Permeability of Canine Cardiac Sarcoplasmic Reticulum Vesicles to K', Na+, H+, and Cl- *

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Cardiac muscle sarcoplasmic reticulum appears to contain channel-like structures that render the membrane permeable to small univalent ions. Canine heart microsomes fractionated according to buoyant density were examined by Millipore filtration, light scattering, and membrane potential measurements. Enzymatic analysis and measurement of D-glucose permeation and Na/Ca exchange systems indicated two membrane fractions suitable for the permeability studies, one enriched in surface membranes with a buoyant density of 1.04–1.11 (10–25% sucrose) and one enriched in sarcoplasmic reticulum with a buoyant density of 1.13–1.15 (30–34% sucrose). Surface membrane vesicles impermeable to [3H]sucrose were largely impermeable to K', Na', and Cl', while sarcoplasmic reticulum vesicles impermeable to [3H]sucrose were readily permeable to K', Na', H', and Cl'. Sarcoplasmic reticulum vesicles were essentially impermeable to Ca2+, Mg2+, choline+, gluconate-, 1,4-piperazinediethanesulfonic acid (Pipes)*, and D-glucose. These results suggest that cardiac muscle sarcoplasmic reticulum contains structures that facilitate the movement of small univalent ions. A possible function of these putative ion-conducting structures may be to allow rapid ion fluxes to counter electrogenic Ca2+ fluxes across sarcoplasmic reticulum during cardiac muscle contraction and relaxation.

Sarcoplasmic reticulum is a highly specialized intracellular membrane system that plays the essential role of releasing and reabsorbing Ca2+ during cardiac muscle contraction and relaxation (1). Few clues are available as to how a muscle action potential results in the release of Ca2+ from sarcoplasmic reticulum (2–4). Studies with membrane vesicle fractions isolated from canine heart muscle have, however, indicated that cardiac muscle sarcoplasmic reticulum, like skeletal muscle sarcoplasmic reticulum, like skeletal muscle sarcoplasmic reticulum, is permeable to K', Na', H', and Cl', suggesting the presence of univalent ion-conducting channels.

MATERIALS AND METHODS

Reagents—The fluorescent dye 3,3'-dipentyl-2,2'-oxacarbocyanine was the generous gift of Dr. Alan S. Waggoner (Amherst College, Amherst, MA). "Cl-, "Na', "Rb", [methyl-3H]choline, [1-14C]glucose, and [fructose-1-14C]sucrose were purchased from New England Nuclear, Boston, MA. [U-14C]Glucose was obtained from ICN Pharmaceuticals, Irvine, CA. Other reagents used were of reagent grade.

Isolation of Membranes—Microsomal membrane fractions derived from canine ventricular tissue were prepared as follows. 50 g of muscle were minced and homogenized in 375 ml of 0.3 M sucrose and 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, pH 7.7, at 4 °C for 60 s in a Waring blender. The homogenate was centrifuged for 12 min at 6,000 rpm (6,800 × g) in a GSA rotor in a Sorvall RC-2 centrifuge. A crude microsomal fraction was obtained from the supernatant by centrifugation for 90 min at 33,000 rpm (90,000 × g) in a Beckman type 35 rotor. The pellet was resuspended in 0.3 M sucrose containing 25 mM KCl and 2.5 mM Hepes, pH 7.2, and placed on a discontinuous sucrose gradient consisting of 10 ml of 5% (w/w), 10 ml of 30%, 10 ml of 50%, and 2.5 ml of 42% sucrose. Sucrose gradient solutions contained 25 mM KCl, 1 mM MgCl2 and 2.5 mM Hepes, pH 7.2. After centrifugation for 16 h at 23,000 rpm (95,000 × g) in a Beckman SW 27 rotor, membranes present at the 0.3 M/25% (Fraction 1), 25–50% (Fraction 2), 30–34% (Fraction 3), and 34–42% (Fraction 4) sucrose interfaces were collected, diluted with 1.5 volumes of 0.6 M KCl, and sedimented by centrifugation for 90 min at 33,000 rpm in a Beckman type 35 rotor. The membranes of Fractions 1–3 were resuspended in 0.3 M sucrose and 1 mM Hepes, pH 7.1. In Fraction 4, only the upper less brownish part of the pellet was recovered. The fractions were quickly frozen and stored at −65 °C before use.

Biochemical Assays—Protein was determined by the procedure of Lowry et al. (18) using bovine serum albumin as a standard.

