Nickel Substitution for Calcium in Excitation-Contraction Coupling of Skeletal Muscle

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ABSTRACT In 1962 Frank (22) reported that the addition of any one of a number of divalent cations, including Ni, to a Ca-free Ringer solution prevented the rapid loss of contractility seen in the absence of external Ca. To investigate further the Ni-Ca substitution, studies were made of ^{45}Ca and ^{63}Ni exchange during contraction and at rest using frog striated muscle. In contrast to ^{45}Ca, it was found that there is no increase of ^{63}Ni uptake associated with a K contracture of the sartorius muscle. The rates of loss of ^{63}Ni and ^{45}Ca from resting toe muscles previously bathed in the respective radioisotopes are not significantly different. Resting and action potentials, after 1 hr in a Ringer solution with Ni replacing Ca, closely resemble these potentials in normal Ca-Ringer's solution. Studies on the syneresis of isolated myofibrils indicate that Ni cannot replace Ca in activating this reaction. It is suggested that Ca is required for at least two steps in E-C coupling: one is the spread of excitation at the sarcolemma and transverse tubular system; the second is the activation of actomyosin ATPase. Conceivably Ni can substitute for Ca in the former but not in the latter.

INTRODUCTION

The essential role of calcium in the coupling of the excitation at the surface of a striated muscle cell with the chemomechanical transduction within the cell is supported by four main lines of evidence (1, 2). First, the potassium-induced contracture response is rapidly and reversibly lost when Ca^{++} is removed from the external medium (3–7). Second, radiocalcium from the medium becomes associated with the muscle cell at an augmented rate during excitation and contraction (8, 9). Third, of all physiologic ions, only Ca^{++} will induce contraction when injected intracellularly (10–13). Fourth, the contraction of isolated myofibrils, the superprecipitation of actomyosin, and the maximal activity of myofibrillar and actomyosin ATPase[1] all require the presence of ionic calcium (14–16).

[1] Abbreviations used are: ATPase, adenosine-5′-triphosphatase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol *bis* (-aminoethylether)-*N*,*N*-tetraacetic acid.

It has been assumed that initiation of contraction occurs when Ca^{++} is released in the vicinity of the myofilaments from some elements of the sarcoplasmic reticulum and relaxation follows depletion of myofibrillar calcium and its reaccumulation in the sarcoplasmic reticulum (15–17). Recent electron microscopic investigations lend support to the idea that longitudinal and terminal cisternal elements of the reticulum accumulate and store calcium (18–20). More direct evidence that Ca^{++} exchange from these sites is accelerated during contraction might be obtained autoradiographically if resolution whithin the dimensions of the sarcomere were possible. Such an investigation has been made by Winegrad (21) using ^{45}Ca, although theoretically, with this isotope (E_{max} = 0.254 Mev) and other radioisotopes of calcium only marginal resolution seems possible.

Frank (22) has observed that certain divalent cations, including Ni^{++}, partially substitute for Ca^{++} in excitation-contraction (E–C) coupling. Therefore, ^{63}Ni (E_{max} = 0.067 Mev) might serve as a tracer of Ca^{++} exchange in this process and permit autoradiographic resolution better than 1 μ. Preliminary to such an application we have compared the kinetics of Ni and Ca exchange in resting and contracting muscle, the effect of substituting Ni for Ca in the medium on the resting and action potentials, and the syneresis of isolated myofibrils in the presence of Ni. We have observed that the resting and action potentials are maintained when Ni is substituted for Ca. However, the uptake of nickel by the fiber from the medium, unlike that of Ca, is apparently not accelerated during contraction. These results presumably limit the usefulness of ^{63}Ni as a tracer for the autoradiographic localization of Ca in E–C coupling but offer further clues to the nature of this process.

MATERIALS AND METHODS

Measurements of radioisotope exchange were performed on isolated sartorius and extensor longus digiti IV muscles of *Rana pipiens* at all months of the year in New York City. Electrical measurements were made on sartorius muscles from *R. pipiens* in February, 1964, in Cambridge, England.

Solutions Solutions were prepared from analytic grade reagents and water, distilled twice, the final distillation in a glass still, and adjusted to pH 7.2 ± 0.1 using a glass electrode. Their compositions are summarized in Table I. All glassware and polyethylene bottles were cleaned with detergent, soaked at least 3 days in 3 % HNO_3 v/v, and rinsed with glass-distilled water. Choline-containing solutions were prepared on the day of use. All other solutions were discarded after 2 wk storage at 4°C or earlier if the pH changed more than 0.1 pH unit.

Calcium-free nickel containing Ringer's solutions were analyzed for calcium by titration with EGTA in the presence of Mg using the calcium indicator 2-hydroxy-1(2-hydroxy-4-sulfo-1-naphthyl-azo)-3 naphthoric acid (23). Calcium could not be detected by this method, the sensitivity of which is 10 μM/liter.

Radioisotopes ^{63}Ni and ^{45}Ca at high specific activities and dissolved in dilute HCl were obtained from Oak Ridge National Laboratories. Both ^{45}Ca (0.254 Mev) and ^{63}Ni (0.067 Mev) emit only β-radiation. For each experiment an aliquot of the HCl solution was pipetted into a platinum crucible, dried under vacuum, and dissolved in the respective Ca- or Ni-Ringer solution at 5 μc/ml and subsequently designated Ni*-R or Ca*-R. For detection of radioactivity 1 ml aliquots in duplicate were placed on silicone-rimmed 1¼ inch aluminum planchets, mixed with one drop of detergent, and dried under a heat lamp. No correction for self-absorption or coincidence loss was necessary for in all cases the dry mass/cm^2 surface was about identical in all planchets. Radioactivity was assayed in a windowless, gas flow counter, in the Geiger range, to within 95% confidence limits of decay statistics. Background varied within 15–18 cpm. Data from all exchange studies have been corrected to a standard specific activity of 10 $\mu\mu$mole Ca or Ni.

