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Permeability of Sarcoplasmic Reticulum Membrane. The Effect of Changed Ionic Environments on Ca^{2+} Release

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Summary. Permeability properties and the effects of a changed membrane potential on Ca^{2+} release of sarcoplasmic reticulum vesicles of rabbit skeletal muscle were investigated by Millipore filtration. The relative permeability of sarcoplasmic reticulum to solutes determined under conditions of isotope exchange at equilibrium and/or under conditions of net flow of solute and water into the vesicles was as follows: sucrose, Ca^{2+}, Mn^{2+} < gluconate$^-$, choline$^+$, Tris$^+$ < methanesulfonate$^-$ < urea, glycerol, K^+, Na^+, Li^+, Cl^-. Transient membrane potentials were induced by rapidly changing the ionic environment of the vesicles. Knowledge of the relative permeation rates of the above ions allowed prediction of the direction and extent of membrane polarization. Osmotic effects in the polarization measurements due to the rapid influx of solute and water into the vesicles were minimized by using media containing a fast (K^+ or Cl^-) and a relatively slow (gluconate$^-$ or choline$^+$) penetrating ion. $^{45}Ca^{2+}$ efflux from vesicles derived from different parts of the sarcoplasmic reticulum structure was not appreciably changed when vesicles were made more positive inside (choline chloride → potassium gluconate) or more negative inside (potassium gluconate → choline chloride). These studies suggest that part or all of the ion-induced changes in sarcoplasmic reticulum membrane permeability, previously interpreted to indicate "depolarization"-induced Ca^{2+} release, may be due to osmotic effects.

In skeletal muscle Ca^{2+} is released into and reabsorbed from the sarcoplasm through the action of a tubular membranous system, the sarcoplasmic reticulum (Ebashi, Endo & Ohtsuki, 1969; Martonosi, 1971; Tonomura, 1972; MacLennan & Holland, 1975). The myofibrils of muscle cells contract when the free Ca^{2+} concentration of the sarcoplasm reaches 10^{-6} to 10^{-5} M and relax when the level of Ca^{2+} falls again below 10^{-7}M through active removal by sarcoplasmic reticulum. The release of Ca^{2+} is triggered by an action potential at the neuromuscular junction which is thought to be communicated to sarcoplasmic reticulum via the T-tubular system (Bastian & Nakajima, 1974; Costantin, 1975;

Franzini-Armstrong, 1975). While a good deal is known about the mechanism of Ca^{2+} uptake (MacLennan & Holland, 1975), few clues are available as to how a muscle action potential induces the release of Ca^{2+} from sarcoplasmic reticulum.

Since T-tubules and terminal cisternae of sarcoplasmic reticulum are in close contact with each other (Peachey, 1965), it has been suggested that depolarization of the T-system may cause a potential change across the sarcoplasmic reticulum membrane which in turn would induce rapid release of Ca^{2+}. Changes in fluorescence intensity of muscle stained with Nile Blue A (Bezanilla & Horowicz, 1975) and birefringence signals (Baylor & Oetliker, 1975) initiated by electrical stimuli have been correlated with potential changes across the sarcoplasmic reticulum membrane. However, the optical measurements failed to answer the important question whether the recorded changes were the cause or the result of Ca^{2+} release from sarcoplasmic reticulum. Attempts to obtain direct experimental evidence have included polarization of isolated sarcoplasmic reticulum vesicles by electrical stimulation or alteration of pH or ionic environment of the media. While direct electrical stimulation of sarcoplasmic reticulum vesicles proved to be ineffective (Miyamoto & Kasai, 1973), release of Ca^{2+} could be induced by chemical means. Nakamaru & Schwartz (1972) reported that an increase in pH from 6.46 to 7.82 resulted in Ca^{2+} release from preloaded vesicles. Skinned muscle fibers contracted when propionate (Ford & Podolsky, 1970) or methanesulfonate (Nakajima & Endo, 1973) anions of the bathing solution were replaced by the presumably more permeant chloride anions. Using similar conditions, Kasai and Miyamoto (1973) observed an increase in Ca^{2+} release from isolated sarcoplasmic reticulum vesicles. The authors concluded that the change in ionic environment might have depolarized the sarcoplasmic reticulum membrane thereby resulting in release of Ca^{2+}.

A more detailed analysis of the molecular processes involved in Ca^{2+} release may require basic information concerning the permeability of the sarcoplasmic reticulum membrane toward Ca^{2+} and other ions. We have now examined the permeability and osmotic behavior of sarcoplasmic reticulum vesicles. We show evidence that changes in ionic composition which cause release of Ca^{2+} (Kasai & Miyamoto, 1973; Nakajima & Endo, 1973) result in massive influx of salt and water into the vesicles thereby causing osmotic swelling and increase in membrane permeability. Further, our data suggest that changes in ionic environment which only polarized the membrane were ineffective in initiating rapid release of appreciable amounts of Ca^{2+} from sarcoplasmic reticulum vesicles.

Materials and Methods

Reagents

Analytical grade reagents were used. PIPES (Piperazine-N,N′-bis [2-ethane-sulfonic acid]), Tris base, EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-N,N′-tetraacetic acid), potassium gluconate, choline chloride, choline bicarbonate were obtained from Sigma Chemical Co. (St. Louis, Mo.), methanesulfonate from Eastman Kodak (Rochester, N.Y.), sucrose (special enzyme grade) from Schwarz/Mann (Orangeburg, N.Y.), [U-^{14}C]-sucrose, [fructose-1-^{3}H]-sucrose, [^{3}H-methyl]-choline, $^{45}Ca^{2+}$, $^{54}Mn^{2+}$, $^{32}P_i$, $^{36}Cl^-$, $^{22}Na^+$ from New England Nuclear (Boston, Mass.) and D-[U-^{14}C]-gluconate from Amersham/Searle (Arlington Heights, Ill.). A choline methanesulfonate stock solution was prepared by adding methanesulfonate to choline bicarbonate to give a pH of 5 followed by boiling for 5 min under N_2 to remove residual bicarbonate.

Preparations

Sarcoplasmic reticulum vesicles used in this study have been characterized previously (Meissner, 1975). They were prepared from rabbit skeletal muscle by zonal gradient centrifugation. Sarcoplasmic reticulum vesicles were resolved into "light", "intermediate" and "heavy" vesicles by isopycnic centrifugation using a 25 to 50% sucrose density gradient. Unless otherwise indicated, vesicles of "intermediate" buoyant density (d = 1.14–1.17) were used in the experiments.

