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Effect of External Sodium and Calcium on Calcium Efflux in Frog Striated Muscle

Carlo Caputo and Pura Bolaños

Centro de Biofísica y Bioquímica, Instituto Venezolano
de Investigaciones Científicas (IVIC), Apartado 1827, Caracas 101, Venezuela

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Summary. The effect of media with different ionic composition on calcium efflux from the dorsal head of semitendinosus muscles of *Rana pipiens* was studied. The reduction in the fractional loss of ^{45}Ca, when going from normal Ringer's solution to an ONa–OCa medium, was 60%. Withdrawal of only Na or Ca from the external medium also caused a significant drop in the fractional loss (33% and 34%, respectively). The effect of different concentrations of Ca (studied in the absence of the external Na) was also studied. It was found that a linear function could describe the relationship between the calcium-dependent calcium efflux and the external calcium concentration. These results indicate that calcium efflux from frog muscle fibers consists of three major components: one that is dependent on the presence of calcium in the external medium, one that is dependent on the presence of sodium in the external medium, and one that persists in the absence of these two cations.

Since the work of Bianchi and Shanes (1960) and of Cosmos and Harris (1961), it is known that calcium efflux from frog skeletal muscles depends on the ionic composition of the external medium. More recently, considerable work has been done on the dependence of calcium efflux on external sodium and calcium in several preparations, such as heart muscle (Reuter & Seitz, 1968), squid giant axon (Blaustein & Hodgkin, 1969; Baker, 1970; Baker & Glitsch, 1973; Baker & McNaughton, 1976; DiPolo, 1973*a*, 1974, 1977), and the giant muscle fiber of the barnacle (Blaustein, Shield & Santiago, 1971; DiPolo & Caputo, 1977).

In view of the importance of calcium transport in vertebrate skeletal muscle, the experiments herein reported were carried out to obtain a more detailed description of the dependence of calcium efflux on external sodium and calcium in frog skeletal muscle. The results obtained are in fair agreement with those reported for other preparations, suggesting that a common mechanism is probably responsible for this type of calcium transport in different tissues.

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Table 1. Composition of solutions

Solution	NaCl	Choline Cl	$CaCl_2$	$MgCl_2$	KCl
Normal Ringer's	115	–	1.8	–	2.5
ONa	–	115	1.8	–	2.5
OCa	115	–	–	2	2.5
ONa – OCa	–	115	–	2	2.5

All solutions were buffered with 2.5 mM Tris pH 7.2.

Materials and Methods

The dorsal head of semitendinosus muscle from *Rana pipiens* was used in all experiments. Usually both the left and right semitendinosus muscles were dissected from two frogs. The dorsal head of each muscle was separated under the microscope taking care to reduce to a minimum the amount of tendon without causing damage to the fibers. Each of the four muscles was tied to a glass rod and immersed in the loading solution. The average muscle wet wt was about 50 mg.

Calcium efflux was measured following the loss of $^{45}Ca^{++}$ from muscles previously loaded with this isotope. The loading solution was prepared by adding solid $^{45}CaCl_2$ to the normal Ringer's solution. The specific activity of the loading solution ranged from 1.8 μCi/ml to 18 μCi/ml. The four muscles of each batch were soaked in the loading solution for two or three hours and washed in a series of tubes, each containing 8 ml of inactive solution. The tubes were rotated by a motor in order to insure stirring. The collection periods were normally of 20 min, except for the first three periods after changes of solution, which were of 10 min. 2 of the 8 ml of each collection sample were pipetted in a counting vial to which 3 ml of Instagel (Packard) were added. The vials were counted in a liquid scintillation spectrometer (Tri-carb, Packard) set for ^{45}Ca counting. At the end of the experiments each muscle was transferred to a vial containing 1 ml of Soluene (Packard).

From the amount of ^{45}Ca leaving the muscle for each collection period and the amount of ^{45}Ca remaining in the muscle at the end of the experiment, the total amount of isotope present in a muscle at the begining of the experiment and at any collection period could be calculated. Efflux is hence presented as the fraction of total ^{45}Ca lost per min. This fraction could be obtained either calculating it for each collection period or by using the slope of the washout curve. In the latter case, the mean fraction over a long period is obtained.

The intracellular space of these muscles was measured in several control experiments with ^{14}C inulin. The composition of the experimental solutions used is given in Table 1. All experiments were carried out at room temperature (19–22 °C).

Results

Curtis (1970), working with single muscle fibers, has shown that calcium efflux in frog muscle can be described by three time constants of about 18, 300, and 882 min respectively, indicating the multicompartmen-

tal nature of these fibers with respect to the calcium distribution. He pointed out that the compartment with intermediate time constant was the one of greater physiological interest, since it alone showed increased calcium turnover when the fibers were stimulated. More recently Kirby, Lindley and Picker, (1975) have found that calcium in frog ELDIV (Extensor longus digiti IV) muscle exchanged with 4 time constants of 21.5 sec, 2.7, 32 and 1244 min.