TABLE I

	Ca-R	Ni-R	Hi K-R	Ca-free R	EDTA-R	Choline-R
	mM	mM	mM	mM	mM	mM
NaCl	111.2	111.2	111.2	111.2	111.2	—
KCl	2.5	2.5	75.0	2.5	2.5	2.5
Choline Cl	—	—	—	—	—	111.2
Na_2HPO_4	1.14	1.14	1.14	1.14	—*	1.14
NaH_2PO_4	0.23	0.23	0.23	0.23	0.23	0.23
$CaCl_2$	1.8	—	—‡	—	—	—
$NiCl_2 \cdot 6H_2O$	—	1.8	—‡	—	—	—
NaEDTA	—	—	—	—	2.0	—

* pH of final solution adjusted to 7.2 by addition of solid Na_2HPO_4.
‡ Neither Ni^{++} nor Ca^{++} was included in the HiK-R used for the tension-recording experiments. For ^{63}Ni contracture-exchange studies 1.8 mM $NiCl_2$ was included in the HiK-R. For ^{45}Ca contracture-exchange studies 1.8 mM $CaCl_2$ was included in the HiK-R.

To compare the kinetics of Ca and Ni movements, measurements of the following parameters were performed:

(*a*) The amount of radioisotope exchange resulting from a potassium-induced contracture (K contracture) in the presence of ^{45}Ca or ^{63}Ni (designated below as contracture-exchange studies); (*b*) the rate of loss of either ^{63}Ni or ^{45}Ca from muscles previously soaked overnight in the respective radioisotope solutions; (*c*) the rate of loss of either ^{63}Ni or ^{45}Ca from muscles presoaked in radioisotope solution 6 min.

Experiments (*b*) and (*c*) are designated below as washout studies.

Contracture-Exchange Studies The design follows that of Bianchi and Shanes (8). Paired sartorii were dissected under the stereomicroscope in Ca-R at about 10°C and mounted at rest length (i.e., sartorius muscle length in situ with leg extended and thigh abducted) with cotton ties to Pyrex glass racks, and equilibrated in Ca-R at 17.0 ± 0.1°C for at least 30 min. Stirring was achieved by vertical oscillation of muscle and rack by a motor-driven cam rotating at 2 revolutions/sec. The rack-

mounted muscles were transferred through a series of experimental solutions of 2.5 cc, contained in test tubes immersed in a water bath maintained at 17°C. After the initial equilibration period in Ca-R, both muscles were placed in Ni-R for 15 min followed by Ni*-R for 5 min. Following exposure to radionickel the muscles were separated and muscle A induced to contract by immersion for 2 min in HiK-R, with 1.8 mM Ni^{++} (no ^{63}Ni). Muscle B (the control) at the same time was bathed in Ni-R (no contracture) for 2 min. At the end of the 2 min both muscles were passed through a series of nonradioactive Ni-R solutions; the total washout period lasted 58 min. After inspection for damaged fibers with the aid of a dissecting microscope, and testing for electrical excitability, the tendons were removed, the muscles blotted on filter paper, and weighed on a torsion balance. Only muscles which contracted briskly and contained less than three visibly coagulated fibers were analyzed for radioactivity. The muscles were dried overnight at 105°C in Pt crucibles and ashed for 24 hr at 580°C. Ash was dissolved in 2 drops of concentrated HCl, diluted to 10 ml with Ni-R, and duplicate 1 ml samples counted as described above. An aliquot of the Ni*-R solution was diluted with Ni-R and counted in an identical manner. From this value the specific activity of the Ni*-R solution could be calculated and used for standardization when the data from different experiments were collected and compared.

Washout Studies To compare the rates of loss of ^{63}Ni and ^{45}Ca from muscles previously soaked overnight in the solutions containing the respective radioisotopes, the following experiment was performed. Four pairs of toe muscles were carefully dissected and mounted on glass racks. One muscle (A) of each pair was placed in Ca*-R, the other (B) in Ni*-R, and both exposed for 14 hr at 6°C to the well stirred solutions. 6°C rather than 17°C was maintained in the interest of preserving the integrity of the muscles during the 14 hr equilibration. Each muscle was placed in nonradioactive solution and remounted on a clean glass rack; the complete procedure for transfer required about 1 hr for all eight muscles. For the next 1.5 hr, the muscles were transferred every 15 min to fresh nonradioactive solutions at 6°C. Muscles in group A were exposed to Ca-R, muscles in group B to Ni-R. At the end of the washout period the radioisotope content of the muscles and solutions was determined as described above. To obtain the isotope content of a muscle after each washout period, the quantity of isotope in the muscle ash was added in reverse order to the isotope lost from the muscle in each bathing solution. The logarithms of these values were then plotted against time.

To compare the movements of Ca and Ni from the muscle cell surfaces the following experiments were performed. A toe muscle was dissected, its widest dimension measured with an ocular micrometer, tied at rest length to a glass rack with cotton thread, and soaked for 6 min in Ca*-R. The muscle was then transferred through a series of nonradioactive Ca-R solutions. At the end of the 10 min washout period the muscle was divided from its proximal and distal tendons, weighed, and ashed; isotope content in the ash was measured as described above. The glass rack (with cotton and tendons still attached) was returned to Ca*-R for another 6 min and passed through a series of nonradioactive solutions in a manner comparable to that described above. Residual radioactivity was eluted from the rack, ties, and tendons by concentrated HCl. The acid was evaporated to dryness and counted after dilution of the HCl residue in Ca-R.

The loss of ^{63}Ni from the toe muscle was studied in a similar manner except that the muscle was exposed to Ni*-R for 6 min and washed out in a series of Ni-R solutions. Solutions were maintained at 17°C.

Isometric Recordings of Potassium-Induced Contractures Toe muscles were mounted with cotton ties in a vertical, cylindrical, glass chamber containing 7 ml of solution at 18–20°C. The lower end of the muscle was fixed while the upper end was tied to an RCA 5734 mechanoelectric transducer. The output of the transducer was coupled through a bridge to a dual beam oscilloscope. Oxygen was bubbled continuously through the chamber via a scintered glass connector in the lower side of the chamber. Solutions were emptied through a wide bore stopcock at the bottom of the chamber, and a fresh solution poured into the top of the cylinder. Emptying and refilling the chamber required 5 sec. Contractures were induced by exposing the muscles to a divalent cation-free Ringer solution to which 75 mM KCl had been added (see Table I). Shortening was never more than 5% of rest length. In early experiments K_2SO_4 rather than KCl was used to raise the potassium concentration, and the (K) × (Cl) product was kept at 300 mM with isotonicity maintained throughout. However, the results were similar to those with the hypertonic HiK-R solution and the latter solution was used in all subsequent experiments. Details of each experiment will be presented with the results.

