Excitation of Skinned Muscle Fibers by Imposed Ion Gradients

III. Distribution of Permeant Ions in Unstimulated and Stimulated Fibers

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ABSTRACT   Ion gradients imposed across an internal membrane system stimulate skinned muscle fibers; to evaluate the sarcoplasmic reticulum (SR) as the primary target site, SR polarization under resting and stimulatory conditions was assessed from fiber uptake of permeant probe ions. Solvent spaces were estimated from simultaneous [^{14}C]urea (U) or [^{3}H]deoxyglucose (DOG) uptake in segments of fibers from bullfrog semitendinosus muscle, skinned by microdissection. The distribution spaces, i.e., virtual solvent volumes at bath concentrations (V_U and V_{DOG}), of these uncharged probes correlated well with the protein content of the same segments, which validated the tracer methodology for volume normalization. The membrane-bounded volume fraction (V_m), derived from the difference between total solvent volume (V_s) and the non–membrane-bounded solvent volume (V_c), was sufficient to detect appreciable SR ion accumulation. The V_m estimated from the difference between V_U and V_{DOG} assayed simultaneously with 2 or 5–6 min exposures was 10–11%, which is consistent with the morphometric volume fraction (mostly SR) in frog fibers; however, the change in this difference after membrane permeabilization corresponded to V_m only 5%. The change in permeant ion distribution space caused by member permeabilization was used to assess SR membrane polarization, assuming the free ions distribute across the intact membrane according to the Nernst ratio. Resting polarization (SR lumen positive) was assessed from [^{14}C]SCN$^-$ or [^{14}C]propionate$^-$ distribution spaces in unstimulated fibers, expressed relative to V_{DOG} (assayed simultaneously). The ratios for (a) [^{14}C]SCN$^-$ space (carrier 2 mM) and (b) [^{14}C]propionate$^-$ space (carrier 120 mM) were not decreased by membrane permeabilization. This indicated that anion distribution was independent of membrane integrity and did not reflect an SR transmembrane potential, although a was more and b was less than 1. Polarization

under stimulatory conditions (lumen negative) was assessed from $^{86}Rb^+$ distribution, before and after an imposed ion gradient (choline Cl replacement of K methanesulfonate (KMes) at constant $[K^+][Cl^-]$) that theoretically could generate a 48-fold transmembrane cation ratio; Ca release was minimized by EGTA. The ratio of $^{86}Rb^+$ space to V_U, >1 in KMes (120 mM K, the effective carrier), was higher in choline Cl (2.5 mM K) but not decreased by membrane permeabilization; this indicated that $^{86}Rb^+$ distribution did not reflect an SR transmembrane potential. Similar results in the presence of valinomycin ruled out the possibility of inadequate $^{86}Rb^+$ equilibration. These results suggest that (a) resting SR polarization is small or absent, (b) a large stimulatory ion gradient does not generate sustained SR polarization concomitant with Ca efflux stimulation in skinned fibers, and (c) ionic stimulation of Ca release does not act primarily by SR transmembrane voltage changes.

INTRODUCTION

During activation of vertebrate twitch fibers, depolarization of the surface and transverse tubule (T tubule) membranes is coupled to increased Ca efflux from the sarcoplasmic reticulum (SR) by intermediate mechanisms that are still under active investigation. One possibility that has been considered is SR depolarization after transmission at the T-SR junction (see Costantin, 1975; Endo, 1977; Stephenson, 1981 for reviews). While early kinetic and morphological studies suggested that the SR lumen was an extracellular extension of the T-tubular system (Dydynska and Wilkie, 1963; Birks and Davey, 1969), and thus polarized with respect to the myoplasm, this interpretation was ruled out by subsequent efflux analysis on single fibers (Neville, 1979) and by the similarity of elemental Na, K, and Cl concentrations in SR and myoplasm in electron probe microanalyses (Somlyo et al., 1977); the intracellular nature of the SR made a simple conducting model unlikely. The possibility that an SR voltage change initiated Ca release also was raised by the size and time course of several optical signals during activation, but subsequent temporal resolution argued against this causal relation (Oetliker, 1982; Baylor et al., 1984).

Another type of support for stimulation by direct SR "depolarization" might be inferred from the ability of ion replacements that produce a negative diffusion potential to induce Ca release in Natori-type and "split" skinned fibers, and in various SR vesicle preparations (see Endo, 1977; Stephenson, 1981b; Martonosi, 1984 for reviews). However, this mechanism has not been demonstrated and alternative mechanisms exist. First, osmotic swelling of the SR can cause Ca release when Cl^- is substituted for a much less permeant anion at constant $[K^+]$ (for example, Meissner and McKinley, 1976; Mobley, 1979; Stephenson, 1985b). Second, the locus of depolarization could be sealed polarized T tubules, as discussed by Costantin and Podolsky (1967) and supported by more recent studies on vesicle preparations (Lau et al., 1979; Ikemoto et al., 1984) and skinned fibers (Donaldson, 1985; Volpe and Stephenson, 1986; Stephenson, 1986; Stephenson and Lerner, 1986).

The question of an SR locus of "depolarization" in skinned fibers also can be addressed by a more direct approach: if imposed ion gradients stimulate the SR directly, its transmembrane potential should be reflected in the Nernst distribution of permeant ions in the SR compartment, which comprises ~9% fiber volume (~36

times the T-tubule volume) in intact frog fibers (Peachey, 1965; Mobley and Eisenberg, 1975). The present studies used this approach to evaluate (*a*) resting SR polarization and (*b*) negative polarization during a sustained ionic stimulation of ^{45}Ca efflux (Stephenson, 1985*b*). Ca release itself was minimized to exclude any secondary electrogenicity of rapid Ca efflux. The results gave no evidence of anion accumulation indicative of an appreciable positive SR resting potential, which is consistent with the elemental distribution in intact fibers (Somlyo et al., 1977), and there was no evidence of cation accumulation indicative of a sustained negative SR potential during the imposed negative diffusion potential.

Preliminary reports of these studies have been presented (Stephenson, 1981*a*, 1985*a*).

METHODS

Fiber Preparation

Microdissection, mounting of skinned fiber segments, and isometric force measurement were carried out as described previously (Stephenson, 1978; 1985*b*), but longer segments from muscle fibers of the bullfrog (*Rana catesbeiana*) were used. In brief, semitendinosus muscles were isolated and suspended in cold low Cl Ringer solution containing (in millimolar): 217 sucrose, 2.5 KCl, 1.8 CaCl₂, 3.1 NaH₂PO₄ plus Na₂HPO₄. A bundle of fibers was transferred to cold paraffin oil and a single fiber was isolated from tendon to tendon; fibers with any visible opacities or membrane defects were rejected. Segments were cut and stored in cold oil until they were skinned by microdissection just before use. The skinned segment was tied with monofilament thread to small stainless steel rods (one was fixed and the other was attached to a leaf-spring photodiode force transducer) for continuous measurement of isometric force and exposure of the segment to bathing solutions; segments that developed force (spontaneously) were rejected. After mounting under oil at ~5°C in the spring-mounted thermoregulated chamber, the temperature was maintained at 19°C during tracer uptake; loading and washout procedures are described below. Triton X-100 treatment (see below) was carried out in a separate lucite chamber at room temperature. Force was recorded on a Honeywell chart recorder (Electronik 193; Honeywell Inc., Minneapolis, MN) or a Linseis chart recorder (2021; Linseis Inc., Princeton Junction, NJ) in the cation uptake studies.

Bathing Solutions

The skinned fiber bathing solutions (pH 7.00) contained (in millimolar): 120 or 122.5 major salt (K propionate, K methanesulfonate (KMES), or choline Cl), 10 imidazole, 5 Na₂ATP, 3 MgSO₄, 1 EGTA, and probe constituents as indicated below. In studies on Cl stimulation at constant [K][Cl], the pH of stock and working solutions was adjusted with Tris base instead of KOH; the solution compositions were matched to previous studies (Stephenson, 1978; 1985*b*). All chemicals were reagent grade. Valinomycin (Sigma Chemical Co., St. Louis, MO) was prepared as a concentrated stock in 95% ethanol and diluted 1:200 to 2 mg/ml final concentration.

