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CHANNEL-MEDIATED Tl^+ FLUXES IN SARCOPLASMIC RETICULUM VESICLES

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The sarcoplasmic reticulum K^+ channel is unusual in that its properties are better known in the reconstituted than in the native membrane. The channel was identified by fusing sarcoplasmic reticulum (SR) vesicles into planar phospholipid bilayers (4). It has been thoroughly studied in this system, and today its cation selectivity, voltage-dependent gating, single-channel conductance, asymmetric orientation in the membrane, and other characteristics are well documented (1–4). The presence of the channel in SR vesicles has been suggested by radioisotope fluxes of monovalent cations (5), but so far, its characterization in the native state has not been possible. Given the high channel conductance in planar bilayers (1), the half time for K^+ flux in SR vesicles is expected to be ~1 ms, much faster than the time resolution attainable by conventional flux techniques. Moreover, the SR membrane in situ is not accessible to direct electrophysiological study. Hence, it still remains to be established whether the channel has been modified by the reconstitution process or how its behavior correlates to the known functions of the SR membrane.

In this report, we apply to SR vesicles a method introduced by Moore and Raftery (6) for monitoring monovalent cation fluxes on the millisecond time scale. A hydrophilic fluorescent probe, pyrenetetrasulfonate (PTS), is trapped inside of SR vesicles. These are then mixed with a solution containing Tl^+, a K^+ analogue that quenches the PTS fluorescence as it enters the vesicles. Given that Tl^+ permeates the SR channel as well as K^+,[1] its rate of entry will be limited by the permeability of other ions in the system, ions that must move to maintain electroneutrality. In the experiments described here, care has been taken to exclude rapidly permeant anions like Cl^-, so that the influx of Tl^+ is determined by the efflux of the cation with which the SR vesicles have been loaded.

METHODS

Isolated SR vesicles (20 mg prot/ml) from rabbit skeletal muscle were equilibrated overnight in a solution containing 100 mM glucose, 100 mM Li^+ (or other cation) glutamate, 10 mM PTS (tetracholine salt), 10 mM MOPS, pH 7.0. Prior to the experiment, the external PTS was removed by passing the SR suspension through a Dowex anion exchange column (Dow Corning Corp, Midland, MI). The vesicles were then diluted to a protein concentration of 0.1–0.2 mg/ml with the same medium, except that PTS was omitted and glucose was added to maintain osmolarity. Immediately after dilution, the vesicles were mixed in a stopped-flow apparatus with a solution of the same composition as the dilution medium, with 50 mM Li^+ replaced by Tl^+.

RESULTS AND DISCUSSION

A typical time course of fluorescence decrease is shown in Fig. 1. Clearly, when the SR vesicles have been loaded with Li^+, most of the Tl^+/Li^+ exchange is completed in the first 100 ms, while if choline is used as the compensating cation, the rate of fluorescence quenching is far slower. It is also observed that in the presence of a blocker of the K^+ channel in planar bilayers, 1,10-bis-guanidino-*n*-decane (bisG10), the initial fluorescence of Li^+ loaded vesicles is larger and decays more slowly.

Fig. 2 shows Tl^+ influx into SR vesicles loaded with different monovalent cations. It is observed that when K^+ is present inside the vesicles, at least 50% of the influx is completed within the mixing time of the stopped-flow apparatus (3 ms). With Li^+-loaded vesicles however, fast and slow relaxations are clearly observed. When the vesicles are made nonselectively permeable to cations by the ionophore gramicidin A, Tl^+ equilibrates with all the

[1]Coronado, R., and C. Miller. Unpublished results.