"Basic" ATPase activity was determined at 25 °C as previously described (19). Assay media contained 10 mM Hepes, pH 7.3, 0.1 mM KCl, 2.5 mM ATP, 6 mM MgCl2, and 1 mM ethylene glycol glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid. Ca2+-loading rates were measured at 25 °C in a buffer solution containing 10 mM Hepes, pH 7.3, 0.1 mM KCl, 5 mM MgCl2, 100 μM CaCl2, 5 mM oxalate, 3 mM ATP, and 0.1 mM concentration of the Ca2+ indicator Antipyrylazo III. The change in absorbance at 651 nm reflected the decrease in free Ca2+ (20). The concentration of the Ca2+-dependent 3P-labeled phosphoenzyme intermediate of sarcoplasmic reticulum was measured at 0 °C by the method of Moore et al. (21). Succinate-cytochrome c reductase activity was estimated at 32 °C according to Fleisher and Fleischer (22).

Isotope Flux and Membrane Potential Measurements—Apparent isotope spaces and efflux rates of microsomes to [3H]glucose, [3Na', [3Rb', and [3Cl' were determined at 22 °C by Millipore filtration as previously described (15). Membrane potentials were generated by gradients of permeant ions between the intravesicular cavity and the medium into which the vesicles were diluted. Membrane potentials

1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid; diO-Cy3-(3), 3,3'-dipentyl-2,2'-oxacarbocyanine.

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(negative inside) were detected by the use of the fluorescent dye 3,3'-
dipentyl-2,2'-oxacarbocyanine iodide as described by McKinney and
Miesmer (16). The sign of the membrane potential is reported ac-
cording to standard convention, i.e. reference (ground) is extravas-
cular. Fluorescence assays were carried out at 15°C under stirring in a
Farrand model 801 fluorometer. Excitation was at 470 nm and emis-
sion was recorded at 495 nm. Vesicle concentrations (approximately
15 μg of protein/ml) were used which produced negligible perturbation
of the fluorescence emission during dilution with incubation
medium.

Light-scattering Measurements—Osmotically induced volume
changes in microsomes were detected by monitoring the changes in
light-scattering intensity at 400 nm at right angle to the incoming
beam using a Farrand model 801 fluorometer. Microsomes (0.1-0.2
mg of protein/ml) were equilibrated at 21°C for about 30 min in a
300 mM sucrose and 10 mM K-Pipes (1,4-piperazinediethanesulfonic
acid), pH 7. Osmotic volume changes were induced at 21°C by adding
to the microsomal suspension, under rapid stirring, 5% of a volume
containing 10 mM K-Pipes, pH 7, and 800 mosm of the test compound.

RESULTS

Properties of Cardiac Microsomal Fractions—Microsomes
were separated into four fractions of differing buoyant density
(Table I). All four membrane fractions displayed properties
characteristic of sarcoplasmic reticulum, i.e. formation of a
[32P]-phosphoenzyme intermediate in the presence of Ca2+ and
accumulation of Ca2+ in the presence of the Ca2+-precipitating
agent oxalate (23, 24). The levels of these two activities
indicated that the sarcoplasmic reticulum content of Fractions
2 and 3 was 2- to 3-fold higher than in Fractions 1 and 4.

Estimation of surface membrane content of the four gra-
dient fractions was complicated by the lack of agreement in
the distribution of enzymatic activities characteristic of this
membrane. All four membrane fractions, with highest levels
being present in Fractions 2 and 3, possessed Mg2+- or Ca2+-
activated ("basic") ATPase activity (Table I), an enzymatic activity
previously shown to be associated with canine cardiac muscle
surface membrane structures with the exception of the
nexus (19). On the other hand, Na/Ca exchange (cf. Fig. 4) and
p-glucose permeation (Fig. 5) rates suggested a higher
surface membrane vesicle content in Fraction 1 than in Fraction
3. Surface membrane-associated enzymes with varying
distribution have also been observed in liver (25) or skeletal
muscle (26, 27). Fractions 1-3 contained small amounts of
inner mitochondrial membranes, as indicated by the succi-
nate- cytochrome c reductase activities. Sarcoplasmic reticu-
lim and surface membranes of Fraction 4 were appreciably
contaminated with inner mitochondrial membranes.

Thin section electron microscopy revealed that Fractions 1 and
3 consisted mainly of membranous, vesicle-like structures
with diameters ranging from approximately 750-3000 nm.
Fraction 2 consisted of membranous, vesicle-like structures
with diameters ranging from approximately 750-3000 nm.
Fraction 2 possessed a number of characteristic features of
sarcoplasmic reticulum. K+, Na+, and C1- permeability of the
membranous fractions isolated from canine heart muscle by sucrose gradient centrifugation
was assessed by Millipore filtration. [3H]Sucrose and [3H]choline were used to
determine the internal volume of intact vesicles. They are
considered impermeant and are retained by the vesicles to a
similar extent (see Table II). [3H]Sucrose (Fig. 3 and [3H]
choline equilibrate across the vesicle membranes during pro-
longed incubation. Using the [3H]sucrose or [3H]choline
spaces as a measure of total internal volume of intact vesicles,
the proportion of vesicles permeable to other ions or solutes
can be determined. Vesicles are considered to be permeable
to a particular ion if an isotope of that ion can be released
within seconds. These vesicles are devoid of radioactivity
within 20-30 s, i.e. the first time point in the Millipore filtra-
tion experiments.

