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Evidence for a K^+, Na^+ Permeable Channel in Sarcoplasmic Reticulum

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Summary. Potassium and sodium cation permeabilities of skeletal sarcoplasmic reticulum vesicles were characterized by means of ^{3}H-choline, $^{22}Na^+$ and $^{86}Rb^+$ isotope efflux and membrane potential measurements. Membrane potentials were generated by diluting K gluconate filled sarcoplasmic reticulum vesicles and liposomes into Tris or Na gluconate media, in the presence or absence of valinomycin, and were measured using the voltage-sensitive membrane probe 3,3′-dipentyl-2,2′-oxacarbocyanine. About 2/3 of the sarcoplasmic reticulum vesicles, designated Type I, were found to be permeable to Rb^+, K^+ and Na^+. The remaining 1/3, Type II vesicles, were essentially impermeable to these ions. The two types of vesicles were impermeable to larger cations such as choline or Tris. Both were present in about the same ratio in fractions derived from different parts of the reticulum structure. Studies with cations of different size and shape suggested that in Type I vesicles permeation was restricted to molecules fitting through a pore with a cross-section of 4–5 Å by 6 Å or more. When vesicles were sonicated, vesicles permeable to K^+ decreased more than those impermeable to K^+. These data suggest the existence of K^+, Na^+ permeable channels which are probably randomly dispersed in the intact reticulum structure at an estimated density of 50 pores/μm^2. The function of the channel may be to allow rapid K^+ movement to counter Ca^{2+} fluxes during muscle contraction and relaxation.

It is now generally accepted that release of Ca^{2+} from sarcoplasmic reticulum is triggered by depolarization of the transverse tubular system (T-system) of skeletal muscle (Ebashi, 1976; Endo, 1977). Parts of the sarcoplasmic reticulum, the terminal cisternae, are in close contact with T-tubules (Peachey, 1965). According to Winegrad (1970) most of the Ca^{2+} required for muscle contraction is released from terminal cisternae of sarcoplasmic reticulum. Ca^{2+} reuptake occurs by way of an ATP-driven pump which appears to be present in all regions of the sarcoplasmic reticulum structure (Meissner, 1975). The exact mechanism by which Ca^{2+} is rapidly released from sarcoplasmic reticulum is unclear. The nature of the signal received by sarcoplasmic reticulum during T-tubule depolarization that triggers Ca^{2+} release is also not well established.

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However, it seems likely that Ca^{2+} release may require or at least be influenced by the movement of other ions across the sarcoplasmic reticulum membrane. Experiments directed at defining the mechanism of Ca^{2+} release, specifically those testing the hypothesis that a change in reticulum membrane potential triggers Ca^{2+} release (Endo, 1977), then require knowledge of sarcoplasmic reticulum membrane permeability to small ions.

In this paper we describe in detail the permeability of sarcoplasmic reticulum vesicles to small cations. We find that one type (designated Type I) is highly permeable to K^+ and Na^+ and certain other small cations, while the second type (Type II) is relatively impermeable to these cations. Data suggest that Type I vesicles contain a K^+, Na^+ permeable pore or channel. Some evidence for the presence of sarcoplasmic reticulum vesicles which differ in their permeability to small cations has been presented previously (McKinley & Meissner, 1977, 1978).

Materials and Methods

Reagents

Gluconic acid (technical grade; Eastman, Rochester, N.Y.) was treated with charcoal before use. Other reagents used were of reagent grade. The fluorescent dye 3,3′-dipentyl-2,2′-oxacarbocyanine was the generous gift of Dr. Alan Waggoner (Amherst College, Amherst, Mass.)

Preparations

Sarcoplasmic reticulum vesicles used in most parts of this study have been characterized previously (Meissner, 1975). They were prepared from rabbit skeletal muscle by zonal gradient centrifugation. Sarcoplasmic reticulum vesicles, resolved into "light", "intermediate", and "heavy" buoyant density vesicles ($d = 1.12$–1.20), were used in the experiments. Vesicles were extracted with 600 mM KCl to remove adhering protein, resuspended in 0.3 M sucrose, quick-frozen, and kept at −60 °C until further use. Sarcoplasmic reticulum vesicles were also purified from rat leg muscle and lobster abdominal muscle as previously described (Meissner, 1974), except that a Beckman SW 27 rotor was used instead of Beckman zonal rotors.

Liposomes were prepared from phospholipids extracted from sarcoplasmic reticulum vesicles purified as described above. Lipids were extracted with chloroform-methanol (2:1 vol/vol) and back extracted to remove nonlipid material (Folch, Lees & Sloane-Stanley, 1957). Neutral lipids were removed by column chromatography using silicic acid (UNISIL-Clarkson Chemical, Williamsport, Penn.) (Fleischer *et al.*, 1962). The phospholipid was dried under argon and suspended in media containing 200 mM of either Na or K gluconate, 5 mM Mg gluconate, 20 μM Ca gluconate, and 10 mM Pipes-Tris, pH 7.0 at 0 °C. In order to create single-walled vesicles, vesicles were sonicated for 90 min at 0 °C

under Ar, nine times the time required for visual clearing to occur (Finer, Flook & Hauser, 1971). Large residual multilayered vesicles were pelleted and removed by centrifugation for 120 min at 40,000 rpm in a 65 Spinco rotor (Barenholz *et al.*, 1977).

Assays

Protein was estimated by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Total phosphorus was measured as an estimate of lipid phosphorus (Meissner & Fleischer, 1971).

Measurement of membrane permeability was carried out at 4 °C as previously described (Meissner & McKinley, 1976). Vesicles were incubated for 4 hr at 0 °C in a large volume (0.5–1.0 mg sarcoplasmic reticulum protein per ml) of unlabeled incubation medium, sedimented by centrifugation for 45 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 μCi/ml of ^{3}H and/or 20 μCi/ml of the other radioisotopes used). The vesicles were kept for 30 to 40 hr at 0 °C before being directly analyzed or stored at −60 °C for later use. The osmolality of the incubation and dilution media was adjusted with the aid of an osmometer (Precision Instruments, Inc.). Vesicles were diluted 400-fold into an unlabeled release medium at 4 °C under rapid mixing. Efflux of the radioactive compounds was monitored at various time intervals by placing 1-ml aliquots on a 0.45 μm HAWP Millipore filter followed by rapid rinsing with unlabeled medium. The time required to execute filtration and rinsing was 20 sec and was taken into account.

Membrane potentials were visualized with the use of the fluorescent dye 3,3′-dipentyl-2,2′-oxacarbocyanine iodide [diO-C_5-(3)] (Sims *et al.*, 1974). Vesicles permeable to K^+ were polarized by dilution from a high K^+ to low K^+ medium containing 1.7 μM of diO-C_5-(3). Fluorescence measurements were made at 0–2 °C under rapid stirring in a Perkin-Elmer MPF-3L fluorometer, equipped with a water cooled cell. Excitation was at 470 nm, and emission was recorded at 495 nm. Both slits were set at 0.52 mm to give half-band widths of 4 nm. The amount of vesicles added (about 17 μg protein/ml or 0.6 μg phosphorus/ml) was chosen so that the fluorescence was little changed when vesicles were diluted into a medium identical to the incubation medium.

The following expedients were employed to deal with the problem of dye binding to glass and quartz surfaces. Dye aliquots were added to the fluorometer cuvette directly from a 5-ml acetone solution containing 1 mM dye, using a Hamilton syringe which was not rinsed between sampling. The solution was kept capped in the dark at 0–2°. The quartz cuvette was seasoned before an experiment by pre-incubation on ice with two changes of K gluconate medium containing 1.7 μM dye. Between runs the cuvette was normally rinsed with distilled water only.

Results

Isotope Exchange Experiments

Apparent isotope spaces and efflux rates of "intermediate" rabbit skeletal sarcoplasmic reticulum vesicles to ^{3}H-choline, $^{22}Na^+$ and $^{86}Rb^+$ were determined by Millipore filtration. $^{86}Rb^+$ was used as a substitute for K^+ because of the lack of a convenient radioisotope for

K^+. Radioisotopes were allowed to equilibrate across the vesicle membranes by prolonged incubation of vesicles in the presence of the radioactive compounds. Vesicles were then diluted into an unlabeled dilution medium with a composition similar to the incubation medium and were collected at various time intervals on Millipore filters. Radioactivity remaining with the vesicles on the filters was determined. As indicated in Fig. 1, sarcoplasmic reticulum vesicles maintained a permeability barrier for ^{3}H-choline. The data is expressed as % of apparent