The loading solutions with probe solutes contained two of the following labeled species: [^{14}C]urea, [^{3}H]deoxyglucose, [^{14}C]SCN, [^{14}C]propionate (Amersham Corp., Arlington Heights, IL; or New England Nuclear, Boston, MA) or ^{86}Rb (New England Nuclear). They were made up to the following activities: 80 μCi/ml ^{3}H, 40 μCi/ml ^{14}C, and 25 μCi/ml ^{86}Rb. Total carrier concentrations were (in millimolar): 1.00 deoxyglucose, 2.07–2.14 urea, 2.03 SCN⁻, 120 propionate⁻ (the major anion in the solution), and 0.08 ^{86}Rb⁺ (+ 120 or 2.5 K⁺,

effectively a carrier). Washout solutions matched the loading solution except for isotope addition.

Isotope Analysis

Aliquots of loading and washout solutions were counted in a three-channel liquid scintillation spectrometer (either Tri-Carb 3330; Packard Instrument Co., Downers Grove, IL; or Inter-technique SL32; Plasir, France), using 10 ml Aquasol (New England Nuclear). Elution by the washout protocol was verified in control experiments by counting the fiber. The distribution spaces were calculated from the ratios of the cumulative isotope eluted in each set of washes from the fiber segment to the concentration of the same isotope in the loading solution, i.e., total disintegrations per minute (dpm) taken up by the fiber segment divided by dpm per nanoliter loading solution gives the virtual volume in the fiber at the concentration of the loading solution. The contributions of each isotope in double-labeled samples were separated by standard methods, setting the spectrometer windows to eliminate ^{3}H or ^{14}C counts from the higher energy channel counting ^{14}C or ^{86}Rb; efficiencies were monitored by the channel-ratio method and activity from each isotope in the lower energy channel was calculated from standard curves on singly and doubly labeled solution aliquots. Efficiencies varied little among the solutions employed. ^{86}Rb loading solution samples usually were counted with the ^{86}Rb washout samples at sufficiently frequent intervals that decay corrections were unnecessary; in a few cases when decay approached 2–3% the count rates of the washout samples were corrected from the bracketing reference solutions.

Tracer Loading and Washout

The skinned segments, mounted isometrically at ~1.1 × slack length under heavy paraffin oil (reagent grade), were exposed as illustrated in Fig. 1 to 10-μl droplets of labeled solutions on a lucite block placed in the mounting well. During loading, the droplet was moved slightly perpendicular to the fiber axis (by translating the chamber) to promote mixing. Exposure was terminated by translating the chamber to remove the droplet from the fiber; the temperature setting was lowered to 2–5°C, and adherent aqueous fluid at the holder tips was removed carefully with small strips of filter paper (GF/C; Whatman Inc. Clifton, NJ), avoiding contact with the fiber. This "blotting" method was developed after preliminary experiments showed that the required exposure of the fiber length to the droplet (Fig. 1) could leave 5–10 nl adherent fluid on the holder tips (assayed by counting the filter strips), a high and variable contamination that could not be eliminated by a brief rinse in washout solution, which also eluted fiber tracer, but could be reduced substantially by the local adsorption. However, residual adherent fluid, including that on the fiber surface itself, remained a source of variability in absolute volume; its effect was minimized by the simultaneous measurement of all ion distribution spaces with a neutral probe distribution space. The filter strips were monitored routinely; a difference in isotope ratio from the loading solution would indicate substantial adsorption of fiber fluid, invalidating the assay, but this rarely occurred after the preliminary study. The fiber tracer was eluted by immersing the segment sequentially in three washes (1.2 ml each) of duration <0.5 s (at 2–5°C), 3 min (at 19°C), and >5 min (at room temperature in the lucite chamber). Essentially all the tracer was eluted in the first two washes. In ion distribution studies, the fibers were loaded first with both neutral probes for 2 min and washed out; then simultaneous uptake of ion and one neutral probe was measured in the same segment, usually under several conditions. After recycled segments were returned to the oil well, adherent aqueous fluid was removed from the holders before exposure to a new droplet. The effect of the internal membranes on tracer distribution was tested in a terminal cycle on segments that had been exposed to 1.2 ml solution with 0.05% Triton-X100 plus 5 mM

EGTA for ~10 min at room temperature to permeabilize the membranes (see Stephenson and Podolsky, 1977). This solution also was monitored to verify completeness of the preceding elution, and the detergent was washed out of the segment before the final uptake measurement. In the stimulation studies most fibers were preequilibrated in a ^{86}Rb-KMes droplet (and the holder tips "blotted") before exposure to a ^{86}Rb-choline Cl droplet, in order to facilitate any rapid SR uptake (see Results).

Results are expressed as the mean ± SEM for the number of measurments in parentheses. The significance of differences was evaluated with Student's t tests; $P < 0.05$ was considered significant.

Protein Analysis

Tracer distribution methodology was evaluated by a comparison of solvent spaces and protein content of the same segment (see Results). Segments to be analyzed for protein content were
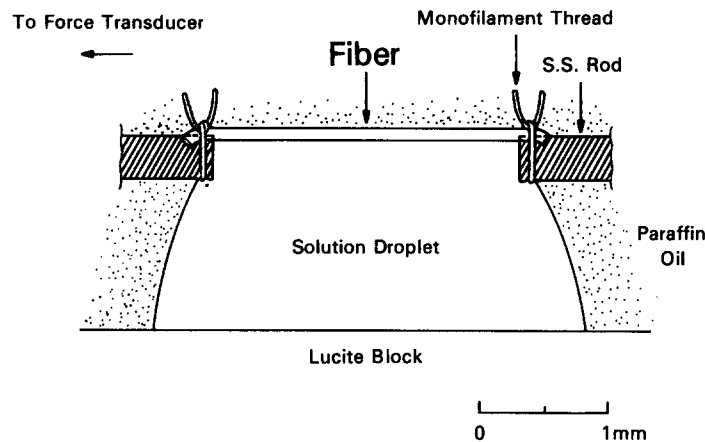

FIGURE 1. Schematic diagram of the experimental setup for exposure of skinned fiber segments to labeled solutions. The fibers were mounted isometrically under heavy paraffin oil between stainless steel rods (attached to micromanipulators). The (aqueous) loading solutions were 10-μl droplets placed on a small lucite block and submerged under the oil in the mounting well of the spring-loaded thermoregulated chamber, which could be translated relative to the fixed fiber segment. See text for further details.

untied from the holders after tracer elution and harvested with small hooks of stainless steel wire. Total protein was analyzed by a micromethod described by Bearden (1978), which uses the differential change in absorbance of Coomassie Brilliant Blue G-250 at two wave lengths (465 and 595 μm) when it binds to protein. The harvested fresh segments were digested in 0.5 N NaOH, neutralized, and diluted with buffered NaCl solution (pH 7.4) containing EDTA and dithiothreitol. Duplicate aliquots were assayed by a slightly modified Bearden procedure, with absorbance measured in a double-beam spectrophotometer (Acta II or III; Beckman Instruments, Inc., Palo Alto, CA) using bovine serum albumin (Sigma Chemical Co.) as a standard (0.5–5 μg/ml). The use of high purity Coomassie Brilliant Blue 250 (Serva Blue G, research grade; Serva Feinbiochemica, Heidelberg, FRG) resulted in larger changes in differential absorbance than those given by Bearden. The differential absorbance and slope of standard curves were larger with bovine serum albumin than with purified rabbit actin, which was kindly supplied by Dr. Lois Greene (data not shown), so that fiber protein content would be ~12% higher if "corrected" to actin.

RESULTS

Validation of Tracer Distribution Measurements

To evaluate the tracer distribution methodology, a series of skinned segments with the largest practical range of lengths was examined for the correlation between the distribution spaces of [^{14}C]urea (V_U) and [^{3}H]deoxyglucose (V_{DOG}), measured simultaneously, and the protein content of the same fiber segment. The linear relation between each distribution space and protein content, with an initial droplet exposure of 5–6 min, is shown in Fig. 2. The correlation of both neutral probe distribution spaces with protein content was excellent ($r = 0.961$ and 0.959, respectively, $n = 18$), and substantially better than the correlation in the same segments of fiber
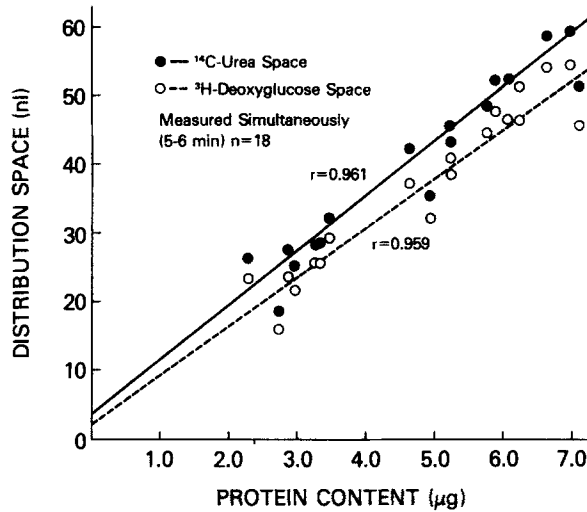

FIGURE 2. Correlation between the distribution space for [^{14}C]urea (filled circles) and for [^{3}H]deoxyglucose (open circles) in 18 skinned fiber segments (measured simultaneously) and the protein content of the same segment. Distribution spaces were measured after an initial 5–6-min exposure to a doubly labeled solution droplet, which was followed by elution of the tracers in 3 (1.2 ml) washes. The segment then was harvested and its protein content was assayed by a Coomassie Blue micromethod (see Methods and text for further details). The correlation coefficients were 0.961 and 0.959, respectively.

volume estimated from length and diameter (measured at 40 and 80× magnification) with protein content ($r = 0.795$). These fibers ranged from 112 to 169 μm in "diameter" (usually the large axis of an elliptical cross-section) and 1.9 to 3.8 mm in length (mean of length between ties and unmounted length). In the subsequent studies lengths were usually 2.1–2.7 mm (and diameters were similar).