Syneresis of Isolated Myofibrils In collaboration with Dr. Annemarie Weber experiments were conducted on the syneresis of isolated myofibrils in the presence of Mg^{++}, ATP, and either Ni^{++} or Ca^{++} or both Ni^{++} and Ca^{++} together. As described by Weber and Herz (24) myofibrils were prepared from rabbit skeletal muscle and washed with Mg-EDTA. Into a calibrated centrifuge tube with a capillary tip were added the following reactants: 4.5 ml of myofibril suspension containing 15.8 mg/ml of protein; 0.5 ml of $CaCl_2$ or $NiCl_2$ solution (the Ni and Ca concentrations were varied in each experiment); 1.0 ml of MgATP giving a final ATP concentration of 3.33 mM. Immediately upon adding ATP the centrifuge tube was placed in a centrifuge, the latter energized, and the time recorded until 3000 *g* was reached. This interval, the incubation time, averaged 45 sec. After spinning 3 min at 3000 *g* the volume of packed myofibrils was measured and recorded.

Resting and Action Potential Measurements In collaboration with Dr. R. H. Adrian resting and action potentials were recorded differentially with intracellular glass microelectrodes using the method of Nastuk and Hogkin (25). The output of the intracellular electrode was fed into a previously calibrated automatic chart recorder, from which resting potentials were later measured. The sartorius muscle, with pelvic bone attached, was mounted in a Lucite dish and stimulated by short cathodal pulses passed between two silver plates at opposite ends of the chamber. Action potentials were recorded photographically from the oscilloscope trace. Microelectrode tip potentials and resistances were measured by the method of Adrian (26). The experiment was conducted at room temperatures of 18–19°C. Solutions were changed with glass pipettes and it is likely that traces of Ca remaining in the chamber were carried over into the Ni-R solution. This Ca contamination was minimized by using a large chamber (approximately 50 ml capacity) and frequently changing the Ni-R solution.

Resting and action potential measurements were made initially with the muscle in Ca-R, 1 hr after transfer to Ni-R, and subsequently after return to Ca-R.

RESULTS

Contracture-Exchange Studies When pairs of sartorius muscles are exposed to radionickel solutions and a K contracture induced in one muscle, it is apparent that the contracture does not alter the rate of uptake of radionickel, as shown in Table II. Results have been normalized for comparison according to the specific activity of the initial radionickel solution. There is no significant difference between the results with the two sets of muscles. Though not presented here, three similar experiments using ^{45}Ca and Ca-R solutions

TABLE II

RADIONICKEL UPTAKE DURING POTASSIUM-INDUCED CONTRACTURES OF ISOLATED SARTORIUS MUSCLES

Contracture	Control
counts/min/mg wet weight	
16.0	12.6
18.7	19.2
13.9	11.8
24.2	38.5
32.2	44.2
20.2	21.5
22.3	28.3
Mean ± SD 21.1 ± 6.6	25.1 ± 12.4

On the left is tabulated ^{63}Ni radioactivity in the ash of muscles induced to contract after exposure to ^{63}Ni. On the right is tabulated ^{63}Ni radioactivity in the ash of the corresponding paired muscle which had also been exposed to ^{63}Ni but not induced to contract. Specific activity, 10 counts/min/$\mu\mu$mole Ni. T = 17°C.

showed a significant increase in the rate of uptake of radiocalcium accompanying a K contracture. The difference in the two sets of muscles was 120 ± 15 cpm/mg (12 ± 1.5 $\mu\mu$mole/mg) of wet weight. In all cases data are presented as mean values plus or minus one standard deviation.

Washout Studies It is conceivable that Ni is released from the muscle at at a faster rate than Ca and thus any increase in the rate of entry of ^{63}Ni associated with the contracture is masked by ^{63}Ni loss during the washout period. To test this possibility paired toe muscles were immersed in ^{63}Ni or ^{45}Ca solutions for 14 hr after which time the rate of loss of radioisotopes from the muscles into nonradioactive solutions was measured. The results are presented in Fig. 1. The initial fast components of the curves were not obtained because the glass holders were changed during the 1st hour after the muscles had been removed from the radioactive solutions. The points represent iso-

tope content associated with the muscles 1–3 hr after removal from the radioisotope solutions. Time as indicated on the abscissa begins 1 hr after removal of the muscles from radioactive solutions. From 1–3 hr, the rate of loss of radionickel or radiocalcium from the muscles is approximately exponential and can be fitted by a straight line on semilogarithmic paper. The number above each set of points is the half-time ($t_{1/2}$) obtained graphically from a visually fitted line. Though the variability is wide between muscle pairs, it is evident that the half-times within a given set do not differ significantly. The mean half-time of Ca release is 9.4 ± 2.4 hr whereas that of Ni is 10.0 ± 2.9