Fig. 2 shows that for vesicles of Fraction 3 after preincuba-
tion for 6 h at 22°C, the apparent isotope spaces for 86Rb+,
32Na+, and 36Cl- were approximately equal and smaller than the
[3H]sucrose space. Comparison of the slopes on the semi-
logarithmic plots indicated that 32Na+ (t1/2 = 2.5 min) passed
through the impermeable vesicles somewhat faster than
86Rb+ or 36Cl- (t1/2 = 5 min). The difference in [3H]sucrose
and the three ion spaces was independent of the time of
preincubation with the radioisotopes (Fig. 3). Preincubation
for 6-12 h or longer was usually required to obtain maximal
radioisotope spaces. Preincubation for 24 h resulted in uni-
formly lower isotope spaces, suggesting a general breakdown of
the permeability barrier.

The apparent [3H]sucrose, [3H]choline, 22Na+, 86Rb+, and
36Cl- spaces and efflux rates of Fractions 1 and 3 are summa-
rized in Table II. In Fraction 1, 32Na+, 86Rb+, and 36Cl-
spaces approached or exceeded the [3H]sucrose and [3H]choline
spaces. In contrast, 32Na+, 86Rb+, and 36Cl- spaces accounted
for a quarter or less of the [3H]sucrose and [3H]choline
spaces of Fraction 3. Our interpretation of the different isotope spaces is that
32Na+, 86Rb+, and 36Cl- were able to efflux within 20-30 s
from a majority of Fraction 3 vesicles because of the presence
of mechanisms that facilitated their rapid release. For the
residual vesicles which trapped these ions, efflux was on the
time scale of minutes, which we believe is characteristic of
nonmediated permeation.

Surface Membrane Vesicle Content of Fractions 1 and 3—
The different permeability behavior of the membrane vesicles
in Fractions 1 and 3 to 86Rb+, 32Na+, and 36Cl- would be
consistent with what is known about their permeability in skel-
etal muscle (16, 17, 28) if Fraction 1 was mainly surface
membranes and Fraction 3 largely sarcoplasmic reticulum.
We tested for the presence of surface membrane vesicles by
measuring the ability of the two fractions to exchange Na+ or
Ca2+ for 4Ca2+ and 32Na+ (29), as well as to preferentially take up
p-glucose over L-glucose (30). The effect of external Ca2+
on 22Na+ efflux was measured by diluting 22Na+-filled vesicles
into KCl media containing either 0.25 mM Ca2+ or the Ca2+

| Fraction | Buoyant density (% sucrose) | Crude microsomal fraction | Sucrose gradient fraction |
|---------|-----------------------------|---------------------------|--------------------------|
| 1       | 10-25                       | 240                       | 2                          |
| 2       | 25-30                       | 12                        | 15                        |
| 3       | 30-34                       | 12                        | 15                        |
| 4       | 34-42                       | 12                        | 15                        |
Permeability of Cardiac Sarcoplasmic Reticulum

Fig. 1. Electron micrographs of cardiac muscle microsomal fractions. Samples were fixed with 2.5% glutaraldehyde in 0.3 M sucrose, pH 7.2, for 15 min in ice and 45 min at 23 °C and sedimented. Pellets were postfixed with 1% OsO₄, embedded, sectioned, and stained as previously described (19). Sucrose gradient Fractions 1 (left) and 3 (right) are shown X 25,000.

TABLE II

| Radioisotope  | Apparent isotope space (μl/mg protein) | Efflux rate (1/2) (min) |
|---------------|----------------------------------------|-------------------------|
|               | Fraction 1 | Fraction 3 | Fraction 1 | Fraction 3 |
| [3H]Sucrose   | 2.7        | 3.0        | >10        | >10        |
| [3H]Choline   | 2.3        | 2.8        | 8          | 4          |
| ⁴⁺Rb          | 3.2        | 0.6        | 6          | 5          |
| ²²Na          | 2.3        | 0.5        | 1.5        | 2.5        |
| ³¹Cl          | 2.2        | 0.7        | ~10        | ~10        |
| D-[¹⁴C]Glucose| 3.0        | 2.9        |             |             |

chelator EGTA at a concentration of 1 mM. Increased initial ²²Na⁺ efflux rates on dilution of the vesicles into the Ca²⁺-containing medium (Fig. 4) suggested that both vesicle fractions were capable of Ca/Na exchange. Vesicles of both fractions also appeared to be capable of Na/Na exchange since an enhanced ²²Na⁺ efflux rate was noted when vesicles were diluted into a medium containing 100 mM NaCl instead of 100 mM KCl. Both Ca²⁺- and Na⁺-mediated ²²Na⁺ efflux could be blocked by prior addition of 1 mM La³⁺, a potent blocker of the cardiac surface membrane Ca/Na exchange system (29). Thus, the ²²Na⁺-impermeable vesicle populations in Fractions 1 and 3 were capable of Ca/Na exchange. However, as shown above (Table II), an important difference was that in Fraction 1 more than two-thirds of the vesicles were impermeable to ²²Na⁺, whereas in Fraction 3, ²²Na⁺-impermeable vesicles made up less than a quarter of the total vesicle population.