The slopes of the regressions were 7.660 for V_U and 7.158 for V_{DOG}, corresponding to protein contents of 0.131 μg/nl solvent space and 0.140 μg/nl solvent space, respectively, which is in a reasonable range. However, these values cannot be equated precisely to the absolute tissue protein concentration; bovine serum albumin is not an absolute standard for total muscle protein (see Methods), and V_U as measured may be slightly larger than the total solvent volume (see below). Similarly, the small intercepts of the regressions, 2.30 nl for DOG and 3.96 nl for urea, suggest that only a small amount of tracer uptake derived from adherent fluid rather than fiber fluid, especially since V_U should be an upper limit (see below).

These results validated the methodology for use of uncharged inert probes to normalize the amount of tissue and estimate solvent volumes in these small preparations, which ranged ~25–40 nl at the lengths usually used. The relationship between distribution spaces and protein content and the difference in distribution space between the two probes (see below) indicated that the measured uptake assayed primarily fiber tracer, without substantial distortion from adherent fluid. These probes, therefore, could be used to normalize the distribution of permeant tracer ions, measured with the same methods.

Solvent Volumes

The neutral probes were also used to confirm that the membrane-bounded solvent fraction (V_m) was large enough to detect the accumulation of charged probes. In intact frog fibers, the total membrane-bounded volume fraction estimated morphometrically is ~11% and the SR volume is 9.1% (Peachey, 1965; Mobley and Eisenberg, 1975); with a similar solvent fraction relatively small SR accumulation ratios could be detected (see Discussion). V_U exceeded V_{DOG}, which was measured simultaneously in the data for Fig. 2, and under appropriate conditions these probes were used to assay total solvent water (V_s) and non–membrane bounded cytosol (V_c) accessible to simple diffusion. Diffusional equilibration of the fibers is rapid and deoxyglucose should enter intact SR relatively slowly (see Discussion); at small fractions of its permeation time V_{DOG} would assay mainly V_c and the difference between V_s and V_{DOG} would reflect V_m (primarily SR).

Difference spaces between V_s and V_{DOG} expressed as percentage of V_s are shown in Fig. 3, with V_s estimated in several ways. The open bars represent data with V_s given by the simultaneously measured V_U; with initial exposures of 2 min, or 5–6 min (the data from Fig. 2) this difference space was 11.0 and 10.4%, which is similar to the morphometric volume fraction. Under these conditions urea and deoxyglucose seemed to be nearly ideal probes for V_s and V_c, but uptake under other conditions necessitated further evaluation.

After the internal membranes of recycled fibers were permeabilized (see Methods), the simultaneously assayed difference space was not zero, as would be expected if deoxyglucose equilibrated with V_m and both were ideal probes. In five segments with the initial 2-min assay, $(V_U-V_{DOG})/V_U$ was decreased by only 5.28 ± 0.64% $(P < 0.001)$ after permeabilization. The significant residual difference space in six permeabilized segments (hatched bar) implies that after recycling and permeabilization V_U overestimated V_s and/or V_{DOG} underestimated V_s. Overestimation by V_U is consistent with reports that the equilibrated urea space exceeds total water in frog and rabbit muscle (see Bozler, 1961); adsorption or binding of only 0.1 mM urea/liter fiber water would add ~5% to V_s (at 2-mM carrier). Final V_U was 1.042 × initial V_U in the five segments above but the change was not significant. With V_s estimated from initial V_U (in the same fiber segment), the residual difference space was smaller and not significant when additional segments were included with V_{DOG} (but not V_U) assayed after permeabilization (adjacent cross-hatched bars). Underestimation by V_{DOG} is also possible; a small fraction of V_s is unavailable to molecules the size of deoxyglucose (Bozler, 1961; D. G. Stephenson et al., 1981). V_{DOG} after permeabilization was slightly but not significantly smaller than initial 2 min V_U in the

same segment (ratio 0.985 ± 0.020 [9]), so as an estimate of V_s it gave an initial V_m, 9.6% (last, stippled bar), similar to the first two bars. This similarity supports the V_m estimated by initial simultaneous V_{DOG} and V_U assay, which also resembles the morphometric V_m in intact fibers. The lower estimate of V_m given by the decrease in $(V_U-V_{DOG})/V_U$ from initial to residual value in recycled permeabilized segments, 5.3%, is subject to several reservations: (a) it could result from overestimation of V_s by final V_U after longer incubation, combined with a slight underestimation by final V_{DOG} (see above); (b) the ultrastructure of skinned frog fibers after aqueous incubation (e.g., Franzini-Armstrong, 1971; Taylor and Godt, 1976) does not suggest a large decrease in SR volume.
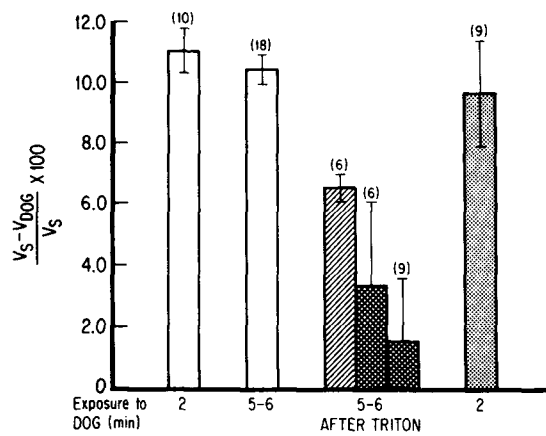

FIGURE 3. The membrane-bounded solvent volume given by the fractional difference (×100) between estimates of total solvent volume (V_s) and the [³H]deoxyglucose distribution space (V_{DOG}) in the same fiber segment. Exposure conditions for V_{DOG} are indicated beneath the bars. Open bars, V_s estimated from the [¹⁴C]urea distribution space (V_U), measured simultaneously with V_{DOG} in initial exposures. Hatched bar, fractional difference between V_U and V_{DOG} measured simultaneously after membrane permeabilization (see text for details). Crosshatched bars, fractional difference between V_s as estimated from V_U with initial 2-min exposures and V_{DOG} after membrane permeabilization (in the same fiber segment); data shown are for the same fiber segments as the hatched bar or including three additional segments with no V_U measurement after permeabilization. Stippled bar, fractional difference between V_s as estimated from V_{DOG} after membrane permeabilization and V_{DOG} with initial 2-min exposures (in the same fiber segment). Results in this and subsequent figures are expressed as the mean ± SEM for the number of determinations in parentheses.

To evaluate V_s changes during recycling that could affect detection of ion accumulation, V_{DOG} assayed simultaneously with anion probes in subsequent cycles was normalized to initial 2 min V_{DOG} in the same fiber segment (Table I). V_{DOG} increased 15% with 10 min of incubation. This is a greater increase than initial V_m, suggesting a V_s increase before membrane permeabilization. V_{DOG} then decreased significantly after permeabilization to values consistent with initial $V_c + V_m$. This final V_{DOG} was stable and reproducible (Table I, footnote). V_U assayed simultaneously with ⁸⁶Rb, in studies with more complex recycling (see below), increased more than in the V_U–V_{DOG} studies. Normalized to the earliest valid assay on the same segment with 2–4-min exposures (initial V_U was unusable for technical reasons), relative V_U in subsequent cycles with altered ionic composition was 1.142 ± 0.036 (9). The pooled rela-

tive V_U, including longer subsequent exposures and total incubations, was 1.187 ± 0.030 (14). Membrane permeabilization decreased the pooled ratio to 1.134 ± 0.025 (9), but this was not significant; the smaller ratio with the same exposure times ($n = 5$) was unchanged. The ratios may include both urea "binding" and increased V_s.

