In vitro Studies of the Gain and Exchange of Calcium in Frog Skeletal Muscle

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ABSTRACT (1) The Ca^{++}, Na^{+}, and K^{+} contents of frog sartorius muscles were found analytically after exposure to various media including some containing labeled Ca^{++}. (2) During storage in media with 100 to 120 mM Na^{+} and 1 mM Ca^{++} both Na^{+} and Ca^{++} are gained while K^{+} is lost; there is a high correlation between Na^{+} and Ca^{++} gains. (3) When Ca^{++} gain occurs from a solution containing labeled Ca^{++} there is also some exchange of the original Ca^{++} with the labeled Ca^{++}. The amount exchanged is considerably less (*e.g.* 50 per cent) than the total amount of labeled Ca^{++} taken up by the tissue. (4) When the external Na^{+} concentration is reduced to 30 mM the amount of labeled Ca^{++} taken up is increased. Part of the increase is attributable to a greater net gain and part to a greater degree of exchange. (5) It is pointed out that muscles which have been loaded *in vitro* with labeled Ca^{++} will not provide a valid measure of the exchangeability of the normal Ca^{++} content present at the time of dissection. (6) Comparison is made between results obtained using Sr^{89} and Ca^{45} as labels for the Ca^{++}. Little, if any, difference is perceptible.

INTRODUCTION

Movement of the Ca^{++} of frog skeletal muscle has been studied both *in vitro* by Cosmos (1), Harris (2), Gilbert and Fenn (3), and Bianchi and Shanes (4), and *in vivo* by Cosmos (1) using Ca^{45} as tracer. However, in some of the work cited (Bianchi and Shanes, 4) analyses for total Ca^{++} are not reported and the observed uptakes of labeled Ca^{++} may there be due to any combination of net Ca^{++} gain and exchange of the original tissue Ca^{++}.

The high Ca^{++} content of muscles which have been kept isolated overnight (Harris, 2) and the increase of Ca^{++} content after isolation noted by Gilbert and Fenn (3) made it of interest to investigate further the changes in Ca^{++} content *in vitro*. The effects of making changes in the ionic composition of the solution were examined both by tracer methods and by analysis.

METHODS

Kinetic Experiments When it is desired to follow the Ca^{++} exchange or the gain of Ca^{++} from the solution by taking successive readings of tissue radioactivity during exposure to a solution having a proportion of labeled Ca^{++}, the use of Ca^{45} is unfavorable. This is because the isotope emits weak beta particles so the emission reaching the counter tube derives from the surface of the tissue. Besides making the readings measures of surface Ca^{++} rather than of total Ca^{++}, the values obtained are subject to errors on account of the variable thickness of the water film on the tissue. For this reason, it was preferred to use Sr^{89} as tracer along with chemical Ca^{++}. This is not the same as using Sr^{89} along with chemical Sr^{++} because the amount of Sr^{++} in the "carrier-free" tracer preparation is extremely small. Although we do not claim that the ratio Sr^{89}/Ca^{++} taken up by the tissue is exactly equal to the ratio holding in the solution, results using either Sr^{89} or Ca^{45} do agree within the "intermuscle scatter" as will be seen later.

The tracer uptake by frog sartorii was followed over periods of 2 to 5 hrs. at 16–22°C. The Ringer's solution used for the kinetic experiments contained 100 or 120 Na^+, 3.5 K^+, 1.0 Ca^{++}, 75.5 or 95.5 Cl^-, 30 mM HCO_3^-; it was agitated with a 95 per cent O_2 + 5 per cent CO_2 mixture. Variations of solution composition are mentioned in the text. To the solution was added a trace of Sr^{89} preparation to provide a count rate of about 20,000 per min. per ml solution.

Readings of tissue radioactivity were made under a Geiger tube. The tissue was given a timed rinse of 5 sec. in inactive solution before each reading.

To find the factor relating tissue radioactivity to the amount of Sr^{89} (used as a measure of Ca^{++}) taken up from the solution the tissue was finally ashed at 500°C. in a platinum planchette and the radioactivity assayed. The result was compared with the measured activity of a dried down portion of the soak solution whose Ca^{++} content was known.

Terminal Experiments In some experiments muscles were soaked in a mixture containing a proportion of Ca^{45} along with chemical Ca^{++}. After ashing, the radioactivity of the ash and that of a dried down 0.1 ml portion of solution were compared. The weights of ash and salts were nearly equal, so self-absorption losses would be similar in each sample.