In the reverse experiment, we studied the effect of extra-vesicular Na⁺ on ⁴⁵Ca²⁺ efflux from vesicles. Vesicles were equilibrated at 22 °C in a medium containing 1 mM ⁴⁵Ca²⁺, 1 mM Mg²⁺, 200 mM K gluconate, and 10 mM K-Pipes, pH 7. Vesicles were then diluted 250-fold into a 200 mM Na or K gluconate medium containing 10 mM K-Pipes, pH 7, 2 mM Mg²⁺, and 1 mM EGTA to maintain external Ca²⁺ at a concentration of less than 10⁻⁷ M. Initial ⁴⁵Ca²⁺ efflux from vesicles of Fraction 1 was appreciably greater in Na⁺ medium than K⁺ medium. At 30 s after vesicle dilution, Na⁺-mediated ⁴⁵Ca²⁺ efflux amounted to 2.4 nmol/mg of protein. For comparison, vesicles of Fraction 3 displayed little Ca/Na exchange activity, with external Na⁺ enhancing ⁴⁵Ca²⁺ release at the 30-s time point by less than 0.5 nmol of Ca²⁺/mg of protein.

Fraction 1 accumulated D-[¹⁴C]glucose with an initial rate nearly twice as great as L-[³H]glucose (Fig. 5). In Fraction 3, D- and L-glucose uptake rates differed by a factor of 1.25 or less, suggesting that most of the D-glucose was nonspecifically taken up. At longer time intervals, similar D- and L-glucose isotope spaces were found. Both were in reasonable agreement with the [³H]sucrose space (2.7 and 3.0 μl/mg of protein for Fractions 1 and 3, respectively, Table II). Together, the ²²Na⁺ and ⁴⁵Ca²⁺ efflux and glucose uptake measurements were in

TABLE II

| Radioisotope  | Apparent isotope space (μl/mg protein) | Efflux rate (1/2) (min) |
|---------------|----------------------------------------|-------------------------|
|               | Fraction 1 | Fraction 3 | Fraction 1 | Fraction 3 |
| [³H]Sucrose   | 2.7        | 3.0        | >10        | >10        |
| [³H]Choline   | 2.3        | 2.8        | 8          | 4          |
| ⁴⁺Rb          | 3.2        | 0.6        | 6          | 5          |
| ²²Na          | 2.3        | 0.5        | 1.5        | 2.5        |
| ³¹Cl          | 2.2        | 0.7        | ~10        | ~10        |
| D-[¹⁴C]Glucose| 3.0        | 2.9        |             |             |
VESICLE SPACES THAT BOTH FORM A PERMEABILITY BARRIER AND ARE ACCESSIBLE

Support of the notion that Fraction 1 was enriched in vesicles derived from the surface membrane of canine heart. Fraction 3 also contained surface membrane vesicles but at a lower proportion than Fraction 1.

LIGHT-SCATTERING MEASUREMENTS—The permeability of Fraction 3 to solutes and ion pairs was further assessed by determining their osmotic behavior. Osmolality was rapidly increased from about 30 to 110 mosm. Transient changes in vesicle volume resulted, as visualized by light-scattering intensity changes in a fluorometer. The effect of osmolality on vesicle size was similar using D-glucose, K gluconate, or choline Cl (Fig. 6). In all three cases, light-scattering signals reached a maximum within 2–3 s, the experimental limit of detection. This rapid initial increase reflected decreased vesicle volumes due to outflow of water (17). The signal then slowly returned to the one observed for control vesicles diluted with 1/10 volume of the initial vesicle medium. The gradual return of the light-scattering signals and thus vesicle volumes to control values was due to influx of the solutes followed by water (17). Re-establishment of equilibrium was similar for D-glucose, K gluconate, and choline Cl (τ a ~ 40–50 s). Since net movement of a salt requires that both ions move across the membrane, the slower ion will be rate limiting. The addition of the K⁺ ionophore valinomycin did not affect the rate of re-establishing equilibrium with K gluconate (Table III), indicating that gluconate was the rate-limiting species.