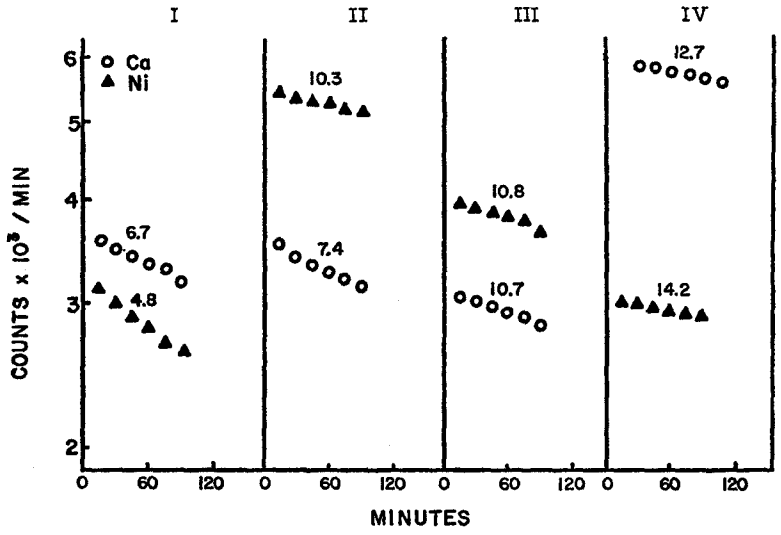

FIGURE 1. The release of ^{63}Ni (▲) and ^{45}Ca (o) from paired toe muscles previously exposed to the respective radioisotopes for 14 hr. Following this exposure muscles were transferred to new glass racks and 1 hr after removal from the radioisotope solution measurement of radioisotope release was begun and continued for 1½ hr. The rapid phases of isotope release, therefore, have not been studied in these experiments. Half-times ($t_{1/2}$) in hours are indicated above each curve assuming a straight line fit. T = 6°C.

hr. This is not a significant difference. This slow release of radiocalcium is assumed to represent exchange of intracellular calcium and has been so measured and interpreted in experiments on frog sartorius muscles by Bianchi and Shanes (27).

Since the slow phases of radioisotope loss from the muscle showed little difference between the two cations, it seemed of interest to investigate the rapid rate of loss of these cations immediately after removal from the radioisotope solutions. The straightforward approach to this problem is difficult because of the large amount of radioisotope adsorption to cotton ties, glass rack, and tendons. To overcome this obstacle, it has been necessary in each experiment to determine how much radioisotope derived from the ties, ten-

dons, and glass and then calculate the contribution from the muscle alone. Thus, the combined rate of loss of radionickel and radiocalcium from muscle, ties, and rack was measured first. After severing the muscle from its tendons the rack, ties, and tendons were reexposed to radionickel or radiocalcium and the washout experiment repeated. The original radiosiotope remaining on the rack, ties, and tendons at the start of the second washout has been measured and its contribution to the second washout found to be insignificant. Typical curves of ^{45}Ca and ^{63}Ni release are presented in Fig. 2. Curve *A* is

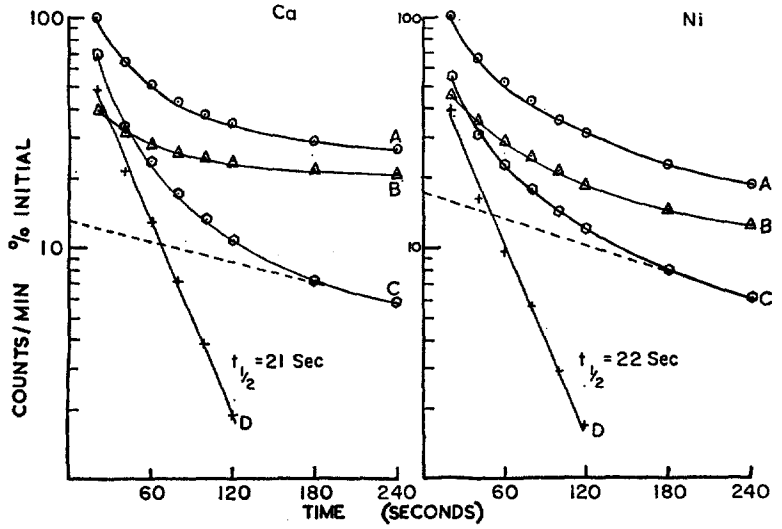

FIGURE 2. The release of ^{63}Ni and ^{45}Ca from toe muscles following a 6 min exposure to the respective radioisotopes. Curve *A* represents the release of ^{45}Ca or ^{63}Ni with the muscle tied to a glass rack. Curve *B* is the curve of washout of ^{45}Ca or ^{63}Ni from the same rack, cotton ties, and tendons after muscle removal and a second exposure to radioisotope for 6 min. Curve *C* is obtained by subtracting *B* from *A*, and curve *D* by subtracting the extrapolated straight line of *C* from earlier time points on the same curve. Curve *C* is assumed to represent radioisotope release from muscle fibers, while *D* is a measure of exchange between medium and intercellular regions. T = 17°C.

the release of radioisotope from the rack + ties + tendon + muscle; curve *B* is the release of isotope from rack + ties + tendon; and curve *C* is obtained by subtracting curve *B* from curve *A*. A straight line has been passed through the last two points and this line subtracted from the rest of curve *C*. A straight line (*D*) results from this subtraction and its half-time is obtained graphically. It has been found from other experiments that the points of curve *C* from 3 min through 10 min fall on a straight line; thus, the extrapolation of a straight line through the last two points on curve *C* is justified. It has been found that the shape of curves *A* and *B* varied widely between different glass racks but the shape of curve *C* was quite comparable for different toe muscles. For

these reasons it is believed that the method does provide a way of eliminating the artifact introduced by glass and cotton ties and measuring the more rapid loss of isotope from the muscle alone.

If one assumes that line D represents cation diffusion from the muscle interspaces, then the diffusion constant (D') can be calculated by the following equation (28):

$$D' = \frac{0.118\, r^2}{t_{1/2}}$$

where r = muscle radius, and it is assumed that the toe muscle can be represented by a cylinder. The experimental values from six muscles are presented in Table III. Since the toe muscle is elliptical in cross-section, rather than circular, the muscle width measured in these experiments with the ocular micrometer (used in the diffusion calculations) is the major axis of

TABLE III

	Radius	$t_{\frac{1}{2}}$	D'
	$cm \times 10^{-2}$	*sec*	$cm^2/sec \times 10^{-6}$
Ni	2.3	19	3.4
	2.0	23	2.0
	2.3	21	3.2
Ca	2.0	20	2.4
	2.6	23	3.5
	2.0	21	2.2

the ellipse. Thus the cross-sectional area of the muscle is less than the authors assumed for the above calculations, and the diffusion constants are too large by approximately a factor of 1.4. The main point to be drawn from these results is that the diffusion times, from the toe muscle interspaces, for the two cations do not differ significantly as measured by this method.

