COMPARISON OF THE KINETICS OF CALCIUM TRANSPORT IN VESICULAR DISPERSIONS AND ORIENTED MULTILAYERS OF ISOLATED SARCOPLASMIC RETICULUM MEMBRANES

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ABSTRACT Knowledge of the functional properties of the protein in oriented multilayers, in addition to vesicular dispersions, of membranes such as the isolated sarcoplasmic reticulum (SR), extends the variety of techniques that can be effectively used in studies of the membrane protein's structure or structural changes associated with its function. One technique requiring the use of oriented multilayers to provide more direct time-averaged and time-resolved structural investigations of the SR membrane is x-ray diffraction. Therefore, the kinetics of ATP-induced calcium uptake by isolated SR vesicles in dispersions and hydrated, oriented multilayers were compared. Ca^{2+} uptake was necessarily initiated by the addition of ATP through flash photolysis of caged ATP, P^3-1-(2-nitro)phenylethyl adenosine 5'-triphosphate, with either a frequency-doubled ruby laser or a 200 W Hg arc lamp, and measured with two different detector systems that followed the absorbance changes of the metallochromic indicator arsenazo III, which is sensitive to changes in the extravesicular $[Ca^{2+}]$. The temperature range investigated was $-2°$ to 26°C. The Ca^{2+} uptake kinetics of SR membranes in both the vesicular dispersions and oriented multilayers consist of at least two phases, an initial fast phase and a subsequent slow phase. The fast phase, generally believed to be associated with the formation of the phosphorylated enzyme, E~P, is kinetically comparable in both SR dispersions and multilayers. The slow phase mathematically follows first-order kinetics with specific rate constants of $\sim 0.6\ s^{-1}$ and $\sim 1.2\ s^{-1}$ for the dispersions at 26°C and multilayers at 21°C, respectively, with the given experimental conditions. The slow phase, generally believed to be associated with the translocation of Ca^{2+} across the membrane profile, appears to be the same process in SR dispersions and multilayers through their virtually identical rate constants and their identical activation energies of 22 ± 1 kcal mol^{-1}. The stoichiometry of ~ 2 mol Ca^{2+}/mol ATP hydrolyzed was measured in dispersions for the slow phase of Ca^{2+} uptake. Photolysis of caged ATP with the lamp and the laser provides comparable results for the Ca^{2+} uptake kinetics in SR dispersions and multilayers. Laser flash photolysis, however, has the advantages of optimal time resolution and effective synchronization of the ensemble of Ca^{2+}-ATPase molecules in the ATP initiated Ca^{2+} transport process.

INTRODUCTION

By studying membrane proteins in an ordered, condensed phase (either in the form of crystals or liquid crystals), in addition to dispersions, one can use a variety of techniques to investigate the membrane protein's structure and any structural changes associated with its function. One technique generally requiring membrane proteins to be in an ordered condensed phase, with which direct detailed structural determinations of the membrane protein may be obtained, is x-ray diffraction complemented with neutron diffraction. However, it is essential to measure the functional properties of the protein in the condensed phase, i.e., its functional capability and characteristics, since the ability of the protein to function, compared with dispersions, could be altered.

An understanding of the process of ATP-induced Ca^{2+} transport across the sarcoplasmic reticulum (SR) membrane requires an investigation of the interrelationship of structural dynamics and function. A direct determination of conformational changes in the Ca^{2+}-ATPase occurring during the Ca^{2+} transport cycle can be accomplished with dynamic x-ray diffraction studies of hydrated, oriented multilayers of isolated SR membranes. Oriented multilayers are necessary for x-ray diffraction experiments to obtain a reasonable level of the structural detail. Separate lipid and protein profile structures have been established at

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~12-Å resolution for the static (resting) state of the SR membrane, through a combination of x-ray and neutron diffraction methods (1; Herbette, L., P. DeFoor, S. Fleischer, D. Pascolini, and J. K. Blasie, manuscript in preparation). Dynamic structural experiments with x-ray diffraction of oriented SR membrane multilayers may be done in one of two ways: (*a*) with time resolution by using appropriate serial time windows for data collection to observe structural changes initiated by ATP or (*b*) with low temperature to slow the Ca^{2+} transport process initiated by ATP and thereby trap an intermediate within the experimental time window. Preliminary time-resolved x-ray diffraction experiments utilizing flash photolysis of caged ATP, P^3-1-(2-nitro)phenylethyl adenosine 5'-triphosphate, have shown that structural changes in the SR membrane do indeed occur during the Ca^{2+} transport process (2–4).