Fig. 2. Measurement of [3H]sucrose, 3Rb⁺, 32Na⁺, and 34Cl⁻ efflux rates and apparent isotope spaces. Sucrose gradient Fraction 3 (8 mg of protein/ml) was incubated for 6 h at 22 °C in media containing (A) 10 mM K-Pipes (pH 7), 1 mM MgCl₂, 0.25 mM [3H]sucrose (●), and 0.1 mM Na⁺EGTA (C) or (B) 10 mM K-Pipes (pH 7), 50 mM [3H]sucrose, 150 mM K gluconate, and 50 mM 3Rb⁺ (▲), or (C) 10 mM K-Pipes, 50 mM [3H]sucrose, 150 mM K gluconate, and 50 mM 32Na⁺ (□). Vesicles were then diluted 500-fold into unlabeled media of identical composition at 22 °C. Aliquots of 0.5 ml were applied to 0.45-μm Millipore filters, rinsed, and the amounts of radioactivity remaining with the vesicles on the filters were determined. [3H]Sucrose, 3Rb⁺, 32Na⁺, and 34Cl⁻ spaces extrapolated back to zero time corresponded to 2.6, 0.6, 0.55, and 0.7 μl/mg of protein, respectively. Essentially identical [3H]labeled spaces and efflux rates were observed in Media A, B, and C.

Fig. 3. Effect of incubation time on [3H]sucrose, 3Rb⁺, 32Na⁺, and 34Cl⁻ spaces. Fraction 3 was incubated at 22 °C for the indicated times in Media A, B, or C of Fig. 2. Vesicles were then diluted and processed as described in Fig. 2. Apparent isotope spaces obtained by back extrapolation to the time of vesicle dilution (cf. Fig. 2) indicate vesicle spaces that both form a permeability barrier and are accessible to the isotopes at the indicated time.

Fig. 4. Effect of extravesicular Na⁺ and Ca²⁺ on 32Na⁺ efflux from vesicles of Fractions 1 and 3. Microsomes were incubated for 6 h at 23 °C in a medium containing 10 mM K-Pipes (pH 7), 0.3 mM sucrose, 1 mM MgCl₂, and 100 mM 32NaCl. They were then diluted into media containing 100 mM NaCl and 1 mM LaCl₃ (C), 100 mM NaCl, 2 mM MgCl₂, and 1 mM EGTA (□), 100 mM KCl, 2 mM MgCl₂, and 1 mM EGTA (●), or 100 mM KCl, 1 mM MgCl₂, and 0.25 mM CaCl₂ (○). All dilution media contained 10 mM K-Pipes (pH 7) and 0.3 mM sucrose. Efflux of 32Na⁺ was determined at 23 °C by measuring radioactivity retained by the vesicles on Millipore filters.

Fig. 5. n-[14C]Glucose and l-[3H]glucose influx into vesicles of Fractions 1 and 3. Microsomes (8 mg of protein/ml) present in 0.3 mM sucrose, 0.1 mM NaCl, 1 mM MgCl₂, and 10 mM K-Pipes (pH 7) were incubated for 5 min at 32 °C. Glucose influx was initiated by the addition of 1/10 volume of 50 mM n-[14C]glucose (□, □) and 50 mM l-[3H]glucose (●, □). At the indicated time points, 7.5 μl of Fractions 1 (□, □) or 3 (○, ○) were diluted into 1.2 ml of ice-cold "stop solution" containing 2 mM MgCl₂, 0.3 mM sucrose, 0.1 mM NaCl, 1 mM MgCl₂, 10 mM D-glucose, 10 mM L-glucose, and 10 mM K-Pipes, pH 7. Two aliquots of this solution (0.5 ml) were rapidly placed on 0.45-μm Millipore filters and rinsed with ice-cold "stop solution." The filters were counted.
Glycerol elicited a suboptimal signal with a lifetime of 2.5 s or less, suggesting that glycerol equilibrated rapidly across the vesicle membranes (Fig. 6). A different light-scattering signal was recorded when the osmolarity of the vesicle medium was increased by the addition of KCl (Fig. 6). In this instance, a signal of intermediate size, but of long lifetime, was observed, indicating the presence of two vesicle populations in Fraction 3 with differing permeability. One appeared to be permeable to KCl, with a transient volume change lasting less than 2-3 s. The remaining vesicles were impermeable to KCl, as indicated by the long lifetime of the signal. These vesicles were impermeable to Cl⁻ since the K⁺ ionophore valinomycin did not alter the signal (Table III).

K⁺, Na⁺, monoethanolamine, and dimethylmethyleneol pass rapidly through the K,Na channel of skeletal sarcoplasmic reticulum vesicles, whereas the somewhat larger cations choline and Tris are impermeable (16). Table III shows that the cardiac vesicles possess an ion pathway comparable to the one in skeletal muscle sarcoplasmic reticulum. Tris-Cl and choline Cl elicited nearly maximal light-scattering signals, indicating that most of the vesicles were relatively impermeable to these two cations. NaCl, monoethanolamine Cl, and dimethylmethyleneol Cl in the presence of valinomycin and sucrose elicited maximal light-scattering signals with a long lifetime (t₁/₂ > 200 s), suggesting that these ions permeated slowly across all of the vesicle membranes (Table III). Light-scattering data of Table III are in good general agreement with radioisotope space and efflux measurements which had also suggested that Fraction 3 contains two vesicle populations with differing permeability to K⁺, Na⁺, and Cl⁻.