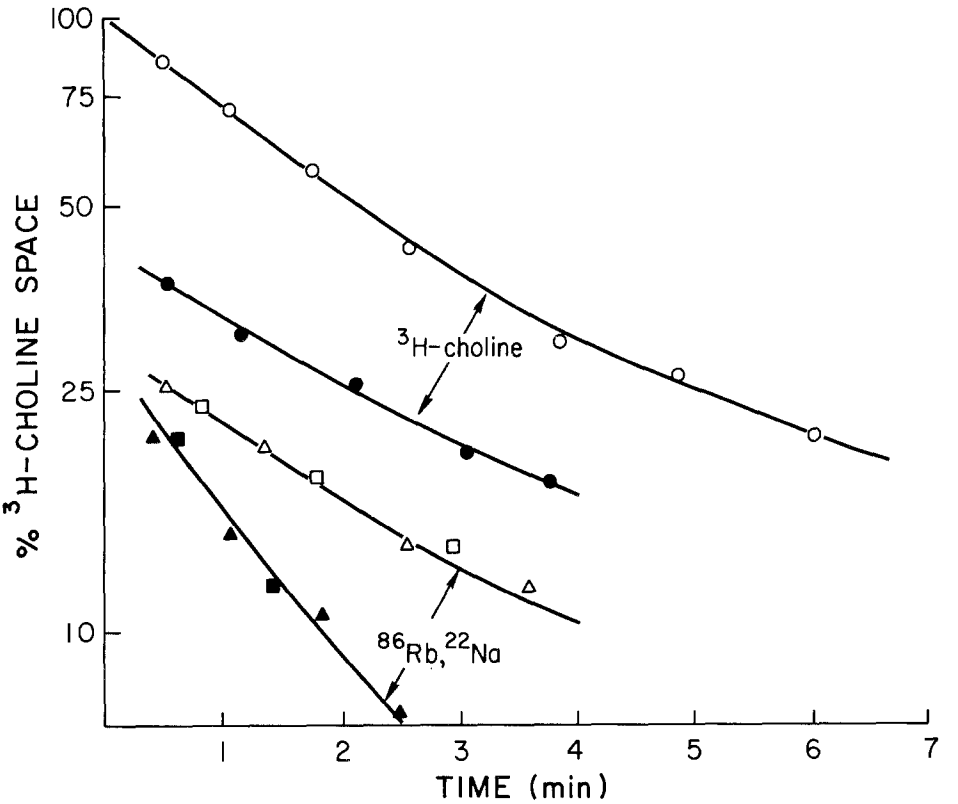

Fig. 1. Measurement of ^{3}H-choline, $^{22}Na^+$ and $^{86}Rb^+$ ion efflux rates and isotope spaces for vesicles filled with choline chloride medium and diluted into either choline chloride or KCl. Sarcoplasmic reticulum vesicles (18 mg protein/ml) were incubated for 24 hr at 0 °C in the following media with ^{3}H-choline and either $^{86}Rb^+$ (□, ■) or $^{22}Na^+$ (△, ▲) added: 200 mM choline Cl, 1 mM $MgCl_2$, 1 mM NaCl, 1 mM RbCl, 20 μM $CaCl_2$ and 5 mM Pipes-Tris, pH 7.0 at 0°. Vesicles were then diluted 200-fold at 4 °C into unlabeled media of identical composition (open symbols), or in which KCl was substituted for choline chloride (filled symbols). 1-ml aliquots were caught on Millipore filters and rinsed. The amounts of radioactivity remaining with the vesicles on the filters were determined. Data are expressed as percent apparent ^{3}H-choline vesicle space which has been determined by extrapolating the choline efflux curve back to zero time. ^{3}H-choline space extrapolated back to zero time corresponded to 3.0 μl/mg protein

^{3}H-choline space obtained by extrapolating the efflux curve back to zero time. A reasonable straight line was obtained on the semilogarithmic plot, suggesting that isotope exchange may be approximated by first order kinetics. Similar apparent isotope spaces were obtained by using ^{3}H-sucrose or ^{14}C-gluconate (Meissner & McKinley, 1976). $^{36}Cl^{-}$ appeared to pass very rapidly since no radioactivity remained within the vesicles 20 sec after dilution into the exchange medium.

Permeability to Na^{+} and Rb^{+} showed a more complex behavior. $^{22}Na^{+}$ and $^{86}Rb^{+}$ and ^{3}H-choline still retained by vesicles after 20 sec passed out of vesicles with a similar rate. However, the two groups differed in an important way in that the apparent isotope spaces for $^{22}Na^{+}$ and $^{86}Rb^{+}$ were only about 1/3 of that for ^{3}H-choline. In these experiments we used 200 mM ^{3}H-choline and 1 mM $^{22}Na^{+}$ and $^{86}Rb^{+}$, but essentially the same results were obtained when 100 mM NaCl or 1 mM choline were used. $^{86}Rb^{+}$ remaining with the filter at 20 sec appeared to be retained by a membrane permeability barrier, rather than being merely bound, since it was fully exchanged within 20 sec when the dilution medium contained the ionophore valinomycin (10^{-8} M). In the presence of the ionophore X537A (10 μg/ml), both $^{86}Rb^{+}$ and $^{22}Na^{+}$ were rapidly released. In concurrent experiments ^{3}H-choline release rates were not appreciably altered by these two ionophores.

Our explanation for the different ion spaces is that there are present two types of vesicles which differ in their permeabilities to Na^{+} and Rb^{+}. About 70 % of the vesicles have exchanged all their Rb^{+} and Na^{+} before the first time point. These vesicles (designated Type I) appear, therefore, to be highly permeable to Rb^{+} and Na^{+}, presumably because they contain a pore for these cations. The remaining 30 % of the vesicle space are vesicles (designated Type II) which do not seem to contain a mechanism for facilitating Rb^{+} or Na^{+} exchange. Rb^{+} and Na^{+} leave these vesicles at the same slow rate characteristic of the much larger choline cation.

Table 1 shows the results of efflux experiments carried out with a crude sarcoplasmic reticulum vesicle preparation and with purified density sub-fractions called "light", "intermediate", and "heavy" sarcoplasmic reticulum vesicles. The sub-fractions are believed to be enriched in membranes derived from the longitudinal sections (light) or the terminal cisternae (heavy) of the intact sarcoplasmic reticulum (Meissner, 1975). Data of Table 1 suggest that in each of these fractions, Type I and II vesicles appear in approximately equal proportions. Apparently, then, the pores or channels allowing Rb^{+} or Na^{+} movement are distributed

Table 1. ^{3}H-choline, $^{22}Na^{+}$ and $^{86}Rb^{+}$ spaces for crude, light, intermediate and heavy sarcoplasmic reticulum vesicles

Vesicle preparation	Space (μl/mg protein)	
	^{3}H-choline	$^{22}Na^{+}$, $^{86}Rb^{+}$
Crude	2.8	1.0
Light	5.8	1.7
Intermediate	3.2	1.1
Heavy	1.4	0.5

A crude preparation of sarcoplasmic reticulum vesicles and sarcoplasmic reticulum vesicles of differing bouyant densities were prepared as previously described (Meissner, 1975). Spaces were determined by measuring isotope effluxes from vesicles as described in Fig. 1. The incubation and dilution media were kept at 0–4 °C and contained (in mM): 200, choline chloride; 10, NaCl; 10, RbCl; 5, $CaCl_2$; 5, $MgCl_2$; 1, sucrose; and 5, Pipes-Tris, pH 7.0. Similar ion exchange rates were observed for the four vesicle preparations.

throughout the bulk of the sarcoplasmic reticulum membrane and are not restricted to a particular region.

Apparent ion spaces were also determined for sarcoplasmic reticulum vesicles prepared from rat leg muscle and from lobster tail flexor muscle. The ^{3}H-choline and $^{22}Na^{+}$ spaces per mg protein were 2.7 and 0.5 μl for rat and 4.0 and 2.7 μl for lobster, respectively.

Osmotic Swelling and Rupture Experiments

Further evidence for K^{+}, Na^{+} permeable and impermeable sarcoplasmic reticulum vesicles was obtained by determining the osmotic behavior of vesicles which were filled with 200 mM choline chloride and small amounts of ^{22}NaCl and ^{86}RbCl and then diluted into either KCl or NaCl media. The impermeant choline trapped inside the vesicles can be expected to generate an osmotic force which must be balanced by an impermeant substance on the outside if vesicles are not to swell and rupture. Both types of vesicles appeared to be permeable to Cl^{-}, as mentioned above. If the major ions on the outside are KCl or NaCl, vesicles permeable to K^{+} and Na^{+} are expected to rupture, as these cations along with chloride and water enter rapidly. Their rupture will be observable as ^{3}H-choline release. In contrast, those vesicles which are able to retain $^{86}Rb^{+}$ for longer times and which are, therefore, believed

to be impermeable to K^+ should not release $^{86}Rb^+$ (or ^{3}H-choline) since KCl cannot rapidly enter these vesicles. Similarly, $^{22}Na^+$ isotope should not be released by NaCl.

An experiment to test these predictions is included in Fig. 1. Vesicles released appreciable amounts of ^{3}H-choline in a rapid initial burst when transferred to KCl or NaCl media. Within 30–60 sec, normal ^{3}H-choline release rates were again observed, suggesting that vesicles had resealed. In contrast to ^{3}H-choline, initial $^{22}Na^+$ and $^{86}Rb^+$ release was not appreciably increased when the ion compositions of the dilution media were changed.

We interpret these results to show that Type I vesicles are permeable to both K^+ and Na^+ since in KCl and NaCl media they rupture, releasing most of their ^{3}H-choline before resealing. Type II vesicles appear to be impermeable to both K^+ and Na^+ since neither Rb^+ nor Na^+ are released by either NaCl or KCl.

