Channel-mediated Monovalent Cation Fluxes in Isolated Sarcoplasmic Reticulum Vesicles

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ABSTRACT The permeability of isolated sarcoplasmic reticulum (SR) vesicles to monovalent cations was studied using a stopped-flow fluorescence quenching technique that permits the measurement of ion fluxes on a millisecond time scale. Approximately 70% of the SR vesicles carry a cation conductance pathway mediating fluxes of Tl^+, K^+, Na^+, and Li^+, but not of choline. Both K^+ and Na^+ equilibrate faster than the 3-ms dead time of the apparatus and Li^+ equilibrates in ~50 ms. These cation fluxes are reduced by a *bis*-guanidinium blocker of the SR K^+ channel previously studied in planar bilayers. The remaining 30% of the vesicles are permeable to these cations on a time scale of seconds. We conclude that the SR K^+ channel is present in a major fraction of vesicles and that its properties in the native membrane are similar to those found in planar bilayers. Moreover, the ion fluxes in fractionated SR vesicles suggest that the channels are distributed along the entire surface of the SR membrane, but in higher concentration in vesicles derived from the terminal cisternae region. From the measured rates of K^+ movement, we calculate a conductance on the order of 10^{-1} S/cm^2 for the SR membrane in situ, which implies that this membrane cannot develop a potential of more than a few millivolts under physiological conditions.

INTRODUCTION

Despite many attempts made to study the coupling between excitation and contraction of skeletal muscle fibers, there is no consensus on the mechanism triggering the release of Ca^{++} from the sarcoplasmic reticulum (SR). Currently there are three theories, all of them based on electrophysiological work in whole or skinned muscle fibers. Chandler et al. (1976) propose a mechanical coupling between T-tubule and SR membranes, while Mathias et al. (1980) envision electric current flow between these membranes as responsible for opening a Ca^{++} permeability pathway in the SR. The third mechanism, chemical coupling, resembles synaptic transmission in which Ca^{++} (Stephenson, 1981) or H^+ (Sho-

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shan et al., 1981) is the transmitter released from the T-tubule and received by the SR. In all these theories, what happens to the SR membrane itself is only vaguely stated, since information on its electrical properties is lacking. Can the SR membrane be depolarized by the T-tubule stimulus? Is Ca^{++} release electrogenic? Does the SR membrane undergo large changes in potential as a result of Ca^{++} release? These are still open questions.

Because the SR membrane is not accessible to conventional electrophysiological techniques, a variety of other approaches has been used to study its electrical properties. Thus, Vergara et al. (1978), working in single muscle fibers, determined SR membrane conductance by monitoring the fluorescence change of Nile blue A, a potential-sensitive dye. Alternatively, the permeability of isolated SR membrane vesicles has been determined by light scattering (Kometani and Kasai, 1978) or by a combination of radioactive tracer flux and potential-sensitive dyes (McKinley and Meissner, 1978). A different technique, also using isolated vesicles, exploited the fusion of these vesicles with voltage-clamped planar lipid bilayers (Miller, 1978). These various approaches yield SR membrane conductances ranging from 10^{-5} S/cm^2 (Vergara et al., 1978; Kometani and Kasai, 1978) or 10^{-4} S/cm^2 (McKinley and Meissner, 1978) to 10^{-1} S/cm^2 (Labarca and Miller, 1981) for K^+ or other monovalent ions (Hasselbach and Oetliker, 1983). This appalling disagreement means that we cannot judge the plausibility of models postulating changes in SR membrane potential or conductance as either a cause or a result of Ca^{++} release. If the SR conductance is in fact low ($<10^{-5}$ S/cm^2), then such voltages could easily arise during Ca^{++} release. If, on the other hand, the conductance is high ($>10^{-2}$ S/cm^2), the SR would always be electrically shunted, regardless of Ca^{++} or other ionic currents across the SR membrane during contractile activation (Oetliker, 1982).

In this report, we introduce yet another approach to determine the conductance of the SR membrane. Like all previous attempts, this one is indirect in that we study isolated SR vesicles, not the SR membrane in situ. But in the present study, we measure the monovalent cation permeabilities of these vesicles directly, and with a time resolution over 100-fold greater than in previous work. The results show that SR membrane vesicles are highly permeable to small monovalent cations such as K^+ and Na^+. The detailed characteristics of this monovalent cation permeability are reminiscent of the corresponding conductance properties of the SR K^+ channel studied in planar lipid bilayers.

METHODS

Special Chemicals

Thallium (I) hydroxide, obtained from Alpha-Ventron (Danvers, MA), was accurately titrated with glutamic acid to produce stock Tl^+ glutamate solutions. 1,3,6,8-Pyrenetetrasulfonic acid (PTS) was obtained as the tetrasodium salt from Molecular Probes (Junction City, OR) and was converted to the tetracholine salt on a Dowex-50 column. To synthesize 1,10-*bis*-guanidino-*n*-decane (*bis*G10), 1,10-diaminodecane (basic form, 100 mM in 50% methanol) was mixed with an equal volume of *S*-methylisothiourea (hemisulfate or chloride salt, 300 mM in water), and the reaction was stirred at room temperature in a fume hood for 2 d, periodically adjusting the pH to 9.5 with NaOH. The *bis*-guanidinated product,

but not the monoguanidinated intermediate, precipitated out, and was washed extensively with cold water, then ethanol and ether, and was dried in a vacuum oven. The product was at least 98% pure, as judged by thin-layer chromatography, a positive Sakaguchi reaction, and an absence of primary amino residues.

Isolation of SR Vesicles

Crude SR vesicles were prepared from the back and leg muscles of albino rabbits. Muscles were shredded in a "La Machine" food processor and stirred for 30 min in the cold, in 3 vol of 0.6 M KCl, 5 mM HEPES, pH 7.4. The suspension was centrifuged at 2,000 *g* for 5 min and the supernatant was discarded. The pellet was resuspended as before and the extraction was repeated. The pellet from the second extraction was homogenized in 3 vol of 0.2 M sucrose, 5 mM HEPES, pH 7.4, for 30 s in a blender and centrifuged at 10,000 *g* for 30 min. The supernatant, filtered through cheesecloth, was centrifuged at 100,000 *g* for 1 h. The pellet was resuspended in homogenization buffer and centrifuged at 10,000 *g* for 15 min. The supernatant containing SR vesicles was centrifuged again for 1 h at 100,000 *g* and the final pellet was resuspended in 0.4 M sucrose, 5 mM HEPES, pH 7.4, at a protein concentration of 20–30 mg/ml. The vesicles were kept frozen at −70°C in small aliquots.

