of the intracellular chloride ions is the same as that of the extracellular chloride ions in Cl^{36}-labeled Ringer's and Ca-free EDTA solutions. The procedure for measuring the radioactivity was the same as that followed to estimate the Cl^{36} efflux.

Membrane Potential

The membrane potential of single muscle fibers was recorded with glass capillary microelectrodes (Ling and Gerard, 1949).

TABLE II

CONTENTS AND INTRACELLULAR CONCENTRATIONS OF NA AND K AFTER 40 MINUTES IN Ca-FREE EDTA RINGER'S AND RINGER'S SOLUTION

	Muscles	Na		K	
		mmole/kg muscle	mmole/kg H_2O	mmole/kg muscle	mmole/kg H_2O
1	Test	27.4	17.1	78.2	116
	Control	27.9	17.9	80.9	120
2	Test	28.8	19.2	77.6	115
	Control	28.4	18.6	78.9	117
3	Test	25.2	13.9	76.6	114
	Control	25.5	14.3	79.3	116
4	Test	24.1	12.2	81.4	121
	Control	24.0	12.1	81.5	121
Mean					
	Test	26.4 ± 1.1	15.6 ± 1.6	78.5 ± 1.0	117 ± 1.0
	Control	26.5 ± 1.0	15.8 ± 1.5	80.2 ± 0.6	119 ± 1.0

± SE of mean.

RESULTS

Efflux of K^{42}

The rate of the K^{42} efflux is shown in record (A) of Fig. 1. In this particular experiment muscles were transferred into a series of beakers at 1 minute intervals. The rate of efflux was practically constant in Ringer's solution. When the solution was changed to Ca-free EDTA solution, it began to increase becoming constant after 5 to 10 minutes. Record (B) of Fig. 1 shows the rate constant of the K^{42} efflux in Ca-free EDTA solution for 40 minutes during which time muscles were transferred into a series of beakers every 5 minutes. The rate constant between 10 and 40 minutes in this solution is 1.5 to 2 times that in Ringer's solution (mean of 6 experiments). The time constant observed in Ringer's solution for the loss of K^{42} is 640 ± 50 minutes (±SE of mean) which agrees well with 680 minutes obtained by Keynes (1954).

Efflux of Cl^{36}

The time constant τ_{Cl} observed in Ringer's solution for the loss of Cl^{36} from muscles soaked either in hot Ringer's or K-rich Ringer's solution ranged between 9 and 21 minutes (7 experiments). The mean value of the time constant was found to be 13 ± 1 minutes (±SE of mean) which is comparable to 11 minutes obtained by Adrian (1961) and 17 minutes obtained by Levi and Ussing (1948). The rate constant of the Cl^{36} efflux began to decrease becoming almost constant after 5 minutes in Ca-free EDTA solution, and was about one-half of that observed in Ringer's solution. Such a change in the relative rate constant was observed regardless of the kind of radioactive soaking solution used (*cf.* Method). An example of the rate of loss of Cl^{36} from the tissue is shown in Fig. 2.

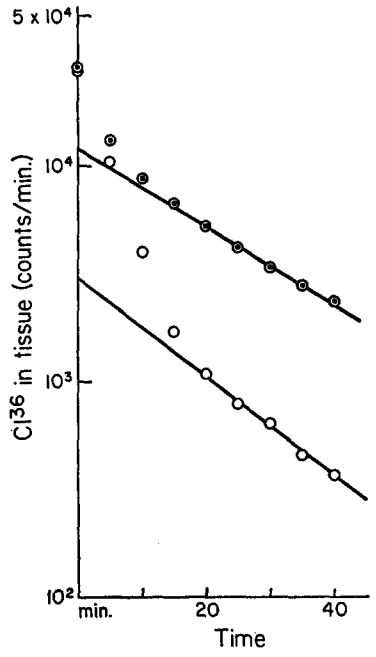

FIGURE 4. Time course of loss of tissue Cl^{36} from a pair of muscles. Test (dotted circles) and control (open circles) muscles were soaked in radioactive Ca-free EDTA solution and Ringer's solution for 40 minutes, respectively. Washout was into nitrate Ringer's solution.