Membrane Potential Measurements—Membrane polarization measurements are another useful means of probing the permeability of vesicles to small cations such as K⁺, Na⁺, or H⁺ (16, 31). The permeability of Fraction 3 to K⁺ was determined in the presence of the impermeable anions gluconate and Pipes (cf. Table III) by measuring the potential that formed during dilution of K⁺-loaded vesicles into media containing low defined K⁺ concentrations. The developed K⁺ diffusion potentials were visualized with the use of the fluorescent dye 3,3'-dipentyl-2,2'-oxacarbocyanine. A characteristic property of this dye is the decrease in fluorescence emission when vesicles become negatively charged inside. The magnitude of fluorescence decrease is roughly proportional to the fraction of vesicles that is polarized (16).

K⁺-loaded vesicles of Fraction 3 elicited no significant change in fluorescence emission when diluted into K⁺ or Na⁺ medium (Fig. 7), indicating that no membrane potential (negative inside) was formed. Dilution of these vesicles into Tris⁺
medium caused a rapid decrease in fluorescence followed by a slow return to the control value. Addition of the K⁺ ionophore valinomycin to Tris⁺ or Na⁺ dilution media resulted in only small increases in the magnitude of the dye signals. The gradual return of the signals and, therefore, the breakdown of the membrane potentials were likely due to the slow inward movement of Tris⁺ (see Table III). In agreement with this interpretation, a rapid breakdown of the potential was observed when the vesicles were rendered permeable to Tris⁺ by the addition of the ionophore X537A (not shown).

Fluorescence measurements suggest that cardiac microsomal vesicles can form a K⁺-diffusion potential, negative inside, when diluted from a K⁺ medium to a Tris⁺ medium containing a low K⁺ concentration. This, in turn, indicated that the vesicles were more permeable to K⁺ than Tris⁺.

Absence of a fluorescence signal in Na⁺ medium suggested the presence of vesicles which did not form a K⁺-diffusion potential because of the rapid exchange of K⁺ for Na⁺ within 1–2 s, the experimental limit of detection. A majority of vesicles of Fraction 3 appeared, therefore, to be permeable to K⁺ and Na⁺, suggesting a K⁺,Na⁺-permeable structure comparable to the one identified in skeletal muscle sarcoplasmic reticulum (16). In support of a similar, or identical, channel structure was the organic cation monoethanolamine behaved like a highly permeant cation in skeletal (16) and cardiac (not shown) sarcoplasmic reticulum.

Increases in fluorescence signal seen in the presence of valinomycin are due to the presence of a K⁺,Na⁺- impermeable vesicle fraction. In the presence of the ionophore, these vesicles were rendered selectively permeable to K⁺ and could, therefore, form a K⁺-diffusion potential. The small increase in fluorescence signals suggested that greater than 85% of the canine heart membrane vesicles in Fraction 3 (as compared to 70% in rabbit skeletal muscle, Ref. 16) were highly permeable to K⁺. Membrane potential measurements are in reasonable agreement with the radiotracer experiments (cf. Table II) considering that the two measurements likely indicate the surface area and the intracellular space of heterogeneously sized vesicle populations, respectively.

Permeability of Fraction 3 to Ca²⁺ and Mg²⁺ was probed by transferring K⁺-filled vesicles to isoosmotic Ca-Pipes or Mg-Pipes media. Fluorescence decreases and return rates were similar to those seen in Tris-Pipes medium (not shown).

In control experiments, we observed a rapid breakdown of the membrane potential in Ca-Pipes medium on addition of the Ca²⁺ ionophore A23187 (1 μg/ml). Thus, membrane potential measurements indicate that Ca²⁺ and Mg²⁺, like Tris⁺, slowly pass across the membranes of the K⁺-permeable vesicles.

A characteristic property of skeletal muscle sarcoplasmic reticulum vesicles is that the fluorescence signals return faster to the base-line when vesicles actively transport Ca²⁺ (14). The faster decay indicates an increase in the rate of K⁺ release during Ca²⁺ transport. In the present study, we initiated Ca²⁺ uptake at 22 °C by transferring K⁺-filled microsomes of Fraction 3 to Tris medium containing 1 mM Mg²⁺, 5 μM Ca²⁺, and 0.5 mM ATP. In a control, ATP was omitted from the dilution medium. Addition of ATP shortened the half-lifetime of the fluorescence signal from 9 to about 3 s (not shown). The effect of ATP on shortening the lifetime of the membrane potential was not eliminated in the presence of 1 μM VO₄⁻, a potent inhibitor of the surface membrane Ca²⁺ pump (52). These results support the suggestion that the K⁺-permeable vesicle population of Fraction 3 originates from sarcoplasmic reticulum.