These ratios had two implications. (a) The modest suggested increases in V_s before permeabilization would not substantially decrease V_m and the detection of ion accumulation. If V_s increased with homogeneous fiber swelling, V_m and sensitivity would be unchanged. If V_m swelled disproportionately, detection sensitivity would increase. Even if increased V_s were associated solely with V_c, and other factors increasing V_{DOG} and V_U were neglected, a 14–18% increase would reduce a 10% V_m to 8.8–8.5%, making only small charge accumulations difficult to detect (see Discussion). (b) A decrease in V_s with membrane permeabilization must decrease total charged probe content, a change in the same direction as dissipation of prior SR ion accumulation; this would complicate the estimation of charge distribution. Therefore, all distribu-

TABLE I

Relative V_{DOG} in Recycled Exposures

Exposure time (min)	Ratio V_{DOG}: initial V_{DOG}*	Ratio V_{DOG} (permeabilized): final V_{DOG}[†]
2	1.040 ± 0.025 (7) (NS)	
5–6	1.132 ± 0.027 (4)	
10	1.151 ± 0.025 (9)	0.951 ± 0.017 (9)
5–6 (permeabilized)	1.110 ± 0.022 (9)	

All ratios refer to V_{DOG} assayed in the same fiber segment, and were significantly different from 1.000 except as marked (NS). Six segments were assayed twice after permeabilization (with V_U or a ^{14}C-labeled anion) and the average used for the ratios above; the ratio of the second to the first assay was 1.026 ± 1.022 (6) and was not significantly different from 1.000, which is an indication of the reproducibility of the method.
*Initial V_{DOG} was measured with a 2-min exposure.
[†]Final V_{DOG} was measured with a 10-min exposure.

tion spaces of charged probes were normalized to simultaneously assayed V_{DOG} or V_U, which empirically monitored random or systematic variations in V_s.

Permeant Anion Distribution in Unstimulated Fibers

If the SR is polarized positive with respect to V_c in resting fibers, permeant anions should accumulate in its luminal space according to the Nernst equation. This possibility was examined from the relative distribution of ^{14}C-labeled SCN⁻ (2 mM total SCN⁻) and propionate⁻ (120 mM total propionate⁻). Anion uptake was assayed simultaneously with V_{DOG} after short and long incubations, and after internal membrane permeabilization to distinguish membrane-bounded accumulation from unrelated effects on the distributions.

Fig. 4 shows the distribution space of [^{14}C]SCN⁻ relative to V_{DOG} after exposure times of 2, 5, or 10 min, or of 5–6 min following membrane permeabilization. The ratio ($\times 100$) decreased from 119.0 after a 2-min exposure to 114.1 after a 10-min exposure (open bars), which is consistent with a slow increase in V_{DOG} above V_c. Within the same set of segments ($n = 6$) the ratio was the same after a 5- or 10-min

incubation. The relative [^{14}C]SCN$^-$ space was unaltered by membrane permeabilization; the ratio after permeabilization (hatched bar) was the same as after a 10-min exposure across intact membranes, even when compared in the same recycled segment (last bar). The absence of any decrease in ratio (mean difference [$\times 100$] $+0.5 \pm 1.1$) indicated that [^{14}C]SCN$^-$ distribution was not attributable to SR membrane polarization. The [^{14}C]SCN$^-$ space relative to V_{DOG} was $>100\%$ at all times; after membrane permeabilization this value was 114.6% and the calculated ratio to V_U (measured in a separate cycle with V_{DOG} and so normalized) was $\sim$108%. These ratios correspond to 0.3 (or 0.16) mM/liter fiber water excess SCN$^-$ at $\sim$2 mM carrier (set low to minimize lyotropic anion effects on the fibers). A plausible basis for excess SCN$^-$ is binding and/or adsorption, which are well documented and implicit in effects on frog muscle (see Dani et al., 1983 and references; Hoek et al., 1980).

FIGURE 4. Estimation of anion accumulation from the distribution space of [^{14}C]SCN$^-$ (V_{SCN}) relative to V_{DOG} ($\times 100$); exposure conditions are indicated beneath. Open bars, simultaneous exposures for increasing times with a 5-min exposure in 2 different series given both pooled and separately to show the similarity between 5- and 10-min exposure in the same series. Hatched bar, simultaneous exposure after membrane permeabilization (see text). Last bar, the difference between

the simultaneously measured relative distribution ($\times 100$) before (10-min exposure) and after membrane permeabilization in the same fiber segment; this small difference was not significant (see text for details).
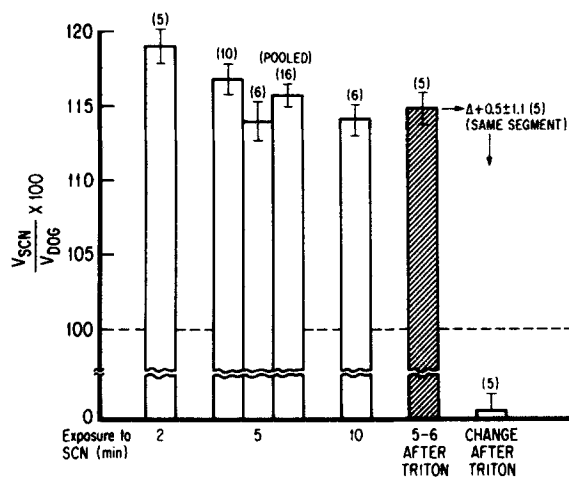
The second anion examined, [^{14}C]propionate$^-$, was a label for the main anion of the bathing solution and therefore at high carrier concentration (which should minimize the contribution of saturable "binding"). K propionate permeates the membranes of isolated SR vesicles with a half-time of 10 s (Kometani and Kasai, 1978). Fig. 5 shows the distribution space of [^{14}C]propionate$^-$ relative to V_{DOG} after exposures of 2 or 10 min (open bars), or of 5–6 min after membrane permeabilization (hatched bar). The ratio ($\times$ 100) was 100% at 2 min, when V_{DOG} reflected primarily V_c, and fell to 95.9% by 10 min, which is consistent with a slow increase in V_{DOG} above V_c. Again, membrane permeabilization did not decrease the ratio, even when comparison was made in the same recycled segment (last bar) where the mean change was $+0.7 \pm 2.0\%$. The calculated ratio of [^{14}C]propionate$^-$ space to V_U (measured separately with V_{DOG} in the same segment and so normalized) was $\sim$90% (data not shown). Evidently, [^{14}C]propionate$^-$ equilibrated with V_s to less than the

bathing medium concentration. A plausible basis for ratios <1.00 is the exclusion of mobile negative charge by Donnan and related effects that would be independent of membrane permeabilization (see Discussion). The relatively small exclusion identified in permeabilized fibers could not have masked appreciable SR accumulation before permeabilization.

Changes in V_{DOG} also could not have masked a decrease in anion accumulation with permeabilization; if the relative [^{14}C]SCN$^-$ spaces before and after permeabilization are normalized to initial V_{DOG} with the data in Table I, accumulated [^{14}C]SCN$^-$ (above its own V_{DOG}) is 0.162 nl/nl initial V_{DOG} in both cases. Similar normalization of the relative [^{14}C]propionate spaces gives a slight increase rather than a decrease after permeabilization. There is no apparent basis for an increase in SCN$^-$ "binding" or decrease in propionate$^-$ (anion) exclusion with permeabilization that each would just compensate a dissipation of SR accumulation. Therefore, the absence of any decrease in the distribution ratio of either permeant anion after membrane permeabilization suggested that no transmembrane concentration gra-
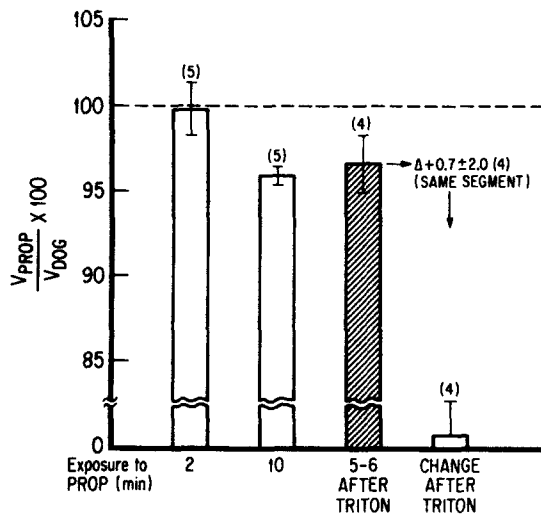

FIGURE 5. Estimation of anion accumulation from the distribution space of [^{14}C]propionate$^-$ (V_{PROP}) relative to V_{DOG} (×100); exposure conditions are indicated beneath. Open bars, simultaneous short and long exposures. Hatched bar, simultaneous exposures after membrane permeabilization. Last bar, the difference between the simultaneously measured relative distribution spaces (×100) before (10-min exposure) and after membrane permeabilization in the same fiber segment; this small difference was not significant.

dient had existed. The absence of anion accumulation across the SR membrane is consistent with the elemental Cl distribution in intact fibers (Somlyo et al., 1977), and implies that resting polarization (lumen positive) was negligible or absent (see Discussion).