Knowledge of the detailed kinetics of the Ca^{2+} transport process is essential for the determination and interpretation of the associated structural changes in the Ca^{2+}-ATPase. Because x-ray diffraction experiments require the use of oriented multilayers and because the water spaces in multilayers are extremely small, with the SR vesicles comprising most of the volume, compared with dispersions, it is imperative to determine the kinetics of Ca^{2+} transport in oriented SR membrane multilayers. Kinetic studies of Ca^{2+} uptake by SR membrane vesicular dispersions afford a reference for comparison with multilayers.

Ca^{2+} uptake by SR membrane vesicles in dispersions and in hydrated oriented multilayers was examined and compared. Flash photolysis of caged ATP, with either a 200 W Hg arc lamp or a frequency-doubled ruby laser initiated the Ca^{2+} transport process. Flash photolysis of caged ATP provided the advantages of higher time resolution, ~10 ms, depending on the temperature, and synchronization of the ensemble of Ca^{2+}-ATPase molecules. Furthermore, with the technique of flash photolysis of caged ATP within the SR membrane multilayers to chemically activate the Ca^{2+}-ATPase, the orientation and ordering of the stacked, flattened SR membrane vesicles in the multilayers were preserved. A time-sharing multiple-wavelength spectrophotometer or absorbance amplifier system processed the absorbance changes of the metallochromic indicator arsenazo III, sensitive to changes in extravesicular [Ca^{2+}]. The temperature range investigated was $-2°$ to 26°C.

Ca^{2+} uptake in both SR dispersions and multilayers was found to consist of at least two distinct kinetic phases, a fast phase and a slow phase. The fast phase, generally believed to be associated with the phosphorylated enzyme, E~P, formation, was kinetically similar in both dispersions and multilayers. The slow phase, generally believed to be associated with the translocation of Ca^{2+} across the membrane profile, was shown to be the same process in dispersions and multilayers because of their comparable rate constants and identical activation energies. This kinetic information concerning the oriented SR membrane multilayers was correlated with the already available results of time-resolved x-ray diffraction experiments.

MATERIALS AND METHODS

Dispersions of sarcoplasmic reticulum vesicles were prepared from the hind legs of rabbits as previously described (5). The protein content was determined according to the Lowry method (6). The Ca^{2+}-ATPase constituted ~67% of the total protein for the SR preparations used in the kinetic study (7). SR preparations with Ca^{2+}-ATPase content of ~90% of the total protein (8), used in the x-ray diffraction experiments, have identical Ca^{2+} uptake rates when Ca^{2+} uptake is expressed in terms of the amount of enzyme that can be phosphorylated (9). But, since the preparation of higher Ca^{2+}-ATPase content tended to lose its viability more rapidly than the one of lower Ca^{2+}-ATPase content over extended periods of time, and since the kinetic experiments generally lasted > 12 h because reproducibility was checked with repeated runs and alternation of controls, the preparation with lower Ca^{2+}-ATPase content was more convenient to study.

Caged ATP was synthesized from ADP morpholidate and 1-(2-nitro)phenylethyl phosphate, essentially as described by Kaplan et al. (10). 1-(2-nitro)phenylethyl phosphate was prepared from the parent alcohol and *o*-phenylene phosphorochloridate by the method used for the synthesis of barium 2-cyano-ethyl phosphate (11). The release time of ATP from caged ATP is determined by the dark reaction, which has a rate constant of 220 s^{-1} at 22°C and pH 7.0 (12). Caged ADP was synthesized as for caged ATP with AMP morpholidate replacing ADP morpholidate. Caged ATP and caged ADP were in 100 mM TES, *N*-[Tris(hydroxymethyl)-methyl-2-amino] ethane sulfonic acid, pH 7.0.

SR vesicular dispersions as prepared (82 μl) contained 40 mM Tris maleate, 8 mM $MgCl_2$, 120 mM KCl, 100 μM arsenazo III, 200 μM glutathione, 2 mg SR protein per ml, 50 μM $CaCl_2$ in addition to the [Ca^{2+}] provided by the SR vesicles, and 1 mM caged ATP for the Hg lamp experiments, or 2.3 mM caged ATP for the laser experiments, pH 7.0. In control experiments caged ADP was used, or the nucleotide or SR was omitted.