Isometric Recordings of Potassium-Induced Contractures The studies by Frank (4) indicated that the rate of loss of the K-induced contracture response in a Ca-free solution could be explained by the diffusion of Ca from the muscle fiber surfaces. These findings have been confirmed and a typical experiment is presented in Fig. 3. Each number at the upper right corner of the tension curves is the area beneath each trace expressed as per cent of the area under curve A. Areas beneath the curves were obtained by weighing cutouts of photographic enlargements of the traces and comparing these to the weights of known areas of the same photographic paper. The area under the contracture curve has been used as an expression of total contractile

output, since it has been shown that such areas are a better measure of contractility than peak tension production (29, 30).

60 sec after removing external Ca^{++} (curve *B*), the contractility has fallen to approximately 50% of the control value (curve *A*). Assuming a diffusion constant of 2.1×10^{-6} cm^2/sec for Ca^{++} in the muscle fiber interspaces, and a cylindrical muscle of radius 2×10^{-2} cm, the concentration of Ca^{++} at a radius corresponding to one-half the cross-sectional area of the muscle is calculated (31) to have fallen to 0.1 of the original value or 0.18 mM within 60 sec after removing Ca^{++} from the external medium. It has been shown by

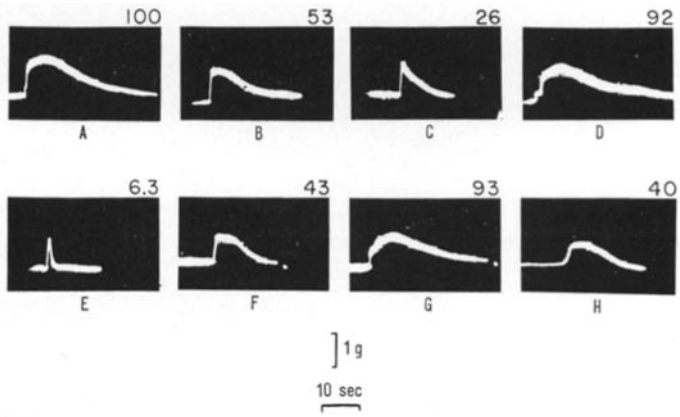

FIGURE 3. Time course of tension development during successive K-induced contractures under varying Ca and Ni contents of bathing medium. Toe muscle, diameter 400 μ; temperature 19°C. Contractures were induced by Hi K-R; Hi-K, divalent cation-free Ringer's solution. Letters *A* through *H* indicate the conditions immediately preceding contracture. Following each contracture the muscle was returned to Ca-R for 10 min. *A*, 10 min in Ca-R (control); *B*, 60 sec in Ca-free R; *C*, 4 min in Ca-free R; *D*, 10 min in Ca-R (control); *E*, 2 min in EDTA-R; *F*, 2 min in EDTA-R and then 30 sec in Ca-R; *G*, 10 min in Ca-R (control); *H*, 2 min in EDTA-R and then 30 sec in Ni-R.

Frank (4) and Curtis (6) that tension production is approximately 50% reduced in 0.1 mM Ca^{++}. Thus, the results are consistent with the interpretation of Frank (4) that the fall in tension production can be explained by the diffusion of calcium from superficial sites on the muscle cell.

The addition of EDTA to a Ca-free medium hastens the failure of the ability to develop tension. 2 min in the presence of EDTA results in a 94% inhibition (curve *E*) of contractility. In confirmation of previous work (4) the brief contraction, as seen in curve *E*, is abolished by substituting choline chloride for NaCl in the medium. When Ca or Ni is replaced in the external medium after a muscle has been immersed for 2 min in EDTA-R, there is a rapid return of the K-induced contracture response. The area under the

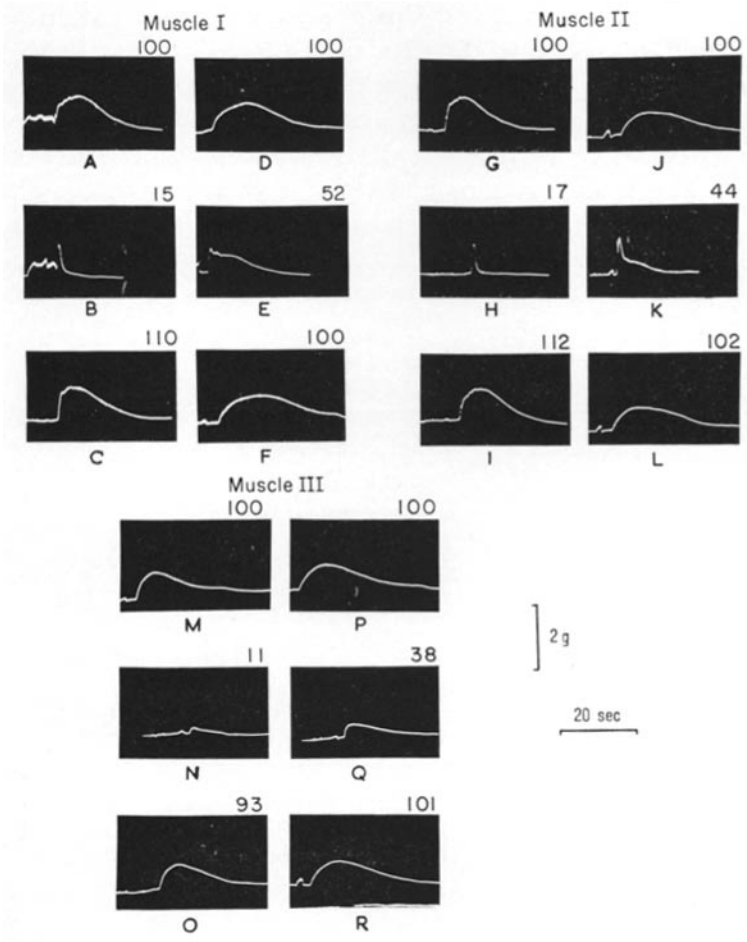

FIGURE 4. Potassium-induced contractures of three toe muscles, I, II, and III, following a presoak in Ca or Ni-R followed by a brief exposure to a solution free of either divalent cation. T = 19°C. For muscle III the NaCl has been replaced by choline Cl. The number at the upper right of each curve expresses in per cent the area under the curve relative to that for the first contracture response in Ni or Ca for that muscle. Results *A* through *R* are in chronologic order and the letters indicate conditions immediately preceding transfer to Hi-K-R.