In the present work we have found that calcium washout from whole muscles after the first hour can be described reasonably well by a single exponential curve, with a time constant of 165 min. Figure 1*a* shows the fractional radioactivity present in muscles previously soaked for 3 hr in a ^{45}Ca loading solution, as function of time; it can be seen that all the points obtained after the first hour of washout can be fitted by a straight line, whose slope is maintained constant for over three hours.

The same result was obtained in other groups of muscles whose washout was followed for more than 4 hr. With a more prolonged washout, however, a slower component became appreciable, after periods which varied with different batches of frogs.

When the line of Fig. 1*a* is extrapolated to zero time and subtracted from the experimental points obtained during the first hour of washout, a second straight line with time constant of 18 min is obtained. The second fast exponential curve describes the ^{45}Ca washout from a compartment which most probably has reached isotopic equilibrium with the loading solution, since its washout time constant is much smaller than the loading time. The first exponential probably combines the three components with small time constants obtained by Kirby *et al.* (1975). This point, however, is of no immediate concern with the results of the present work, since the effect of external ions on the Ca efflux to be described was always tested after at least a 2-hr washout period, and at this time only the slower exponential component was evident.

The amount of ^{45}Ca associated with the slower exponential is only a fraction of the total (labeled and unlabeled) intracellular calcium. Furthermore, the time constant of this slow exponential is greater than the loading time. Thus, isotopic equilibrium with all intracellular calcium cannot be assumed. On the other hand, the time constant of this slower exponential is 165 min which agrees well with the results of Bianchi and Shanes (1960) and Bianchi (1961) obtained with whole sartorious muscles, and less well with the values of the intermediate exponential (time constant 300 min) obtained by Curtis (1970) on single muscle fibers. In several experiments, with longer washout periods, a third and still slower

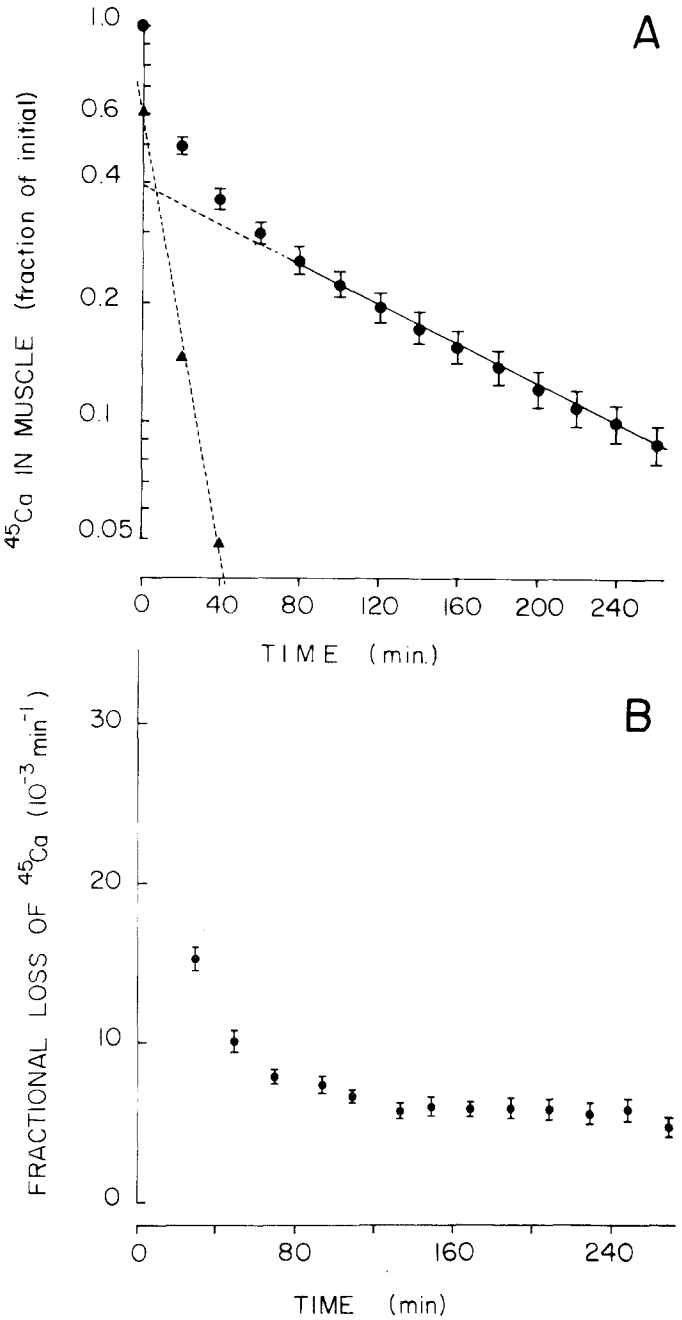

Fig. 1. (*a*): ^{45}Ca in muscle as a function of time for the first 4 hr of washout. The points represent the mean ±SEM for 4 muscles. The triangles were obtained by substracting from the experimental points the corresponding values obtained extrapolating the slow exponential line to zero. (*b*): Efflux of ^{45}Ca expressed as rate coefficient. This shows the same results of *a*. Note that after the first 90 min the rate coefficient remains almost constant. In other cases (*see* Fig. 4) a sustained decline with time was observed