Oriented SR membrane multilayers were made by sedimenting 1 mg SR protein onto Mylar (0.001 inch thick, DuPont Co., Wilmington, DE) from a 2-ml solution of 40 mM Tris maleate, 8 mM $MgCl_2$, 120 mM KCl, 50 μM arsenazo III, 1.75 mM glutathione, 25 μM $CaCl_2$ in addition to the [Ca^{2+}] associated with the SR protein, pH 7.0. The centrifugation speed was 23,000 rpm with a swinging bucket rotor (Beckman Instruments, Inc., Palo Alto, CA), time 1–1.5 h, and temperature 7°C. After sedimentation of the multilayers, the supernatant was discarded, and any excess solution on the multilayer surface was carefully removed. The Mylar supporting the multilayers was then glued to a small glass slide and suspended in a vial with a saturated $ZnSO_4 \cdot 6H_2O$ solution. After <1 h, 130 nmol caged ATP was gently added in the dark from a pipette to the surface of the multilayers and was allowed to diffuse within the layers of the stacked flattened SR vesicles. Controls consisted of multilayers with caged ADP or no nucleotide. The multilayers were partially dehydrated overnight in the vial via equilibration with a saturated $ZnSO_4 \cdot 6H_2O$ solution. The temperature during preparation of the multilayers was 3–7°C. These conditions for hydrated, oriented SR membrane multilayer formation are generally optimal for producing extensive lamellar x-ray diffraction.

The Mg^{2+}, Ca^{2+}, and caged ATP concentrations were chosen to be considerably higher than the Ca^{2+} -ATPase concentration, to permit several turnovers of the Ca^{2+} transport cycle. A large Mg^{2+} concentration also effectively helped to prevent Ca^{2+} binding to ATP in appreciable amounts. A high buffer concentration served to hold the pH constant upon photolysis of the caged compound, which produces protons. Glutathione was essential to protect the Ca^{2+}-ATPase from possible reaction with the ketone photolysis product (10, 13).

For dispersion experiments, a 100 μl quartz cell in a temperature-

controlled chamber was used with a 5-mm path length for the measuring light beam and a 2-mm path length for the photolyzing light beam perpendicular to the measuring beam. With oriented multilayers, the glass slide was mounted in the temperature- and humidity-controlled chamber so that the photolysis light was incident at an angle of ~60° to the surface of the multilayers. A sketch of the chamber appears in Fig. 1 showing the geometry of the measuring and photolyzing light beams.

Two light sources were used in the photolysis of the caged compounds. One light source was a 200 W Hg arc lamp. A shutter was placed on the lamp and another on the photomultiplier tube (PMT) in the detection system. The shutters were operated by a shutter control box so that the PMT shutter could be closed while the lamp shutter was open, thus protecting the PMT during the flash. In dispersion experiments, the lamp flash was ~270 ms and the PMT was blocked for ~580 ms. The lamp was used with no filter and gave a pulse of ~165 mJ over the broad spectrum. However, in multilayer experiments, an Oriel 5165 filter (Oriel Corp., Stratford, CT) (passing mostly 300–370 nm) was placed on the lamp and a quartz light pipe transmitted the photolysis light to the multilayers. A 1-s lamp flash, ~6 mJ, was used.

The other light source utilized was a frequency-doubled ruby laser (Korad Dept., Electronics Div. Union Carbide Corp., Santa Monica, CA) that was passively Q-switched with vanadyl phthalocyanine in nitrobenzene, ~0.16 OD at 694 nm. A potassium dihydrogen phosphate (KDP) crystal doubled the frequency of the ruby laser light. $CuSO_4$ solution served as a filter to block the 694 nm pulse and pass the 347 nm pulse. A neon laser (Metrologic Instruments Inc., Bellmawr, NJ) was used in alignment of the optical system. The frequency-doubled beam had an energy range of 20–30 m J. The energy was measured with a Gentec energy meter (Gentec Inc., Quebec, Canada) and a Tektronix 564 storage oscilloscope (Tektronix, Inc., Beaverton, OR) triggered by the laser. A quartz slide reflected a small fraction of the 347 nm light to a photodiode (PD) (RCA Electro-Optics & Devices, Lancaster, PA) also connected to the storage oscilloscope. The signal from the photodiode was calibrated with that from the energy meter. Hence, during experiments when the sample chamber was in the 347 nm beam, the laser energy used for photolysis could be checked and maintained above 20 mJ for each experiment by using the photodiode.