Ca^{++} Analysis The ashed muscle after assay of radioactivity was dissolved in dilute nitric acid and made up to 2 ml. Of this a 0.5 ml portion was used after further dilution for Na^+ and K^+ analysis with an E.E.L. flame photometer. A 1 ml portion was used for Ca^{++} analysis with a flame photometer consisting of a Hilger monochromator set to 4226 Å, Beckman oxyhydrogen atomizer-burner, and photomultiplier tube. If the solution was used directly it so lowered the flame temperature that the general emission became less and a negative reading of photocurrent was obtained. This was obviated by admixing one-third part by volume of propanol with the solution and with all the Ca^{++} standard solutions. The Ca^{++} content of the 1 ml used for analysis was about 0.1 μmol. Accuracy is estimated as ±5 per cent. Appropriate corrections were made for Na^+ and K^+ interference on the Ca^{++} reading.

RESULTS

Kinetic Experiments Uptake of tracer from the bathing solution commences rapidly and continues with diminishing rate. The form of the uptake-time curve can be seen in the first part of Fig. 3. It is convenient to plot the uptake against the root of the time of immersion because this allows the continuous increase at later times to be shown. It seems likely that the Ca^{++} diffuses into the muscle from a rapidly established surface deposit because after

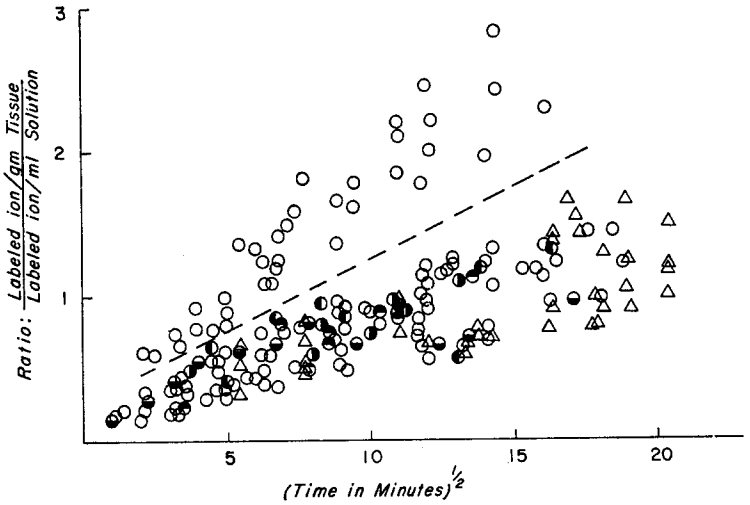

FIGURE 1. The ratios, labeled ion per gram tissue/labeled ion per milliliter solution, determined using either Sr^{89} or Ca^{45} as tracer at 18–22°C. Various external Ca^{++} concentrations were used. ○, 1 mM Ca^{++} using Sr^{89}, ◓, 2 mM Ca^{++} using Sr^{89}, ◑, 4 mM Ca^{++} using Sr^{89}, ◐, 0.8 mM Ca^{++} using Ca^{45}, △, 2 mM Ca^{++} using Ca^{45}, ◒, 2 mM Ca^{++} using Ca^{45} (taken from Gilbert and Fenn (3), Table IV). Points below the dashed line refer to freshly dissected muscles bathed in a medium containing 3 or 12 mM K^+. Points above the dashed line refer to muscles which were either (*a*) stored 3 to 4 hrs. before use; (*b*) immersed in a solution without added K^+ ions; (*c*) immersed in a solution with 1 μg/ml strophanthin (and 3 mM K^+).

the initial gain the process runs linearly with the root of the time (Fig. 3). Eggleton, Eggleton, and Hill (5) have made use of the square root plot in treating the time course of diffusion into muscle.

The uptake experiments made with either Sr^{89} or Ca^{45} (terminal experiments) as tracer can be divided into three groups. The first of these was made on freshly dissected muscles and with solutions containing 3.5 or 12 mM K^+ and 100 or 120 mM Na^+. The results are plotted in Fig. 1 and fall below the dashed line. By expressing the uptakes of labeled ion as the ratio labeled ion per gm tissue/labeled ion per ml solution, the values fall within the range of a

factor of two. There is no trend to permit differentiation between points obtained in media with 1, 2, or 4 mM Ca^{++} present. Also the results of our own and of Gilbert and Fenn's (3) Ca^{45} experiments are distributed within the scatter of the Sr^{89} experiments. The latter fact justifies the use of Sr^{89} as tracer in this work in which the range of Ca^{++} concentration used was limited.