exponential did appear after 5 hr. The appearance of this third component put some limitation on the experimental procedure that could be followed, in the sense that all the solution changes had to be completed before the appearance of this component.

As mentioned above, however, it is the compartment associated with Curtis's second exponential that is the more important physiologically and the one on which we will focus our attention.

Figure 1*b* shows the rate coefficient of calcium efflux from the same muscles as in Fig. 1*a*, obtained from the fractional loss of ^{45}Ca per unit time. The value of this rate coefficient, as expected, agrees well with the value obtained considering the slope of the slow exponential of Fig. 1*a*.

The rate coefficient could be considered a measure of the Ca efflux, since Ca efflux = rate coefficient × Ca in the exchangeable compartment. Since uncertainties exist about the Ca concentration in the exchangeable compartment, no attempt is made to present the data in terms of calcium efflux directly.

Effect of External Sodium and Calcium on Calcium Efflux

Figure 2 shows the effect of applying external sodium- and calcium-free solution on calcium efflux. This figure shows a plot of the rate coefficient for two muscles, one maintained in normal Ringer's solution throughout the experiment, and the other exposed for 90 min to a ONa − OCA solution after the first 120 min of washout in normal Ringer's. It can be seen that the rate coefficient of calcium loss is appreciably diminished, this effect being reversible when normal conditions are restored after a transient increase in the rate coefficient. Table 2 summarizes the results obtained in similar experiments with 14 muscles. The mean value ±SEM of the fractional rate coefficient obtained in normal Ringer's solution was $10.3 \pm 3.4 \times 10^{-3} \cdot \text{min}^{-1}$. In ONa − OCa solution, it was reduced to 4.1 ± 1.7, and on returning to normal Ringer's it was 10.6 ± 5. In this table are shown the rate coefficients of ^{45}Ca obtained in normal Ringer's in ONa − OCa Ringer's and again in normal Ringer's. The results obtained can be thus expressed as test/((control$_1$ + control$_2$)/2). For comparative purposes they are also expressed as test/control$_1$, since in several other experiments to be reported later it was not always possible to return the muscle to the original solution. The means of these ratios are, respectively, 0.40 and 0.39. Therefore, assuming that the calcium concentration in the exchangeable compartment is not

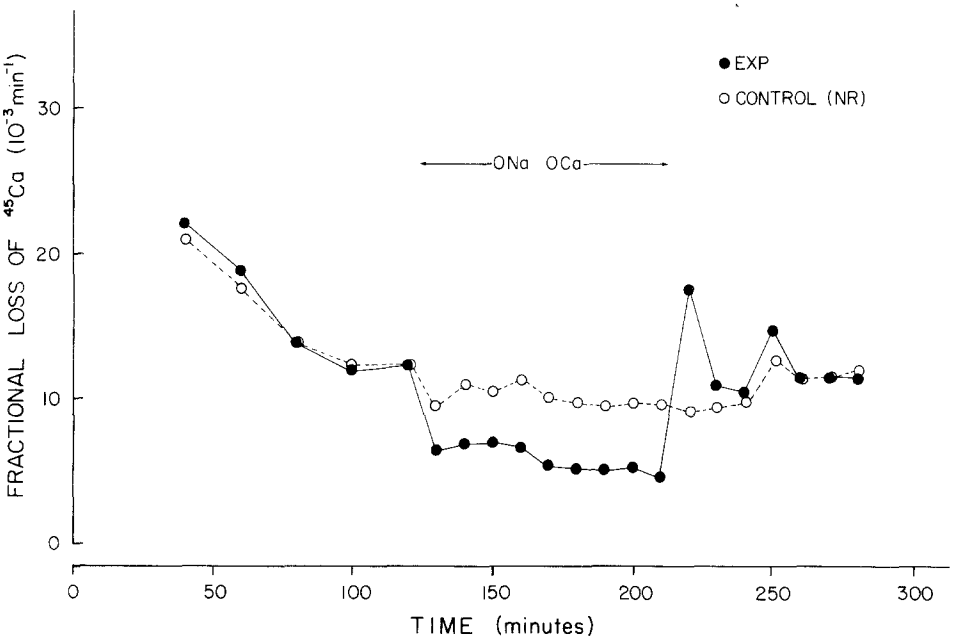

Fig. 2. Effect of ONa−OCa medium on the rate coefficient of Calcium efflux from muscle. Shown is the result of an experiment in which paired muscles were used, one as control (open circles) and the other exposed to the ONa−OCa medium