Use of DiO-C_5-(3) to Visualize Membrane Potentials

In the next section we will show that the fluorescent dye 3,3′-dipentyl-2,2′oxacarbocyanine (diO-C_5-(3)) (Sims *et al.*, 1974) can be used to demonstrate the formation of membrane potentials in sarcoplasmic reticulum vesicles. Initial calibration experiments were carried out with liposomes prepared from sarcoplasmic reticulum phospholipid in 200 mM K gluconate medium. A membrane potential was formed by diluting liposomes more than 500-fold into a medium containing either Tris gluconate or Na gluconate. The dilution media contained, in addition to the dye, valinomycin to make the lipid bilayer selectively permeable to K^+ and various concentrations of K^+ to control the magnitude of the formed membrane potential. Phospholipid vesicles were sufficiently impermeable to Tris or Na^+ to allow measurement of the emission fluorescence spectrum of the dye in the presence of polarized vesicles. Curve *a* of Fig. 2 shows the emission spectrum of the dye alone in solution. Curve *b* shows the emission spectrum after the addition of vesicles under polarizing conditions. A shift to longer wavelengths was observed when vesicles were added. The third spectrum (curve *c*) was obtained after the K^+ gradient had been allowed to collapse so that the vesicles were no longer polarized. Collapse of the K^+ gradient resulted in an increase in emission fluorescence. Spectra in the presence of unpolarized samples (as in curve *c*) also could be obtained by diluting vesicles directly into a K

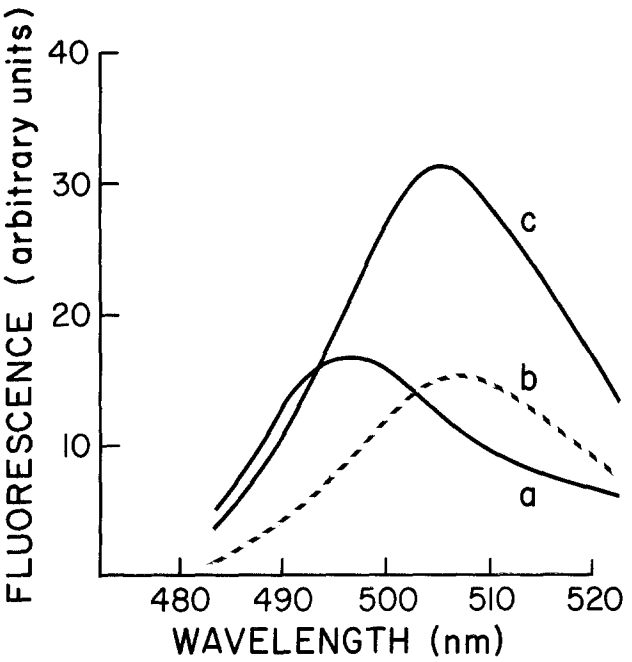

Fig. 2. Fluorescence emission spectra of diO-C_5-(3) in the presence of polarized and nonpolarized liposomes. Liposomes were prepared by suspending sarcoplasmic reticulum phospholipid (200 μg phosphorus/ml) in a 200-mM K gluconate medium as described in *Methods*. Fluorescence emission spectra were recorded at 2°. Excitation was at 470 nm. Dilution media contained 200 mM Na gluconate, 1.3 mM K gluconate, 1 mM Mg gluconate, 20 μM Ca gluconate, 10 mM Pipes-Tris, pH 7.0, 8 nM valinomycin and 1.7 μM diO-C_5-(3). Spectrum *a* was recorded before the addition of liposomes. Spectrum *b* was obtained within 3 min after liposomes (final concentration 0.66 μg phosphorus/ml) had been polarized by adding them to the above medium. The concentration of valinomycin was then raised to 0.33 μM to speed up collapse of ion gradients (*cf.* Fig. 3). Spectrum *c* was recorded when ionic gradients had decayed and there was no longer a change in fluorescence emission. Spectrum *c* could be also obtained by diluting liposomes into K gluconate medium

gluconate medium. Thus, in agreement with a previous report (Sims *et al.*, 1974), we found that formation of a membrane potential (negative inside) resulted in a decrease of the fluorescence emission of diO-C_5-(3).

When dye responses were measured as described above, a linear relationship was observed between decrease in fluorescence emission at 495 nm and the logarithm of K^+ concentration in the dilution media (Figs. 3 and 4). It follows then that fluorescence signals seen under our conditions were proportional to the magnitude of the developed membrane potential. Fluorescence signals slowly returned to values seen for nonpolarized liposomes (Fig. 3). Apparently, membrane potentials were slowly reduced as Tris or Na^+ moved into the vesicles.

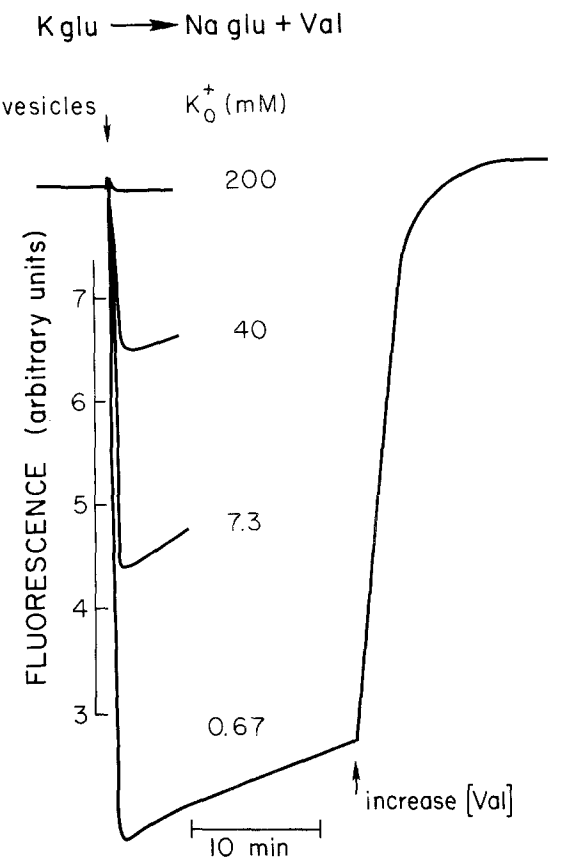

Fig. 3. Time course of fluorescence change of diO-C_5-(3) when K^+ filled liposomes were diluted into Na^+ media containing valinomycin and various concentrations of K^+. Fluorescence emission was recorded at 495 nm while excitation was at 470 nm. At the time indicated by the upper arrow, 10 µl of a liposome suspension present in a 200 mM K gluconate medium (*cf. Methods*) was added under stirring to 3 ml of a medium containing indicated amounts of K gluconate, and Na gluconate at a concentration so that the sum of Na and K gluconate was 200 mM, 1 mM Mg gluconate, 20 µM Ca gluconate, 10 mM Pipes-Tris, pH 7.0, 8 nM valinomycin, and 1.7 µM diO-C_5-(3). In the lowest curve the concentration of valinomycin was increased to 1.7 µM to speed up depolarization of the vesicles. The amount of liposomes used was chosen so that fluorescence emission seen after Na^+ and K^+ gradients had completely decayed was equal or close to the fluorescence emission of the dye seen before addition of vesicles

The size of the dye response was dependent on the amount of polarized vesicles present (Fig. 5). K gluconate-filled phospholipid vesicles were added in different amounts to Na gluconate media containing the dye and valinomycin. The total amount of vesicles was kept constant by adding a complementary amount of Na gluconate-filled vesicles, which being already equilibrated in a Na gluconate medium were not

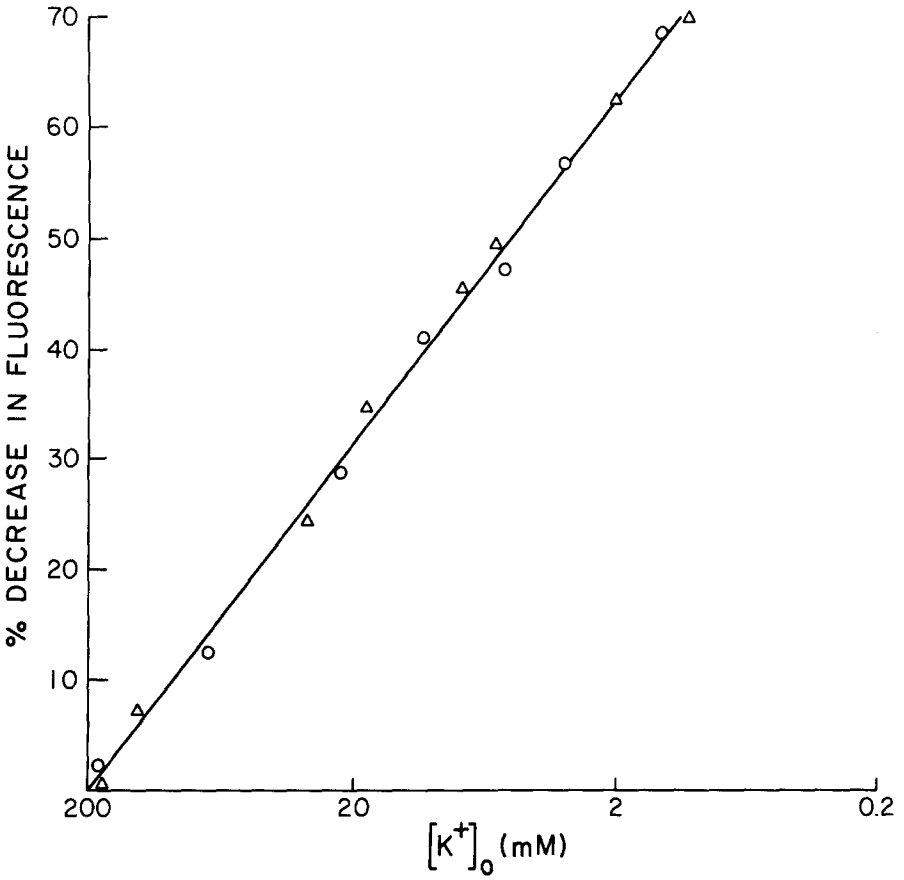

Fig. 4. Effect of liposome ion gradients on fluorescence emission of diO-C_5-(3). Maximal decrease in fluorescence emission observed within 5–10 sec after the addition of liposomes is plotted against the logarithm of K^+ concentration in the dilution media (*cf.* Fig. 3). Experiments were carried out as described in the legend of Fig. 3 using either Na (○) or Tris (△) gluconate as the major ions in the dilution media. Decrease in fluorescence is expressed as % of initial fluorescence seen before the addition of liposomes

polarized. Fig. 5 shows that the size of dye response increased with the amount of active vesicles present, although the relationship was not exactly proportional. It should, therefore, be possible to use the dye measurements to estimate in a preparation the fraction of vesicles in which a membrane potential change has been induced.