Assays

Protein was estimated by the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

Measurement of membrane permeability involved prolonged incubation of sarcoplasmic reticulum vesicles with radioactive compounds followed by dilution of the vesicles into a "release" medium. Efflux of radioactive material was terminated by Millipore filtration. Vesicles were initially transferred to a medium of known composition. They were incubated for 4 hr at 0 °C in a large volume (0.5–1.0 mg sarcoplasmic reticulum protein per ml) of unlabelled incubation medium, sedimented by centrifugation for 30 min at 35,000 rpm in a Beckman 42.1 rotor, and resuspended in a small volume (20–25 mg protein/ml) of incubation buffer containing radioactive compounds (80 μC/ml of ^{3}H and/or 20 μC/ml of the other radioisotopes used). The vesicles were kept for 30 to 40 hr at 0 °C before directly analyzed or stored at −60 °C before use. The osmolality of the incubation and release media was adjusted with the aid of an osmometer (Precision Instruments, Inc.). Permeability measurements were carried out at 23 °C. After incubation for 5 min at 23 °C, vesicles were diluted 400-fold into an unlabelled release medium under rapid mixing. Efflux of the radioactive compounds was monitored at various time intervals by placing 1 ml aliquots on a 0.45 μ HAWP Millipore filter followed by rapid rinsing with release medium. The time required to execute filtration and rinsing was 20 sec and was taken into account. In order to stop $^{45}Ca^{2+}$ efflux at time intervals shorter than 20 sec, La^{3+} was used. The ability of La^{3+} to block Ca^{2+} efflux was determined (*cf.* Fig. 1). The radioactivity retained on the filters was counted in 4.5 ml of a scintillation liquid which completely dissolved the filters. The fluid contained 60 g naphthalene, 4.2 g 2,5-diphenyloxazole, 180 mg 1,4-bis-[2-(5-phenyloxazolyl)] benzene and 70 ml water in 900 ml dioxane. Counting of singly and doubly labeled samples was carried out in a Nuclear Chicago Mark II Liquid Scintillation System, using mini vials.

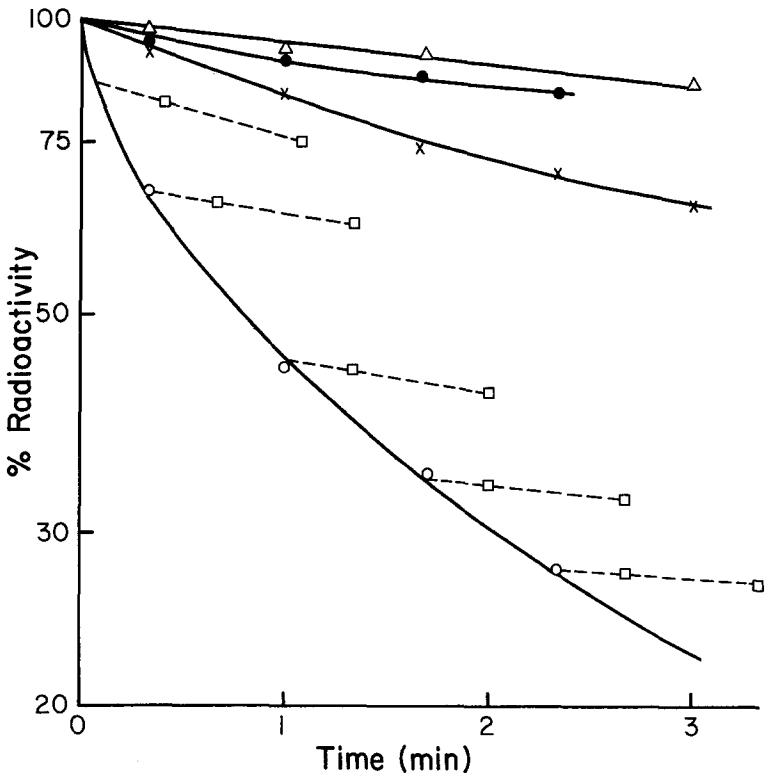

Fig. 1. $^{45}Ca^{2+}$ and ^{3}H-sucrose efflux from sarcoplasmic reticulum vesicles. Vesicles were filled with $^{45}Ca^{2+}$ and ^{3}H-sucrose by incubating them for 36 hr at 0 °C in 5 mM PIPES–10 mM Tris (pH 7.0 at 23 °C), 10 mM $^{45}Ca^{2+}$, 10 mM ^{3}H-sucrose, 2 mM Mg^{2+}, 0.1 mM EDTA and 0.2 M choline chloride. The vesicles were diluted 400-fold into a release medium containing 5 mM PIPES–10 mM Tris (pH 7.0), 2 mM Mg^{2+}, 0.1 mM EDTA, 0.2 M choline chloride and either 10 mM Ca^{2+} ($^{45}Ca^{2+}$: –×–×–, ^{3}H-sucrose: –△–△–), 1 mM EGTA ($^{45}Ca^{2+}$: –○–○–), or 10 mM La^{3+} ($^{45}Ca^{2+}$: –●–●–). Efflux of $^{45}Ca^{2+}$ and ^{3}H-sucrose was determined by measuring the radioactivity retained on the filter. In a second type of experiment, La^{3+} was added to vesicles already present in release media containing 1 mM EGTA. At 5, 20, 60, 100 and 140 sec a tenth volume of 100 mM La^{3+} present in release medium was added. 10 and 50 sec after the addition of La^{3+}, 0.5 ml aliquots were placed on the filters and rinsed for 10 sec with release medium containing 10 mM La^{3+} (–□–□–)

The functional integrity of the vesicles was ascertained by determining their capability to accumulate Ca^{2+} in the presence of ATP. A loss of only 10–25% of their Ca^{2+} uptake and Ca^{2+} loading capacities [measured in the absence and presence of the Ca^{2+} precipitating agent oxalate (Meissner, 1975)] indicated to us that prolonged incubation at 0°C had not appreciably impaired the permeability barrier of the vesicles to Ca^{2+}. Efflux rates of the radioisotopes are expressed as percent release of radioactivity with time. The radioactivity present initially in the vesicles was obtained by first plotting the log of radioactivity retained by the vesicles on the Millipore filters as a function of time and then extrapolating back to the zero time value, which was set equal to 100%. The permeability measurements were carried out at least in duplicate with more than 3 time points each. ^{3}H-sucrose

and $^{45}Ca^{2+}$ permeability of the vesicles varied somewhat with the preparation. For a given preparation the standard errors were generally less than $\pm 15\%$ of the amount of released radioactivity.