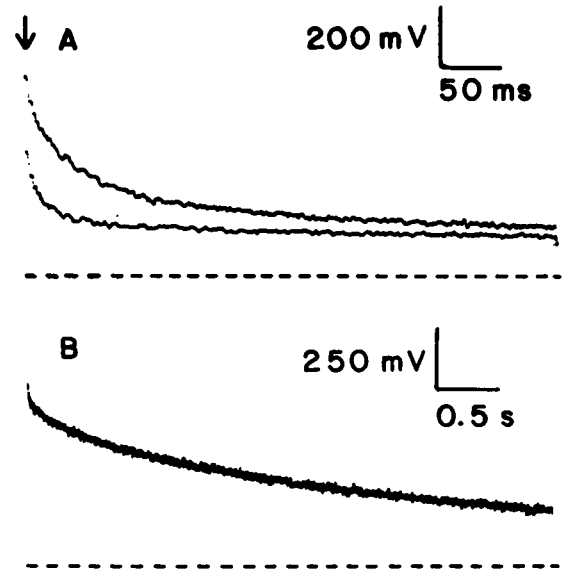

FIGURE 1 Tl^+ quenching of PTS fluorescence. Vesicles loaded with PTS were mixed 1:1 in a stopped-flow apparatus with a solution containing 50 mM Tl^+ glutamate, and the decrease in fluorescence was recorded. Excitation and emission wavelengths were 355 nm and 405 nm. The arrow indicates the experimental zero time. *A*, Bottom trace: vesicles were loaded in a medium containing 100 mM Li^+ glutamate. Top trace: same vesicle preparation, except that 0.25 mM bisG10 was added 30 min before the quenching experiment. *B*, Tl^+ influx into vesicles preloaded in 100 mM choline glutamate. The dotted lines indicate the final fluorescence value.

vesicles in <3 ms. If the vesicles are preincubated with the channel blocker bisG10, the fast phase of the Tl^+/Li^+ exchange is considerably retarded.

Table I summarizes the rate constants determined for several Tl^+ exchange experiments. For comparison, the maximum channel conductance for each of the ions tested has been included. In all cases studied, the exchange can be

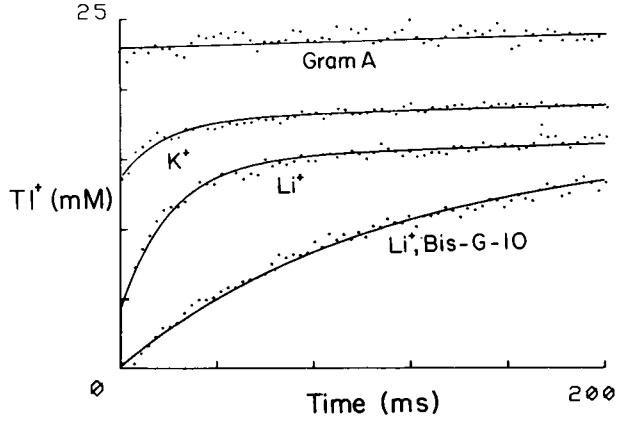

FIGURE 2 Time course of Tl^+ influx into SR vesicles. Fluorescence has been converted into Tl^+ concentration by suitable calibration. Vesicles were loaded with the glutamate salts of the indicated ions. The continuous line represents the fitting of the data with a double exponential relaxation. The top trace corresponds to an experiment in which vesicles loaded with Li^+ glutamate were treated with 1.25 μg/ml gramicidin A before the experiment.

TABLE I
IONIC SELECTIVITY AND INHIBITION OF CATION FLUXES IN ISOLATED SR VESICLES

		$k_{fast}(s^{-1})$		$k_{slow}(s^{-1})$	
	γ_{max}(pS)	−bisG10	+bisG10	−bisG10	+bisG10
K^+	240	>300 (0.71)	26 (0.40)	0.51 (0.28)	0.43 (0.39)
Na^+	77	>300 (0.70)	26 (0.48)	0.52 (0.29)	0.54 (0.42)
Li^+	8	42 (0.35)	14 (0.56)	0.40 (0.34)	0.60 (0.43)
TEA	~2	0.7 (0.13)	0.9 (0.20)	0.04 (0.60)	0.05 (0.50)
Chol	<1	0.9 (0.20)	1.3 (0.20)	0.07 (0.58)	0.06 (0.56)

Rate constants were obtained by fitting the data to a double exponential. In the experiments indicated, 0.25 mM bisG10 was added to the vesicle suspension 30 min before mixing. The fraction of Tl^+ exchanged with the given rate is indicated in parentheses. The maximal channel conductance, γ_{max}, was measured directly in planar bilayer experiments (1, 3).

described by at least two rate constants: fast and slow. Ions known to permeate the SR K^+ channel (K^+, Na^+, Li^+) have a slow exchange component with a rate constant of ~0.5 s^{-1}. The amount of Tl^+ slowly exchanged is also comparable in these three cases (30%). In vesicles loaded with K^+ or Na^+, the remaining 70% of the Tl^+ is exchanged instantly, i.e., within the mixing time of the apparatus. In the case of Li^+, at least two fractions of fast Tl^+/Li^+ exchange can be distinguished: 30% is instantaneous, while another 30% has a rate constant 100 times larger than the slow exchange. In vesicles preincubated with bisG10, the slow component is not affected in any of the cases studied. However, the rate of the fast exchange of Tl^+ for K^+, Na^+ or Li^+ becomes measurable and the fast Tl^+/Li^+ exchange rate is reduced. In contrast, the slow TEA and choline fluxes are not affected by the presence of bisG10.