Permeant Cation Distribution

The ability of permeant anion replacements to induce Ca release from skinned or split fibers could be attributed to direct "depolarization" of the SR (for reviews, see Endo, 1977; Stephenson, 1981b; Martonosi, 1984). In the absence of appreciable positive resting polarization (see above), generation of a transmembrane potential (lumen negative) would be required. An SR membrane voltage change was assessed from the ability of stimulatory ion replacements to generate concomitant SR accu-

mulation of a permeant cation. (The inverse anion exclusion ratios would be less detectable, see Discussion.)

The distribution space of ^{86}Rb$^+$ was measured during unstimulated and stimulatory conditions that were based on previous studies; choline Cl replacement of KMes at constant $[K^+][Cl^-]$ product stimulates a large ^{45}Ca efflux that is reduced to a small Ca^{2+}-insensitive component in the presence of EGTA (Stephenson, 1985b). Stimulation in EGTA was chosen because (a) efflux stimulation is sustained for at least 1 min and (b) any secondary SR transmembrane potential change due to a large rapid Ca release would be avoided (see Discussion). When the resting solution, 120 mM KMes + 2.5 mM choline Cl, was replaced by 120 mM choline Cl + 2.5 mM KMes, the stimulatory Cl^- and K^+ gradients (120/2.5) theoretically could produce nearly 100 mV diffusion potential (lumen negative) and a 48-fold permeant cation accumulation across the SR membrane, with sufficiently impermeant counterions. Much smaller Nernst accumulation ratios across the SR would be detectable (see Discussion).

^{86}Rb$^+$ uptake was measured simultaneously with V_U, and the fiber segments were recycled to measure the relative distribution spaces in KMes or choline Cl solution under several conditions. Results were expressed as the ratio ^{86}Rb$^+$space:V_U ($\times 100$). Exposure time under stimulatory conditions was based on the following considerations: (a) both diffusional and transmembrane equilibration of ^{86}Rb$^+$ were required, (b) stimulation of Ca^{2+}-insensitive ^{45}Ca efflux is sustained for at least 1 min under similar conditions (Stephenson, 1985b), and (c) a causal membrane potential change was assumed to have similar minimum duration (see Discussion). To minimize diffusional delays, almost all segments were preexposed to ^{86}Rb$^+$ + [^{14}C]urea in KMes solution to equilibrate fiber ^{86}Rb$^+$ and removed to the oil phase (see Methods) before exposure to ^{86}Rb$^+$-choline Cl solution.

Under resting conditions, the ^{86}Rb$^+$ distribution ratio after 2–4 min exposure to labeled KMes solution was 109% in the first set of experiments (Fig. 6), as shown by the first bar. This ratio, like propionate$^-$ exclusion, is consistent with Donnan-type effects (see Discussion). ^{86}Rb$^+$ distribution under stimulatory conditions (in choline Cl) was examined with several protocols. Preexposed segments (see above) were exposed to ^{86}Rb-choline Cl solution for 0.5 min ($n = 1$), 1 min ($n = 5$) or 2 min ($n = 5$). The preexposure times in ^{86}Rb$^+$-KMes, 0.5–3.5 min, gave total ^{86}Rb$^+$ exposures of 2–4 min. The effect of prior Ca loading (with unlabeled Ca-EGTA buffer) was checked in four segments. No significant differences (or trends) in the distribution ratio were seen with these protocol variations, so the results were pooled. The mean ^{86}Rb$^+$ distribution ratio in choline Cl, shown by the second (stippled) bar in Fig. 6, was 130%. The significantly higher ratio in choline Cl was independent of membrane integrity, since it was not decreased after membrane permeabilization (third bar, Fig. 6). When the ratio before and after permeabilization was compared in the same segment, usually with identical exposure protocols, the difference ($\times$ 100) was $+0.5 \pm 1.9\%$, i.e., very small, positive, and not significant (last bar). The inability of membrane permeabilization to dissipate accumulation indicated that an SR transmembrane potential was not the basis of the higher ^{86}Rb$^+$ distribution ratio in choline Cl. The increase must result from membrane-independent factors; for example, the decrease in effective carrier concentration for ^{86}Rb$^+$, when $[K^+]$

changes from 120 mM in KMes solution to 2.5 mM in choline Cl solution, could unmask specific binding (<0.5 mM/liter V_s).

A second study was performed to rule out the possibility that Rb^+ permeability was rate-limiting to measurement of a cation redistribution, although isolated SR vesicles have high K^+ and Rb^+ permeability (Meissner and McKinley, 1976; Kometani and Kasai, 1978). The main $^{86}Rb^+$ experiments were repeated with the K^+/Rb^+ ionophore valinomycin (Pressman, 1976) in all labeled solutions (2 mg/ml). All segments were exposed to $^{86}Rb^+$-KMes solution for 2 min to assay resting distribution and then recycled; to assay under stimulatory conditions they were preexposed to $^{86}Rb^+$-KMes solution for 1 min and then to $^{86}Rb^+$-choline Cl for 1 min. As seen in Fig. 7, the $^{86}Rb^+$ distribution ratio was slightly larger in KMes solution (first bar) and
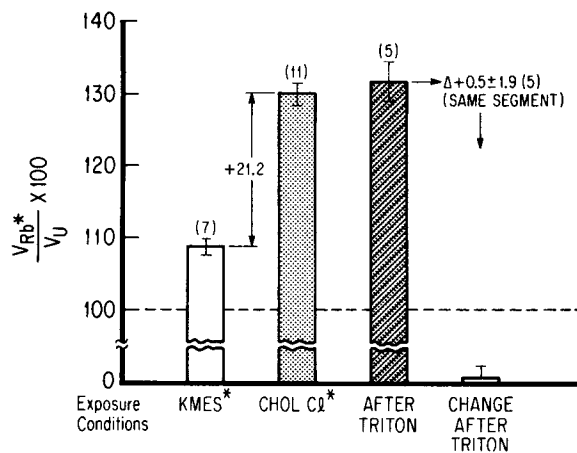

FIGURE 6. Estimation of cation accumulation from the distribution space of $^{86}Rb^+$ (V_{Rb^*}) relative to [^{14}C]urea (V_U) measured simultaneously under the exposure conditions indicated below. Open bar, ^{86}Rb-KMes solution (unstimulated), 2–4-min exposures. Stippled bar, ^{86}Rb-choline Cl solution (stimulated), 0.5–2-min exposures usually after preincubation in ^{86}Rb-KMes (without elution); see text for details. Hatched and stippled bar, ^{86}Rb-choline Cl solution after membrane permeabilization (see text for details). Last bar, the difference between simultaneously measured relative distribution spaces ($\times 100$) in choline Cl solution before and after membrane permeabilization in the same fiber segment; this small difference was not significant.

slightly smaller in choline Cl solution (second bar) than in the first series, so the difference between resting and stimulatory conditions in the valinomycin study actually was smaller rather than larger. The essential results were the same in the presence of valinomycin. The higher $^{86}Rb^+$ ratio in choline Cl again was not decreased by membrane permeabilization (third bar); the change in the $^{86}Rb^+$ distribution ratio after permeabilization with the same protocol in the same fiber segment (last bar) was $+0.25 \pm 2.0\%$, i.e., very small, positive, and not significant.

Again, there is no apparent basis for increases with permeabilization of specific binding or Donnan-related cation accumulation that would just compensate dissipation of SR accumulation; such an increase would also be opposite in charge to any decreased anion exclusion effect. Therefore, these results showed no suggestion of $^{86}Rb^+$ accumulation indicative of SR membrane polarization under the stimulatory conditions, even when Rb^+ permeability could not be rate limiting. The pooled change in distribution ratio after membrane permeabilization from the two studies was $+0.38 \pm 1.30\%$ (10).