Results

Permeability of Sarcoplasmic Reticulum Vesicles to $^{45}Ca^{2+}$ and ^{3}H-Sucrose

As indicated in Fig. 1, sarcoplasmic reticulum vesicles maintained a permeability barrier for $^{45}Ca^{2+}$ and ^{3}H-sucrose when incubated for 36 hr in a buffer containing 0.2 M choline chloride. Similar efflux rates were observed when media were used containing KCl, K gluconate or Tris methanesulfonate instead of choline chloride. Ca^{2+} efflux from the vesicles could be modified by placing the vesicles in release media containing EGTA or La^{3+} instead of 10 mM Ca^{2+} (Fig. 1). At a low external Ca^{2+} concentration, achieved by the addition of 1 mM EGTA to the release medium, an increase of the $^{45}Ca^{2+}$ efflux rate was observed. $^{45}Ca^{2+}$ efflux was reduced to a level close to that of ^{3}H-sucrose when the release medium contained 10 mM La^{3+}. On the other hand, Ca^{2+} efflux rates were not appreciably changed when the free Mg^{2+} concentration in the Ca^{2+}-free release medium was varied from 0.2 mM to 25 mM (not shown). La^{3+} was also found to effectively block further Ca^{2+} efflux when added to the vesicles after they had been transferred to a Ca^{2+} free medium. Use of La^{3+} therefore permitted us to stop Ca^{2+} release at shorter time intervals than would have been possible with the use of the Millipore filtration technique alone. Further, by diluting the vesicles directly into a release medium containing 10 mM La^{3+}, the amount of $^{45}Ca^{2+}$ initially trapped within the vesicles could be estimated with higher accuracy. In agreement with a recent report (Meissner, 1975) but in contrast to an earlier study (Weber, 1971), addition of 1 mM ATP to a Ca^{2+} free release medium did not alter appreciably the permeability of passively loaded vesicles to Ca^{2+}. The $^{45}Ca^{2+}$ inside the vesicles was available for rapid efflux since $^{45}Ca^{2+}$ was fully released within 20 sec when the release medium contained the ionophore X537A (25 μg/ml). In contrast to $^{45}Ca^{2+}$ efflux, permeability of the vesicles to ^{3}H-sucrose was not significantly changed when Ca^{2+} was replaced in the release medium by EGTA or La^{3+}.

Osmotic Fragility

The osmotic fragility of sarcoplasmic reticulum vesicles was tested by altering the osmotic strength of the release medium. Fig. 2 shows

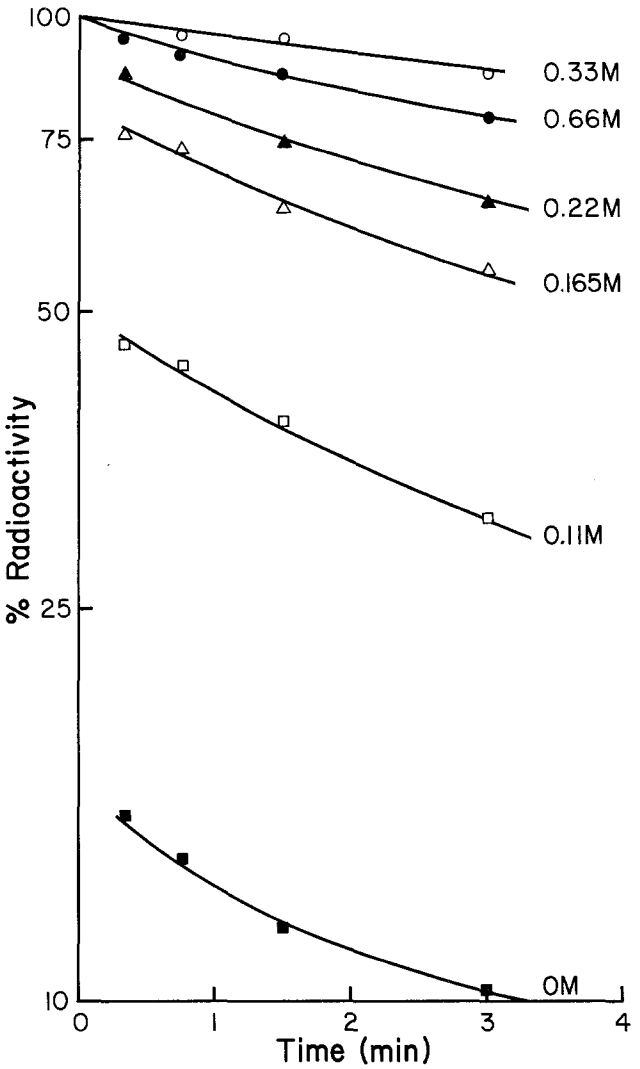

Fig. 2. Efflux of ^{3}H-sucrose from sarcoplasmic reticulum vesicles diluted into media of different osmolality. Vesicles were incubated for 40 hr at 0 °C in a 1 mM PIPES–2 mM Tris buffer (pH 7.0 at 23 °C) containing 0.33 M sucrose. They were then diluted 400-fold into a 1 mM PIPES–2 mM Tris buffer containing 0.66 M Sucrose (–●–●–), 0.33 M sucrose (–○–○–), 0.22 M sucrose (–▲–▲–), 0.165 M sucrose (–△–△–), 0.11 M sucrose (–□–□–) or no sucrose (–■–■–). The 100% value for sucrose content was determined by diluting vesicles into 0.33 M sucrose and by extrapolating to zero time. The intravesicular ^{3}H-sucrose space (3.0 ± 1.0 μl/mg protein) calculated from the zero time value was in good agreement with those obtained by a centrifugation method (Duggan & Martonosi, 1970)

that ^{3}H-sucrose efflux was slow when vesicles containing 0.33 M sucrose were transferred into media of isoosmolal or higher sucrose concentration. Dilution of the vesicles into media of lower sucrose concentration

resulted in increased ^{3}H-sucrose leakage within the first 20 sec. The efflux rates then returned to those observed under isoosmolal conditions. Apparently the vesicles quickly reformed a permeability barrier once the internal osmotic pressure had dropped sufficiently. The concentration of sucrose retained inside the vesicles was somewhat higher than that of the release medium. The apparent difference in osmolality maintained after 20 sec between the inside and outside of the vesicles ranged between 50–100 mosm. We also tested the osmotic fragility of vesicles which were suspended in media containing 10 mM ^{3}H-sucrose, 10 mM $^{45}Ca^{2+}$ and either 0.2 M KCl, K methanesulfonate, choline Cl, or choline methanesulfonate. $^{45}Ca^{2+}$ and ^{3}H-sucrose efflux rates again increased when the osmolality of the media was lowered (not shown). The actual amounts of $^{45}Ca^{2+}$ and ^{3}H-sucrose released were somewhat dependent on the composition of the media and were in general lower than those shown in Fig. 2 for ^{3}H-sucrose. A burst of $^{45}Ca^{2+}$ release was also observed by Kasai and Miyamoto (1973) when sarcoplasmic reticulum vesicles incubated with 0.3 M salt were washed with a medium of low osmolality.