Alternatively, to fractionate SR vesicles according to density (Meissner, 1975), microsomes obtained by the method of Fernandez et al. (1980) were loaded on a discontinuous sucrose density gradient consisting of the following steps: 50, 40, 35, 27.5, and 25% (wt/vol) sucrose, 20 mM MOPS, pH 7.0. After spinning for 14 h at 25,000 rpm in a SW27 rotor, the following fractions were collected: heavy (50–40% interface), intermediate (40% layer), light (40–35% interface), and T-tubules (27.5–25% interface). The fractions were diluted with 20 mM MOPS, pH 7.0, and centrifuged at 100,000 *g* for 1 h, and the pellets were resuspended at 10–30 mg/ml in 0.3 M sucrose, 20 mM MOPS, pH 7.0. The fractions were kept frozen at −70°C.

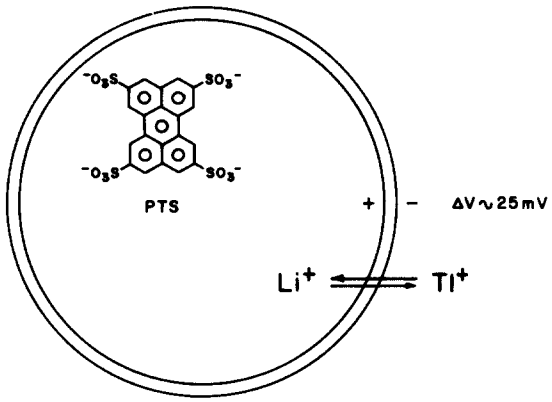
Cation Fluxes Determined by Fluorescence Quenching

Passive fluxes of monovalent cations across SR vesicle membranes were followed at high time resolution by the stopped-flow fluorescence quenching method developed by Moore and Raftery (1980), with substantial modifications. SR vesicles were loaded with PTS, a fluorescent, hydrophilic dye, and mixed in a stopped-flow spectrophotometer with a solution containing Tl^+, a K^+ analogue that quenches PTS fluorescence. Tl^+ entry into the vesicles was followed by monitoring the decrease in fluorescence of the trapped dye. For this method to give reproducible and interpretable results, it was necessary to pay attention to two points. First, the movement of Tl^+ should be restricted to exchange for the "test cation" preloaded within the vesicles; therefore, in all our experiments glutamate was the only anion present, since it is impermeant on the time scale of the cation fluxes. Second, it was necessary to correct the fluorescence quenching for the small amount of PTS probe on the outside of the vesicles; this was done using methionine as an external quencher.

The detailed procedure is described below, using Li^+ as an example of "test cation." SR vesicles were washed and resuspended at ~20 mg/ml, in a medium containing 100 mM glucose, 100 mM Li^+ glutamate, 10 mM MOPS-*N*-methyl-D-glucamine, pH 7.0, with or without 5 mM $MgSO_4$. Choline-PTS was added to a final concentration of 8–10 mM and the suspension was then incubated 16 h on ice. Determinations of the equilibrium spaces of sucrose, methionine, and PTS show that this is sufficient time to load all vesicles to equilibrium. Immediately before the experiment, the external PTS was removed by passing an aliquot of the suspension (20–50 μl) through an anion exchange column (0.2

ml Dowex 1 × 4–200, glutamate form), equilibrated with the loading medium and pretreated with bovine serum albumin to reduce nonspecific protein binding. Over 99% of the external PTS was removed by this procedure. The sample was eluted with the same incubation medium, except that the glucose concentration was increased to keep the osmolarity inside and outside of the vesicles constant.

The vesicles thus loaded with PTS and Li^+ and diluted to 0.1–0.2 mg/ml were immediately mixed in a stopped-flow apparatus (dead time 3 ms) with an equal volume of solution of the same composition, except that 50 mM of the Li^+ had been replaced by 50 mM Tl^+. Fig. 1 shows a schematic diagram of the vesicle suspension immediately after



	In (mM)	Out (mM)
PTS^{-4}	10	–
Choline	40	–
Li^+	100	75
Tl^+	–	25
$Glutamate^-$	100	100
Glucose	100	150

FIGURE 1. Ionic conditions at the time of mixing. Schematic diagram depicting the ionic concentrations inside and outside of the SR vesicles at the time of mixing in the stopped-flow apparatus. The protein concentration was 0.1–0.2 mg/ml; other conditions are indicated in Methods. The arrows indicate only the rapidly permeant species. The voltage across the membrane at this time was calculated from the relative ion permeability measured in planar bilayer experiments (see Discussion).

mixing. Each experiment was performed in triplicate; in each case, the fluorescence was recorded until a stable baseline was reached. The PTS fluorescence (λ_{excit} = 355 nm) was monitored with a 2C Wratten gelatin filter (Eastman Kodak Co., Rochester, NY). The photomultiplier current, after amplification, was stored on FM tape and later analyzed on a MINC 11/23 computer (Digital Equipment Corp., Maynard, MA).

To convert the fluorescence signal to intravesicular Tl^+ concentration, we used the Stern-Volmer relationship:

$$F_o/F = 1 + K_{SV}\,[Tl^+], \tag{1}$$

where F_o and F are the unquenched and quenched fluorescence values, respectively, and

K_{SV} is the Stern-Volmer constant (Eftnik and Ghiron, 1981). Under all conditions tested, F_o/F varied linearly with Tl^+ concentration up to at least 30 mM, with K_{SV} ranging from 30 to 50 M^{-1}. Thus, after determining K_{SV} for each experiment from the final fluorescence (corresponding to 25 mM Tl^+), we calculated the internal Tl^+ concentration following the time course of fluorescence decrease. In each experiment, care was taken to subtract the external fluorescence as described below. In this way, the internal Tl^+ concentration was determined with <10% error and with excellent reproducibility from experiment to experiment.

Over the course of a series of experiments, significant leakage of trapped PTS occurs, and it was essential to correct for this. The correction was conveniently done by using an external quencher, methionine, which is impermeant on the time scale of the rapid cation

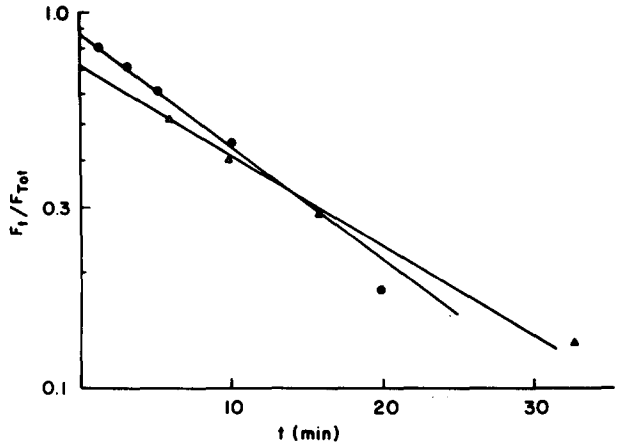


FIGURE 2. PTS efflux from SR vesicles. SR vesicles were loaded with 8 mM PTS as indicated in Methods. Immediately after removal of the free PTS, the vesicles were diluted to 0.04 mg/ml with the same medium. At the times indicated, 1.5-ml aliquots were filtered through Millipore filters (HAWP, 0.45 μm) and the fluorescence $F(t)$ appearing in the filtrate was determined (closed circles). Alternatively, the vesicles were diluted to 0.1 mg/ml and, at the indicated times, mixed in the stopped-flow apparatus with a methionine solution as in Methods. From the fluorescence instantaneously quenched, the amount of external probe was calculated (triangles).