The second group of labeled ion uptake results was obtained from muscles from which K^+ loss had been promoted. The points fall above the dashed line of Fig. 1. Some are from runs made in K^+-free solution, some with media containing 1 μg/ml strophanthin, and some using muscles which had been stored for some hours in a solution with low K^+ concentration before use. All these treatments lead to loss of cellular K^+. Curves relating K^+ loss to time

TABLE I

CATION ANALYSES OF FROG SKELETAL MUSCLE (±s.d.)

Treatment	Contents in μmol/gm wet tissue			No. of analyses
	Ca^{++}	K^+	Na^+	
Fresh (Jan.-Mar.)	1.45±0.03	95.5±6.6	24.9±2.1	19
Stored 2–5 hrs. in Ringer's solution	1.95±0.25	83.0±1.2	33.6±5.4	12
Stored 6 hrs. in K^+-free Ringer's solution at 4°C.	3.7±1.20	60.3±6.7	43.3±2.0	6
Stored 16 hrs. in K^+-free Ringer's solution at 4°C.	6.2±1.30	53.4±9.5	51.9±5.4	12
Stored overnight in K^+-free Ringer's solution at 4°C. and placed in 10 mM K^+ Ringer's solution for 5 hrs.	2.8±0.30	90.0±5.0	33.0±5.0	4

have been given for K^+-free and strophanthin media by Edwards and Harris (6, Fig. 6) and for 2 mM K^+ solution by Harris (2, Fig. 7). When previous storage in a K^+-free solution was compared with storage in a 3.5 mM K^+ medium using paired muscles, it was found that the K^+-free treatment led to greater Ca^{++} uptake than did the use of normal solution. (At 2 hrs the former had 1.94 μmol labeled Ca^{++}/gm and the latter only 1.44 μmol labeled Ca^{++}/gm.)

Analytical Experiments Since it appeared that an increased Ca^{++} uptake was likely to be associated with a loss of cellular K^+ we examined the cation analysis of muscles after various periods of storage. Table I, line 1, shows that freshly dissected muscles have the lowest Ca^{++} and Na^+ contents associated with the highest K^+ content. Storage for 2 to 5 hrs. in the ordinary Ringer's solution (3.5 mM K^+; 100 mM Na^+) leads to loss of some 12 μmol K^+/gm with

gain of 0.5 μmol Ca^{++}/gm and gain of Na^+ (line 2). Storage in K^+-free solution accentuates the changes which increase with time (lines 3 and 4), changes which seem to be partially reversed with increased K^+ in the bathing solution (line 5). Since the Na^+ and Ca^{++} contents of stored muscles were widely scattered, it seemed worthwhile to plot the Na^+ contents of muscles against the respective Ca^{++} contents (Fig. 2). A high degree of correlation pertains; the correlatlon coefficient is 0.95 for 42 pairs of values.

Ca^{++} Exchange and Net Gain The results show that a net gain of Ca^{++} is to be expected when muscle K^+ is lost with Na^+ gain. Under such conditions the labeled Ca^{++} uptake will include both net gain and such exchange of the original Ca^{++} as takes place. It is clear that any net gain is of the same specific

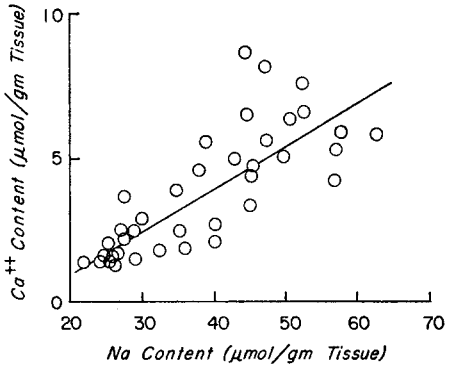

FIGURE 2. Points obtained by analysis for Ca^{++} and Na^+ of freshly dissected muscles and of muscles stored for various times before analysis. The regression line is Ca^{++} = 2.97 + 0.146 (Na^+—34.4); this was calculated from forty-two pairs of values not all of which are shown.