Permeation Rates of Solutes

The relative permeability of sarcoplasmic reticulum to a number of solutes was determined by tracer flux (isotope exchange) and sucrose release measurements. Tracer flux experiments were carried out under conditions of equilibrium between the outside and inside of the vesicles. Low concentrations of labeled ions or sucrose were used in the presence of 0.2 M KCl to minimize charge effects (Duggan and Martonosi, 1970). Reasonably straight lines were obtained on semilog plots suggesting that isotope exchange may be approximated by first order kinetics (Fig. 3). Sucrose and the divalent cations Ca^{2+} and Mn^{2+} did not readily penetrate the membrane. The percent of radioactivity exchanged with time was largely independent of concentrations between 0.1–10 mM Ca^{2+} or 1–25 mM sucrose. Larger univalent ions (gluconate, choline) and phosphate anions passed through the membrane with an intermediate rate, while small univalent ions (Na^{+}, Cl^{-}) passed quickly. When the vesicles were equilibrated in the presence of $^{22}Na^{+}$ or $^{36}Cl^{-}$ (at 1 mM and 0.2 M concentration, respectively), less than 5% of the expected radioactivity remained within the vesicles 20 sec after dilution into the exchange medium. Apparently, $^{22}Na^{+}$ and $^{36}Cl^{-}$ penetrated the membrane too rapidly to allow measurement of their exchange rates with the use of the

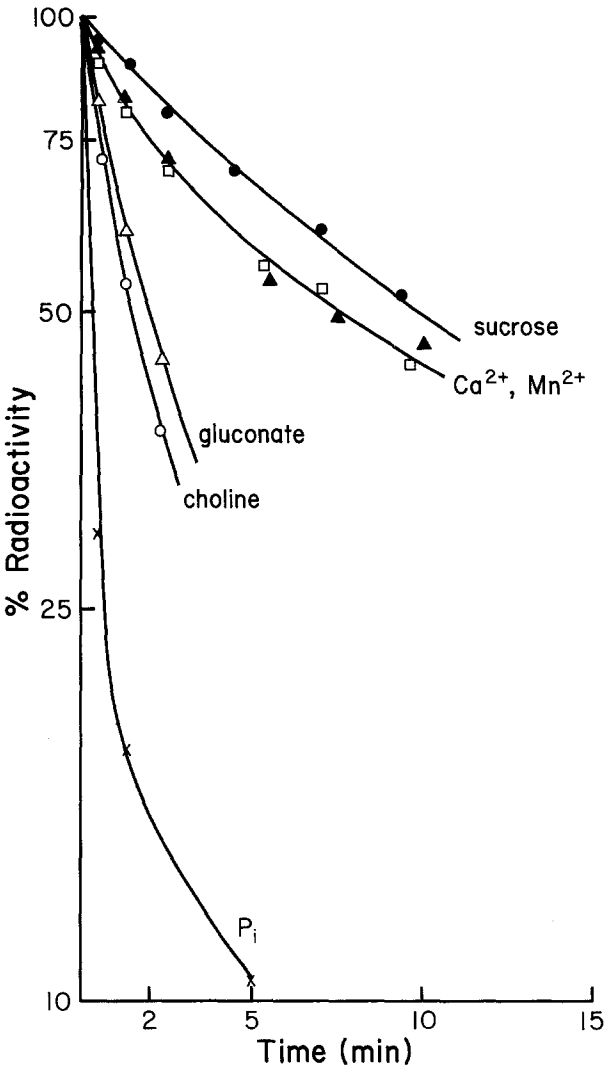

Fig. 3. Radioisotope exchange measurements with sarcoplasmic reticulum vesicles. Vesicles were equilibrated for 40 hr at 0 °C with various radioactive compounds in the presence of 0.2 M KCl, 1 mM ^{3}H- or ^{14}C-labelled sucrose, 2 mM $MgCl_2$, 0.1 mM EDTA and 5 mM PIPES–10 mM Tris, pH 7.0. They were then diluted into a medium of identical composition but containing no radioisotopes. Exchange rates were determined at 23 °C by measuring the radioactivity retained by the vesicles on the filters (*cf. Methods*). The radioactive compounds used were: 1 mM $^{45}Ca^{2+}$ (–□–□–), 1 mM $^{54}Mn^{2+}$ (–▲–▲–), 1 mM ^{14}C-gluconate (–△–△–), 1 mM $^{32}P_i$ (–×–×–), all measured in the presence of 1 mM ^{3}H-sucrose, and 1 mM ^{3}H-choline (–○–○–) measured in the presence of 1 mM ^{14}C-sucrose. Similar ^{3}H- or ^{14}C-sucrose exchange rates (–●–●–) were observed in all cases. ^{3}H- or ^{14}C-sucrose, ^{14}C-gluconate, ^{3}H-choline and $^{32}P_i$ intravesicular spaces extrapolated back to zero time corresponded to 3.0 ± 0.4 µl/mg protein