fluxes. Before and after a Tl^+ quenching experiment, several determinations were carried out by mixing the vesicles with a solution containing 100 mM methionine, 100 mM Li^+ glutamate, 50 mM glucose, and 10 mM MOPS, pH 7.0. The instantaneous quenching measured the amount of PTS that had leaked out of the SR vesicles during the time after passage over the Dowex column. That this is a reliable assay is shown in Fig. 2, which demonstrates that efflux of PTS from the vesicles is determined identically either by methionine quenching or by direct fluorimetric measurement of free external PTS. As the figure shows, the half-time for PTS leakage is ~15 min, and we were careful to perform all stopped-flow measurements well within this time after passing the loaded vesicles over the Dowex column.

We also used methionine as a slowly permeant quencher to assess the validity of the PTS quenching method. Fig. 3 shows that identical time courses of methionine flux are measured using PTS quenching or direct determination of [^{14}C]methionine efflux by Millipore filtration. This control experiment further shows that the presence of PTS

inside the vesicles does not alter the membrane permeability to methionine; this is also true of [^{14}C]sucrose fluxes (data not shown).

Ion Fluxes by Light-Scattering Changes after Osmotic Shock

The technique described by Kometani and Kasai (1978) was followed: SR vesicles in 10 mM MES, pH 6.5, were mixed in a stopped-flow apparatus with a solution of higher osmolarity. The resulting shrinking and reswelling of the vesicles were followed as changes in the light scattered at 90° (λ = 450 nm).

Planar Bilayer Experiments

Macroscopic conductances in bilayers formed from decane solutions of phosphatidylethanolamine/phosphatidylcholine were done according to Miller (1978), after fusion of SR vesicles into the bilayers. Aqueous media in the bilayer system were chosen to reproduce the asymmetric conditions in the SR vesicle flux determinations.

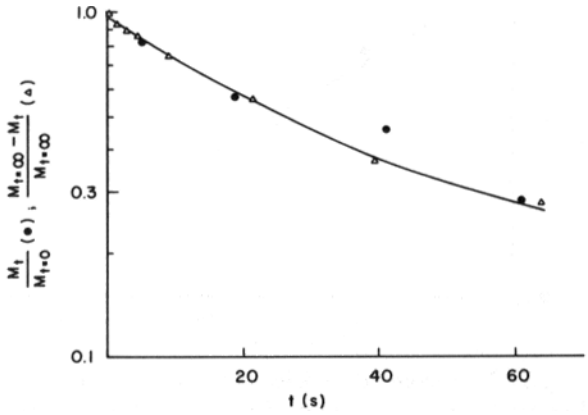


FIGURE 3. Methionine flux. Methionine efflux (circles): SR vesicles loaded with [^{14}C]methionine were diluted 120 times in unlabeled medium, at room temperature. At the indicated times, an aliquot was filtered through a Millipore filter (HAWP, 0.45 μm) and washed with 15 ml of the same medium, and the radioactivity remaining in the filter was determined. Methionine influx (triangles): SR vesicles loaded with PTS were mixed in the stopped-flow apparatus with a solution containing methionine. The influx of methionine was calculated from the fluorescence decrease as in Methods.

RESULTS

Ion Fluxes Measured by Tl^+ Entry

When SR vesicles loaded with PTS are mixed with a solution containing Tl^+, a time-dependent decrease in fluorescence ensues, as internal Tl^+ concentration increases to its equilibrium value (Garcia and Miller, 1984). Typical results for Tl^+ influx are shown in Fig. 4, *A* and *B*. In *A*, the exchange of Tl^+ for K^+, Li^+, or choline is shown at short times (0.25 s). The data have been fitted with two exponentials. Clearly, at least two components can be observed: slow and fast. In the case of K^+, the rate of exchange of the fast component is "instantaneous," i.e., faster than the time resolution of the system (3 ms). This fast component of the K^+/Tl^+ exchange accounts for 70% of the total intravesicular space; the

remaining 30% of the space is eventually filled, but with a time constant on the order of several seconds. With Li^+ as the internal cation, an instantaneous Tl^+ entry is followed by a clearly resolvable component with a time constant of 25 ms. As with K^+, ~70% of the internal space is rapidly accessible to Li^+, while the remaining entry occurs on a much slower time scale. With choline as the internal

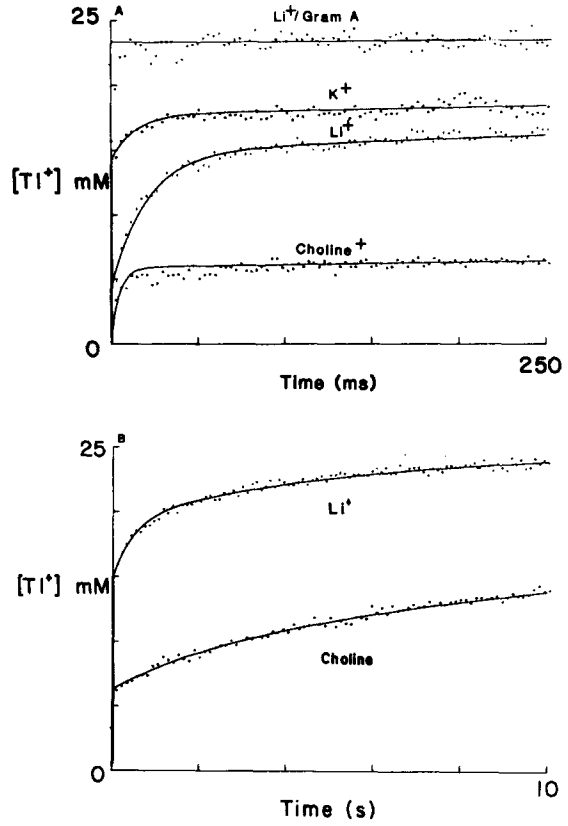


FIGURE 4. Tl^+ influx into SR vesicles. SR vesicles loaded with PTS and the indicated "test cation" were mixed in the stopped-flow apparatus with a solution containing Tl^+. The concentration of Tl^+ inside the vesicles was calculated from the fluorescence decay as indicated in Methods. The experimental points have been fitted with two exponentials (solid lines) by the method of least squares. (*A*) Fast time course. The top trace corresponds to one experiment in which vesicles loaded with PTS and Li^+ were incubated with 1.25 μg/ml gramicidin A for 10 min prior to the experiment. (*B*) Long time course: 10 s of fluorescence decay have been analyzed.

cation, a very small, rapid Tl^+ influx is seen, but the bulk of the exchange occurs over a much slower time scale than with the group IA cations. Addition of gramicidin A to the Li^+-loaded vesicles results in instantaneous Tl^+ entry into the entire intravesicular space. A similar instantaneous influx into the entire space of K^+-loaded vesicles was observed in the presence of valinomycin (data not shown). Neither gramicidin added to choline-loaded vesicles nor valinomycin

added to Li^+-loaded vesicles had any effects on Tl^+ influx (data not shown). Table I summarizes the rate constants for the fast and slow phases of Tl^+ exchange under a variety of conditions. Notice the marked ion selectivity ($K^+ \sim Na^+ > Li^+ \gg$ choline) at short times, while the slow exchange is rather nonselective. The Tl^+/Li^+ exchange rates are not significantly affected by addition of either 1 mM EGTA or 0.1 mM Ca^{++}.