Muscle I. *A*, 10 min in Ca-R (Ca control); *B*, 10 min in Ca-R, then 2 min in Ca-free R and Ni-free R; *C*, 10 min in Ca-R; *D*, 10 min in Ni-R (Ni control); *E*, 10 min in Ni-R, then 2 min in Ca and Ni-free R; *F*, 10 min in Ni-R (Ni control).

Muscle II. *G*, 10 min in Ca-R (Ca control); *H*, 10 min in Ca-R, then 4 min in Ca-free R and Ni-free R; *I*, 10 min in Ca-R (Ca control); *J*, 10 min in Ni-R (Ni control); *K*, 10 min in Ni-R, then 4 min in Ni-free R and Ca-free R; *L*, 10 min in Ni-R (Ni-control).

Muscle III. *M*, 10 min in Ca-R (Ca control); *N*, 10 min in Ca-R, then 2 min in Ca-free R and Ni-free R; *O*, 10 min in Ca-R (Ca control); *P*, 10 min in Ni-R; *Q*, 10 min in Ni-R, then 2 min in Ca-free R and Ni-free R; *R*, 10 min in Ni-R (Ni control).

tension curve is approximately 40% of the control values within 30 sec of replacement of Ca-R or Ni-R after 2 min in EDTA-R.

Since the fast phase of Ni and Ca exchange, presented in Fig. 2, suggested that the diffusion of these two divalent cations in the toe muscle interspaces was not significantly different, it was desirable to test whether a muscle presoaked in Ni-R had the same rate of loss of contractility in the absence of Ca^{++} and Ni^{++} as a muscle presoaked in Ca-R. Such an experiment is presented in Fig. 4. The results indicate that the loss of contractility in a Ca-free medium has a slower time course if the muscle had been preexposed to Ni^{++} rather than to Ca^{++}.

TABLE IV

	Reactants*					Volume of centrifuged myofibrils	
	Myofibrils	MgATP	$CaCl_2$‡	$NiCl_2 \cdot 6H_2O$			
	gm/liter	*mM*		*mM*		*ml*	
I	14.5	3.33	—	—	3.7		
II	14.5	3.33	0.033	—	0.95		
III	14.5	3.33	—	0.045	3.5	+ 0.4 μmole $CaCl_2$ / recentrifuged	0.75§
IV	14.5	3.33	—	0.15	3.0		
V	14.5	3.33	—	0.30	3.0	+ 0.4 μmole $CaCl_2$ / recentrifuged	0.65§

* Total volume in each tube, 6.0 ml.

‡ Myofibrils have been washed with MgEDTA to remove exchangeable calcium bound to the protein. No preparations were absolutely Ca-free (14). The ATP had a Ca contamination of 0.06 μmole Ca/20 μmole ATP.

§ Following centrifugation, 0.4 ml of 1 mM $CaCl_2$ was added to tubes III and V, incubated for a further 45 sec, and recentrifuged. This addition of $CaCl_2$ diluted all other reactants 6.25%.

Syneresis of Isolated Myofibrils in the Presence of Nickel The foregoing studies of radioisotope exchange indicate that nickel exchange between medium and muscle is not accelerated by a K-induced contracture in the manner that calcium exchange is accelerated (8). The time course of Ni^{++} substitution for Ca^{++} in E-C coupling (see Fig. 3 *H*) is consistent with an action of Ni near the surface of the muscle cells, rather than at the myofibrils. Previous workers (23, 32) have demonstrated that the syneresis of isolated myofibrils and the superprecipitation of actomyosin require the presence of ionic calcium in addition to Mg^{++} and ATP. We have tested with the isolated myofibril preparation whether Ni can substitute for Ca at the latter site by noting whether Ni^{++} can activate myofibrillar contraction in the absence of Ca^{++}. The results are presented in Table IV. It is clear that nickel cannot substitute for calcium in the syneresis of myofibrils. In the absence of added calcium (row I) the volume of the centrifuged myofibrils is 3.7 ml.

When calcium is added (row II), the volume is reduced to 0.95 ml, indicating in vitro contraction of the myofibrils. If Ni^{++} is added instead of Ca^{++} (rows III, IV, V), no significant syneresis occurs. The addition of calcium after nickel still results in syneresis. Thus it is concluded that nickel cannot replace calcium in activating myosin ATPase during the myosin and actin interaction. In the concentration range of 0.045-0.3 mM nickel does not inactivate this enzyme within the 10 min incubation time studied. These results complement the finding of Seidel and Gergely (33) that Ni^{++} cannote reactivate the ATPase activity of calcium-free myofibrils.

Resting and Action Potential Measurements The action potential obtained from a sartorius muscle after bathing 1 hr in Ni-R is presented in Fig. 5. Control traces in Ca-R before and after exposure to Ni^{++} are also presented

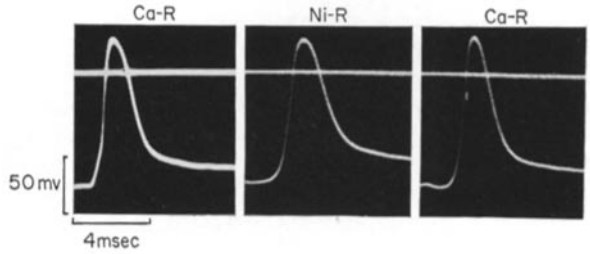

FIGURE 5. Intracellularly recorded action potentials of sartorius muscle fibers before, during, and after substitution for Ca^{++} of Ni^{++} in the Ringer solution. The action potential on the left was recorded 1 hr after muscle immersion in Ca-R, the center recording after 1 hr in Ni-R, and the final record after a 15 min return to Ca-R. T = 18–19°C.

in Fig. 5. The amplitude of the action potential is only slightly decreased in Ni-R and the overshoot is not significantly different from the controls. The maximum rate of rise of the action potential declines in Ni-R to 170 v/sec from 480 v/sec, the mean for the action potentials in Ca-R before and after the exposure to Ni-R. There is a comparable slowing of the falling phase and an increase in the negative afterpotential in Ni-R. Action potentials similar to those in Ni-R also are seen if one-half of the Ni^{++} is replaced by Ca^{++}, giving 0.9 mM of each divalent cation. A similar study of the effect of Ni^{++} on the electrical properties of frog skeletal muscle has been conducted by Kobayashi (34). Our results confirm this author's findings. These changes in the action potential of skeletal muscle shown above are strikingly different from those obtained at the node of Ranvier in nerves exposed to similar concentrations of Ni^{++} (35–38).