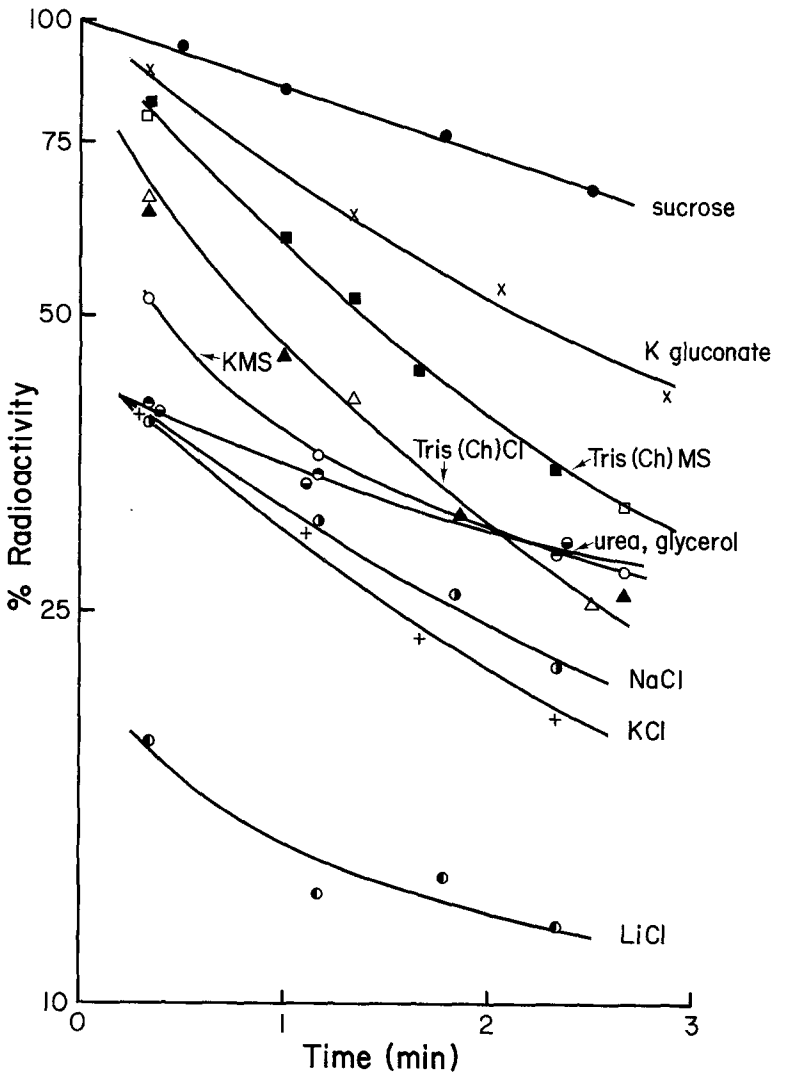

Fig. 4. The effect of various solutes on the efflux of ^{3}H-sucrose from sarcoplasmic reticulum vesicles containing 0.33 M ^{3}H-sucrose. Vesicles were incubated for 40 hr at 0 °C in a 1 mM PIPES–2 mM Tris buffer (pH 7.0 at 23 °C) containing 0.33 M ^{3}H-sucrose. ^{3}H-sucrose efflux was initiated by diluting them into a 1 mM PIPES–2 mM Tris buffer containing either 375 mosm sucrose (–●–●–), K gluconate (–×–×–), Tris (–□–□–) or choline (–■–■–) methanesulfonate, Tris (–△–△–) or choline (–▲–▲–) chloride, K methanesulfonate (–○–○–), KCl (–+–+–), NaCl (–◑–◑–), LiCl (–◐–◐–) urea (–⊖–⊖–) or glycerol (–⊖–⊖–)

Millipore filtration technique. Permeability of vesicles to sucrose was similar in all of the ion exchange experiments suggesting that the small amounts of tracer used did not appreciably alter overall membrane permeability.

Above we have presented evidence that sarcoplasmic reticulum vesicles are osmotically active (Fig. 2) and that the membrane is selectively permeable (Fig. 3). One would then expect that placement of the vesicles into a medium containing a faster penetrating solute than was present in the original medium would result in net influx of solute and water. In order to lower the internal osmotic pressure, the vesicles would have to release the solute originally present inside the compartment. Fig. 4 indicates that vesicles equilibrated with 0.33 M ^{3}H-sucrose, a rather slow penetrating substance, rapidly release ^{3}H-sucrose when diluted into media containing the test solutes at 375 mosm concentration. Comparison of ^{3}H-sucrose leakage rates with the radioisotope exchange rates indicated reasonable agreement between tracer flux and bulk movement of the solutes across the membrane. Thus, a solution containing two ions which can readily penetrate the membrane (e.g. Na^+ and Cl^-) was effective in causing rapid release of ^{3}H-sucrose. The initial burst of ^{3}H-sucrose release was followed by a slower ^{3}H-sucrose leakage rate suggesting that a normal permeability barrier was reestablished within 20 sec after transfer of the vesicles into the release medium. When experiments were carried out using one fast and one slow penetrating ion (e.g. choline Cl instead of NaCl or K gluconate instead of KCl), increased ^{3}H-sucrose leakage rates were also observed but the initial burst of ^{3}H-sucrose release was reduced or not present. ^{3}H-sucrose release measurements were also carried out to obtain information about the relative permeability of some additional neutral and ionized solutes (Fig. 4). Essentially, two groups of solutes could be distinguished. Urea, glycerol, KCl, NaCl and LiCl caused a rapid and massive efflux of ^{3}H-sucrose. Tris HCl, choline Cl and choline methanesulfonate were only moderately effective in increasing the initial ^{3}H-sucrose efflux rate. K methanesulfonate was between these two groups. Assuming that influx of ions is largely determined by the slower of the two penetrating ions present in the test medium, the ^{3}H-sucrose release experiments suggest the following order of increasing permeation rates: sucrose < gluconate < Tris, choline < methanesulfonate < Na^+, K^+, glycerol, urea < Li^+, Cl^-.

Effect of Membrane Polarization of $^{45}Ca^{2+}$ and ^{3}H-Sucrose Efflux

We have reexamined the effect of "membrane polarization" on Ca^{2+} release from sarcoplasmic reticulum (Ford & Podolsky, 1970; Kasai & Miyamoto, 1973; Nakajima & Endo, 1973). Vesicles were incubated

in a medium containing choline chloride and were then transferred into a release medium containing isoosmolal KCl. As shown above (Figs. 3 and 4), K^+ will traverse the membrane more rapidly than choline$^+$, thus changing the membrane potential in such a way that the inside of the membrane becomes more positive with respect to the outside. As indicated in Fig. 5, there occurred a rapid initial burst of $^{45}Ca^{2+}$ and ^{3}H-sucrose release. Within 20 sec, measured efflux rates were again normal. An initial burst occurred even though 10 mM La^{3+} (instead of 1 mM EGTA) was present at zero time in the release medium or was added several seconds later. However, the amounts of Ca^{2+} released were significantly lower than those observed in the absence of La^{3+}. Thus La^{3+} appeared to block the subsequent slower Ca^{2+} efflux, just as in control experiments where no change in ionic environment had taken place (cf. also Fig. 1).

Table 1 summarizes a set of similar experiments in which vesicles were incubated and diluted into various media composed of either Tris-HCl, Tris methanesulfonate, KCl or K methanesulfonate. A significant increase in membrane permeability was observed in five of the eleven given combinations of incubation and release media. Of these five, one was predicted to polarize the membrane more negative inside, while three were assumed to make the membrane more positive inside. The increase in membrane permeability did not appear therefore to be dependent on the direction of membrane polarization. Another important finding was that $^{45}Ca^{2+}$ efflux paralleled ^{3}H-sucrose permeation.

One interpretation of the membrane polarization experiments described in Fig. 5 and Table 1 is that increased membrane permeability was caused by osmotic swelling and possibly rupture of vesicles because of influx of solutes and water. To test this possibility, release media were made hypertonic by supplementing them with a slow penetrating solute such as sucrose. As shown in the last two columns of Table 1, addition of 0.11 or 0.25 M sucrose to the release media suppressed the initial burst in $^{45}Ca^{2+}$ and ^{3}H efflux suggesting that increased membrane permeability was at least in part due to osmotic effects. When both the incubation and release media contained 0.1 M sucrose, release occurred at slightly reduced levels (not shown). However, an initial increase in $^{45}Ca^{2+}$ and ^{3}H-sucrose release was again observed.