Fig. 4*B* shows the exchange on a 10-s time scale. Here, fluxes of K^+, Na^+, and Li^+ cannot be distinguished from each other, and only the 30–40% "slow fraction" is resolved. With choline as the counterion, several components of the flux can be seen, but the bulk of the entry is slow; this is also the case with other large organic cations such as tetraethylammonium and *N*-methylglucamine (data not shown). It is clear in the figure that the rate of this slow Tl^+ influx is similar

TABLE I

Rate Constants of Tl^+ Exchange

	f_{inst}	k_{fast}	f_{fast}	k_{slow}	f_{slow}
		s^{-1}		s^{-1}	
K^+	0.71	>300*	—	0.51	0.28
Na^+	0.70	>300*	—	0.52	0.29
Li^+	0.30	42	0.35	0.40	0.34
Li^+ + 1 mM EGTA	0.33	37	0.38	0.62	0.28
Li^+ + 1 mM Ca^{++}	0.42	43	0.25	0.37	0.32
TEA	0.13	50	0.16	0.10	0.70
Choline	0.05	54	0.16	0.18	0.78
N-methylglucamine	0.12	41	0.10	0.20	0.77

The rate constants of the fast and slow exchange, k_{fast} and k_{slow}, were determined by computer fitting the data with a double exponential as in the legend to Fig. 4. Each value represents the mean of at least 10 determinations, with an SEM of <5%. The fraction of Tl^+ exchanged with each rate (i.e., instantaneous, fast, or slow) is indicated by f_{inst}, f_{fast}, and f_{slow}.

* Faster than the time resolution of the measurement.

whether the internal cation is "fast" (Li^+) or "slow" (choline). The main difference between the "fast" and "slow" ions is that 60–70% of the Tl^+ influx is completed before the first experimental point for the small inorganic ions, compared with 20% for choline. The slow exchange thus behaves as nonspecific leakiness of the vesicle membrane.

These results suggest that 60–70% of the SR vesicles possess some kind of rapid transport mechanism that allows Tl^+, K^+, Na^+, and Li^+ to permeate, but not larger cations such as choline. The remaining 30% of the vesicles have no such pathway, which agrees with a previous proposal by McKinley and Meissner (1978). Since it is known (Miller, 1978; Coronado et al., 1980) that SR vesicles inserted into planar bilayer membrane display an ionic channel allowing conduction of K^+, Na^+, and Li^+, but not of choline, it is tempting to propose that this "SR K^+ channel" is the system responsible for these rapid fluxes. The remainder of this study will attempt to test this proposal.

Effect of K^+ Channel Blockers on Tl^+ Flux

If the fast exchange of Tl^+ for K^+ or Li^+ is indeed channel mediated, we would expect that in the presence of a channel blocker the rate of exchange will be reduced. For these experiments, we use *bis*G10, a guanidinium analogue of *bis*-quaternary ammonium alkane blockers (Miller, 1982) and one of the best blockers of the K^+ channel, as studied in the planar bilayer (C. Miller, unpublished data). Fig. 5 shows the result of an experiment in which vesicles in the presence of K^+ have been preincubated with *bis*G10. The blocker reduces the fast Tl^+ influx rate, but has no effect on the slow "leak." A similar effect is observed in the presence of Na^+ or Li^+ or for the small fraction of fast exchange of the slowly permeable cations (Table II). Several minutes of preincubation of the vesicles with blocker is required for inhibition to be observed.

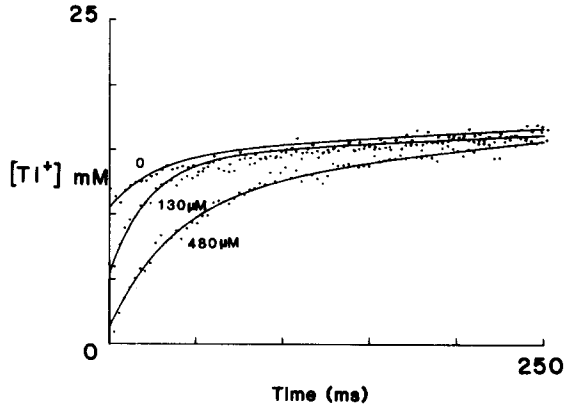


FIGURE 5. Effect of K^+ channel blocker on Tl^+ influx. SR vesicles in K^+ medium were incubated with the indicated concentrations of *bis*G10 for at least 10 min before the experiment. The continuous line corresponds to a double-exponential fitting of the data.

So far, we have not differentiated between K^+ and Na^+ fluxes because they are both faster than the system's time resolution. However, under conditions of partial block by *bis*G10, there is a clear selectivity of K^+ over Na^+, both of which are still substantially faster than Li^+ (Fig. 6), which is in harmony with the selectivity sequence of the K^+ channel conductance. At higher blocker concentrations (>0.25 mM), the three ions tested reached approximately the same minimum rate of Tl^+ entry.

It is important to compare quantitatively the inhibition by *bis*G10 of the channel-mediated planar bilayer conductance and of the vesicle fluxes. In Fig. 7 we can see that the inhibition is similar in both cases: the concentration of *bis*G10 for half-inhibition of the bilayer conductance is 50 μM, which is in good agreement with the 75 μM required for half-inhibition of the rate of Tl^+ entry. This agreement is seen when *bis*G10 is added to the *trans* side of the planar bilayer, i.e., opposite to the side to which SR vesicles are added. The compound

TABLE II

Effect of 0.25 mM bisG10 on Tl^+ Influx

	k_{fast}		k_{slow}	
	s^{-1}		s^{-1}	
	−	+	−	+
K^+	>300	26	0.51	0.43
Na^+	>300	26	0.52	0.54
Li^+	42	14	0.40	0.60
TEA	50	31	0.10	0.16
Choline	54	28	0.18	0.19
N-methylglucamine	41	28	0.20	0.22