Influx of Na^{24}

As seen in Table I the ratio of the Na^{24} influx during 0 to 5 minutes in Ca-free EDTA solution to that in Ringer's solution is slightly reduced. It can also be seen that the ratio during 0 to 10 minutes is practically unchanged. An increased influx, however, can be seen between 30 and 40 minutes, the total uptake of Na^{24} being about 4 times greater than that in Ringer's solution during this period (Fig. 3).

Intracellular Na and K

The intracellular concentrations of sodium and potassium ions were calculated according to the method proposed by Boyle *et al.* (1941). It was assumed that the ratio of the intracellular to the extracellular space remained constant in the test solution since no appreciable changes in the wet weights were observed between the test and control muscles after 40 minutes' soaking. As seen in Table II, there are no appreciable changes in the intracellular potassium and sodium concentrations.

TABLE III

INTRACELLULAR CONCENTRATION OF CHLORIDE IONS IN MUSCLE FIBER AFTER 40 MINUTES IN Ca-FREE EDTA SOLUTION

No. experiment	Intracellular radioactivity of Cl^{36} In test muscle fiber / In control muscle fiber	Intracellular chloride concentration In control muscle	In test muscle
		mmole/kg H_2O	*mmole/kg H_2O*
1	4.0_0	2.9_4	$11._8$
2	4.4_7	4.0_2	$18._3$
3	3.5_4	5.4_6	$19._3$
4	4.3_5	3.2_6	$14._8$
5	4.3_5	2.1_8	9.4_0
6	5.8_0	2.1_0	$12._2$
7	2.2_2	8.3_0	$19._7$
Mean	$4.1_1 \pm 0.4_0$	$4.0_4 \pm 0.7$	$15._6 \pm 1.1_3$

±SE of mean.

Intracellular Cl

The total radioactivity of Cl^{36} which entered into the intracellular space of the test muscle is about 4 times greater than that of the control muscle (Fig. 4 and Table III). As seen in Table III, the intracellular chloride concentration in Ringer's solution ranged between 2 and 8 mmole/kg H_2O, the mean value being 4 mmole/kg H_2O. These figures were obtained without a diffusion correction (*cf.* Adrian, 1961), and are similar to those obtained by Adrian (1961).

Membrane Potential

In general the membrane potential of individual fibers dropped rapidly to 40 to 50 mv within 10 to 20 minutes in Ca-free EDTA solution. The value of the potential then dropped gradually and became constant (25 to 30 mv) upon further immersion (Koketsu and Noda, 1962) (Fig. 5).

DISCUSSION

Assuming that the specific activity of the ion fluxes across the membrane from the radioactive phase to the non-radioactive one is the same as that of the radioactive phase, the radioactive ion fluxes can be written as,

$$J^* = -K_e C_i^* \tag{1}$$

$$J'^* = K_i C_o^* \tag{2}$$

where J denotes the efflux per unit area of cell surface, J', the influx, C_i, the intracellular ion concentration, C_o, the extracellular ion concentration,

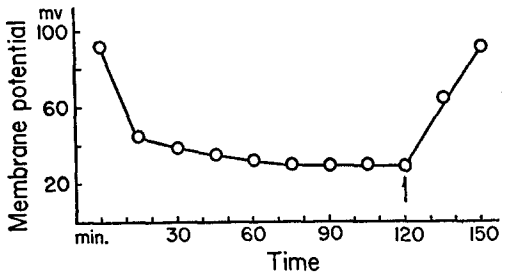

FIGURE 5. Time course of changes in membrane potential of a muscle in Ca-free EDTA solution. Solution was changed to Ringer's solution (arrow) after 120 minutes. Each point indicates the mean value of membrane potential measured from 6 different fibers at random.

K_e and K_i, the rate constants for the efflux and the influx, respectively, and the asterisk refers to the radioactivity.

According to the constant field theory (Goldman, 1943), the rate constants are expressed as follows:—

$$K_e = P\frac{ZFV}{RT}\frac{1}{1 - \exp(-ZFV/RT)} \tag{3}$$

$$K_i = P\frac{ZFV}{RT}\frac{1}{\exp(ZFV/RT) - 1} \tag{4}$$

where P denotes the membrane permeability to this particular ion, R, the gas constant, T, the absolute temperature, Z, the valency of the ion, F, Faraday's constant and V, the membrane potential.

In writing equations (3) and (4), it has been assumed that the inward and outward movements of an ion species are independent of each other, and that in applying these equations to determine the membrane permeabilities to ions, the effluxes of potassium and chloride ions and the influx of sodium ions are due only to their own electrochemical forces.