Table 2. Effect of external sodium and calcium on calcium efflux

Muscle reference	Fractional loss of calcium (min^{-1}) × 10^{-3}			Ratio	
	1 NR	2 ONaOCa	3 NR	2/1	2:(1+3)/2
49	12.5	4.7	12.0	0.38	0.38
50	10.7	4.3	12.0	0.40	0.38
51	10.4	3.9	11.2	0.38	0.36
52	10.8	4.3	11.4	0.40	0.39
53	12.9	5.5	17.0	0.43	0.37
54	14.5	4.9	17.3	0.34	0.31
55	11.1	4.9	12.4	0.44	0.42
56	12.7	5.4	14.2	0.43	0.40
61	5.4	1.5	2.5	0.28	0.38
62	5.3	1.6	2.8	0.30	0.40
63	5.7	2.2	4.2	0.39	0.44
64	5.5	1.9	4.1	0.35	0.40
194	13.5	6.4	12.9	0.47	0.48
196	13.1	6.5	14.3	0.50	0.47
	10.29 ±	4.14 ±	10.59 ±	0.39 ±	0.40 ±
SD	3.37	1.71	5.1	0.06	0.04

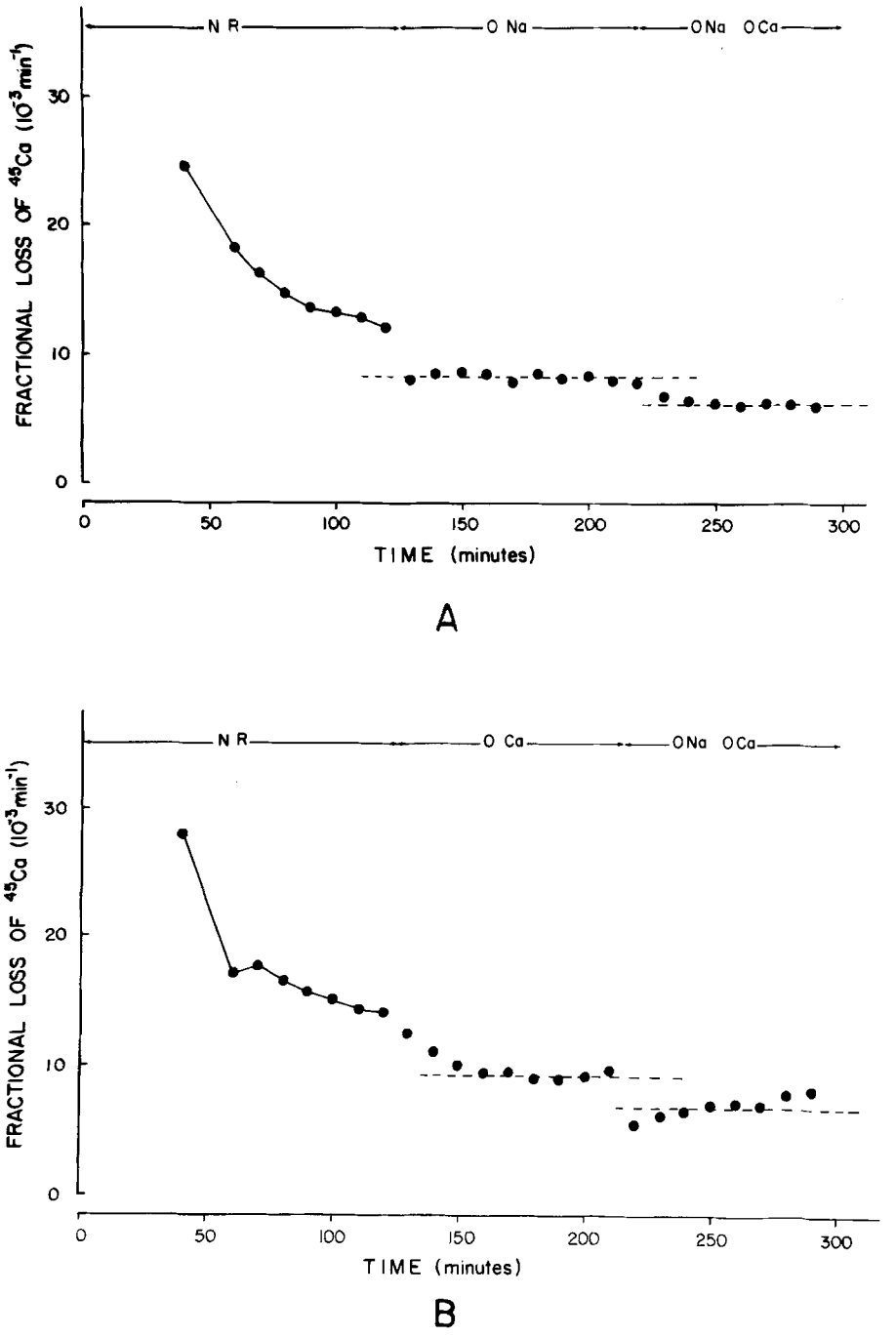

Fig. 3. (*a*): Effect of ONa and then ONa−OCa media on the rate coefficient of calcium efflux from 1 muscle. Table 3 shows the results of several similar experiments. (*b*): Effect of OCa and then ONa−OCa media on the rate coefficient of calcium efflux from 4 muscles. *See* Table 3 for similar experiments

changed during the experiment, about 60% of the total calcium efflux appears to be dependent on external sodium and calcium.

Experiments were also carried out in which either the external sodium or the external calcium was removed before removing the other ion. The results are shown in Fig. 3*a* and *b* and Table 3. In Fig. 3*a*, calcium washout was started in normal