DISCUSSION

These studies show that solvent volumes in skinned fibers can be estimated from the uptake of labeled neutral probes and that this method can be applied to assess the accumulation of permeant ions as a reflection of SR transmembrane polarization. The effect of membrane permeabilization on ion distribution ratios was used to evaluate the role of internal membrane-bounded accumulation. Since permeabilization did not decrease any of the ratios of ion to solvent space, the results do not support either positive SR polarization at rest or a sustained negative potential difference with large ion gradients that can produce sustained ^{45}Ca efflux stimulation. They suggest, therefore, that the mechanism responsible for this stimulation is not a change in SR transmembrane potential.

Solvent Spaces

Accurate normalization was required for the assessment of ion accumulation, but the size of the preparation (normally <50 nl) makes methods using wet and dry
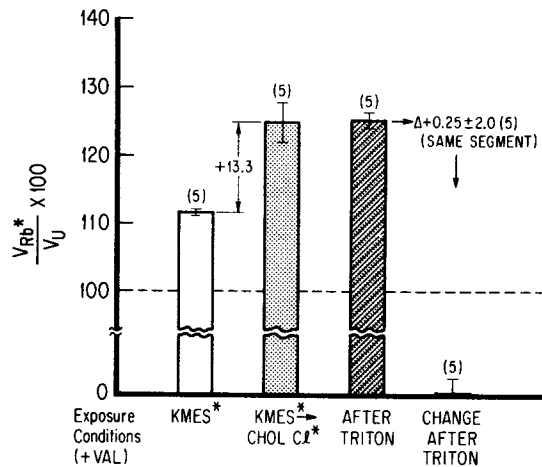

FIGURE 7. Estimation of cation accumulation in the presence of valinomycin (2 mg/ml) from the relative distribution of ^{86}Rb$^+$ and V_U measured simultaneously. Open bar, ^{86}Rb-KMes solution (unstimulated), 2-min exposures. Stippled bar and hatched and stippled bar, all fiber segments were exposed to ^{86}Rb-choline Cl for 1 min after preexposure to ^{86}Rb-KMes for 1 min (without elution). Last bar, the difference between simultaneously measured relative distributions (×100) in choline Cl before and after membrane permeabilization in the same fiber segment; this small difference was not significant.

weights or total protein content impractical for routine use. Microprotein analysis on fiber segments with a widened range of lengths was used to evaluate normalization by solvent volume measurements from the distribution space of neutral labeled probes. The excellent correlation between V_U or V_{DOG} assays and protein content of the same segments validated the procedures developed (Fig. 2). Simultaneous uptake of a neutral probe and permeant ion then provides, at minimum, a measure of changes in accumulation. Neutral probe normalization methods also can be applied to ^{45}Ca flux studies for improved assay of influx and absolute Ca efflux.

Under limited conditions, these probes could also be used to assess V_m from the fractional difference between estimates of V_c and V_s. V_c was estimated from V_{DOG}

after a brief initial exposure; rapid deoxyglucose equilibration with V_c and small SR permeation in 2 min are expected from the small fiber radius and the relatively slow entry of sugars into SR vesicles. From a cylindrical cross-section of radius 50 μm and the apparent fiber diffusion coefficient of sorbitol, 3×10^{-6} cm²·s⁻¹ (Kushmerick and Podolsky, 1969), diffusional equilibration of deoxyglucose should be practically complete in <10 s (Hill, 1928). Although SR vesicles are not impermeable to sugars (Duggan and Martonosi, 1970), the half time for glucose permeation by a light scattering assay is 25 min (Kometani and Kasai, 1978) and for sucrose tracer exchange it is 10 min (Meissner and McKinley, 1976), so diffusional equilibration of deoxyglucose should be widely separated from membrane permeation with 2 min of incubation. Consistent with slow permeation, the ratios of anion space to V_{DOG} decreased slightly between 2 and 10 min (Figs. 4 and 5). V_s estimated from V_{DOG} after membrane permeabilization did not differ significantly from V_U after an initial 2-min exposure; V_m calculated from this estimate and from the (simultaneous) V_U-V_{DOG} differences after an initial 2- or 5–6-min exposure averaged 10.1% (9.6–11%, Fig. 3), which is in good agreement with morphometric measurements in intact fibers (Peachey, 1965; Mobley and Eisenberg, 1975). The decrease in simultaneous V_U-V_{DOG} difference after membrane permeabilization from the initial 2-min exposure corresponded to an appreciably smaller apparent V_m of 5.3%; this estimate is considered less plausible (see Results), but the present data do not rule it out.

Appreciable ion accumulation in such V_m would be detectable from the distribution ratio of ion/neutral probe, even with some increase in V_s during recycling (see Results). Simultaneous assay then can be used to assess polarization of the SR compartment, which contributes most of the 11% membrane-bounded volume in intact frog fibers (Peachey, 1965; Mobley and Eisenberg, 1975); T tubule volume is only 0.3% and mitochondrial volume 1.6%. The compartments in skinned fibers shown schematically in Fig. 8 are designated (1) for V_c (MFS, myofibrillar and intermyofibrillar solvent), (2) for SR solvent volume, and (3) for T tubule solvent volume. The distribution space, by definition, is the virtual volume (V_x) that would contain the solute (x) at the bath concentration, so the ratio of distribution spaces (R_f) is equal to the average concentration ratio of the fiber to the bathing medium. The average ratio is determined by the concentration ratios (R) of each compartment weighted by its actual solvent volume (V), and a term (X) for any "bound" solute:

$$V_x/V_s = R_f = R_1(V_1) + R_2(V_2) + R_3(V_3) + \cdots + X_{bound} . \tag{1}$$

This equation can be simplified to focus on the relationship between SR accumulation and R_f with respect to measurable changes that could result from membrane permeabilization, which should not alter R_1 and X_{bound}. If only compartments (1) and (2) are considered, and $R_1 = 1$,

$$R_f = (V_2/V_s)(R_2 - 1) + 1. \tag{2}$$

With 10% V_2 (see above), $R_2 = 6, 3, 1.4$, or 1.2 would give an R_f ($\times 100$) of 150, 120, 104, or 102%, respectively. Even if increased V_s during recycling had reduced V_2 maximally (see Results), an R_2 of 1.4 still would give an R_f ($\times 100$) of 103.5 to 103.4%. SR exclusion of like charge would change R_f much less than accumulation of opposite charge, the condition primarily tested; R_2 values of $^1/_6$, $^1/_3$, or $^1/_{1.4}$ (with

10% V_2) would give R_f values of 91.7, 93.3, or 97.1%, respectively, with a minimum at 90%. If V_2 were only 5% (see above), an R_2 equal to 6, 3, 1.4, or 1.2 would give R_f ($\times 100$) values of 125, 110, 102, or 101%, respectively.

Permeant Anion Distribution in Resting Fibers

Transmembrane potentials have been assessed from permeant ion distribution in organelles, single cells, and tissues (e.g., Pollard et al., 1976, and references; Hoek et al., 1980), based on the principle that the equilibrium ratio of the ion across the membrane monitors the transmembrane potential according to the Nernst equation. It is assumed that distribution is passive in a quasiequilibrium system, the concentration ratio approximates the activity ratio, and the ion is sufficiently permeant to equilibrate, or that corrections can be made for deviations. In the examples given above, R_2 values of 6, 3, 1.4, or 1.2 for monovalent ions correspond to transmembrane potential differences of 45, 27.7, 8.5, or 4.6 mV, respectively.

Previous studies provide evidence that the SR is not an extracellular compartment. Isolated SR vesicles are very permeable to Na^+, devoid of a Na^+/K^+ pump,
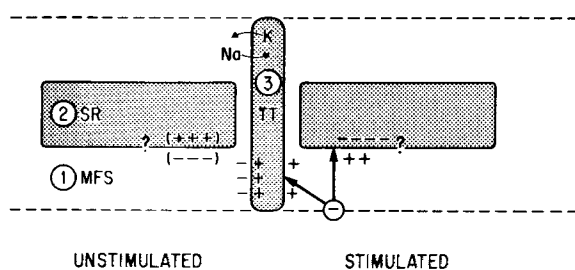

UNSTIMULATED   STIMULATED

FIGURE 8. Schematic representation of the solvent spaces and possible polarization tested in the skinned fiber preparation. The non–membrane-bounded compartment, directly accessible to diffusional equilibration (labeled 1, MFS), includes myofibrillar solvent surrounding the myofilaments and intermyofibrillar solvent. The membrane-bounded compartments are labeled 2, SR, to indicate luminal SR solvent space; and 3, TT, to indicate luminal T tubule solvent space. Left side, unstimulated; right side, stimulated by choline Cl replacement of KMes. See text for discussion.

and highly permeable to Cl^-. An appreciable transmembrane potential thus should be reflected in Cl^- accumulation, but the SR elemental Cl concentration in intact frog fibers, determined by electron microprobe analysis, does not exceed that of myoplasm (Somlyo et al., 1977). It was important to verify the implication of this elemental distribution with resting anion distribution in the skinned fiber. In particular, an appreciable resting transmembrane potential (lumen positive) would raise the possibility that depolarizing ion gradients stimulate by reducing without reversing the polarity and it would alter the design of studies on charged probe distribution during stimulation.