Proton Permeability—Proton permeability of Fraction 3 was investigated as previously described for skeletal muscle and liver microsomal fractions (31). Vesicles present in Tris-

Pipes buffer at pH 6.2 were transferred to Tris-Pipes buffer at pH 7.8. Fig. 8 shows that the H⁺ gradient thus established generated a transient membrane potential. Addition of the H⁺-carrier carbonyl cyanide p-trifluoromethoxyphenylhydrazone did not significantly affect the overall magnitude of the fluorescent signal, suggesting the presence of membranes intrinsically permeable to H⁺ (or OH⁻).

Additional evidence for H⁺ permeability was provided by investigating the effect of opposing H⁺ and K⁺ gradients on fluorescence emission of diO-C₃(3). In Experiments 1-4 of Table IV, we determined the ability of an opposing H⁺ gradient to nullify membrane potentials generated by a K⁺ gradient. Dilution of K⁺-containing vesicles into Tris⁺ media of equal pH yielded fluorescence decreases of 18% at pH 6.2 and 12% at pH 7.8. Dilution from K⁺ medium at pH 6.2 to Tris⁺ medium at pH 7.8 resulted in an increased fluorescence signal (25%). In the latter case, K⁺ and H⁺ gradients were of equal magnitude.

**Table IV**

Effect of opposing H⁺ and K⁺ gradients on fluorescence emission of diO-C₃(3)

| Experiment | Vesicle medium | Dilution medium | Fluorescence decrease |
|------------|----------------|-----------------|-----------------------|
| 1          | K, pH 6.2      | Tris, pH 6.2    | 16                    |
| 2          | K, pH 6.2      | Tris, pH 7.8    | 25                    |
| 3          | K, pH 7.8      | Tris, pH 6.2    | 1                     |
| 4          | K, pH 7.8      | Tris, pH 7.8    | 12                    |
| 5          | Tris, pH 6.2   | Tris, pH 7.8    | 14                    |
| 6          | K, pH 7.8      | Na, pH 7.8      | 0                     |
| 7          | Tris, pH 6.2   | Na, pH 7.8      | 0                     |
direction and similar magnitude. No significant K\(^+\)-H\(^+\) exchange could, therefore, occur in vesicles permeable to both K\(^+\) and H\(^+\), and an optimal membrane potential was formed (31). In contrast, an opposing pH gradient (K\(^+\), pH 7.8 \rightarrow Tris\(^-\), pH 6.2) essentially eliminated the fluorescence signal, indicating that vesicles were capable of rapid K\(^+\)-H\(^+\) exchange and of lowering their internal K\(^+\) concentration to that of the surrounding medium within 1-2 s, the experimental limit of detection.

Experiments 5-7 of Table IV describe the reverse experiment, namely, the effect of opposing K\(^+\) and Na\(^+\) gradients on a membrane potential generated by a H\(^+\) gradient. Dilution of vesicles from Tris\(^-\) medium at pH 6.2 to Tris\(^-\) medium at pH 7.8 generated a H\(^+\)-induced membrane potential which decreased the fluorescence emission of diO-C5-(3) by 14%. Replacement of external Tris\(^-\) by K\(^+\) or Na\(^+\) nullified the membrane potential, indicating that K\(^+\) and Na\(^+\) could rapidly enter all of the H\(^+\)-permeable vesicles. Thus, together the data of Table IV suggest that the majority of the vesicles in Fraction 3 were permeable to K\(^+\), Na\(^+\), and H\(^+\).

**DISCUSSION**

We have used three techniques to measure the membrane permeability of a cardiac muscle microsomal fraction enriched in sarcoplasmic reticulum. The Millipore filtration technique using radioactively labeled compounds is ideal for determining the vesicle spaces and exchange or efflux rates of relatively impermeant solutes. Light-scattering measurements provide similar information and have the additional advantage of allowing us to measure ion pair and solute fluxes on a time scale of 2-3 s, as compared to 20-30 s using Millipore filtration. Finally, membrane polarization measurements prove useful in evaluating the free cation permeability of isolated membrane vesicles. If should be noted, however, that different vesicle parameters were measured by the three techniques. Radioisotope and membrane polarization measurements reflect internal vesicle spaces and surface areas of the polarized vesicles, respectively.