Rate constants of Tl^+ influx in the absence (−) or presence (+) of 0.25 mM *bis*G10. The rate constants were calculated by a double-exponential fitting of the data as in Fig. 4. For K^+, Na^+, and Li^+, each value represents the mean of at least 10 determinations, with an SEM of <10%. For the remaining cations (TEA, choline, *N*-methylglucamine), values are from triplicate determinations.

does block from the *cis* side as well, but with an at least threefold-lower affinity. In experiments in which SR vesicles were mixed in the stopped-flow apparatus with a solution containing 500 μM *bis*G10, no effect on the Tl^+/Li^+ exchange rate was observed. The lack of an observable effect from the external side of the vesicles suggests an asymmetry similar to the one observed for the K^+ channel incorporated in planar bilayers, and it suggests that the *cis* side of the planar

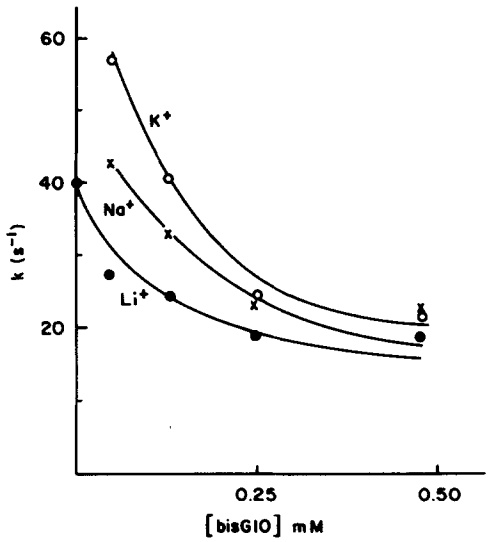


FIGURE 6. Titration of *bis*G10 effect. The rate of the fast component of Tl^+ influx was determined as a function of *bis*G10 concentration in vesicles loaded with K^+, Na^+, or Li^+ as indicated. The vesicles were incubated with the blocker from 10 to 30 min prior to the experiment. The rapid phase of Tl^+ influx in K^+- or Na^+-loaded vesicles is too fast to be measured in the absence of blocker.

bilayer is equivalent to the exterior of the vesicle. However, since we fail to observe any inhibition under these conditions, the possibility exists that the channel has undergone some minor modification upon insertion into the planar bilayer.

Tl^+ Exchange in Fractionated SR

Previous studies have shown that when microsomes obtained from skeletal muscle are fractionated by density, membranes derived from different regions of the SR can be separated (Meissner, 1975). Therefore, in order to study the distribution of channels, we examined the rate of Tl^+/Li^+ exchange in the heavy (H),

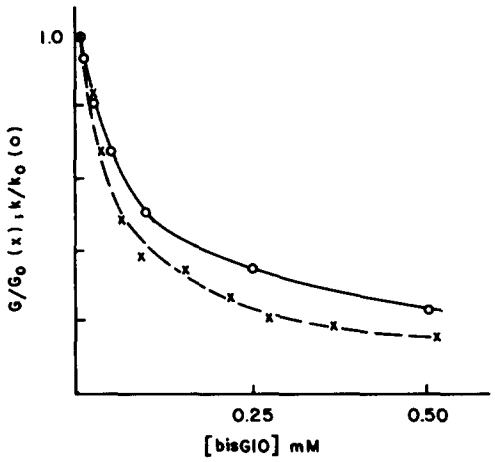


FIGURE 7. Comparison of the effect of *bis*G10 in planar bilayers and vesicles. Circles: relative macroscopic conductance of a planar bilayer as a function of *bis*G10 concentration. The ionic conditions were as follows: *cis* side: 75 mM Li^+ glutamate, 25 mM Tl^+ glutamate, 10 mM MOPS, pH 7.0; *trans* side: 100 mM Li^+ glutamate, 10 mM MOPS, pH 7.0. The blocker was added to the *trans* chamber, in small aliquots. Crosses: the relative rate of fast Tl^+ influx into Li^+-loaded vesicles is plotted as a function of *bis*G10. The vesicles were incubated with the indicated concentration of the blocker at least 10 min prior to the experiment.

intermediate (I), and light (L) fractions from the SR. Meissner (1975) has shown that the H and L fractions are derived primarily from the terminal cisternae and longitudinal tubules, respectively. The flux results, shown in Fig. 8, tell us that the three fractions studied have qualitatively similar behavior, i.e., that both fast and slow exchanges are present and that *bis*G10 inhibits the fast exchange. It is also observed that there are quantitative differences among the fractions, with the heavy vesicles displaying faster exchange rates than the light vesicles. Moreover, a systematically lower fraction of "rapid" vesicles is seen in the light (40%) than in the heavy (60%) SR. The data have been summarized in Table III. These differences do not result from size differences in the three vesicle fractions. All fractions display average diameters of 0.15 μm in negatively stained electron

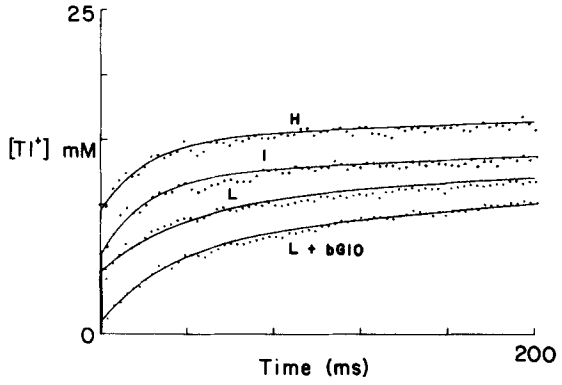


FIGURE 8. Tl^+ influx into SR fractions. SR vesicles fractionated according to density were loaded with PTS in Li^+ medium, as indicated in Methods. The rate of Tl^+ influx was determined for the heavy (H), intermediate (I), and light (L) fractions and the data were fitted with two exponentials (continuous line). Also shown is one experiment in which light vesicles were incubated with 0.23 mM *bis*G10 prior to the experiment (L + *bis*G10). Similar *bis*G10 inhibition is also seen with the H and I fractions (data not shown).

microscopic images (data not shown). Therefore, assuming that the channels behave similarly in the different fractions, we conclude that the channels, though present in both terminal cisternae and longitudinal tubule regions of the SR, are preferentially concentrated in the former.

Light-Scattering Changes Do Not Measure Cation Fluxes

In previous studies, changes in light scattering following osmotic shock have been used to measure ion permeabilities of SR vesicles (Kometani and Kasai, 1978). These studies arrived at a very different conclusion than those obtained here: that K^+ and Na^+ permeabilities are rather low in SR vesicles, indeed much lower than the Cl^- permeability. We therefore carried out similar experiments to assess the validity of the light-scattering method. When vesicles are mixed with a solution of higher osmolarity, they shrink and reswell, the rate of reswelling

TABLE III

Rate Constants of Tl^+/Li^+ Exchange in SR Fractions

	k_{fast}	f_{i+f}	k_{slow}	f_{slow}
	s^{-1}		s^{-1}	
Total SR	20.4	0.58	0.66	0.41
H	>300	0.59	0.68	0.41
I	47.6	0.47	0.62	0.52
L	25.4	0.42	0.64	0.57

Tl^+ influx was determined in unfractionated SR vesicles (total SR), or in heavy (H), intermediate (I), and light (L) fractions. The rate constants, k_{fast} and k_{slow}, were calculated by fitting the data with two exponentials. The sum of the instantaneous and fast fractions of Tl^+ exchange is indicated as f_{i+f}, while f_{slow} corresponds to the fraction of Tl^+ slowly exchanged.

depending on the permeability of the slowest-moving ion. The changes in volume are followed as changes in 90° light scattering.