Ringer's, (NR), and after 115 min the muscle was exposed first to an ONa solution and then an ONa and OCa solution. The experiment of Fig. 3*b* was similar, except that the exposure to the OCa solution preceded the one to the ONa, OCa

Table 3. Effect of OCa, ONa, and OCa – ONa media on calcium efflux

	Fractional loss of calcium (min^{-1}) × 10^{-3}					Ratio		
	1 NR	2 OCa	3 ONa	4 OCaONa	5 NR	2/1	3/1	4/1
9	7.5	4.6	–	1.5	4.6	0.61	–	0.20
10	6.5	4.2	–	1.3	4.7	0.65	–	0.20
11	5.5	2.7	–	1.5	5.0	0.49	–	0.27
12	5.0	4.7	–	1.3	5.5	0.94	–	0.26
209	17.0	11.1	–	–	–	0.65	–	–
210	14.9	9.5	–	5.5	–	0.64	–	0.37
211	13.1	7.6	–	–	–	0.58	–	–
212	16.0	11.6	–	8.4	–	0.73	–	0.53
217	15.3	9.6	–	6.4	–	0.63	–	0.42
218	14.3	10.4	–	6.8	–	0.73	–	0.48
219	15.3	10.4	–	4.1	–	0.68	–	0.27
220	13.2	7.3	–	5.5	–	0.55	–	0.42
57	7.3	–	3.7	1.7	4.2	–	0.51	0.23
58	9.8	–	6.6	2.3	4.0	–	0.67	0.23
59	8.1	–	5.4	1.7	3.6	–	0.67	0.21
60	7.7	–	4.5	2.2	3.9	–	0.58	0.29
197	12.3	–	10.3	–	–	–	0.84	–
198	13.0	–	8.6	–	–	–	0.66	–
201	15.5	–	7.7	–	–	–	0.50	–
202	15.5	–	8.6	–	–	–	0.55	–
203	15.2	–	8.8	–	15.9	–	0.58	–
204	15.2	–	9.4	–	14.3	–	0.62	–
205	15.5	–	11.2	–	–	–	0.72	–
206	16.3	–	11.9	–	–	–	0.73	–
207	15.9	–	12.1	–	–	–	0.76	–
208	17.0	–	10.4	–	–	–	0.63	–
213	12.4	–	10.3	5.7	–	–	0.83	0.46
214	13.1	–	7.9	6.7	–	–	0.60	0.51
215	15.7	–	11.2	–	–	–	0.71	–
216	13.0	–	10.5	–	–	–	0.81	–