activity as that in the outside medium; the additional amount of labeled ion not accounted for by the net gain of Ca^{++} can only have exchanged with the initial Ca^{++} of the muscle. In order to find the exchange it will be necessary to subtract the net gain from the total uptake of labeled Ca^{++}. Accordingly a number of tracer experiments were made in which analyses were also made of control muscles taken at the time of dissection. When both sartorii were to be in test solution, the semitendinosus was taken as control. Previous experiments done in this laboratory by one of us (E. J. H.) have shown that the Na^+ and K^+ contents and K^+ exchanges in this latter muscle were comparable to those in the sartorius. The Ca^{++} analyses indicate that the same relationship holds true with this ion. The difference Ca^{++}/gm tissue in the muscle after immersion (Table II, column 2) minus Ca^{++}/gm tissue in the control muscle (Table II, column 3) provides a figure for the net Ca^{++} gain (Table II, column 4). This gain was deducted from the total Ca^{45} uptake deduced from

the tracer measurement (Table II, column 5). This procedure is subject to the errors of two analyses and the intermuscle Ca^{++} variability but the figures do show that commonly about half the tracer uptake is ascribable to net gain. Hence only half the tracer uptake represents exchange. If one wishes to know the effect of changed conditions on the *exchange* of the original Ca^{++}, it is necessary to measure total Ca^{++} changes as well.

TABLE II

A. Net Ca^{++} gains and labeled Ca^{++} uptakes during storage in solution with 1 mM Ca^{++}/liter, 100 mM Na^+/liter, and 3.5 mM K^+/liter at 20°C.*

Time of exposure, *hrs.* (1)	Total Ca^{++} contents of test muscle (2)	Total Ca^{++} contents of fresh muscle (3)	Net Ca^{++} gain (4)	Total labeled Ca^{++} uptake (5)	Ca^{++} exchanged (6)	
					Actual (5)-(4)	As per cent‡ $\frac{(5)-(4)}{(3)}$
			Tissue, *μmol/gm*			
2	1.88	1.72	0.16	0.30	0.14	8
2.5	1.94	1.48	0.46	0.72	0.26	17.5
3.3	2.09	2.02§	0.07	0.47	0.40	20
6.4	1.88	1.48	0.40	0.82	0.42	28.5

B. Net Ca^{++} gains and labeled Ca^{++} (Ca^{45}) uptakes during storage in a mixture with 1 mM Ca^{++}/liter, 30 mM Na^+/liter, 3.5 mM K^+/liter, and 4.8 per cent sucrose. Muscles are paired with those of the respective times in *A*.

2	2.86	1.72	1.14	1.59	0.45	26
2.5	1.91	1.48	0.43	1.14	0.71	48
3.3	2.49	2.02§	0.47	1.54	1.07	53
6.4	2.18	1.48	0.70	1.47	0.77	52

* Single pairs of muscles.
‡ Of original Ca^{++}.
§ Muscle from starved frog, K^+ content 80 μmol/gm, not used in computation of mean for fresh muscle in Table I.

In order to test the effect of raised external Ca^{++} concentration on the exchange of the original Ca^{++}, eight experiments were made using a solution having 11 mM Ca^{++}. After 2 hrs.' exposure the Ca^{++} contents of the muscles were found to be between 4 and 5 μmol/gm tissue, part of which is extracellular. Of the original 1.4 to 1.5 μmol Ca^{++}/gm less than 20 per cent had become exchanged; therefore, the major effect of the high concentration used was merely to add Ca^{++} to the tissue.

Effect of Reduced Na^+ Concentration Previous work with heart tissue had shown that reduction of the external Na^+ concentration led to an increased uptake of tracer Ca^{++} (Niedergerke and Harris, 7). Similar experiments have