Fig. 9*A* shows a typical experiment in which SR vesicles in 10 mM MES, pH 6.5, 1 mM EGTA, and 1 mM $CaCl_2$ have been mixed with either 100 mM KCl or 100 mM glucose. The increase in light scattering is due to the shrinking of the vesicles induced by the osmotic shock (Kometani and Kasai, 1978). Even though the osmolarity of the glucose solution is half that of KCl, the vesicles apparently shrink more in glucose than in KCl, a result comparable to others already published (Kometani and Kasai, 1978; Meissner and McKinley, 1982).

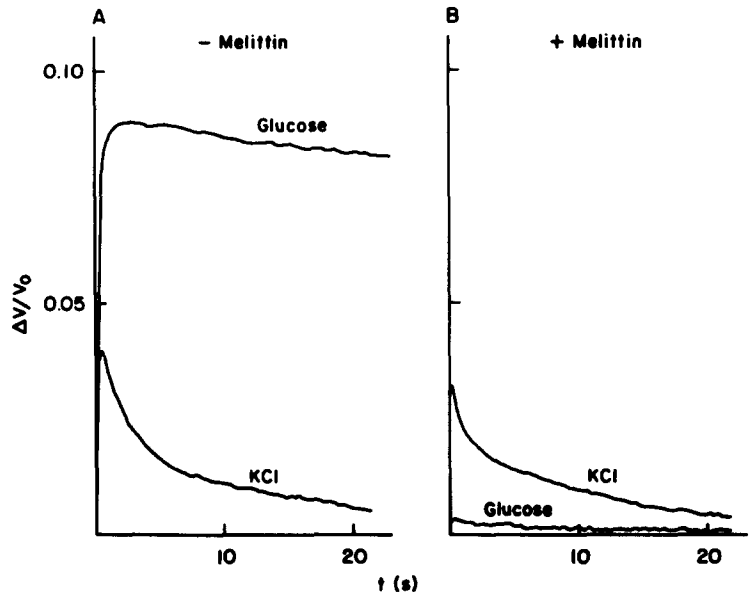


FIGURE 9. Light scattering of SR vesicles. SR vesicles in 1 mM EGTA, 1 mM $CaCl_2$, 10 mM MES, pH 6.5, at ~0.3 mg/ml, were mixed in a stopped-flow apparatus with a solution supplemented with 100 mM KCl or 100 mM glucose as indicated in the figure. The increase in light scattering corresponds to vesicle shrinking due to the osmotic shock and the return to baseline to their reswelling. The experiment was done either in the absence (*A*) or presence (*B*) of 40 μg/ml melittin. The vesicles were incubated with melittin at room temperature for 30 min before the experiment.

A very striking result is observed in Fig. 9*B*. In this experiment, the SR suspension is treated with melittin, a small peptide from bee venom that forms nonselective pores (Hanke et al., 1983) and makes SR vesicles permeable to solutes like glucose, Ca^{++}, and methionine, as seen by radioactive tracer fluxes (data not shown). As expected, SR vesicles treated with melittin do not respond to a glucose osmotic shock, presumably because glucose permeates at least as rapidly as does water. However, the light-scattering response to a KCl osmotic shock is almost unaffected by melittin. Our interpretation of these results is that the observed changes in the light scattered by an SR suspension are not solely due to volume changes of the vesicles. It is possible that other membrane

properties, such as refractive index, are altered by the change in ionic strength rather than in osmolarity and that this is reflected in the light-scattering behavior. Therefore, since the results obtained with this type of experiment depend on the conditions of the medium, we conclude that they cannot be validly used to measure permeabilities of SR vesicles, at least on a time scale of 1 s and faster.

DISCUSSION

The basic motivation of this work has been to measure the permeability of the SR membrane to small cations in as direct a way as possible, in hopes of settling two standing questions about the participation of SR membrane potential changes during excitation-contraction coupling. The first question concerns the value of the SR membrane conductance, estimates of which have ranged over four orders of magnitude. The second question concerns the function of the SR K^+ channel in the native membrane; whereas this channel has been extensively studied by fusing SR vesicles into planar lipid bilayers, its function in the SR membrane itself has never satisfactorily been demonstrated. We cannot claim to have answered these questions unequivocally, since all the work here was carried out on isolated SR membrane vesicles, not on the native membrane in situ. But we can claim to have performed a direct measurement of SR vesicle permeability to several small cations; moreover, we will argue that the specific properties of this permeability—its ion selectivity, its inhibition by *bis*G10, and the values of specific membrane conductance derived from it—all behave as if it were mediated by the K^+ channel characterized previously in planar bilayer studies.

The most fundamental and striking observation here is that the movement of inorganic monovalent cations across the SR vesicle membrane is rapid: on the order of tens of milliseconds for Li^+ and faster than 3 ms for K^+ and Na^+. The time scale of these passive fluxes may be compared with those reported for other solutes permeating the SR: Cl^-, 1 s (Kometani and Kasai, 1978); phosphate, 10 s (Meissner and McKinley, 1976; Kometani and Kasai, 1978); choline, glutamate, and methionine, 100 s (Meissner and McKinley, 1976; and this paper); Ca^{++} and sucrose, 1,000 s (Meissner and McKinley, 1976). Thus, small inorganic cations equilibrate across the SR vesicles membrane many orders of magnitude more rapidly than other solutes of various chemical types. In our preparations, only ~70% of the internal SR space is accessible to this fast ionic flux; the cations enter the other 30% of the space on the time scale of a few seconds. We therefore conclude that 70% of the SR vesicles carry a specific permeability pathway for these ions, while the remaining 30% lack such a pathway. This conclusion is in complete agreement with McKinley and Meissner (1978), who originally postulated two such SR vesicle populations on the basis of radioactive flux studies of monovalent cations.

The bulk of this discussion will be concerned with the cation flux into the "rapid flux" vesicle population. It was originally our suspicion that this flux may be mediated by the K^+ channel described in planar bilayers, and we consider that this idea has been largely confirmed. There are three characteristics of this flux that are strongly suggestive of the K^+ channel's behavior in planar bilayers: the specific membrane conductance derived from the flux rates, the ionic

selectivity of the flux, and the inhibition of the flux by the channel blocker *bis*G10.