Probably more pertinent to excitation-contraction coupling is the area under the action potential which increases in Ni-R to approximately 20% above the mean of the areas under the action potentials recorded in Ca-R

before and afterwards. A more relevant analysis, according to Sandow et al. (39), would be a comparison of the area under each curve between mechanical threshold and "mechanical saturation," but the influence of the conditions of these experiments on these latter two parameters has not been determined and it cannot be assumed that mechanical threshold and mechanical saturation remain unchanged when Ni is substituted for Ca in the medium.

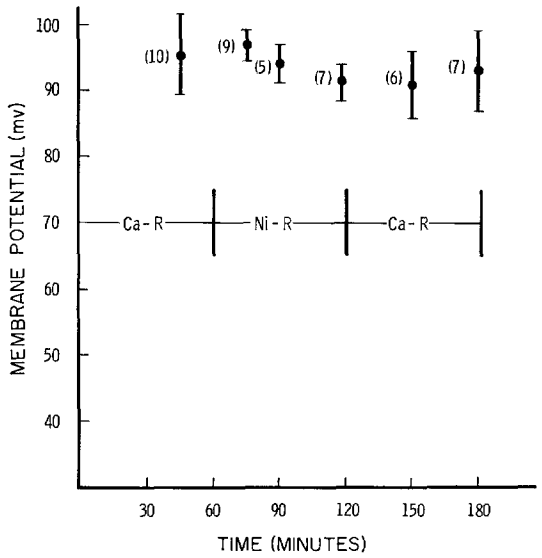

FIGURE 6. Intracellularly recorded resting membrane potentials of a sartorius muscle before, during, and after substitution of Ni^{++} for Ca^{++} in the Ringer solution. Mean potentials ± one standard deviation. The number beside each mean value is the number of fibers impaled. Ni-R solution changed twice at 60 min and then once every 15 min thereafter. T = 18–19°C.

Resting potentials from the same muscle are presented in Fig. 6. After 1 hr in Ni-R the resting potential is 90.9 ± 3.4 mv, which is not significantly different from the control values in Ca-R obtained in the present study or in previously published work (25). The graph does suggest a trend downward in Ni-R but this has not been examined for more prolonged periods in Ni-R.

Two other findings will be mentioned in passing, although not relevant to the principal considerations. First, the excitatory threshold for direct electrical stimulation approximately doubles in Ni-R. Second, a muscle will not contract when stimulated through its nerve in Ni-R. This block to indirect stimulation is present in Ringer's solution containing 0.9 mM each of Ni^{++} and Ca^{++}. The block is rapidly reversible suggesting a surface action of the Ni. Presumably this is a block of neuromuscular transmission since Ni, at this concentration, does not block nerve conduction (36). At similar concentrations zinc has been reported to block neuromuscular transmission (40).

DISCUSSION

The failure to observe an increased uptake of nickel by frog muscle during a K-induced contracture is in marked contrast with the increased calcium

uptake under similar experimental conditions. Radiocalcium uptake, in the present study, averaged 12 ± 1.5 $\mu\mu$mole/mg of wet muscle weight. This is in essential agreement with the value of 13 $\mu\mu$mole/mg obtained by Bianchi and Shanes (8). Half-times for the slow phase of radionickel and radiocalcium release (Fig. 1) from toe muscles at 6°C averaged 9.7 hr. This value might be compared with the $t_{1/2}$ of 6.3 hr suggested by Gilbert and Fenn (41) for the entry of ^{45}Ca and the time constant ($t_{1/e}$) of 8 hr obtained by Bianchi and Shanes (27) for the loss of ^{45}Ca. These figures are reasonably consistent when account is taken of muscle and temperature differences; the authors cited above worked at temperatures above 20°C and with the sartorius muscle.

Milligan (30) has recently calculated the diffusion constant for Ca movement in toe muscle interspaces using the area under contracture curves as a measure of contractile output after varying times in Ca-free solutions. He has calculated a diffusion constant of 2×10^{-6} cm²/sec which is to be compared with the value of 2.1×10^{-6} cm²/sec obtained in the present study by an independent approach.

It has been suggested by Frank (22) that Ni, and other divalent cations capable of maintaining contractility in the absence of Ca, act by releasing or displacing bound Ca which is then free to enter the muscle cell during excitation. However, if such were the case, one would expect the rate of disappearance of contractility in a Ca-free medium to be the same whether a muscle had been preexposed to either Ni-R or Ca-R. In both cases the diffusion of Ca^{++} from the muscle would be the rate-limiting factor. The results presented in Fig. 4 rule out this possibility, for the rate of loss of the response to elevated external K^+ concentrations, in a divalent cation-free medium, is slower if the muscle has been preexposed to Ni-R rather than to Ca-R. Two possible explanations for this finding are (*a*) Ni^{++} slows the rate of loss of Ca^{++} from a superficial location required in E-C coupling; or (*b*) Ni^{++} can completely substitute for Ca^{++} in one step of the coupling reaction, presumably at a membrane site, the sarcolemma or transverse tubular membranes, but the binding of Ni^{++} at this site is greater than the binding of Ca. Unpublished results from the authors' laboratory indicate that the net rate of loss of ^{45}Ca from a frog muscle is increased, at least transiently, in the presence of Ni^{++}. Thus, one would suspect, until contrary evidence is available, that Ni^{++} does not impede Ca^{++} release from the muscle fiber and that the first explanation is less likely than the second. That Ni^{++} may be bound to membranes more tightly that Ca^{++} is probable when one considers the relative positions of the two cations in the electrochemical potential series. Indeed, in a recent investigation (42) comparing Ca, Mg, and Ni-phospholipid complexes, it has been found that the dissociation constants of nickel phospholipid complexes are significantly smaller than similar complexes formed by Ca or Mg. The possibility that divalent cations may be adsorbed to the membrane surface and