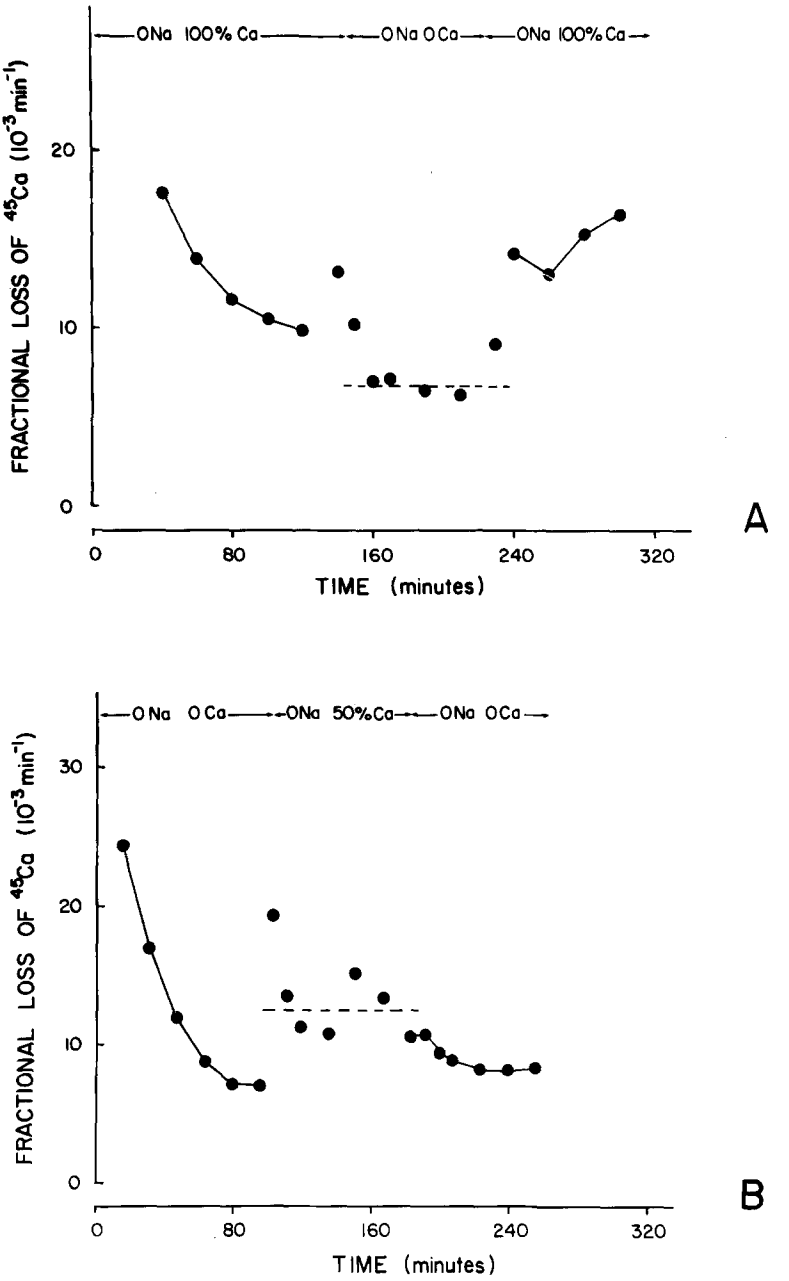

Fig. 4. (*a*): Effect of external calcium on the calcium efflux. The experiment was carried out in the absence of external sodium and the efflux measurement was started in a solution containing full Ca (1.8 mM). The reduction in the rate coefficient after removal of the external Ca, and its increase after restoring it, are shown. (*b*): Effect of external calcium on calcium efflux in the absence of external sodium. The experiment is similar to that shown in *a*, except that the Ca efflux was measured starting in a medium containing OCa

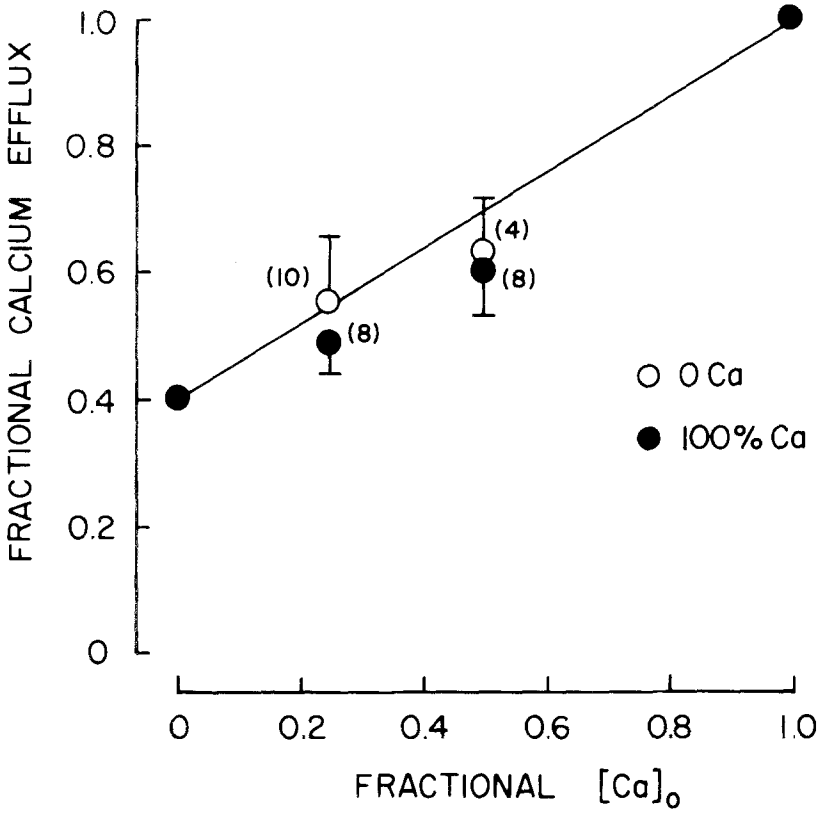

Fig. 5. Effect of external calcium on the calcium efflux. The points were obtained in the absence of external sodium and represent the mean ±SDM. from different experiments similar to those shown in Fig. 4*a* and *b*. The abscissa shows the ratio of the rate coefficients obtained with low Ca and full Ca. The ordinate represents the external Ca concentration. The black dots represent the results of experiments similar to that of Fig. 4*a*, in which the muscles were initially bathed in a solution containing 100% Ca, and later exposed to solution with decreased Ca concentration. The open circles represent results of experiments similar to that of Fig. 4*b*, in which the initial solution was one containing OCa