Previously it was found that purified sarcoplasmic reticulum consists of vesicles of differing buoyant density (Meissner, 1975). Three subfractions, "light", "intermediate" and "heavy" vesicles accounting for 10–15%, ~75% and 10–15% of the vesicles, respectively, were isolated

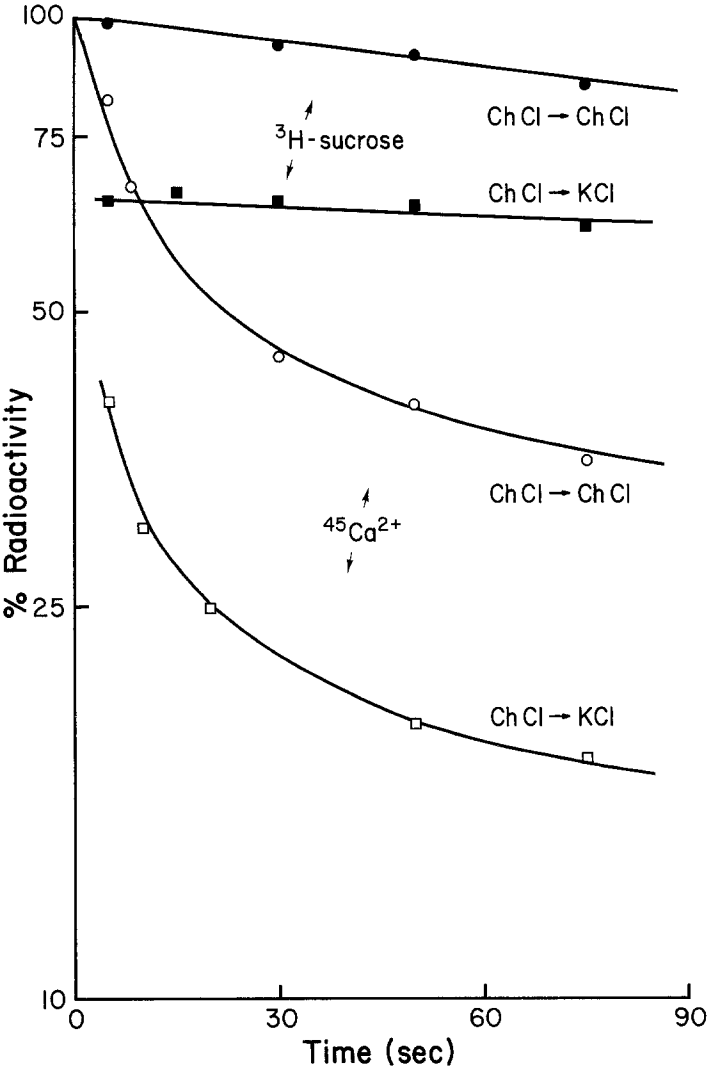

Fig. 5. $^{45}Ca^{2+}$ and ^{3}H-sucrose efflux from sarcoplasmic reticulum vesicles which were transferred from choline chloride to KCl. Vesicles were incubated for 36 hr at 0 °C in a 5 mM PIPES–10 mM Tris buffer (pH 7.0 at 23 °C) containing 10 mM $^{45}Ca^{2+}$, 10 mM ^{3}H-sucrose, 2 mM Mg^{2+}, 0.1 mM EDTA and 375 mosm choline chloride. They were then diluted 400-fold into release media containing 5 mM PIPES–10 mM Tris (pH 7.0), 10 mM sucrose, 1 mM EGTA, 2 mM Mg^{2+}, 0.1 mM EDTA and either 400 mosm choline chloride (-○-○-, -●-●-) or KCl (-□-□-, -■-■-). The percent of $^{45}Ca^{2+}$ (-○-○-, -□-□-) and ^{3}H (-●-●-, -■-■-) radioactivity retained by the vesicles is given. The 100% values for ^{3}H-sucrose and $^{45}Ca^{2+}$ content were estimated by diluting vesicles into the choline Cl release medium containing in addition 10 mM La^{3+} and by extrapolating to zero time. Five and 10 sec time points were obtained by adding one tenth volume of 100 mM La^{3+} present in release medium to the vesicles at 5 or 10 sec. Ten and 50 sec after the addition of La^{3+}, 0.5 ml aliquots were placed on filters and rinsed for 10 sec with release medium containing 10 mM La^{3+}. Slow $^{45}Ca^{2+}$ and ^{3}H-sucrose efflux rates were observed in the presence of 10 mM La^{3+} permitting estimation of 5 and 10 sec time points by extrapolation (*cf.* Fig. 1). It may be noted that corresponding $^{45}Ca^{2+}$ and ^{3}H-sucrose efflux data were obtained when vesicles were placed on a Millipore filter and then rinsed for 5 or 10 sec with a medium containing either choline Cl or KCl

Table 1. $^{45}Ca^{2+}$ and ^{3}H-sucrose efflux from sarcoplasmic reticulum vesicles diluted into media of different composition and osmolality

Incubation medium	Release medium	Radioisotope	Expected Membrane potential (inside → outside)	[Sucrose] in release medium 10 mM	110 mM	250 mM
				% release in 5 sec		
TrisMS	TrisMS	^{3}H-sucrose		1	4	
		$^{45}Ca^{2+}$	(0)	9	10	
	Tris HCl	^{3}H-sucrose		2	4	
		$^{45}Ca^{2+}$	(−)	12	13	
	KMS	^{3}H-sucrose		21	4	
		$^{45}Ca^{2+}$	(+)	40	20	
	KCl	^{3}H-sucrose	(?)	42	21	7
		$^{45}Ca^{2+}$		70	45	15
Tris HCl	Tris HCl	^{3}H-sucrose	(0)	3	5	
		$^{45}Ca^{2+}$		10	9	
	KCl	^{3}H-sucrose	(+)	31	7	
		$^{45}Ca^{2+}$		58	25	
	KMS	^{3}H-sucrose	(+)	20	8	10
		$^{45}Ca^{2+}$		49	36	27
KMS	KMS	^{3}H-sucrose	(0)	3	2	
		$^{45}Ca^{2+}$		7	7	
	KCl	^{3}H-sucrose	(−)	27	5	4
		$^{45}Ca^{2+}$		37	19	16
KCl	KCl	^{3}H-sucrose	(0)	4		
		$^{45}Ca^{2+}$		15		
	Tris HCl	^{3}H-sucrose	(−)	5		
		$^{45}Ca^{2+}$		18		

Sarcoplasmic reticulum vesicles were incubated in media containing 5 mM PIPES–10 mM Tris (pH 7.0 at 23 °C), 10 mM $^{45}Ca^{2+}$, 10 mM ^{3}H-sucrose, 2 mM Mg^{2+}, 0.1 mM EDTA and either 375 mosm Tris methanesulfonate (Tris MS), KCl, Tris HCl or K methanesulfonate (KMS). The vesicles were then diluted into media containing 5 mM PIPES–10 mM Tris, 10 mM sucrose, 1 mM EGTA, 0.1 mM EDTA, 2 mM Mg^{2+} and 400 mosm of the indicated salt solution. Five sec after transfer of the vesicles into the release medium, a solution of 100 mM La^{3+} in release medium was added to a final concentration of 10 mM La^{3+} (*cf.* Fig. 1). The sucrose concentration of some of the release media was increased from 10 to 110 or 250 mM. The direction of membrane polarization as predicted from the relative permeation rates of the ions (*cf.* Figs. 3 and 4) is given. In one case, using incubation and release media containing Tris methanesulfonate and KCl, respectively, the permeation data did not allow a clear prediction concerning the sidedness of the induced membrane potential.