thus alter the electric field on the opposite side has been suggested by A. F. Huxley (see Frankenhaeuser and Hodgkin, 43). It is probable that the strength of this adsorption will be a function of ionic size and electronegativity of the cations and will follow the order (44):

$$Mg^{++} < Ca^{++} < Mn^{++} < Fe^{++} < Co^{++} < Ni^{++} < Cu^{++}.$$

The intracellular electrode recordings demonstrate that Ni^{++} can prevent the fall in resting membrane potential seen in a calcium-free medium. It can substitute for Ca^{++} in maintaining a relatively normal action potential. Spontaneous contractions which develop when external Ca^{++} concentration is reduced are also prevented by adding Ni^{++} to the surrounding medium. Luttgau (7) has shown that qualitatively, the changes in Ca^{++} concentration and changes in membrane potential have similar effects not only on the sodium-carrying system (43) but also on the system that controls contractility. The shifts in mechanical and electrical thresholds produced by alterations in the Ca concentration, can be altered similarly by larger changes in the Mg concentration (7, 45). From the electrical recordings of Figs. 5 and 6, Ni^{++} appears to have effects on the sodium-carrying system qualitatively similar to those of Ca and Mg (34). Furthermore, it is probable that Ni, Mg, and Ca have qualitatively similar effects on the mechanical threshold as well (7, 46). It has been proposed by Luttgau (7) that Ca may influence the shift of an activator of contraction in a manner similar to the effect of Ca on the permeability of sodium at the plasma membrane. Our results can be reconciled with such an hypothesis if it is assumed that Ni can substitute for Ca in regulating the movement of an "activator substance" but Ni itself cannot be this substance.

However, such an hypothesis provides no information regarding the cellular location of such an activator compound nor the position to which it shifts during E-C coupling. At present one might suggest five cellular locations for Ca action during the initiation of contraction. The first would be at the sarcolemma; the second, along the walls of the transverse tubules; third, at the junction between transverse tubules and terminal cisternae of the sarcoplasmic reticulum; fourth, within longitudinal elements of the sarcoplasmic reticulum; and fifth, at the myofibrils. Although no direct morphological evidence is available for the sites of nickel action, the present results suggest that such sites must be in rather rapid exchange with the extracellular fluids. This would exclude a nickel for calcium substitution in the cellular locations listed fourth and fifth above.

In confirmation of Frank (4) it has been found that the K-induced contracture which disappears rapidly in the absence of external Ca^{++}, is quickly restored and maintained for at least 3-4 hr when Ni^{++} is added to the Ca-free

Ringer solution. Although contractility is maintained when Ni^{++} is substituted for external Ca^{++}, there is no increase in ^{63}Ni uptake accompanying the K-induced contractures. Furthermore, Ni cannot substitute for Ca in activating the MgATP-induced syneresis of isolated myofibrils or in stimulating myofibrillar ATPase activity (33). One is led again to conclude that Ni^{++} exerts its action external to the myofibril compartment of the muscle cell.

Strong evidence is now available relating the ionized Ca concentration in the vicinity of the myofilaments quantitatively to contraction and relaxation (14, 15). It is now widely assumed that active transport of Ca into the cisternae of the sarcoplasmic reticulum reduces the concentration of ionized Ca at the sites of actomyosin ATPase. Since electron microscopic studies of muscle fixed in the presence of a Ca precipitant (oxalate) (18–20) have demonstrated calcium oxalate crystals in only one region of the reticulum, the terminal cisternae, it is plausible to assume that Ca storage and perhaps uptake occur at this site. Autoradiographic evidence (21) has been presented suggesting ^{45}Ca movement from the middle of the I band to the A-I junction during contraction, but the resolution is not sufficient to decide whether Ca is released from the transverse tubules or terminal cisternae during muscle activation.

This comparison of ^{45}Ca and ^{63}Ni exchange during K-induced contracture supports the conclusion that the augmented Ca exchange during contracture does not represent Ca diffusing into direct contact with the contractile elements. A muscle can be induced to contract repeatedly in the absence of external Ca^{++} provided another divalent cation such as Ni^{++} is provided in the extracellular fluid. Uptake of Ni is not augmented during contracture and Ni cannot substitute for Ca in activating actomyosin ATPase. Thus, the interpretation of ^{45}Ca movement, first measured by Bianchi and Shanes (8), presently remains unclear. Indeed, recent publications by Bianchi and coworkers (47, 48) have pointed out the disparity between Ca influx and the area beneath the contracture-tension curves.

Our results lead us to conclude that Ca recycles within the muscle fiber during shortening and relaxation. For at least 3-4 hr the Ca content in the fiber is sufficient to maintain contractility in the absence of an external source of this cation. Our conclusion is compatible with the suggestion put forward previously by Weber et al. (15) that calcium recycles between the longitudinal sarcoplasmic reticulum and the myofibrils during contraction and relaxation.

Since light and electron microscopic studies (49–51) suggest that the fluid within the T-tubules is in direct continuity with the extracellular fluid, it may be assumed that Ni rather rapidly diffuses to the membranes of the T-tubules as well as to the sarcolemma. The role of divalent cations in propagated or electrotonic spread of depolarization along the T-tubules and their influence on the permeability of these tubules remain to be investigated. Recent workers (52, 53) have implied a role in E-C coupling for the sites where the T-tubules

are apposed to the membranes of the terminal cisternae but here again, no direct experimental information is available. The present results are consistent with an action of Ni at this location, although the mechanism of such an action remains unclear. Ni may substitute for Ca in a part of the excitation-contraction sequence involving these sites.

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