It is well known that the ion efflux corresponding to the slow phase of the desaturation curve obeys the equation,

$$J^* = -kS^* \tag{5}$$

where S^* denotes the total intracellular radioactivity and k, the rate constant for the loss of radioactivity from the cell.

When using muscles, we may write,

$$k = \frac{1}{\tau} = K_e\overline{(a/v)}f \tag{6}$$

where $\overline{(a/v)}$ denotes the mean of the surface area divided by the volume of fibers in the muscle, f, the correction factor as a function of the geometry of the fibers in a muscle, and τ, the time constant for the loss of radioactivity from a muscle. The value of f may be regarded as being in the order of unity in view of the fact that the time constants for the losses of K^{42} (or Na^{24}) from a single fiber and from a muscle are of the same order (Hodgkin and Horowicz, 1959 *a*). Assuming that the values of $\overline{(a/v)}$'s as well as the values of f's are almost equal between a pair of muscles, the relative change in the membrane permeability can be determined by the relative change in the rate constant and the value of the membrane potential. In the present study, the relative changes in the membrane permeability were estimated on the basis of the foregoing assumption.

According to Hodgkin and Horowicz (1959 *b*), $P_K:P_{Na}:P_{Cl}$ is 1:0.01:2 and $P_K = 1 - 2 \times 10^{-6}$ cm/sec. in normal Ringer's solution; these values were calculated from the observed membrane potential value. It has been pointed out that there is a discrepancy between the calculated ion flux and the ion flux observed with the tracer method (Hodgkin and Horowicz, 1959 *b*; Adrian, 1961). However, the tracer method was used in the present studies since it was more reasonable to use the ion flux data directly for estimating the permeability.

By comparing Equations 3 and 6 we obtain the expression for the relative permeability P_{Cl}/P_K, provided the geometrical factor is a constant:

$$P_{Cl}/P_K = (\tau_K/\tau_{Cl}) \exp (FV/RT) \tag{7}$$

If the values $\tau_K = 640$ min., $\tau_{Cl} = 13$ min., and $V = -90$ mv are inserted into Equation 7, $P_{Cl}/P_K = 1.4$ which agrees fairly well with the value obtained by Hodgkin and Horowicz (1959 *b*).

By comparing Equations 1 and 3 with Equations 2 and 4, we may derive,

$$P_{Na}/P_K = -(J'^*_{Na}C_{iK}/J^*_K C_{oNa}) \exp (FV/RT) \tag{8}$$

It has been recently reported that the sodium ion influx is 3.5 ± 0.4 pmole/cm² sec. and the potassium efflux is 8.8 ± 1.2 pmole/cm² sec. (±SE of mean) in normal Ringer's solution (Hodgkin and Horowicz, 1959 *a*). By inserting these values into Equation 8, the value $P_{Na}/P_K = 0.01$ is obtained. Thus, we obtain the relative permeability $P_K:P_{Na}:P_{Cl} = 1:0.01:1.4$ in Ringer's solution.

The equation for the change in the relative permeability is obtained from Equations 3 and 6 as follows:—

$$\frac{P_1}{P_2} = \frac{k_1 V_2 \exp(-ZFV_1/RT) - 1}{k_2 V_1 \exp(-ZFV_2/RT) - 1} \tag{9}$$

where $k_1(k_2)$, $P_1(P_2)$, and $V_1(V_2)$ indicate the rate constant of efflux, the membrane permeability, and the membrane potential in the control (test) solution, respectively. The relative change in the membrane permeability can be evaluated from the relative change in the rate constant and the membrane potential according to Equation 9. Since the rate constants of the potassium and chloride effluxes were constant between 10 and 40 minutes in Ca-free EDTA solution and since the change in the membrane potential with time was slow, an approximate calculation was tentatively applied to estimate the changes in the membrane permeability; the value of the membrane potential during this period was taken as 40 mv. It was found that (*a*) the rate constant of the potassium efflux was increased 1.5 to 2 times indicating that the potassium permeability was about 40 to 50 per cent of the normal value, whereas (*b*) that of the chloride efflux was reduced to about one-half indicating that the chloride permeability was not appreciably changed.