These kinetics of Tl^+ entry support the idea first proposed by McKinley and Meissner (5) of a heterogeneous vesicle population in which only 70% of the vesicles have cation-selective channels. In the experiments described here, 70% of the Tl^+ flux was found to be fast and sensitive to bisG10, suggesting the presence of K^+ channels. Furthermore, the measured cation exchange rates of this fraction are compatible with the absolute single channel conductance in planar bilayers. Assuming that a "typical" SR vesicle carries five channels on the average (2), the expected rate constant of Tl^+/Li^+ exchange for a vesicle with five of these channels would be 50 s^{-1} (7), in excellent agreement with the found value of 42 s^{-1}. Under the same conditions, the rate constant for K^+ exchange would be 1,500 s^{-1}, faster than the time resolution attainable here. Comparison of the rate constants of the bisG10-sensitive fractions give us a selectivity sequence similar to the one obtained for the K^+ channel in planar membranes: $K^+ \sim Na^+ > Li^+ \gg$ TEA ~ choline.

In conclusion, the cation fluxes measured directly in native SR vesicles are compatible with the properties of the K^+-selective channel studied by fusing SR vesicles to planar bilayers. Specifically, its ionic selectivity, conduc-

tance, and bisG10 blocking seem not to have been modified by the reconstitution process. The results also conform to the previous proposal of heterogeneity in channel distribution (5), with 30% of the vesicles having no channels and the rest having one or more. Furthermore, the fact that blocking by bisG10 was observed only after it had been loaded into the vesicles supports the idea of an asymmetric channel that can be blocked only from the inside of the membrane.

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Zn, MULTIPLE ACTIVATION PROCESSES, AND INACTIVATION DELAYS

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It is now well established that the activation and inactivation processes of the sodium conductance, g_{Na}, are coupled together in some way (Goldman, 1976; Armstrong and Bezanilla, 1977). Less clear is the nature of the coupling.

For *Myxicola* axons the coupling seems to be sequential, i.e., at least some fraction of the channels must open before they can inactivate (Goldman and Kenyon, 1982). Inactivation development, determined in this preparation with a two-pulse procedure, proceeds with an initial delay, as expected if inactivation develops subsequent to a precursor process; this precursor (delaying) process has the properties of Na activation.

In an extensive series of experiments in *Myxicola*, the time to peak g_{Na} during the conditioning pulse was found to be proportional to the inactivation delay, as required if inactivation follows sequentially on activation. This demonstrates that the potential dependence of the time constant of the delay process is the same as that of the activation process (Goldman and Kenyon, 1982). The potential dependence of the time constants of inactivation, τ_c and τ_h, differ significantly. Neither the normalized $\tau_h(V)$ nor $\tau_c(V)$ fit the inactivation delay vs. potential data, while the normalized $\tau_m(V)$ describes them well. In a less extensive series of determinations, the extracted time constant of the delay process, $\tau_{delay}(V)$, was found to be in quantitative agreement with $\tau_m(V)$.

We present here new results from *Myxicola* which firmly establish the close correspondence between inactivation delay and Na activation by showing that external Zn selectively slows both processes in parallel.

RESULTS

Fig. 1 presents a typical inactivation determination in the presence of Zn (5 mM). Peak Na currents during a series of fixed test steps in clamped potential were obtained by subtraction of current records obtained with identical protocols in Na-free artificial sea water (ASW). Each test step was preceded by a conditioning potential step of fixed amplitude but varying duration and a 6-ms step back to the holding potential. The peak currents during each of the test steps normalized to that during an unconditioned test step are shown as a function of conditioning step duration.

Inactivation delay, determined operationally as the time at which the unconditioned I_{Na} value intersects the τ_c exponential (solid curve), was 1.081 ms, while for another determination on this same axon under identical conditions, but without Zn, it was 0.594 ms: an increase in delay of 1.82-fold in Zn. Correspondingly, the time-to-half maximum of g_{Na} ($t_{1/2}$) during the conditioning pulse in this same axon increased nearly identically (1.81-fold) in 5 mM Zn, from 0.775 to 1.40 ms. 1 mM Zn had no effect.

These Zn effects on the Na kinetics cannot be attributed solely to changes in surface potential. If the changes in $t_{1/2}$ are assumed to arise entirely from surface potential effects, then τ_c should have increased from this change by