medium. As shown in Table 3, the fractional loss of Ca obtained in the ONa, OCa solution is the same regardless of whether the muscles were first exposed to the ONa or the OCa medium. Yet the drop in the rate coefficients appears to be greater in the muscles exposed either to ONa or OCa before exposure to the ONa, OCa medium, than in those exposed directly to the latter solution, as could be inferred comparing the mean reduction to 39% shown in Table 2 and that to 33% shown in the last column of Table 3. This difference, however, is not statistically significant since P for these two means has a value near 0.05.

Sodium and Calcium-Dependent Fraction of the Calcium Efflux

Another series of experiments was carried out to study the effect of changing external concentrations of calcium on the calcium efflux in the absence of external sodium. These experiments were carried out in two different manners. In one, shown in Fig. 4*a*, the muscles were exposed to a solution containing 100% Ca and ONa, then Ca was reduced to different values (OCa for the case shown in this figure), and then the solution with full Ca was restored again. In the second type of experiments the muscles were first exposed to a solution containing ONa – OCa and then to solutions with increasing Ca concentration followed by return to ONa – OCa (*see* Fig. 4*b*).

The results of several experiments similar to those of Fig. 4 are shown in Fig. 5, which demonstrate the effect of external Ca on Ca efflux. The ratio between the rate coefficient for Ca efflux obtained with the test Ca concentration and that obtained with the initial Ca concentration (black dots 100% Ca, empty circles OCa) is plotted *vs.* the test Ca concentration, expressed as the fraction of the normal Ca concentration (1.8 mM). It can be seen that the points obtained with the different concentrations of Ca fall on a straight line.

Discussion

The results reported here show that about two thirds of the calcium efflux from frog striated muscle depend on the presence of external sodium and calcium. They confirm and extend the work of previous authors working with this preparation (Bianchi & Shanes, 1960; Cosmos & Harris, 1961; Watson & Winegrad, 1973). These results can be interpreted in terms of sodium and calcium activated fractions of the calcium efflux. A mechanism of this type has been shown to operate in several different preparations, thus suggesting that this exchange system is probably a very general one. From the results presented here, it appears that the Ca efflux in frog muscle consists of three components, one sensitive to external Ca, one sensitive to external Na, and one that persists in the absence of these two cations in the external medium. Each of these components represents roughly one third of the total Ca efflux.

There is evidence that calcium uptake is increased after exposure to sodium-free media in both frog skeletal muscle (Cosmos & Harris, 1961) and in dialyzed barnacle muscle fibers (DiPolo, 1973*b*). The fact that the

drop in the Ca efflux occurs early after the solution change could be explained by assuming new steady-state conditions under which the extra Ca entering the fibers is either pumped out or sequestered in special regions inside the fiber (e.g., mitochondria and sarcoplasmic reticulum).

In view of this, the possibility should be considered according to which the effect of sodium-free solution on the Ca efflux could be due in part to isotope dilution. However, when external Na is reduced in the absence of external Ca, no isotope dilution should occur, yet the effect on Ca efflux is about as large as that in the presence of external Ca.

Therefore, it seems more likely that the effect of reducing the external Na on the Ca efflux is due to inhibition of the Na−Ca exchange.

Regarding the effect of Ca-free solutions on the Ca efflux, it is known that Ca deprivation causes depolarization of the muscle fiber membrane (Curtis, 1963; Argibay & Caputo, 1971). This depolarization is probably caused by changes in the relative membrane conductance to different ions.

The drop in the Ca efflux observed in the OCa medium could be attributed to such a mechanism, assuming that Ca permeability decreases as a result of membrane depolarization. This explanation is, however, unlikely since the fraction of the Ca efflux sensitive to external Ca is reduced when the concentration of this ion is reduced from its normal value (1.8 mM) to one tenth of it. From the work of Curtis (1963) it is known that the depolarizing effect of lowered Ca solutions occurs with concentrations lower than 100 μM. Furthermore, in the present experiments Ca was substituted by Mg, in which case one would expect the depolarization caused by Ca-free media to be reduced.