Membrane Potential Changes in Sarcoplasmic Reticulum Vesicles

Knowledge of the behavior of the fluorescent dye diO-C_5-(3) and of the various ion exchange rates allowed us to create and record mem-

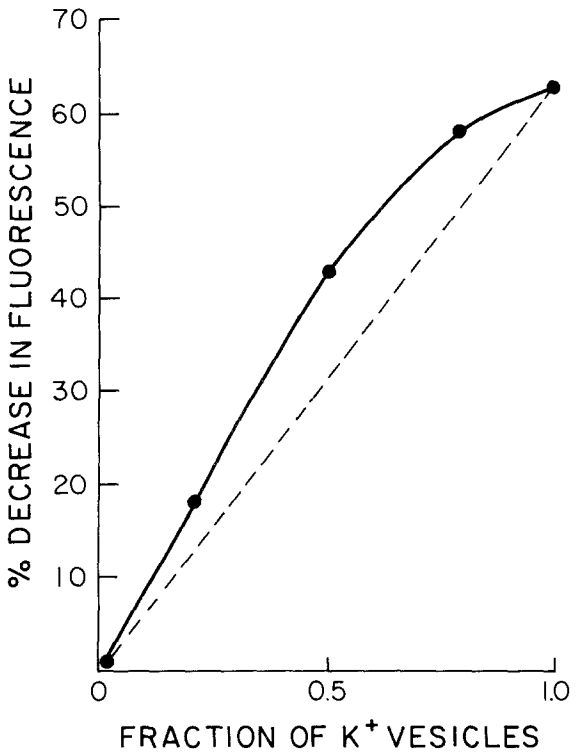

Fig. 5. Dependence of fluorescence emission of diO-C_5-(3) on the amount of polarizable liposomes. Liposomes were prepared containing either Na gluconate or K gluconate as the major ions *(cf. Methods)*. These were diluted into a Na gluconate medium containing 2 mM K gluconate as described in Fig. 3. Nonpolarizable Na gluconate-filled liposomes and polarizable K gluconate-filled liposomes were added simultaneously in various proportions so that the final concentration of phospholipid in the fluorometer cuvette was 0.66 μg phosphorus/ml. The fraction of phospholipid contributed by K^+-filled vesicles is indicated on the abscissa

brane potentials (negative inside) in sarcoplasmic reticulum vesicles and to use these measurements to obtain additional information about the K^+ and Na^+ permeability of the vesicles. Membrane potentials were formed by filling vesicles with K gluconate and diluting them into media containing either K gluconate (control), Tris gluconate or Na gluconate. A relatively slowly penetrating anion, gluconate, was used (Meissner & McKinley, 1976). Dilution media contained the dye and variable amounts of K^+ to obtain membrane potentials of different magnitudes. When vesicles were diluted into K gluconate or Na gluconate media, no or only small transient changes in fluorescent emission were observed suggesting that no significant membrane potentials were formed (not shown). How-

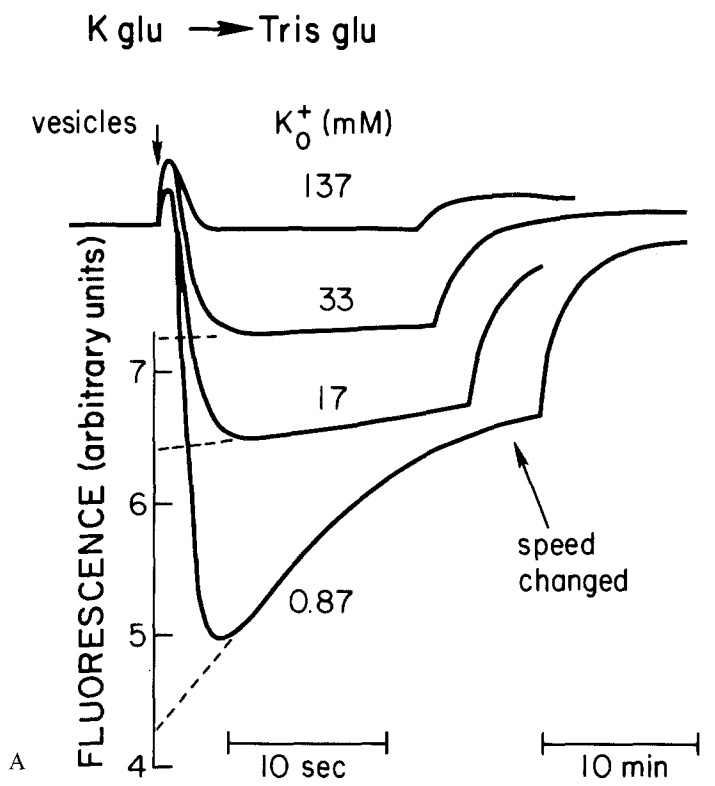

Fig. 6. Effect of changing ionic environments of sarcoplasmic reticulum vesicles on fluorescence emission of diO-C_5-(3). The graphs show the time course of fluorescence changes when K^+-filled sarcoplasmic reticulum vesicles were diluted into Tris gluconate media (*A*), Na gluconate media plus 0.6 μM valinomycin (*B*), or Tris gluconate media plus 0.6 μM valinomycin (*C*) in the presence of the indicated concentrations of K^+ in the dilution media. Vesicles (4 μl) suspended in 200 mM K gluconate medium *(cf. Methods)* (16.4 mg protein/ml, 400 μg phosphorus/ml) were diluted into 3 ml of media containing the indicated amounts of K gluconate, and Tris (or Na) gluconate at a concentration so that the sum of K and Tris (or Na) gluconate was 200 mM, 10 mM Pipes-Tris, pH 7.0, 1 mM Mg gluconate, 20 μM Ca gluconate and 1.7μM diO-C_5-(3). In *A*, only Type I vesicles are expected to be polarized; in *B*, only Type II; and in *C*, both Types I and II

ever, when vesicles were diluted into Tris gluconate medium, in the presence or absence of valinomycin, or Na gluconate medium in the presence of valinomycin, there occurred a rapid decrease in fluorescence (Fig. 6). Within 1 hr, fluorescent signals returned to values seen when vesicles were diluted into K gluconate medium. As in liposome preparations, the magnitude of fluorescence signals was dependent on the K concentration in the dilution media. When the decrease in fluorescence was plotted against the logarithm of $[K^+]$, three fairly straight lines with different slopes were obtained (Fig. 7).

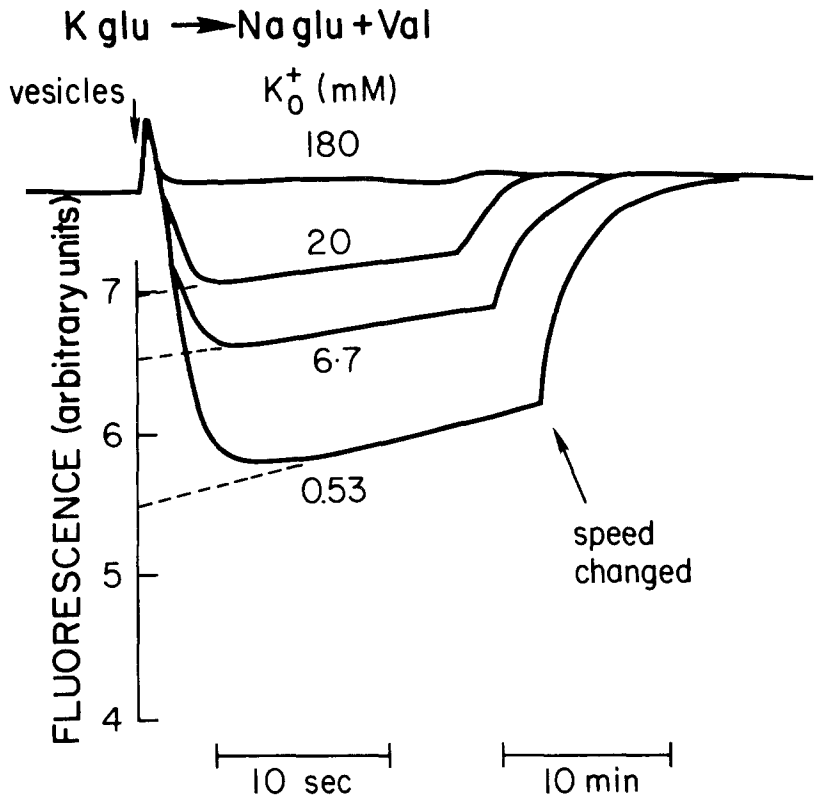

Fig. 6B

Dye responses seen in Fig. 6 are consistent with the existence of two types of sarcoplasmic reticulum vesicles, one type being permeable and the other being impermeable to K^+ and Na^+. Fluorescence signals seen on transfer of K gluconate vesicles to Tris gluconate media indicate the presence of a fraction of vesicles which is intrinsically permeable to K^+ (Type I vesicles). Changes in fluorescence signals seen when valinomycin is included in the Tris gluconate dilution media suggests the presence of another fraction of vesicles not intrinsically permeable to K^+ (Type II vesicles). A consequence of high K^+ and Na^+ permeability of Type I vesicles would be that external Na^+ and internal K^+ should rapidly exchange so that within a short time no K^+ and Na^+ gradients would be left to generate a diffusion potential. In contrast, Type II vesicles, which are impermeable to both small cations, would retain their K^+ and Na^+ gradients. Apparently, these gradients did not result in a diffusion potential unless vesicles were made selectively permeable to K^+. The fluorescence response seen in Na gluconate media in the presence of valinomycin, then, should be solely due to Type II vesicles.