As summarized below, radioisotope flux measurements indicated that Fraction 1 is predominantly made up of surface membrane vesicles, whereas Fraction 3 consisted of about 80% sarcoplasmic reticulum vesicles and 20% surface membrane vesicles. Fractions 1 and 3 differed appreciably in their permeability to \(^{86}\)Rb\(^+\), \(^{22}\)Na\(^+\), and \(^{36}\)Cl\(^-\). About 80% of \([H]\)sucrose-and [H]choline-impermeable vesicles of Fraction 3 were readily permeable to these ions, while a similar portion of Fraction 1 was impermeable to \(^{86}\)Rb\(^+\), \(^{22}\)Na\(^+\), and \(^{36}\)Cl\(^-\). The ability of Na\(^+\)-impermeable vesicles to exchange \(^{22}\)Na\(^+\) for Ca\(^{2+}\) or Na\(^+\) suggested that the Na\(^+\)-impermeable vesicles in Fractions 1 (80%) and 3 (20%) were mainly derived from the surface membrane structures of cardiac muscle. In agreement with this interpretation was the enhanced ability of the Na\(^+\)-impermeable enriched vesicle Fraction 1 to differentiate between Na\(^+\) and L-glucose uptake.

A majority of skeletal muscle surface membrane vesicles are impermeable to Cl\(^-\) (28), whereas both skeletal muscle sarcoplasmic reticulum (16, 17) and liver endoplasmic reticulum (15) membranes are permeable to Cl\(^-\). The presence of a significant portion of Cl\(^-\)-impermeable vesicles in Fraction 1 is in accord with our contention that this fraction is enriched in surface membrane vesicles. The higher "basic" ATPase activity of Fraction 3, an enzyme characteristic of the surface membrane (19), may be due to an uneven in vivo distribution resulting in vesicle fractions with different enzyme contents. A similar phenomenon has been observed in other tissues such as liver (25) and skeletal muscle (26, 27). Na\(^+\)-impermeable vesicles did not originate from inner mitochondrial membranes since Fractions 1 and 3 had low succinate cytochrome c reductase activities.

Recent studies have contrasted the ion permeability of skeletal muscle sarcoplasmic reticulum and liver endoplasmic reticulum vesicles (15, 16). About two-thirds of skeletal muscle sarcoplasmic reticulum vesicles contain a K,Na channel, rendering them highly permeable to K\(^+\), Rb\(^+\), Na\(^+\), and small organic cations such as monoethanolamine\(^+\). The remaining one-third lack the K,Na channel and are, therefore, relatively impermeable to these cations. K\(^+\),Na\(^+\)-impermeable vesicles are thought to arise as a consequence of a limited number of channels in the in vivo skeletal muscle reticulum structure (about 50/\(\mu\)m\(^2\)) (16). Both K\(^+\),Na\(^+\)-permeable and -impermeable vesicles are permeable to protons (37) and chloride (16), suggesting a higher density of H\(^+\)- and Cl\(^-\)-conducting structures.

Liver microsomes resemble sarcoplasmic reticulum vesicles in that they are permeable to small univalent ions. They appear to lack, however, H\(^+\) and K,Na channels characteristic of skeletal muscle sarcoplasmic reticulum (15). Another important difference between these membranes is that liver microsomes are readily permeable to several small biologically relevant solutes and ions including Na\(^+\), monovalent cations including Na\(^+\), monoethanolamine\(^+\), and H\(^+\). It also contained a highly active Ca\(^{2+}\) transport system characteristic of sarcoplasmic reticulum.

K\(^+\), Na\(^+\), and Cl\(^-\) equilibrated within 3 s, the experimental limit of detection of the light-scattering and membrane potential techniques. Using an average vesicle diameter of 0.15 \(\mu\)m (Fig. 1), the permeability coefficient of the vesicles for K\(^+\), Na\(^+\), and Cl\(^-\) can be calculated to be greater than 10\(^{-9}\) cm/s. This greater proportion of K\(^+\),Na\(^+\)-permeable vesicles in cardiac (70%) versus skeletal muscle (50%) sarcoplasmic reticulum preparations indicates that heart reticulum has at least as many K,Na channels as skeletal reticulum.

The physiological function of the H\(^+\)- and K\(^+\),Na\(^+\)-permeable structures in the sarcoplasmic reticulum membrane of cardiac and skeletal muscle is unclear at present. Miller (33) made the interesting observation that the K,Na channel displays single channel conductance fluctuations between an "open" and "closed" state when skeletal muscle sarcoplasmic reticulum vesicles were fused with planar bilayers. Ca\(^{2+}\) transport and ion flux measurements have led us to propose that the H\(^+\) and K,Na channels of skeletal muscle sarcoplasmic reticulum act as a complementary part of the Ca\(^{2+}\) transport system by allowing rapid H\(^+\) and K\(^+\) movement to counter Ca\(^{2+}\) fluxes during muscle relaxation (14). In addition, rapid H\(^+\) and K\(^+\) fluxes may allow cardiac and skeletal muscle sarcoplasmic reticulum to release Ca\(^{2+}\) during muscle contraction.

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