K^+ Channel Conductance and SR Vesicle Fluxes

We would like to know if the values of cation fluxes measured in the SR vesicles by the Tl^+ quenching method are consistent with the single channel conduction properties of the SR K^+ channel determined in planar bilayers. Since there are several inevitable uncertainties in the calculations involved, it is necessary to stress that we take these merely as order-of-magnitude estimates, not as precise values of SR membrane conductances. Furthermore, because of these uncertainties, we make the calculations in two separate ways as an internal check.

First, we calculate the time constant, τ, of zero-voltage equilibrium exchange in the vesicles for K^+, Na^+, and Li^+ that would be expected from the known K^+

TABLE IV

Comparison of Vesicle Flux and Single Channel Conductance

		τ		G	
	γ	Calc	Obs	Flux	Bilayer
	pS	*ms*		*S/cm²*	
K^+	130	1.3	<3	$>3 \times 10^{-1}$	4×10^{-1}
Na^+	55	3.1	<3	$>3 \times 10^{-1}$	1×10^{-1}
Li^+	6	29	24	1×10^{-2}	2×10^{-2}

Single channel conductances, γ, were measured in 100 mM salt, symmetrical solutions (Coronado et al., 1980). The time constant for equilibrium exchange, τ, was calculated from Eq. 2 (calc) and is compared with the observed values of the fast fraction of Tl^+ exchange in SR vesicles (obs). Alternatively, the SR membrane conductance for each cation, G, was estimated either from the observed Tl^+ influx rate via Eq. 3 (flux) or from the single channel conductance via Eq. 5 (bilayer).

channel conductances. Assuming (*a*) that the SR vesicles comprise a homogeneous population of radius r, with N channels per vesicle, (*b*) that at zero voltage a channel has a probability p of being in its open state, (*c*) that at ion concentration c the single channel conductance is γ, and (*d*) that ionic movements are independent, with no electroneutral "exchange flux" present, we can write (Miller, 1984):

$$\tau = 4\pi F^2 c r^3 / 3RTN\gamma p. \quad (2)$$

We apply this treatment to an idealization of the SR vesicles: a population of 0.075-μm-radius vesicles, each containing four channels, each with a probability of being open of unity. These parameters are not chosen arbitrarily, but are estimated averages from electron microscopy (data not shown), the number of channels inserted into planar bilayers in an "average" fusion event, and the probability of the channels being open at the moment of insertion into the bilayer (Labarca et al., 1980).

The expected time constants for equilibrium exchange are shown in Table IV. Thus, in an idealized equilibrium exchange experiment, both K^+ and Na^+

should equilibrate more rapidly than the 3-ms time resolution of our measuring system, while Li^+ equilibration should be easily resolvable. Our experimental method does not permit flux measurements under strictly equilibrium exchange conditions. We require the presence of ion gradients and a nonzero voltage (Fig. 1), and these change with time during the approach to equilibrium. But we would argue that the conditions of the measurement (small gradient of the test ion, small potential) are near enough to equilibrium exchange conditions that the above calculation is valuable as an indicator of the expected time scale of the cation flux. Indeed, the observed flux rates are in good agreement with those expected from equilibrium exchange. The excellence of this agreement is undoubtedly fortuitous, since the vesicles are certainly not homogeneous in size and the flux relaxations are not monoexponential. Nevertheless, the agreement between the expected and observed absolute flux rates can be taken as an indication of the kinetic competence of the SR K^+ channel, as described in planar bilayers, to account for the permeability of native SR vesicles to small cations.

A second way to test the consistency of the vesicle flux rates with the channel conductance values is to calculate the expected vesicle membrane conductance from the measured fluxes, but without assuming equilibrium exchange conditions or independent ion movement. If J_i is the net efflux of the ith "test cation" under the conditions shown in Fig. 1, we can write the conductance per unit area of vesicle membrane, G_i, for the ith ion:

$$G_i = FJ_i/(V_m - V_i), \tag{3}$$

where V_m is the voltage across the vesicle membrane, and V_i is the Nernst potential for the ith cation. Eq. 3 is valid during the entire time course of the flux experiment, but all of its parameters are time dependent. The equation can be used by noting that at zero time, immediately after the rapid mixing, the values J_i, V_m, and V_i are known with reasonable certainty. The flux at zero time can be estimated from the initial rate of Tl^+ influx directly:

$$J_i = (r/3)\, d[Tl^+]/dt \quad (\text{at } t = 0). \tag{4}$$

Similarly, V_i is given by the Nernst equation from the known concentration ratio of the ith ion immediately after the rapid mix (Fig. 1), and is -7 mV in all experiments here. Finally, V_m cannot be measured directly, but it can be estimated by establishing asymmetric ionic conditions of Tl^+ and the ith ion as in Fig. 1 in planar bilayer experiments, and measuring reversal potentials. In such experiments, we have found that the Tl^+ permeability is close to that of K^+ and Na^+, and is about eightfold higher than that of Li^+. Accordingly, we estimate the vesicle membrane potentials at zero time to be ~0 mV for K^+, +3 mV for Na^+, and +25 mV for Li^+.

We can thus calculate the expected membrane conductances for these three ions (Table IV). Only Li^+ permeates slowly enough for us to estimate a value for the vesicle membrane conductance, 1×10^{-2} S/cm^2; for K^+ and Na^+ we arrive at lower limits of 10^{-1} S/cm^2.

How do these values compare with those expected from the known single

channel conductances? We can estimate the specific conductance of a vesicle containing N channels easily, using the parameters of Eq. 2:

$$G_i = pN\gamma_i/4\pi r^2. \quad (5)$$

Again (Table IV) assuming $r = 0.075$ μm, $p = 1$, and $N = 4$, we expect a vesicle conductance for Li^+ of 1.7×10^{-2} S/cm^2, which is entirely consistent with the calculation from the flux rates. Conductances for Na^+ and K^+ are 10–30-fold higher than this. Our estimate of Li^+ conductance is most likely a lower limit, since it ignores the fact that a substantial fraction of the fast flux is "instantaneous," i.e., more rapid than 3 ms (Fig. 4). (This instantaneous fraction may represent a population of vesicles carrying more than four channels.)

The conclusion from these two types of calculation is that the measured fluxes in the "fast fraction" of the SR vesicle preparation are quantitatively in harmony with those expected from the measured single channel conductances after fusion of vesicles into planar bilayer membranes. These two calculations are independent of one another, each relying upon different assumptions: the first upon the validity of approximating the stopped-flow measurements with equilibrium exchange conditions, and the second upon making a reasonable guess of the vesicle voltage immediately after mixing. That both of these approaches lead to the same calculated values lends credibility to the basic conclusion here: that a conductance pathway reminiscent of the SR K^+ channel resides in a major fraction of SR vesicles.