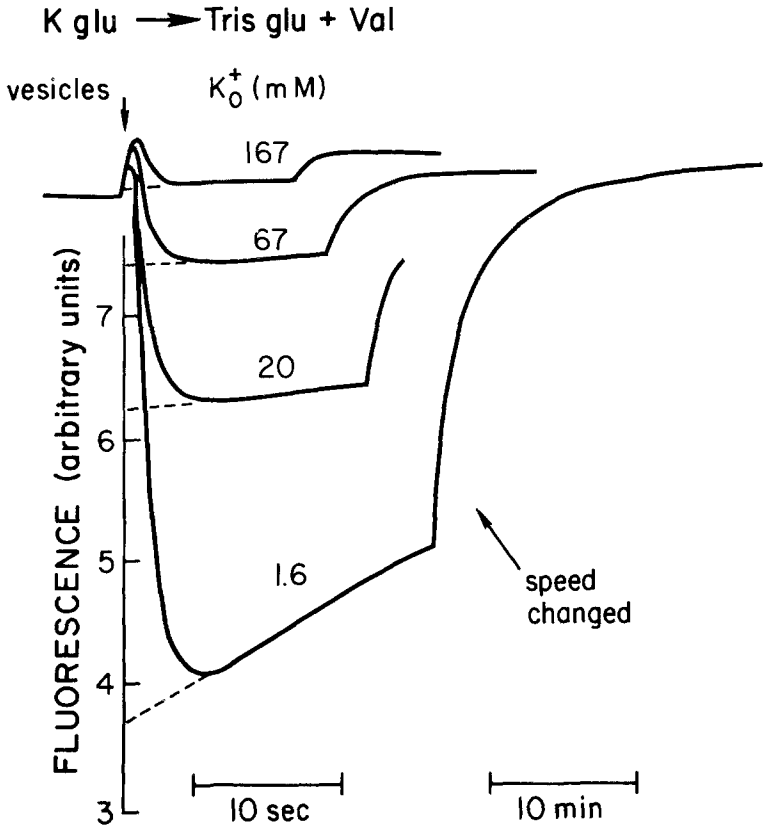

Fig. 6C

Fluorescence signals assigned to Type I and II vesicles correlated with the ion spaces of the two types of vesicles as determined from tracer flux measurements. In agreement with a lack of perfect proportionality between dye response and the amount of polarizable vesicles, (*cf.* Fig. 5), the sum of fluorescence signals for Type I and II vesicles measured separately was somewhat greater than the signal seen for both types polarized together in Tris gluconate medium containing valinomycin. It was also of interest that the size of the fluorescence signals were similar for phospholipid and sarcoplasmic reticulum vesicles (Type I and II), provided a comparable amount of phospholipid was present (*cf.* Figs. 4 and 7).

The ability of K^+, Na^+ permeable vesicles to retain Ca^{2+} was tested by isolating vesicles which were able to accumulate Ca oxalate when incubated with ATP and 5 mM oxalate. Samples were centrifuged into a sucrose gradient in order to separate by way of their greater density those vesicles which had accumulated Ca oxalate from those containing

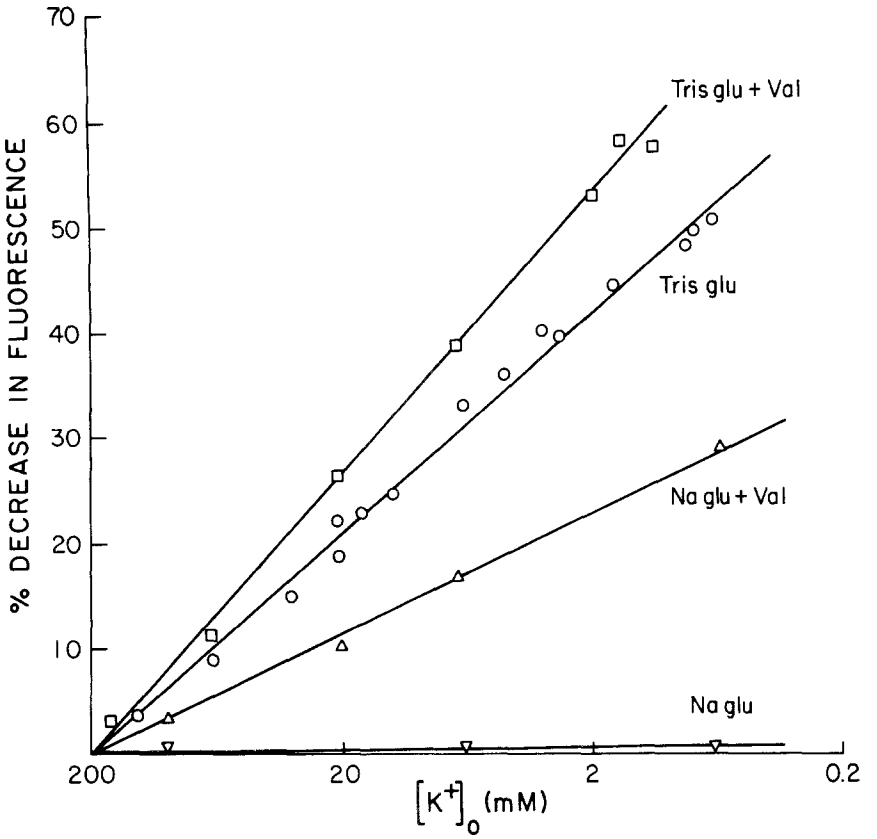

Fig. 7. Effect of Type I and II vesicle membrane potentials on fluorescence emission of diO-C_5-(3). Curves shown in Figs. 6*A–C* were extrapolated back to zero time, as indicated by the dotted lines. Extrapolated fluorescence changes, i.e., the decrease in fluorescence emission observed on addition of vesicles, were then plotted against the logarithm of K^+ concentration in the dilution media. Fluorescence changes observed in Tris gluconate, Na gluconate (plus valinomycin) or Tris gluconate (plus valinomycin) media are expected to be obtained from Type I, Type II, or both Type I+II vesicles, respectively

little or none (Meissner & Fleischer, 1971). About one third of the protein was recovered in the pellet fraction. Fractions were then dialyzed to remove most of their Ca oxalate, and their ability to form membrane potentials was assessed as outlined in Table 3. The vesicle fraction which had accumulated Ca oxalate produced virtually identical fluorescence changes corresponding to Type I and Type II vesicles as did the control, unfractionated material (*cf.* Fig. 6). Therefore, it appeared that the pore or channel permitting high K^+ permeability does not allow Ca^{2+} efflux to an extent that would prevent accumulation of Ca^{2+} in the presence of ATP.

Estimation of the Size of K, Na Permable Channel

Permeability of sarcoplasmic reticulum vesicles to cations of different sizes and shapes has been tested. Cation permeabilities were determined by comparing membrane potentials formed in the presence of various cations. Vesicles filled with 200 mM K gluconate were diluted into Tris gluconate or Na gluconate (plus valinomycin) media which contained 0.67 mM K^+ plus 20 mM of the test cation. Resulting membrane potentials were then recorded using the dye diO-C_5-(3). As shown above, an increase of K^+ concentration from 0.67 to 20 mM greatly attenuated the dye signal in both the Tris gluconate and Na gluconate media (Figs. 6 and 7). Permeability of a cation, then, should be easily determined by recording its ability to change the size of the fluorescent signal. In control experiments it was established that all cations tested were impermeable in phospholipid vesicles. Cations fell into two groups (Table 2). Cations in the first group, and also K^+, Rb^+ and Cs^+, acted as permeant cations in Type I vesicles, in that they attenuated the dye signal in the same manner as K^+. In all cases, the dye signal reached a minimal value within 3 sec (Fig. 8). Cations in the second group acted as impermeant ions. In their presence the dye response was almost identical to that seen with 0.67 mM K^+. Cations in both groups behaved as impermeant ions when Na gluconate dilution media were used, suggesting that all of them were impermeant in Type II vesicles.

It seems likely that the cations passed through the same pore or channel since they only crossed the membrane of K^+ permeable vesicles. The channel appeared to discriminate between cations of different sizes and shapes. For example, dimethylaminoethanol was permeant, whereas, trimethylaminoethanol (choline) was impermeant. Apparently, the presence of an extra methyl group in trimethylaminoethanol made this molecule too bulky to allow its passage through the pore. Considering the van der Waals' dimensions of these molecules (Table 2), it would appear that permeation through the channel is restricted to cations with one dimension not exceeding 4 to 5 Å and the other being 6 Å or greater.