The lipophilic [^{14}C]SCN$^-$ anion, at 2 mM carrier, is taken up rapidly to ratios over 100% that are not decreased by membrane permeabilization (Fig. 4); the mean change from V_{SCN} relative to V_{DOG} at 10 min in the same (recycled) segment is a very small insignificant increase, indicating that SCN$^-$ distribution is independent of membrane integrity. With [^{14}C]propionate$^-$, at 120 mM carrier, anion exclusion from V_s is observed. The R_f for [^{14}C]propionate$^-$ relative to V_{DOG} at 10 min. ~95%,

is also unchanged after membrane permeabilization (Fig. 5). The exclusion evidently is independent of membrane integrity, and is consistent with the Donnan-type effects related to the net negative charge of myofibrillar proteins observed in various skinned and/or permeabilized muscles by microelectrode and ion distribution measurements (e.g., Scordilis et al., 1975; Hincke, 1980; D. G. Stephenson et al., 1981; Godt and Baumgarten, 1984; Bartels and Elliott, 1985). If R_f were the weighted mean of Donnan-type anion exclusion and an opposing membrane-bound accumulation, it still would have decreased after membrane permeabilization.

The absence of any decrease in R_f after permeabilization showed that neither anion was accumulated detectably by an inside-positive transmembrane potential. Since both changes in R_f were small and positive ($+0.5 \pm 1\%$ and $+0.7 \pm 2\%$) no more than a few millivolts would have been undetected in a 10% SR compartment. If the SR compartment were only 5%, this uncertainty would be doubled (see above). The uncertainty of the measurements could also include accumulation in a very small volume fraction of sealed polarized T tubules.

Permeant Cation Distribution under Stimulatory Conditions

$^{86}Rb^+$ accumulation was measured under resting conditions in KMes and during choline Cl replacement, imposing a negative diffusion potential that stimulates ^{45}Ca efflux in the absence or presence of EGTA (Stephenson, 1985b). The stimulation of Ca^{2+}-insensitive ^{45}Ca efflux (in EGTA) is sustained for at least 1 min and the causal potential change was assumed to have a similar duration, as discussed below. An appreciable potential change of this duration in a compartment the size of the SR should be detectable as $^{86}Rb^+$ accumulation. The key comparison was $^{86}Rb^+$ accumulation under stimulatory conditions before and after membrane permeabilization. Stimulation in the presence of EGTA also excludes a substantial Ca release, in order to evaluate SR potential change as a primary stimulus. Therefore, the results do not address the question of an SR potential change secondary to Ca release, as was suggested by potentiometric dye signals in intact fibers (see Baylor et al., 1984) and by the effects of valinomycin on changes in SR elemental K after a brief tetanus (Kitazawa et al., 1983).

In KMes, R_f values for $^{86}Rb^+$ relative to V_U are above 100% (Figs. 6 and 7), which would be expected from Donnan-related accumulation with a myofibrillar potential of -4.4 mV in frog muscle (Godt and Baumgarten, 1984). Mitochondrial cation accumulation, which could increase R_f many fold (e.g., Hoek et al., 1980), must be negligible under these experimental conditions. The higher R_f values in choline Cl (2.5 mM K^+) are not dissipated by membrane permeabilization, in either the absence or presence of valinomycin, i.e., they do not depend on membrane integrity. Preincubation in labeled KMes and the study with valinomycin ensured that ^{86}Rb approached steady state accumulation during incubation. In all assays with valinomycin, choline Cl exposure was limited to 1 min, the minimum duration of the sustained efflux stimulation. The pooled change in R_f after membrane permeabilization in the two studies, $+0.38 \pm 1.30\%$ (10), suggests that any induced negative SR membrane potential would have been less than a few millivolts with a 10% SR compartment. If the SR compartment were only 5% this uncertainty would be doubled (see above).

The cation accumulation studies were designed with the assumption that the potential change of interest is concomitant with the ionic stimulation of the Ca^{2+}-insensitive ^{45}Ca efflux, which does not decline during the 1-min measurement period (Stephenson, 1985b); this component is similarly sustained at physiological Mg^{2+} (Stephenson, 1989) and correlates with the much larger Ca^{2+}-dependent release under varying conditions (see Stephenson, 1987). If the ion gradients acted by direct SR polarization, the hypothesis under evaluation, SR efflux stimulation would not be expected to long outlast the potential change; the longest time constant of the putative SR Ca release channel in SR vesicles is <100 ms even when activated by Ca^{2+} and adenine nucleotide in the absence of Mg^{2+} (Smith et al., 1985). T tubule-mediated stimulation also does not long outlast the causal potential change; potassium contractures in intact fibers terminate abruptly upon return to normal $[K^+]_o$ (Hodgkin and Horowicz, 1960) and $[Ca^{2+}]$ signals stimulated by voltage-clamp pulses in cut fibers turn off in milliseconds with repolarization (see Melzer et al., 1986 and references) even with small stimuli and Ca release.

The results do not exclude a brief SR polarization that has decayed on the time scale of these studies. In SR vesicles, choline Cl replacement of K glutamate can induce transient voltage-sensitive dye signals (Beeler et al., 1981), which is consistent with dissipative counterion fluxes that would also attenuate the maximum potential changes (Meissner and McKinley, 1976). Such a transient polarization would not account for the observed minimum duration of undiminished ^{45}Ca efflux stimulation in the skinned fiber. This sustained stimulation is consistent, however, with gradients prolonged by lower counterion permeability of T tubules. A substantial decrease in T tubule polarization (lumen positive to myoplasm) would have little effect on fiber $^{86}Rb^+$ distribution.

Implications of the Permeant Ion Distributions

The present results suggest that resting SR polarization is zero or negligible, and any negative SR transmembrane potential induced by the imposed ion gradients is at most very small on the time scale of the sustained stimulation. If the stimulatory gradients do not generate an appropriate transmembrane potential change, SR "depolarization" is unlikely to be the direct stimulus for Ca release in skinned fibers and the response is not evidence for such a coupling mechanism in intact fibers.

This interpretation is consistent with circumstantial evidence for an alternative mechanism, stimulation by depolarization of sealed polarized T tubules. The T tubules, in contrast to the SR, contain a Na^+/K^+ pump that is inhibited by cardiac glycosides, which is required for ionic gradients and the polarization of sealed T tubules (Lau et al., 1979). Stimulation of skinned fibers by current pulses under oil (Costantin and Podolsky, 1967) by depolarizing ion gradients at constant $[K^+][Cl^-]$ product (Donaldson, 1985), and by Na^+-K^+ ionophores (Volpe and Stephenson, 1986) all are inhibited by suitable pretreatment with cardiac glycosides. Stimulation by depolarizing ion gradients is also potentiated by perchlorate anion, which potentiates charge movements and contractile activation in intact fibers, and is selectively inhibited in highly stretched fibers (Stephenson, 1989). This mechanism implies that stimulation follows the same T-SR coupling pathway as that in intact fibers.

REFERENCES

Bartels, E. M., and G. F. Elliott. 1985. Donnan potentials from the A- and I-bands of glycerinated and chemically skinned muscles, relaxed and in rigor. *Biophysical Journal.* 48:61–76.

Baylor, S. M., W. K. Chandler, and M. W. Marshall. 1984. Calcium release and sarcoplasmic reticulum membrane potential in frog skeletal muscle fibres. *Journal of Physiology.* 348:209–238.

Bearden, J. C., Jr. 1978. Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. *Biochimica et Biophysica Acta.* 533:525–529.

Beeler, T. J., R. H. Farmen, and A. N. Martonosi. 1981. The mechanism of voltage-sensitive dye responses on sarcoplasmic reticulum. *Journal of Membrane Biology.* 62:113–137.

Birks, R. I., and D. F. Davey. 1969. Osmotic responses demonstrating the extracellular character of the sarcoplasmic reticulum. *Journal of Physiology.* 202:171–188.