Ionic Selectivity

The K^+ channel as studied in planar bilayers displays substantial ionic selectivity in its conduction properties (Coronado et al., 1980), with single channel conductances in 0.1 M salt of K^+ (130 pS) > Na^+ (60 pS) > Li^+ (5 pS). (Cs^+ shows complex properties of both conduction and block and will not be considered here.) As shown in Table I, the fast fluxes into SR vesicles qualitatively mimic this ionic selectivity. Both K^+ and Na^+ permeate too rapidly to measure, and at least 20-fold faster than does Li^+.

Larger cations that do not permeate the K^+ channel, such as choline, leave the "fast fraction" vesicles at least 1,000-fold more slowly than does K^+. One unsolved puzzle apparent in Fig. 4*A* is that a small fraction (~20%) of the Tl^+ influx in choline-loaded vesicles proceeds on a time scale of tens of milliseconds, much faster than the bulk choline flux. The same phenomenon is observed with all the slowly permeant ions. We do not understand this effect. The amount of Tl^+ moving into the vesicles is too large to be accounted for by uncompensated ion flux; it is possible that it represents exchange of Tl^+ for H^+, but we have not seriously tested this suggestion, and we have ignored this small fraction in our analysis. Overall, we conclude that the ion selectivity of the rapid vesicle fluxes is consistent with that of the single channel conductances of the K^+ channel in planar bilayers.

Block by bisG10

The SR K^+ channel in planar bilayers has been shown to be competitively inhibited, or "blocked," by many organic cations, such as tetraethylammonium

and other quaternary amines (Coronado and Miller, 1982; Miller, 1982). Most of these, unfortunately, are very weak blockers that inhibit only in the millimolar range. We have found that *α,ω-bis*-guanidino-*n*-alkanes are the highest-affinity blockers yet encountered (C. Miller, unpublished data), when used on the K^+ channel in planar bilayers. The results presented here show that the compound *bis*G10 inhibits the rapid fluxes of K^+, Na^+, and Li^+, but does not substantially affect the slow-fraction fluxes of these ions, or of choline or methionine. Moreover, the concentration dependence of the *bis*-G10 inhibition of flux parallels its reduction of macroscopic conductances in the planar bilayers under identical ionic conditions. We therefore again conclude that the rapid flux behaves as though it were mediated by the SR K^+ channel.

Furthermore, our findings suggest that the blocker is effective only on the internal side of the SR membrane. For *bis*G10 to inhibit the cation fluxes, it must be preincubated for several minutes with the SR vesicles; independent studies of fluxes of *bis*-cationic alkanes (data not shown) establish that molecules of this type do in fact permeate the SR membrane on the time scale of 1–5 min. This proposal is also consistent with the fact that these blocking compounds are more effective from the side of the planar bilayer opposite to the side on which the SR vesicles are added, and with our view that the *cis* side of the channel in the planar bilayer is most likely equivalent to the outside of the SR membrane (Miller, 1978).

The Connection to Muscle Physiology

The arguments above lead to the strong conclusion that the K^+ channel detected in planar bilayer membranes after fusion of SR vesicles is present in a major fraction (50–70%) of these vesicles, and that its properties in the native vesicle membrane are not grossly different from those detected in planar bilayers. The results presented here do not directly address the question of the channel's behavior in the SR membrane in the muscle. But of the many kinds of flux phenomena described in isolated SR vesicles, only the cation fluxes documented here operate on the same time scale as muscle contraction in vivo. Therefore, we can, with more confidence than in the past, attempt to describe several important physiological properties of the SR membrane, assuming that the vesicle membrane properties are valid approximations of the situation in vivo.

The first major conclusion is that the SR membrane conductance is large. Since channel-containing vesicles make up 50–70% of the SR membrane area, the calculated K^+ conductance of the SR membrane is ~0.1 S/cm^2, an extremely high value. This estimate leads to a second conclusion: that at rest the SR membrane maintains zero membrane potential. Of all ions present, K^+ would be the overwhelming determinant of potential because of its high conductance and high concentration. Because this ion is present at equal concentrations on both sides of the membrane (Somlyo et al., 1981), the potential would be set near zero.

Third, we conclude that because of the high K^+ conductance of the SR membrane, it will always be clamped near zero voltage, even during the peak of Ca^{++} release. Oetliker (1982) has shown that an SR membrane of this conduct-

ance could not be polarized by more than 7 mV by ionic currents due to Ca^{++} release.

Fourth, we conclude that the K^+ channels are not localized in any particular region of the SR, but are found in the terminal cisternae as well as in the longitudinal tubule regions. Although some heterogeneity in the distribution is observed, we would have expected to see more dramatic differences in the flux behavior of vesicles from the heavy and light SR fractions, if an absolute localization had been the case.

According to these arguments, then, we view the SR as a very high conductance membrane, always shunted near zero voltage. The function of the K^+ channel is seen to be merely a "leak" to allow Ca ion to be released from and taken up into the SR lumen at high rates, as needed by the demands of the contraction and relaxation cycle. Without such a leak, Ca^{++} release would seriously charge the membrane potential and thus might become self-limiting.

This view of the SR membrane in situ is seriously at odds with several other proposals concerning excitation-contraction coupling. Experiments using voltage-sensitive dyes (Vergara et al., 1978) have postulated very large changes in membrane potentials during Ca^{++} release, and an SR membrane conductance of 6×10^{-5} S/cm^2, three orders of magnitude lower than that estimated here. The primary uncertainty in these experiments is that the dye may be tracking not potential, but rather Ca ion bound to myofilaments (Oetliker, 1982), or other unknown changes (Baylor et al., 1983). Similarly, the postulate of a high conductance spread uniformly over the SR membrane does not appear to be consistent with the "electrically active" SR membrane required by the model of Ca^{++} release developed by Mathias and colleagues (1980). Furthermore, our picture contradicts the previous report that K ion equilibrates across the SR membrane slowly, on the order of 10 s, much more slowly than does Cl^- ($\tau_{1/2}$ = 0.5 s) (Kometani and Kasai, 1978). Although we did not measure anion fluxes in this work, our rates of K^+ movement are almost three orders of magnitude faster than those based on light-scattering studies. In view of the artifacts encountered with the light-scattering method (Fig. 9), we do not consider those conclusions to be valid. A final, and, we think, the most serious inconsistency with the present conclusions, arises from electron microprobe studies of frog muscle fibers (Kitazawa et al., 1983, 1984). The basic result arguing against a high "resting" K^+ conductance is the finding that valinomycin apparently increases the rate of K^+ moving into the SR to compensate Ca^{++} release during a sustained tetanus.

We consider ourselves justified, on the basis of this work, in asserting that the K^+ channel described in planar bilayers is present in the SR membrane as a major ionic conduction pathway. Whether its full potential for conferring conductance on the SR membrane in vivo is expressed, or whether its activity is suppressed by physiological control mechanisms, can only be answered by future work on excitation-contraction coupling in the muscle cell itself.

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