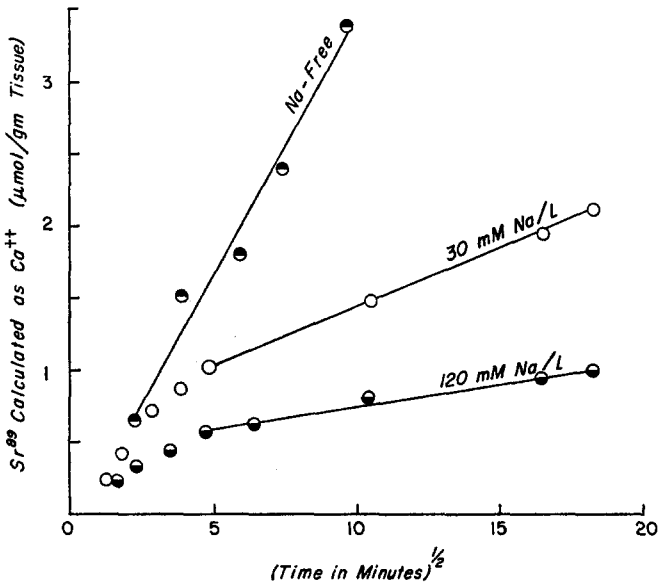

FIGURE 3. Comparison of Ca^{++} uptake (as measured by Sr^{89}) from media with 120 mM (◒), 30 mM (○), and no added Na^{+} (◓) present. 1 mM Ca^{++} was present in all the solutions used. Paired muscles were used for 120 and 30 mM experiments. Temperature, 21°C. The lines are drawn straight.

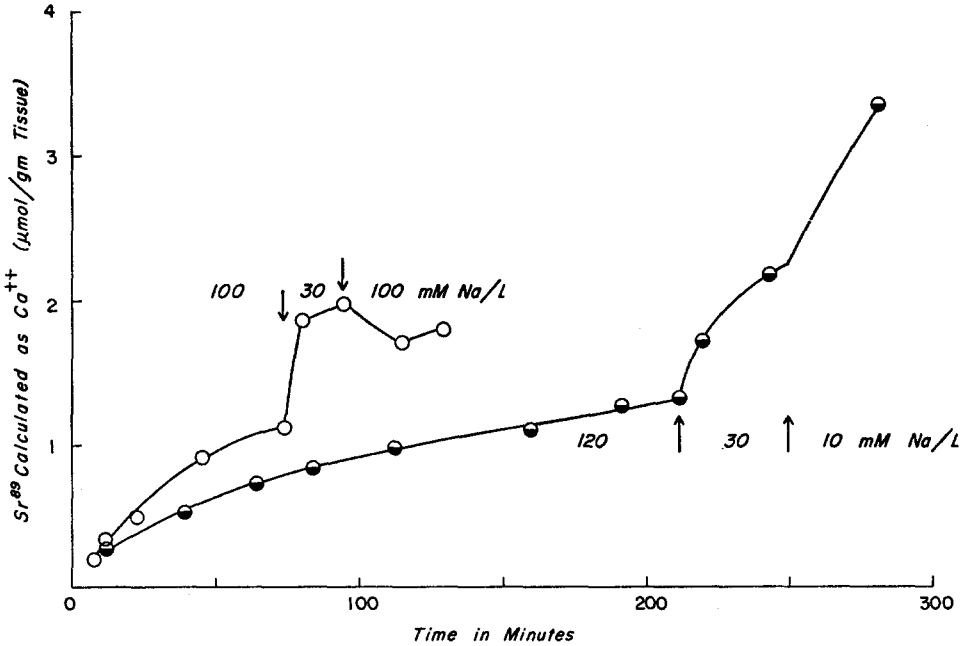

FIGURE 4. The effect of changing the Na^{+} concentration during exposure to a constant 1 mM Ca^{++} concentration (with Sr^{89}). The lines have no special significance.

now been made on sartorius muscles. The test medium contained 30 mM $NaHCO_3$, 1 mM $CaCl_2$, a trace of Sr^{89}, and 4.8 gm/100 ml sucrose. This had to be freshly prepared and kept in contact with 95 per cent O_2 + 5 per cent CO_2 mixture.

The greater uptake of tracer by a muscle immersed in this solution is shown in Fig. 3 with the uptake by the paired muscle in a solution with 120 mM Na^+ salt. In other experiments it was found that uptake in Na^+-free sucrose solution was still greater than that in the sodium-containing solutions: one of these is illustrated in Fig. 3. In Fig. 4 the result of reducing the Na^+ concentration of the solution during exposure to 1 mM Ca^{++} is shown. In the longer experiment two steps were used, namely from 120 to 30 mM Na^+, and then from 30 to 10 mM Na^+ using sucrose to maintain tonicity. In the shorter experiment and in another not illustrated a change from 30 to 100 mM Na^+ was made. This increase of external Na^+ concentration led to a rapid loss of between 0.5 and 0.6 μmol Ca^{++}/gm tissue which is not much less than the amount of the rapid gain after reduction of Na^+ concentration. Combined analytical and tracer experiments (using Ca^{45}—see Methods) showed that the net gain of Ca^{++} and the tracer uptake are both increased in the low Na^+ solution (compare part B, Table II, with part A). The net gain does not solely account for the increased tracer uptake; there is an increased exchange of the tissue Ca^{++} in the low Na^+ solution (see last columns in parts A and B).