If one does not consider the possibility of isotope dilution, and assumes that the effect of abolishing the external sodium and calcium are due to interference with a Na−Ca exchange mechanism, it appears that this mechanism in frog skeletal muscle could account for only 70% of the Ca efflux. It would therefore be of interest to characterize the residual compartment of the Ca efflux since it appears to be too large to be a passive flux.

It would also be of interest to know the membrane potential dependence of this calcium extrusion mechanism and the source of the energy necessary for its functioning.

Regarding the energy source for this extrusion mechanism, Baker & Glitsch (1973); Baker and McNaughton (1976), and DiPolo (1974, 1977), working with squid axons, and DiPolo and Caputo (1977), working with barnacle muscle fibers, have found that a sizeable amount of this

exchange mechanism is dependent on the presence of ATP. One point of interest is whether the proper functioning of this exchange system is relevant to the contractile activity of frog muscle fibers. For instance, it is known that in barnacle muscle fibers a decrease of external sodium concentrations causes such an increase in the calcium influx that the fiber develops contractures (DiPolo, 1973*b*). Glitsch, Reuter and Scholz (1970) have found that contractile activity in frog heart muscle is enhanced under the same conditions, probably due to an increased myoplasmic-free calcium concentration. In the case of frog skeletal muscle, no such effects are evident (C. Caputo, *unpublished observations*), probably due to a greater calcium buffering capacity of the sarcoplasmic reticulum in this preparation.

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References

Argibay, J., Caputo, C. 1971. Effect of calcium deprivation on frog skeletal muscles at different pH values. *J. Membrane Biol.* **4**:68

Baker, P.F. 1970. Sodium-calcium exchange across the nerve cell membrane. *In:* Calcium and Cellular Function. A.W. Cuthbert, editor. p. 96. MacMillan, London

Baker, P.F. 1972. Transport and metabolism of calcion ions in nerve. *Prog. Biophys. Mol. Biol.* **24**:177

Baker, P.F., Glitsch, H.G. 1973. Does metabolic energy participate directly in the Na^+ dependent extrusion of Ca^{2+} ions from squid giant axons? *J. Physiol. (London)* **233**:44

Baker, P.F., McNaughton, P.A. 1976. Kinetics and energetics of calcium efflux from intact squid giant axons. *J. Physiol. (London)* **259**:103

Bianchi, C.P. 1961. The effect of caffeine on radiocalcium movement in frog sartorius. *J. Gen. Physiol.* **44**:485

Bianchi, C.P., Shanes, A.M. 1960. The effect of the ionic milieu on the emergence of radiocalcium from tendon and from sartorius muscle. *J. Cell. Comp. Physiol.* **56**:67

Blaustein, M.P., Hodgkin, A.L. 1969. The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol. (London)* **200**:497

Blaustein, M.P., Shield, C.E., Santiago, F. 1971. Sodium ions and calcium fluxes in giant barnacle muscle fibers. *Biophys. Soc. 15th. Ann. Meet. Abstrs.* 141*a*

Cosmos, E., Harris, E.J. 1961. *In vitro* studies of the gain and exchange of calcium from frog skeletal muscle. *J. Gen. Physiol.* **44**:1121

Curtis, B.A. 1963. Some effects of Ca-free choline-Ringer solution on frog skeletal muscle. *J. Physiol. (London)* **166**:75

Curtis, B.A. 1970. Calcium efflux from frog striated muscle fibers. *J. Gen. Physiol.* **55**:243

DiPolo, R. 1973*a*. Calcium efflux from internally dialyzed squid giant axons. *J. Gen. Physiol.* **62**:575

DiPolo, R. 1973*b*. Sodium dependent calcium efflux in dialyzed barnacle muscle fibers. *Biochim. Biophys. Acta* **209**:25

DiPolo, R. 1974. Effect of ATP on the calcium efflux in dialyzed squid giant axons. *J. Gen. Physiol.* **64**:503

DiPolo, R. 1977. Characterization of the ATP-dependent calcium efflux in dialyzed squid giant axons. *J. Gen. Physiol.* **69**:795

DiPolo, R., Caputo, C. 1977. The effect of ATP on calcium efflux in dialyzed barnacle muscle fibers. *Biochim. Biophys. Acta* **470**:389

Glitsch, H.G., Reuter, H., Scholz, H. 1970. The effect of the internal sodium concentration on calcium fluxes in isolated guinea-pig auricles. *J. Physiol. (London)* **209**:25

Kirby, A.C., Lindley, B.D., Picken, J.R. 1975. Calcium content and exchange in frog skeletal muscle. *J. Physiol. (London)* **253**:37

Reuter, H., Seitz, N. 1968. The dependence of calcium efflux from cardiac muscle on temperature and external ion composition. *J. Physiol. (London)* **195**:451

Watson, P.T., Winegrad, S. 1973. A possible sodium-calcium exchange in skeletal muscle. *Fed. Proc.* **32**:374 *(Abstr.)*