According to the ionic theory, the depolarization occurring in Ca-free EDTA solution would be associated with a large increase in the membrane permeability to sodium ions. In the present studies, the approximate changes in the sodium permeability in this solution were estimated by measuring the Na^{24} uptake. By making assumptions similar to those applied in deriving Equation 9, the following equation is obtained,

$$\frac{P_1}{P_2} = \frac{J_1'^* V_2 \exp(ZFV_1/RT) - 1}{J_2'^* V_1 \exp(ZFV_2/RT) - 1} \tag{10}$$

The value $J_2'^*/J_1'^*$ can be roughly estimated from the ratio of the total uptake of radioactivity of the test muscle to that of the control muscle (*cf.* Method). From calculations in which the value of the membrane potential, between 30 and 40 minutes in Ca-free EDTA solution, was taken as 35 mv, and the Na^{24} uptake was increased 4 times, the membrane permeability to sodium ions was found to be increased approximately 8 times.

It was found in the present experiment that P_{Na} increased 8 times, while

P_K decreased to about one-half and P_{Cl} remained unchanged. If $P_K:P_{Na}:P_{Cl}$ in normal Ringer's solution is taken as 1:0.01:1.4, the value of $P_K:P_{Na}:P_{Cl}$ between 30 and 40 minutes in Ca-free EDTA solution will be 1:0.16:2.8.

The intracellular contents of sodium and potassium ions showed no appreciable change whereas the intracellular chloride concentration increased about 4 times; *i.e.*, to 15 mmole/kg H_2O after 40 minutes in Ca-free EDTA solution. The values of Na_o, Na_i, K_o, K_i, Cl_o, and Cl_i in Ca-free EDTA solution, therefore, would be 122.4 mM, 15.6 mM, 2 mM, 117 mM, 114 mM, and 15 mM, respectively.

On the basis of the foregoing facts, the value of the membrane potential can be calculated according to Goldman's equation,

$$V = \frac{RT}{F} \ln \frac{K_o + (P_{Na}/P_K)Na_o + (P_{Cl}/P_K)Cl_i}{K_i + (P_{Na}/P_K)Na_i + (P_{Cl}/P_K)Cl_o} \qquad (11)$$

The calculated membrane potential is about −49 mv, deviating from the observed value (*ca.* −35 to −30 mv). If the intracellular chloride concentrations in Ringer's solution and in Ca-free EDTA solution were taken as 3.1 mM (*cf.* Adrian, 1961) and 12 mM (4 times increase), respectively, the calculated membrane potential would be −52 mv. It must, however, be kept in mind that since the changes in P_{Na} were estimated from the influx of Na^{24} in an unsteady state and since no corrections were made for back diffusion, this estimate would be an approximate one. Furthermore, the presence of any exchange diffusion (Ussing, 1949) was not considered in the present experiment. It may be also pointed out that a slight modification of the value of the membrane permeability to sodium, potassium, and chloride ions in Ringer's solution results in a fairly good agreement between the calculated and the observed membrane potential.

If the drop of the membrane potential in Ca-free EDTA solution is actually a function of the changes in the relative membrane permeability to different ions, it is conceivable that depolarization is related to a large selective increase in the membrane permeability to sodium ions. It is likely that such a selective increase is caused by the removal of membrane calcium in view of the evidence that membrane calcium is dissociated in Ca-free EDTA solution (Koketsu and Miyamoto, 1961; Kimizuka and Koketsu, 1962). In other instances, it has been suggested that the removal of calcium from the membrane is responsible for the sudden increase in the membrane permeability to sodium ions during the production of the action potential (Brink, 1954; Frankenhaeuser and Hodgkin, 1957; Shanes, 1958; Tobias, 1958; Koketsu, 1961). It may be of interest to note that both the production of the action potential and the membrane depolarization involve the removal of calcium ions as well as the selective increase in the membrane permeability to sodium ions.

The mechanism of depolarization during the initial 10 minutes in Ca-free EDTA solution would be difficult to explain in terms of the constant field theory inasmuch as the muscle was clearly in an unsteady state. If the increase in the permeability to sodium ions were responsible for membrane depolarization, the sodium influx would be expected to increase appreciably during this period since the value of the membrane potential was markedly low. Contrary to expectations the present experimental results show that there is no appreciable increase in the sodium influx. This would imply that the increase in sodium permeability follows depolarization rather than precedes it. The alteration of the membrane property caused by the removal of membrane calcium with EDTA may also be responsible for the increase in the membrane permeability.

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