Estimation of the K^+ Permeability Coefficient

Our techniques allowed measurement of the rate of ion movement for the slow-moving, relatively impermeant ions. Flux rates for choline or Tris (for Type I vesicles) could be calculated from isotope efflux rates or

Table 2. Fluorescence emission changes in the presence of various organic cations

Cation	% Fluorescence decrease		
	Type I	Type II	Liposomes
Tris	55	36	71
$K^{+c,g}$	21	12	25
Group A	21	36	71
Group B	55	36	71

Fluorescent assays were carried out essentially as described in the legend of Fig. 6 with the following modifications. Dilution media contained 20 mM of the listed cation, 0.67 mM K gluconate and either 180 mM Tris gluconate or 180 mM Na gluconate to assay for cation permeability of Type I vesicles or Type II vesicles and liposomes, respectively. All measurements were carried out at least in duplicate and were within $\pm 3\,\%$ of the values given. Fluorescent changes extrapolated to zero time are given. Estimates of the van der Waals dimensions (in Ångstrom units) of some of the molecules are given below. The following salts of the cations listed below were used: (c) chloride, (g) gluconate, or (s) sulfate.

Group A	Dimension (Å)	Group B	Dimension (Å)
Sodium[c,g]	1.9	Tetramethylamine[c]	5.3 × 5.5 × 6
Cesium[c]	3.4	Trimethylamino-ethanol[c] (choline)	5.3 × 5.7 × 7
Methylamine[c]			
Ethylamine[c]			
Hydrazine[c]			
Monoethanolamine[c]		Triethanolamine[c]	
Guanidine[c]	3.1 × 6 × 6	Tetraethylamine[c]	
Methylguanidine[s]	3.8 × 6 × 7.5	Tris(hydroxymethyl)-aminomethane[c,g] (Tris)	5 × 6.5 × 7
Aminoguanidine[s]	3.7 × 6 × 7.5		
Dimethylamino-ethanol[c]	4.5 × 5.7 × 7		

from the rebound portion of the dye response curves. Estimating vesicles to be 1,000 Å in diameter (Meissner, 1975) and assuming choline permeated by a first order process with a measured half-time of about 100 sec (Fig. 1), the choline permeability coefficient was estimated to be on the order of 10^{-8} cm/sec.

The permeability coefficient for K^+ has been estimated by comparing the relative permeability for K^+ to that of choline or Tris. The relative

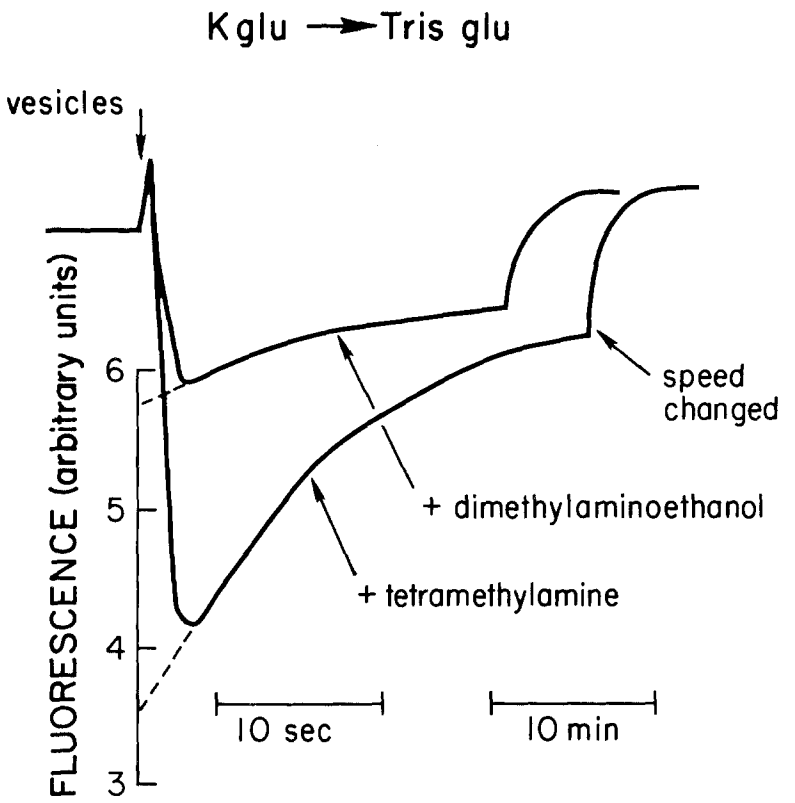

Fig. 8. Fluorescence emission of diO-C_5-(3) in the presence of dimethylaminoethanol and tetramethylammonium. The graph shows the time course of the fluorescence change for sarcoplasmic reticulum vesicles diluted into Tris gluconate (Type I signal) in the presence of 20 mM of the indicated organic cations. Fluorescence assays were carried out as described in the legend of Fig. 6, except that dilution media contained 180 mM Tris gluconate, 0.67 K gluconate, and either 20 mM dimethylaminoethanol chloride or 20 mM tetramethylammonium chloride

permeability for K^+ and Tris, or "selectivity", of Type I vesicles containing the cation pore was assessed from an analysis of the membrane potential generated by large K^+ gradients in the presence of high concentrations of Tris or choline. In Fig. 9 membrane potentials are given that would be expected to be formed across membranes whose relative permeabilities for Tris and K^+ ranged from 0.1 to 0, as calculated using the Goldman-Hodgkin-Katz equation. It can be seen that the greater the selectivity of the membrane for K^+, the greater the absolute magnitude of the potential that should be formed at low concentrations of K^+ in the dilution media. The hypothetical curves in Fig. 9 may be compared with the data of Figs. 4 and 7, where it was shown that the size of the

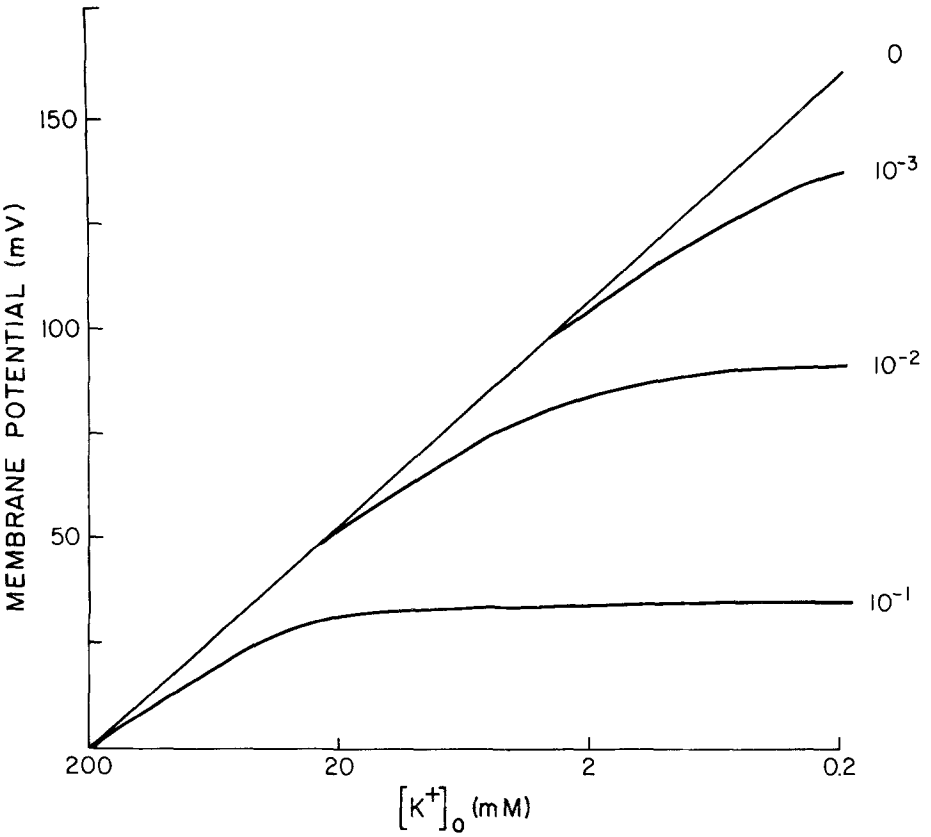

Fig. 9. Membrane potentials as a function of external K^+ concentration at four different permeability ratios for Tris to K^+. Membrane potentials have been calculated according to the equation:

$$\Delta V = -54\,\text{mV}\log\frac{P_K[K]_i + P_{Tris}[Tris]_i + P_{glu}[glu]_o}{P_K[K]_o + P_{Tris}[Tris]_o + P_{glu}[glu]_i}$$

using $[K]_i = [glu]_o = [glu]_i = 200$ mM; $[Tris]_i = 10$ mM; $[K]_o + [Tris]_o = 200$ mM; P = permeability coefficient of subscript ion. The permeabilities of Tris and gluconate, previously found to be similar for sarcoplasmic reticulum vesicles (Meissner & McKinley, 1976), have been set equal to simplify calculations. Permeability ratios for Tris to K^+ used in the calculations are indicated at the right of each curve

fluorescence response for lipsomes and sarcoplasmic reticulum vesicles was proportional to the logarithm of the K^+ concentration in the dilution media. Since it can be assumed that liposome permeability is highly selective for K^+ in the presence of small amounts of valinomycin, the dye response appeared to be proportional to the size of the formed membrane potential for both liposomes and sarcoplasmic reticulum vesicles. Therefore, it would appear that Type I vesicles were at least 100

times more permeable to K^+ than to Tris. Analysis of experiments using choline gluconate instead of Tris gluconate showed that K^+ was also at least 100 times more permeant than choline. Since the permeability of Type I vesicles to choline was about 10^{-8} cm/sec, it follows that the K^+ permeability coefficient for Type I vesicles was greater than 10^{-6} cm/sec.