Table 2. The effect of a changed ionic environment on $^{45}Ca^{2+}$ efflux from light, intermediate and heavy sarcoplasmic reticulum vesicles

Composition		Light SR vesicles	Intermediate SR vesicles	Heavy SR vesicles
Incubation medium	Release medium	% Release in 5 sec		
K gluconate	K gluconate	9	4	3
K gluconate	Choline Cl	15	15	5
Choline Cl	Choline Cl	4	3	12
Choline Cl	K gluconate	12	9	10

Sarcoplasmic reticulum vesicles were incubated in media containing 5 mM Pipes–10 mM Tris (pH 7 at 23 °C), 10 mM $^{45}Ca^{2+}$, 2 mM Mg^{2+}, 0.1 mM EDTA and either 375 mosm K gluconate or choline chloride. The vesicles were then diluted into media containing 5 mM PIPES–10 mM Tris, 1 mM EGTA, 0.1 mM EDTA, 2 mM Mg^{2+} and either 400 mosm K gluconate or choline chloride. Five sec after transfer of the vesicles into the release medium a solution of 100 mM La^{3+} in release medium was added to a final concentration of 10 mM La^{3+} (*cf.* Fig. 1). The amounts of Ca^{2+} retained by the vesicles were 80, 80 and 120 nmoles Ca^{2+}/mg protein for light, intermediate and heavy vesicles, respectively. These data were obtained using La^{3+} and extrapolating back to zero time. Ca^{2+} uptake capacities of light, intermediate and heavy vesicles were previously found to be 90, 120 and 120 nmoles Ca^{2+}/mg protein, respectively (Meissner, 1975).

from a sucrose gradient and characterized. Morphological data suggested that light and heavy vesicles may have been derived from the longitudinal sections and terminal cisternae of sarcoplasmic reticulum, respectively. According to Winegrad (1970), longitudinal sections and terminal cisternae are preferentially involved in the reabsorption and release of Ca^{2+}, respectively, during the relaxation-contraction cycle of skeletal muscle. It was therefore of interest to see how an alteration in membrane potential would influence Ca^{2+} efflux from the three types of vesicles. Changes in membrane potential were induced with the use of media containing a fast penetrating ion (K^+ or Cl^-) and a relatively slow penetrating counter ion (gluconate$^-$ or choline$^+$). Use of the two slow ions should minimize osmotic effects since only a moderate increase in the amount of intravesicular ions would be expected to occur (*cf.* Figs. 3 and 4). The amounts of Ca^{2+} retained by the filtered and washed vesicles were close to the Ca^{2+} uptake capacities of the vesicles (measured in the absence of oxalate and in the presence of ATP) (Meissner, 1975). Thus, vesicles which were close to maximally loaded with Ca^{2+} were used in these experiments. Table 2 shows that $^{45}Ca^{2+}$ efflux from the various vesicle fractions was not significantly altered when the membranes were

made more positive inside (choline chloride → potassium gluconate) or more negative inside (potassium gluconate → choline chloride). Similarly slow Ca^{2+} efflux rates were observed when membrane "depolarization" experiments (potassium gluconate → choline chloride) were performed with intermediate vesicles which were actively loaded using 5 mM ATP and 50 μM $^{45}Ca^{2+}$ (Meissner, 1975) and which were diluted into release media containing 1 mM ATP.

Discussion

This study shows that the sarcoplasmic reticulum membrane is selectively permeable. A relatively high barrier was found for sucrose and divalent cations (Ca^{2+}, Mn^{2+}), while small neutral solutes (urea, glycerol) and univalent ions (K^+, Na^+, Li^+, Cl^-) readily passed through the membrane. Larger univalent cations (Tris, choline) and anions (gluconate, methanesulfonate) traversed the membrane with an intermediate rate. As is characteristic of a closed compartment with a semipermeable membrane, sarcoplasmic reticulum was found to increase its permeability to small molecules and ions, perhaps by osmotic swelling or a transient rupture of the membrane, when placed in hypotonic solutions or in media containing a faster penetrating solute than was present inside the vesicle. The ionic environment of the vesicles was manipulated to induce transient membrane potentials across the sarcoplasmic reticulum membrane. We found that Ca^{2+} efflux from the vesicles was not significantly increased when osmotic effects were minimized.

The permeability properties of the sarcoplasmic reticulum membrane have been previously investigated to a limited extent. Low passive $^{45}Ca^{2+}$ efflux rates comparable to those observed in this study have been reported by Makinose and Hasselbach (1965), Weber, Herz and Reiss (1966), Duggan and Martonosi (1970), and Inesi, Millman and Eletr (1973). Evidence for the permeation of anions such as fluoride, oxalate, orthophosphate and pyrophosphate was provided by the observation that these ions potentiate Ca^{2+} accumulation by serving as precipitating agents for the transported Ca^{2+} (Hasselbach & Makinose, 1963; Martonosi & Feretos, 1964). With the use of a centrifugation method, Duggan and Martonosi (1970) found that sarcoplasmic reticulum vesicles form a permeability barrier against larger molecules such as inulin (MW ~ 5,000) while sucrose, urea, acetate, chloride, and citrate fully penetrated the interior of the vesicles within one hour. We found it

necessary to carry out our experiments for a shorter time in order to distinguish between the permeation rates of small solutes.

It is well accepted that many cells and subcellular organelles will behave as good osmometers. For example, red blood cells will swell when placed into a hypotonic medium because of water uptake. They will change initially from a biconcave disk to an almost perfect shape. In this way they can increase their volume without appreciably changing the surface area. Further osmotic stress will finally result in disruption of the membrane and release of hemoglobin. Seeman (1967) observed that the erythrocyte membrane acquired holes within the first 15–25 sec after initiation of hypotonic hemolysis. Sarcoplasmic reticulum vesicles appeared to behave similarly when placed into hypotonic media or solutions containing a faster penetrating solute than was present in the original medium. A fast and a slow efflux component could be distinguished (Figs. 2, 4 and 5). Whether the sarcoplasmic reticulum membrane actually formed holes during the initial release phase is not known at present. Curvature of the semilogarithmic plot of the slow efflux component (Figs. 2, 4 and 5), seen also under conditions of equilibrium (Fig. 3), would be consistent with the presence of heterogeneously sized vesicles (Meissner, 1975) which possibly vary in membrane permeability or osmotic fragility.