Ca^{2+} uptake was measured by following the changes in the absorbance spectrum of arsenazo III, which has a Ca^{2+} on and off time of ~2–5 ms, with the wavelength pair difference 660–690 nm (14) using a time-sharing spectrophotometer system (15) with ~20 ms time resolution. Light passed from a Quartzline lamp (General Electric Co., Cleveland, OH) through an air-driven (200 Hz) filter wheel (with 660 and 690 nm interference filters), collimating lens, and light pipe into the chamber to the sample. The light from the sample exited via another light pipe in the chamber to the PMT (EMI Gencom Inc., Plainview, NY model 9798). The demodulator subtracted and averaged the voltage signals from the PMT. A chart recorder and storage oscilloscope recorded the signal.

In addition, several dispersion experiments with the laser involved the use of an absorbance amplifier. A Wratten 29 filter (Eastman Kodak Co., Rochester, N.Y.) replaced the filter wheel and a 660 nm filter was put on the PMT. The PMT voltage was sent to the absorbance amplifier (Johnson Foundation, University of Pennsylvania), and the signal was recorded on a chart recorder (~100 ms response time).

The amount of ATP released from caged ATP was determined (*a*) with the luciferin/luciferase assay (16), and (*b*) through a comparison of the Ca^{2+} uptake by SR initiated with photolysis of caged ATP and direct addition of various concentrations of ATP itself. The amount of photolytic release of ADP from caged ADP was determined by the luciferin/luciferase assay after the ADP was converted to ATP using a buffer solution at pH 8.0 containing Mg^{2+}, phosphoenol pyruvate, and pyruvic kinase. The quantum yield for ATP and ADP release from the respective caged nucleotide was found to be identical in solutions within the experimental error.

Ca^{2+} calibrations were established separately for the SR dispersions and multilayers, over the temperature range considered. In the Ca^{2+} calibration for dispersions, a 1-cm path length, 2-ml cell was used instead

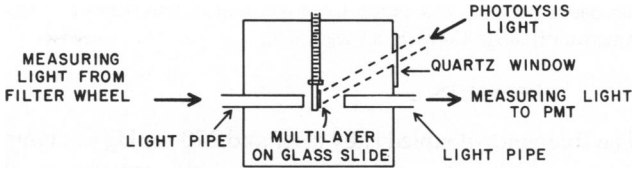

FIGURE 1 Temperature controlled chamber with mounted SR multilayer. Photolysis light from the Hg arc lamp or the frequency-doubled ruby laser was incident at an angle of ~60° to the multilayer surface and ~30° to the measuring light entering the PMT, to minimize the scattering of the photolysis light to the PMT.

of the 5-mm path length, 100-μl cell to avoid inaccuracies inherent in the addition of very small volumes to small volumes. Beer's law was then applied to simply calibrate the arsenazo III in the 100 μl cell.

The consistency of the Ca^{2+} calibration and the amount of photolytic release of ATP from caged ATP was tested in dispersions using the established mole to mole ratio of 2:1 for Ca^{2+} transported to ATP hydrolyzed (17–19). The 2:1 ratio was applicable because the concentrations of Ca^{2+} and ATP, each not greatly in excess of the concentration of the Ca^{2+}-ATPase, were limiting, and only a small Ca^{2+} concentration gradient was established, as indicated by negligible Ca^{2+} leakage after exhaustion of ATP. Use of the 2:1 ratio with calcium uptake obtained upon direct addition of known amounts of ATP confirmed the calcium calibration. Therefore, the calcium calibration and the 2:1 ratio were applied to the calcium uptake obtained upon flash photolysis of caged ATP to check the previously determined photolytic conversion of caged ATP to ATP.