DISCUSSION

The main purpose of this paper is to emphasize that mere observation of uptake of labeled Ca^{++} unaccompanied by analytical data does not furnish information for the proper interpretation of results dealing with the movements of Ca^{++} either in the form of exchanges or net gains. Tracer uptake experiments in which no changes of the Ca^{++} content of tissues are detected, clearly indicate that the labeled ion gained has entered into a true exchange with the original Ca^{++} of muscle as shown by Cosmos (1) in experiments following stimulation of frog muscles *in vivo*. In this same paper, it should be noted that large increases in radiocalcium following stimulation of muscles *in vitro* were also accompanied by large net gains in Ca^{++}. Without the complete analytical data one could easily misinterpret the increases in radiocalcium observed in the latter experiments as being entirely the result of an exchange of tissue Ca^{++} for radioactivity in the medium.

In the present *in vitro* experiments, it is noted that increases in tracer uptake may be brought about by a number of factors; these are conveniently divided into (*a*) those which act by causing K^+ loss and (*b*) those which increase the capacity of the tissue to accommodate Ca^{++} ions in other ways. The Na^+ con-

centration in the solution is of major importance; it seems to act on the Ca^{++} content by different mechanisms at the upper and lower extremes of concentration. High Na^+ concentration promotes loss of K^+ from the tissue (*e.g.* Table 7, Shaw, Simon, Johnstone, and Holman, 8) and as this happens, both Na^+ and Ca^{++} are gained. Here one can say that the Ca^{++} is entering what had been the K^+ space. Low Na^+ concentration has little effect on the K^+ content of the tissue; it leads, however, to gain of Ca^{++} because competition for sites is reduced. Here the Ca^{++} can be regarded as entering what had been Na^+ occupied space. With both factors operating, it appears that there will be a certain external Na^+ concentration at which the cell Ca^{++} will remain minimal. This Na^+ level will then be equal to the highest level at which the cell can maintain a constant K^+ content.

Experiments made on muscles which have been loaded with labeled Ca^{++} *in vitro* for this reason become of little direct relevance to the behavior of fresh muscles. For example, in the muscles used by Harris (2) it is likely that there had been gain of Ca^{++} during the loading and so influences on the true Ca^{++} self-exchange might be expected to be masked. It would be useful to know how to keep isolated muscles with their original Ca^{++} content in a solution for the purpose of introducing labeled Ca^{++} by exchange. We made some tests of modified media without obtaining a satisfactory one. Use of a proportion of protein (albumin 5 gm/100 ml) led to binding of the Ca^{++} in the solution to the extent of one-half to two-thirds. Although this reduced the tracer Ca^{++} uptake it only did so to the same extent as would the same reduction of Ca^{++} ion concentration. The most effective change which was found consisted in reducing the Na^+ concentration in the solution from 120 to 100 mM. Although the lower Na^+ concentration sometimes led to swelling, its use permitted a better maintenance of normal (fresh) Na^+ and Ca^{++} contents than did 120 mM Na^+; even so there are appreciable electrolyte changes as shown in the tables.

It was pointed out by Gilbert and Fenn (3) that mechanical damage increases the Ca^{++} uptake and one can perhaps generalize this to include other kinds of damage, a common symptom being the loss of cellular K^+.

Ca^{++} can be gained even when K^+ loss is slight for there seems to be a Na^+–Ca^{++} competition of the kind found in heart muscle (Neidergerke and Harris, 7). The additional Ca^{++} uptake taking place in low Na^+ media may indicate that the two ions Na^+ and Ca^{++} can alternatively occupy the same anionic sites. It is noteworthy that the uptake occurs in a matter of about 10 minutes (Figs. 3 and 4), so it is unlikely to require movement deep into the cell. That the exchange of the muscle Ca^{++} is increased in the low Na^+ solution (Table II B, last column) can be explained if we suppose that entry to the original part of the muscle Ca^{++} takes place through the region within which the Ca^{++}–Na^+ competition occurs.

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