Effect of Membrane Polarization on the Influx Rate of Impermeant Ions

The rates with which the fluorescence signals returned to the base line in Fig. 6 should provide a measure of the rate of collapse of the K^+ gradient. Since a lowering of the internal K^+ concentration depends on compensatory entry of Na^+ (Type II vesicles) or Tris cation (Type I

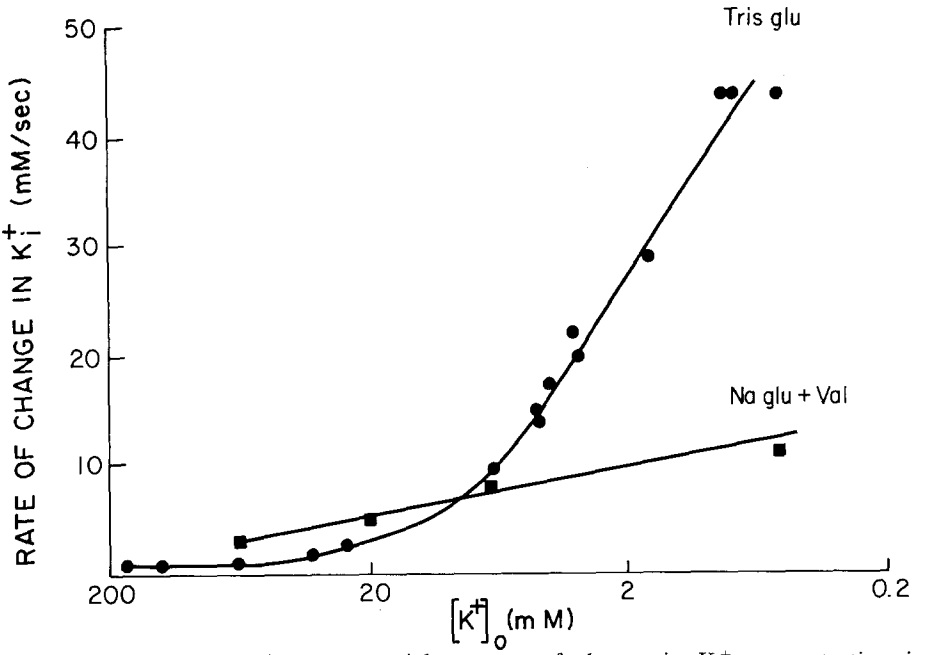

Fig. 10. Effect of membrane potential on rate of change in K^+ concentration in sarcoplasmic reticulum vesicles. Sarcoplasmic reticulum vesicles filled with K gluconate were diluted into Tris gluconate or Na gluconate (plus valinomycin) media as described in the legend of Fig. 6. The initial rate of change in K^+ concentration in vesicles was calculated according to the equation $\frac{dK_i}{dt} = \frac{2.3 \times K_i(t)}{\alpha} \times \frac{dF}{dt}$ where dF/dt represents the initial slope of fluorescence rebound curves (broken lines in Figs. 6*A* and *B*), $K_i(t)$ gives internal K^+ concentration of vesicles at time t ($K_i = 200$ mM at $t = 0$), and α is a proportionality factor which was obtained from slopes of lines in Fig. 7. α was equal to 21 % and 13 % for Type I and Type II vesicles, respectively

vesicles), the kinetics of the rebound portion of the fluorescence changes should indicate the rate of entry of these relatively impermeant cations from the dilution media.

These rates have been calculated from the initial slopes of the rebound portion of curves such as those in Fig. 6. In Fig. 10 the results are plotted as a logarithmic function of the K^+ concentration in the dilution media. At an external K^+ concentration of 100 mM or more, corresponding to the creation of a membrane potential not far from zero, K^+ concentration inside the vesicles changed with a rate of about 0.6 mM/sec in Type I vesicles present in Tris media. Using a choline gluconate dilution medium, an initial change in K^+ concentration of 2–3 mM/sec was calculated. These rates are in reasonable agreement with those for choline efflux (0.4–2 mM/sec) obtained from isotope exchange experiments for different preparations (*cf.* Fig. 1). The rate with which K^+ gradients collapsed in Type I vesicles and, therefore, the movement of impermeant ions was greatly increased when greater membrane potentials were created during dilution. For example, decrease in external K^+ concentration from 60 to 0.6 mM resulted in a more than 40-fold increase of K^+ outflow. Under comparable conditions, K^+ outflow from Type II vesicles increased only about fourfold. Different permeability behavior of the two types of vesicles raises the possibility that the structure of the K^+, Na^+ permeable channel in sarcoplasmic reticulum may be changed by a membrane potential.

Sonication Experiments

The presence of K^+, Na^+ permeable and impermeable vesicles in the three sarcoplasmic reticulum sub-fractions can be easily explained by making three assumptions: *in vivo* sarcoplasmic reticulum contains channels for Na^+ and K^+; these channels are randomly distributed throughout the bulk of the reticulum; and the density of the channels is insufficient for all vesicles to possess them. During homogenization some vesicles would then be obtained which have channels while other vesicles would have none. A decrease in vesicle size and, therefore, an increase in the number of vesicles should result in a shift toward a higher proportion of K^+, Na^+ impermeable vesicles.

To test this hypothesis we have decreased the size of the vesicles by sonication and measured their isotope spaces and ability to form membrane potentials under the conditions already outlined. As expected for a

Table 3. Apparent isotope spaces and fluorescence signals of sonicated sarcoplasmic reticulum vesicles

Sonication time (min)	Type I	Type II	Type II / Type I
	Space (μl/mg protein)		
0	2.5	1.2	0.48
5	0.32	0.40	1.3
25	0.15	0.33	2.2
	% Fluorescence decrease		
0	32	27	0.85
5	4	22	5.5
25	4	22	5.5

Vesicles present in a 200 mM K gluconate medium (*cf.* Methods) were sonicated for 0, 5 or 25 min in a Mettler sonicator bath at 0°. Apparent Type I and II vesicle spaces were determined by measuring ^{3}H-choline and ^{22}Na exchange rates and spaces as described in the legend of Fig. 1. Fluorescent assays were done as described in the legend of Fig. 6 using 2 mM K gluconate in the dilution media.

population of smaller vesicles, total internal vesicle space, as measured by the amounts of trapped ^{3}H-choline decreased appreciably (Table 3). Most of this decrease was due to a loss of Type I vesicle space. In polarization experiments, fluorescent signals elicited by Type I vesicles were drastically reduced while those assigned to Type II vesicles were only slightly affected.

Our results show that the proportion of Type II vesicles was increased during sonication, but they cannot tell us whether the absolute amount of membrane incorporated into Type II vesicles was increased. Sonication might have damaged membranes so that some did not reseal successfully. An apparent indication that this occurred to some extent was a three- to fourfold increase in Ca^{++} stimulated ATPase activity after sonication. This circumstance prevents ruling out an alternate interpretation, that Type I vesicles of K^{+}, Na^{+} permeable channels may have been selectively destroyed, rather than that Type I vesicles were converted into Type II vesicles.

Discussion

This study has shown that purified skeletal muscle sarcoplasmic reticulum vesicles are composed of two types which differ in their

permeability to small cations. Vesicles designated Type I are relatively impermeable to larger cations such as choline or Tris, but are at least 100 times more permeable to small cations such as K^+. Type II vesicles differ from Type I vesicles in that Na^+, Rb^+ and K^+ permeate across their membranes as slowly as choline or Tris. It, therefore, appears that Type I vesicles contain a channel for small cations. K^+, Na^+ permeable vesicles constitute approximately 2/3 of the vesicles, while K^+, Na^+ impermeable vesicles account for the remainder.

Most of the experiments reported in this study have been carried out with an intermediate density vesicle fraction obtained from rabbit skeletal muscle. Previous freeze-fracture and trypsin digestion studies with this vesicle fraction indicated that the predominant protein of sarcoplasmic reticulum, the Ca^{2+} ATPase, projects only toward the outside of essentially all vesicles (Meissner, 1978). It is therefore unlikely that either Type I or II vesicle is inside-out.

The osmotic rupture induced by KCl and the ability of K^+ gradients to generate membrane potentials suggest that K^+ and Na^+ permeation is likely free rather than being an obligate exchange process. The latter process would be electrically silent, if it exchanged ions on a one-for-one charge basis, as seems to be the case for the anion carrier of red blood cells (Gunn, 1972). The most economical explanation for Na^+ and K^+ permeability of sarcoplasmic reticulum vesicles would be that both cations move through the same channel. While separate channel structures cannot be ruled out, our data would suggest that these two structures must stay together during homogenization. In previous Millipore filtration experiments carried out at 23 °C (Meissner & McKinley, 1976), the presence of Na^+ impermeable vesicles was not recognized. At 4 °C ion permeation rates were diminished, allowing a more reliable measurement of ion spaces.