Bozler, E. 1961. Distribution of nonelectrolytes in muscle. *American Journal of Physiology.* 200:651–655.

Costantin, L. L. 1975. Contractile activation in skeletal muscle. *Progress in Biophysics and Molecular Biology.* 29:197–224.

Costantin, L. L., and R. J. Podolsky. 1967. Depolarization of the internal membrane system in the activation of frog skeletal muscle. *Journal of General Physiology.* 50:1101–1124.

Dani, J. A., J. A. Sanchez, and B. Hille. 1983. Lyotropic anions: Na channel gating and Ca electrode response. *Journal of General Physiology.* 81:225–281.

Donaldson, S. K. B. 1985. Peeled mammalian skeletal muscle fibers: possible stimulation of Ca^{2+} release via a transverse tubule–sarcoplasmic reticulum mechanism. *Journal of General Physiology.* 86:501–525.

Duggan, P. F., and A. Martonosi. 1970. Sarcoplasmic reticulum. IX. The permeability of sarcoplasmic reticulum membranes. *Journal of General Physiology.* 56:147–167.

Dydynska, M., and D. R. Wilkie. 1963. The osmotic properties of striated muscle fibres in hypertonic solutions. *Journal of Physiology.* 169:312–329.

Endo, M. 1977. Calcium release from the sarcoplasmic reticulum. *Physiological Reviews.* 57:71–108.

Franzini-Armstrong, C. 1971. Studies of the triad. II. Penetration of tracers into the junctional gap. *Journal of Cell Biology.* 49:196–203.

Godt, R. E., and C. M. Baumgarten. 1984. Potential and K^+ activity in skinned muscle fibers: evidence against a simple Donnan equilibrium. *Biophysical Journal.* 45:375–382.

Hill, A. V. 1928. The diffusion of oxygen and lactic acid through tissue. *Proceedings of the Royal Society, Series B (Biological Sciences).* 105:39–96.

Hinke, J. A. M. 1980. Water and electrolyte content of the myofilament phase in the chemically skinned barnacle fiber. *Journal of General Physiology.* 75:531–551.

Hodgkin, A. L., and P. Horowicz. 1960. Potassium contractures in single muscle fibres. *Journal of Physiology.* 153:386–403.

Hoek, J. B., D. G. Nicholls, and J. R. Williamson. 1980. Determination of the mitochondrial protonmotive force is isolated hepatocytes. *Journal of Biological Chemistry.* 255:1458–1464.

Ikemoto, N., B. Antoniu, and D. K. Kim. 1984. Rapid calcium release from the isolated sarcoplasmic reticulum is triggered via the attached transverse tubular system. *Journal of Biological Chemistry.* 259:13151–13158.

Kitazawa, T., A. P. Somlyo, and A. V. Somlyo. 1983. The effects of valinomycin on ion movements across the sarcoplasmic reticulum in frog muscle. *Journal of Physiology.* 350:253–268.

Kometani, T., and M. Kasai. 1978. Ionic permeability of sarcoplasmic reticulum vesicles measured by light scattering method. *Journal of Membrane Biology.* 41:295–308.

Kushmerick, M. J., and R. J. Podolsky. 1969. Ionic mobility in muscle cells. *Science.* 166:1297–1298.

Lau, Y. H., A. H. Caswell, M. Garcia, and L. Letellier. 1979. Ouabain binding and coupled sodium, potassium, and chloride transport in isolated transverse tubules of skeletal muscle. *Journal of General Physiology.* 74:335–349.

Martonosi, A. N. 1984. Mechanisms of Ca^{2+} release from sarcoplasmic reticulum of skeletal muscle. *Physiological Reviews.* 64:1240–1320.

Meissner, G., and D. McKinley. 1976. Permeability of sarcoplasmic reticulum. The effect of changed ionic environments on Ca^{2+} release. *Journal of Membrane Biology.* 30:79–98.

Melzer, W., M. F. Schneider, B. J. Simon, and G. Szucs. 1986. Intramembrane charge movement and calcium release in frog skeletal muscle. *Journal of Physiology.* 373:481–511.

Mobley, B. A. 1979. Chloride and osmotic contractures in skinned frog muscle fibers. *Journal of Membrane Biology.* 46:315–329.

Mobley, B. A., and B. R. Eisenberg. 1975. Sizes of components in frog skeletal muscle measured by methods of stereology. *Journal of General Physiology.* 66:31–45.

Neville, M. C. 1979. The extracellular compartments of frog skeletal muscle. *Journal of Physiology.* 288:45–70.

Oetliker, H. 1982. An appraisal of the evidence for a sarcoplasmic reticulum membrane potential and its relation to calcium release in skeletal muscle. *Journal of Muscle Research and Cell Motility.* 3:247–272.

Peachey, L. D. 1965. The sarcoplasmic reticulum and transverse tubules of the frog's sartorius. *Journal of Cell Biology.* 25:209–231.

Pollard, H. B., O. Zinder, P. G. Hoffman, and O. Nikodejevics. 1976. Regulation of the transmembrane potential of isolated chromaffin granules by ATP, ATP analogs, and external pH. *Journal of Biological Chemistry.* 251:4544–4550.

Pressman, B. C. 1976. Biological applications of ionophores. *Annual Review of Biochemistry.* 45:501–529.

Scordilis, S. P., H. Tedeschi, and C. Edwards. 1975. Donnan potential of rabbit skeletal muscle myofibrils. I. Electrofluorochromometric detection of potential. *Proceedings of the National Academy of Science.* 72:1325–1329.

Smith, J. S., R. Coronado, and G. Meissner. 1985. Sarcoplasmic reticulum contains adenine nucleotide–activated calcium channels. *Nature.* 316:446–449.

Somlyo, A. V., H. Shuman, and A. P. Somlyo. 1977. Elemental distribution in striated muscle and the effect of hypertonicity. Electron probe analysis of cryosections. *Journal of Cell Biology.* 74:828–857.

Stephenson, D. G., I. R. Wendt, and Q. F. Forrest. 1981. Non-uniform ion distributions and electrical potentials in sarcoplasmic regions of skeletal muscle fibres. *Nature.* 289:690–692.

Stephenson, E. W. 1978. Properties of chloride-stimulated ^{45}Ca flux in skinned muscle fibers. *Journal of General Physiology.* 71:411–430.

Stephenson, E. W. 1981a. Solute distribution in skinned muscle fibers. *Biophysical Journal.* 33:152a. (Abstr.)

Stephenson, E. W. 1981*b*. Activation of fast skeletal muscle: contributions of studies on skinned muscle fibers. *American Journal of Physiology.* 240(*Cell Physiology* 9):C1–C192.

Stephenson, E. W. 1985*a*. Permeant cation distribution in skinned muscle fibers. *Biophysical Journal.* 47:377*a*. (Abstr.)

Stephenson, E. W. 1985*b*. Excitation of skinned muscle fibers by imposed ion gradients. I. Stimulation of ^{45}Ca efflux at constant [K] [Cl] product. *Journal of General Physiology.* 86:813–832.

Stephenson, E. W. 1986. Perchlorate potentiates the stimulation of skinned muscle fibers by ion gradients. *Biophysical Journal.* 49:191*a*. (Abstr.)

Stephenson, E. W. 1987. Mechanisms of stimulated ^{45}Ca efflux in skinned skeletal muscle fibers. *Canadian Journal of Physiology and Pharmacology.* 65:632–641.

Stephenson, E. W. 1989. Excitation of skinned muscle fibers by imposed ion gradients. IV. Effects of stretch and perchlorate ion. *Journal of General Physiology.* 93:173–192.

Stephenson, E. W., and S. S. Lerner. 1986. Stretch inhibition of stimulation of ^{45}Ca release from skinned muscle fibers by depolarizing ion gradients. *Journal of General Physiology.* 88:56*a*. (Abstr.)

Stephenson, E. W., and R. J. Podolsky. 1977. The influence of magnesium on chloride-induced calcium release in skinned muscle fibers. *Journal of General Physiology.* 60:17–35.

Taylor, S. R., and R. E. Godt. 1976. Calcium release and contraction in vertebrate skeletal muscle. *In* Symposium XXX of the Society for Experimental Biology, Calcium in Biological Systems. C. J. Duncan, editor. Cambridge University Press, Cambridge. 361–380.

Volpe, P., and E. W. Stephenson. 1986. Ca^{2+} dependence of transverse-tubule–mediated calcium release in skinned muscle fibers. *Journal of General Physiology.* 87:271–288.