A dramatic change in membrane permeability in response to a muscle action potential is expected to occur in sarcoplasmic reticulum *in vivo*. Examination of Ca^{2+} movement during muscle contraction in the presence of the Ca^{2+} indicators murexide (Jöbsis & O'Connor, 1966) and aequorin (Ashley & Ridgway, 1970) has shown that Ca^{2+} release from sarcoplasmic reticulum is a rapid process which occurs before and is nearly completed by the time tension develops. Assuming that 0.1 to 0.2 μmole Ca^{2+}/g muscle is released in 5 msec when muscle activity is initiated (Weber & Herz, 1963) and using a sarcoplasmic reticulum content of 5 mg protein/g muscle (Meissner, Conner & Fleischer, 1973), a release rate of 4–8 μmoles Ca^{2+}/mg protein × sec is calculated. It is of interest that the passive Ca^{2+} efflux rate into a Ca^{2+} free medium was only 0.5–1.0 nmoles/mg protein × sec or 0.2–0.4 pmole/cm^2 × sec for vesicles having an average diameter of 750 Å. For comparison, Ca^{2+} flux rates of 0.1–0.2 pmole/cm^2 × sec have been reported for resting nerve (Blaustein & Hodgkin, 1969) and muscle (Bianchi & Shanes, 1959) where a positive equilibrium potential for Ca^{2+} (120–150 mV) provides a large driving force for inward Ca^{2+} movement. In both tissues Ca^{2+} influx is considerably increased during an action potential. To account for

the large differences in sarcoplasmic reticulum permeability during muscle activity, it is tempting to speculate that the membrane contains specific Ca^{2+} channels which open and close in response to an external stimuli. The presence of a specific Ca^{2+} channel has been inferred from lipid bilayer experiments where a detergent treated, inactive Ca^{2+} pump protein preparation increased the conductance of the bilayer in the presence of Ca^{2+} (Shamoo & MacLennan, 1974). Passive Ca^{2+} movement through a channel (or carrier) in native sarcoplasmic reticulum would be also consistent with our observation that Ca^{2+} efflux into a Ca^{2+} free medium was reduced by Ca^{2+} or La^{3+} when these two ions were added to the outside of the vesicles.

A rapid change in ionic environment of isolated sarcoplasmic reticulum vesicles, which has been thought to induce a transient change in membrane potential, has been found to promote membrane permeability to Ca^{2+} (Kasai & Miyamoto, 1973). We have found that manipulation of ionic environments may result in osmotic swelling and possibly rupture of sarcoplasmic reticulum vesicles. Vesicles were highly permeable to small univalent ions Na^+, K^+ and Cl^-. As a consequence, transfer of vesicles from a potassium methanesulfonate medium to one containing KCl resulted in rapid release of Ca^{2+}, as previously reported (Kasai & Miyamoto, 1973), and ^{3}H-sucrose. Rapid efflux of the two solutes could be suppressed by making the release medium hypertonic. It appears then that part if not all of the Ca^{2+} release is due to osmotic effects rather than to the presence of a membrane potential.

Ion-induced Ca^{2+} release has also been observed in skinned muscle fibers (Ford & Podolsky, 1970; Nakajima & Endo, 1973). As in isolated sarcoplasmic reticulum vesicles, replacement of methanesulfonate (or propionate) ions by Cl^- was found to induce rapid release of Ca^{2+} from the internal membrane system of skinned fibers. Interestingly, this ion-induced Ca^{2+} release could be inhibited by 40 mM or higher concentrations of sucrose (Thorens & Endo, 1975). Although the authors offer no explanation for this result, it seems to be consistent with the interpretation that part or all of the Ca^{2+} release observed in the absence of sucrose is caused by osmotic forces. Some differences appear to exist between isolated vesicles and skinned fibers when K^+ is replaced by the less permeant $Tris^+$ (Costantin & Podolsky, 1967; Nakajima & Endo, 1973). Nakajima and Endo reported that such an ion replacement caused muscle contraction in fibers heavily loaded with Ca^{2+}. However, no response was observed in fibers which were lightly loaded with Ca^{2+}. These latter experiments were carried out with a partially skinned muscle

fiber preparation which reportedly was free of resealed T-tubules. Ca^{2+} release caused by electrical stimulation of the T-tubules could thus be largely eliminated.

Knowledge of the relative permeation rates of a number of solutes allowed us to design experiments in which we could temporarily polarize the membrane without encountering massive influx of solute within the duration of the experiment. Two such conditions were obtained when the solutes potassium gluconate and choline chloride were used. Data of Figs. 3 and 4 suggest that the presence of the relatively slow penetrating ions gluconate$^-$ or choline$^+$ in the release media may decrease the rate of solute influx to the vesicles. Further, the use of solutes with similar permeation rates, like K gluconate and choline Cl, should diminish the occurance of a significant increase in the amount of intravesicular ions since as one solute moves into the vesicles, the other will move out with a similar rate. At the same time, replacement of potassium gluconate by choline chloride was expected to result in a more rapid passage of K^+ and Cl^- than of choline$^+$ and gluconate$^-$ thereby making the membrane negative inside with respect to the outside. Reversal of the order of the solutes was expected to alter the membrane potential in the opposite direction. The data in *Results* (Section- *Permeation Rates of Solutes*) suggest that K^+ and Cl^- fluxes are on the order of ten times faster than those of gluconate$^-$ and choline$^+$. Accordingly, an initial change in membrane potential of approximate 50 mV (either negative or positive inside) can be calculated according to the constant field equation of Hodgkin and Katz (1949). Since the two slow ions could also move across the membrane, the potential caused by such a change in ionic environment would be expected to be only transient. Using "light", "intermediate" and "heavy" sarcoplasmic reticulum vesicles, Ca^{2+} efflux was found to take place near the level of the control, i.e. when the ionic environment had not been altered (Table 2). These experiments raise the question whether a change in membrane potential in itself is effective in inducing rapid release of Ca^{2+}.

The permeability and electrochemical properties of sarcoplasmic reticulum in intact muscle are not known due to its inaccessibility to direct ion flux and electrophysiological measurement. For example, it is not even clear whether a potential difference exists across the sarcoplasmic reticulum membrane in relaxed muscle or what the magnitude and direction of polarization would be. Disruption of muscle and use of media having a different composition than the sarcoplasm during the isolation of skinned muscle fibers and sarcoplasmic reticulum vesicles may well

result in loss or alteration of a membrane potential. It follows that in order to be able to study the Ca^{2+} release mechanism of sarcoplasmic reticulum *in vitro,* it may be first necessary to reestablish a membrane potential similar to that present in muscle.

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