Both K^+, Na^+ permeable and impermeable vesicles appeared to be highly permeable to chloride. This would imply that small anions can pass the sarcoplasmic reticulum membrane by a separate pathway.

Recent work by a number of investigators is in accord with the presence of a cation channel in sarcoplasmic reticulum. Kasai and Miyamoto (1976) found that Na^+ and K^+ spaces corresponded to about 60 % of the sucrose space of their sarcoplasmic reticulum vesicle preparations. No interpretation for these different solute spaces was given. Miller and Racker (1976) observed that fusion of sarcoplasmic reticulum vesicles with a lipid bilayer resulted in an increase of its K^+, Na^+ and Cl^- conductances. Their preliminary studies indicated that conductance in-

creases for K^+ and Na^+ were approximately equal. While protein incorporation often results in an indiscriminate increase in lipid bilayer conductance, it is reasonable to assume that at least part of the conductance increases were due to the K^+, Na^+ permeable channel in sarcoplasmic reticulum. Use of some of the organic cations listed in Table 2 may help to assess nonspecific and specific conductance increases.

We have attempted to estimate the size of the K^+, Na^+ permeable channel of sarcoplasmic reticulum by assessing its permeability to a variety of cations. The approach was in principle the same as that used by Hille (1971, 1972, 1973). His studies suggested that the Na^+ channel may form an oxygen-lined pore of about 3 by 5 Å in cross-section and that the K^+ channel consists of a circle of oxygen atoms having a diameter of about 3 Å. Our ionic selectivity measurements suggest that the K^+, Na^+ permeable channel in sarcoplasmic reticulum has a somewhat greater dimension than the K^+ and the Na^+ channels of nerve. For example, the K^+ channel of nerve could select against hydroxylamine or methylamine cations. The Na^+ channel discriminated against methylamine or methylguanidine cations. All of these cations readily permeated across the membrane of Type I vesicles (Table 2). Especially striking was the observation that dimethylaminoethanol behaved as a permeant cation in Type I vesicles, while cations with only slightly greater van der Waals dimensions, like trimethylamino-ethanol, were impermeant. Among this latter group were also tris(hydroxymethyl)aminomethane (i.e., Tris), and tetramethylamine, a primary and quarternary amine, respectively. Disallowing hydrogen bond formation to oxygens lining the channel, this organic cation selectivity can be explained in geometrical terms by assuming that K^+, Na^+ permeable channel forms a rectangular hole with a width of 4 to 5 Å and a length greater than or equal to 6 Å.

It has been proposed that K^+ and Na^+ channels of nerve membrane lose most of their hydration shell when passing through the narrowest part of their respective pores (Bezanilla & Armstrong, 1972; Hille, 1972). This is allowed because the energy required to dehydrate the ions is compensated for by a favorable interaction with the pore walls, which are, therefore, postulated to be lined with oxygen atoms. Because of its wider dimensions, K^+ and Na^+ could pass through the K^+, Na^+ permeable channel of sarcoplasmic reticulum in a more fully hydrated state. Provision of some polar interaction with the passing ions would then be less of a rate limiting step. A possible consequence would be that the cation channel in sarcoplasmic reticulum is less selective, since

specific interaction of the pore walls with dehydrated ions may confer some selectivity against ions which have a smaller dimension than the pore. For example, K^+ channel of nerve membrane is about 100 times less permeable to the smaller Na^+ than to K^+ (Hille, 1973). We could show that both cations readily permeated Type I vesicles, but our experimental approaches were limited in that they did not allow us to determine the exact permeation ratios of fast-moving ions. We could only determine permeation ratios of relatively slow-moving ions. An additional energetic factor that has to be considered, is that, as the valency of ions increases, the size of their hydration shell becomes greater and their dehydration becomes energetically less favorable. This property might allow the K^+, Na^+ permeable channel to discriminate against Ca^{2+} and other divalent cations.

It appears unlikely that the K^+, Na^+ permeable channel is permeable to Ca^{2+}. Previously, we showed that dilution of vesicles filled with choline chloride and diluted into KCl media induced rapid release of ^{3}H-sucrose and $^{45}Ca^{2+}$ (Meissner & McKinley, 1976). Therefore, it appears the releasable Ca^{2+} is contained in vesicles having the K^+, Na^+ channel (*cf. Results,* section *Osmotic Swelling and Rupture Experiments*). Since this Ca^{2+} is normally only very slowly exchangeable, it appears that Ca^{2+} does not quickly pass through the channel. The ability of Type I vesicles to accumulate Ca oxalate, reported here, also speaks against a high permeability of the channel to Ca^{2+}. To further test Ca^{2+} permeability, the polarization experiments using diO-C_5-(3) might be used, provided they were done in the presence of an impermeant anion other than gluconate; the complexation of Ca^{2+} by gluconate made our present procedure inadvisable for testing Ca^{2+} permeability of Type I vesicles.

Type I vesicles do not appear to be simply "leaky" vesicles, since both types of vesicles have similar permeability to gluconate, choline, or sucrose (Meissner & McKinley, 1976). Also, a similar proportion of the two types of vesicles is found in crude sarcoplasmic reticulum preparations or highly purified preparations consisting of either light, intermediate, or heavy vesicles. Our present working hypothesis is, therefore, that *in vivo* sarcoplasmic reticulum contains K^+, Na^+ permeable channels which are randomly dispersed.

Vesicles in our preparations are typically about 1,000 Å in diameter (Meissner, 1975), which corresponds to about 50 vesicles per μm^2 of surface area. If the density of pores randomly distributed in *in vivo* sarcoplasmic reticulum were 50 per μm^2 and vesicles were of uniform size,

it may be calculated that about 1/3 of the vesicles obtained during homogenization contain no pore (Type II vesicles) while the remainder (Type I vesicles) would be expected to have one pore (1/3 of the vesicles), two pores (1/6 of the vesicles) or more than two pores (1/6 of vesicles). Some support for this interpretation has been provided by the sonication experiments which indicated that a decrease in vesicle size favored the formation of Type II, K^+, Na^+ impermeable vesicles. For comparison, freeze-fracture studies (Packer *et al.*, 1974; Scales & Inesi, 1976) suggest that sarcoplasmic reticulum membrane contains 5000 or more Ca^{2+}-ATPase protein molecules/μm^2. The density of Na^+ and K^+ channels in nerve membrane has been estimated to range from 2–400 pores/μm^2 in different species (Armstrong, 1975).

Recently, electron probe X-ray microanalysis of muscle has indicated that the concentrations of K^+, Na^+ and Cl^- in the lumen of the reticulum are approximately the same as in the cytoplasm (Somlyo, Shuman & Somlyo, 1977). This suggests that there are no large gradients in the free concentration of these ions. Assuming that *in vivo* sarcoplasmic reticulum is permeable to these ions, as are vesicles derived from it, it seems that the resting membrane potential across the reticulum membrane is not far removed from zero.

A likely physiological function of K^+, Na^+ permeable channels would be to minimize charge and osmotic effects during Ca^{2+} release and uptake. Rapid movement of Ca^{2+} in and out of sarcoplasmic reticulum during muscle relaxation and contraction requires an equally fast movement of an equivalent amount of charge in the opposite direction. Otherwise, a membrane potential would be rapidly formed which would limit further Ca^{2+} movement. This charge transfer could be provided for by co-movement of chloride or counter-movement of K^+. The larger the free permeability of sarcoplasmic reticulum to small anions or cations, the smaller would be the size of the potential which could be generated by Ca^{2+} movement. K^+ counter-movement would seem to be most advantageous since K^+ is the prevalent ion in sarcoplasmic reticulum and the sarcoplasm (Somylo *et al.*, 1977). To prevent the formation of a large membrane potential, either two K^+ would have to pass the membrane in a direction opposite to that of Ca^{2+}, or two Cl^- and one Ca^{2+} would have to move in the same direction. Accordingly, K^+ would appear to minimize osmotic effects since counter-movement of K^+ would result in net transfer of only one ion per Ca^{2+} translocated while Cl^- co-movement would lead to that of three ions.

Size of the potential which could be generated during electrogenic

Ca^{2+} pumping or release will depend on the free permeability of sarcoplasmic reticulum to other ions. In isolated sarcoplasmic reticulum vesicles, Ca^{2+} uptake has been measured to proceed initially with a rate of 60–70 nmol Ca^{2+}/mg protein × sec (Inesi & Scarpa, 1972). We found that K^+ can cross the membrane with a rate that is at least 5 times greater than that of Ca^{2+} pumping, suggesting that no significant potential changes may be produced during muscle relaxation even if the Ca^{2+} pump were entirely electrogenic. On the other hand, Ca^{2+} release may occur with a rate of 4–8 μmol Ca^{2+}/mg protein · sec if it is assumed that 0.1 to 0.2 μmol Ca^{2+}/g muscle is released in 5 msec (Weber & Herz, 1963) and using a sarcoplasmic reticulum content of 5 mg protein/g muscle (Meissner, Conner & Fleischer, 1973). Ca^{2+} release may then be too fast to be immediately and completely compensated for by K^+ inward movement. *In vivo* studies have indicated that this may be the case. Changes in fluorescence intensity of muscle stained with Nile Blue A (Bezanilla & Horowicz, 1975) and birefringence signals (Baylor & Oetliker, 1975) seen during muscle contraction have been correlated with potential changes across the sarcoplasmic reticulum membrane.

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