Because a direct Ca^{2+} calibration was not possible in the case of the multilayer, the calibration was based on the assumed 2:1 mol/mol ratio of Ca^{2+} transported to ATP hydrolyzed, and obtained through measurements of Ca^{2+} uptake initiated with known amounts of ATP. Two methods were used. One involved complete photolysis of caged ATP and was corrected with a caged ADP control. Multiple light flashes that did not initiate further Ca^{2+} uptake ensured that the photolytic release was complete. The result was checked with a second method that involved direct addition of ATP in the same amount as the caged ATP previously used. This method was corrected for dilution caused by direct addition. Because the multilayers were made as described above, except for the concentrations of arsenazo III, Ca^{2+}, and glutathione (100 μM, 50 μM, and 3.5 mM, respectively), a correction was made to compensate for the different Ca^{2+}-dye equilibrium conditions in the kinetic experiments.

The total concentration of calcium in the multilayer experiments was not known because the total calcium concentration is the amount added to the reaction medium plus an unknown amount associated with the SR vesicles. Therefore, it is not certain whether the ATP concentration or the Ca^{2+} concentration is limiting in the calcium transport reactions. If the concentration of ATP is limiting, considering the photolytic release of ATP in multiple flashes, then the use of the assumed ratio of 2:1 for Ca^{2+} transported to ATP hydrolyzed was appropriate for the calcium calibration. However, on the other hand, if the calcium is limiting, then the calcium calibration values would mark an upper limit.

The controls, photolysis of caged ADP, photolysis in the absence of nucleotide and in the absence of SR, collectively tested the effects of nucleotide addition and energy dissipation due to the photolysis flash. Photolysis of caged ADP, which does not produce a substrate for Ca^{2+} uptake by SR vesicles, checked the combined effects of nucleotide addition, photolysis products, and energy dissipation due to the light flash. The effect of the light flash on the dye was examined separately through photolysis of the reaction medium without caged nucleotide. Photolysis of caged ADP in the absence of SR determined the effects of both the photolysis flash and release reaction on the dye. McCray et al. claim that the ATPase is not damaged by 347 nm light and that caged ATP does not bind to the ATPase (12).

Arsenazo III was obtained from Sigma Chemical Co. (St. Louis, MO).

Ionophore A23187 was purchased from Calbiochem-Behring Corp., American Hoechst Corp. (San Diego, CA).

RESULTS

The frequency-doubled ruby laser and 270 ms Hg arc lamp flashes produced somewhat comparable conversion of caged ATP to ATP or of caged ADP to ADP, i.e., ~ 2% of 2.3 mM ≅ 46 μM with the laser, ~6% of 1 mM ≅ 60 μM with the lamp, in experiments with SR membrane vesicular disperisons. The temperature increase was < 0.1°C in the disperisons or multilayers upon flash photolysis and 0.5°C in the dispersions exposed to the Hg arc lamp radiation. In both dispersions and multilayers a second similar light flash produced the same Ca^{2+} transport kinetics as did the first one.

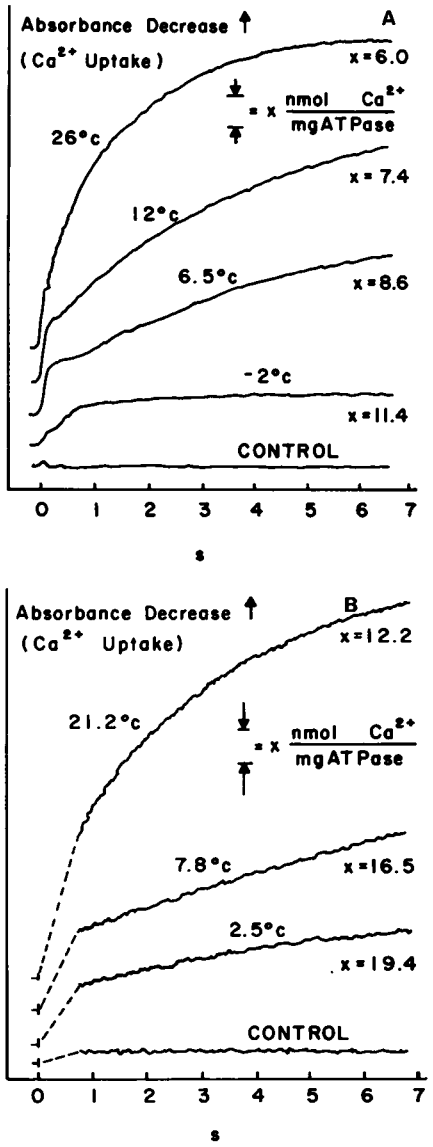

FIGURE 2 Typical experimental curves for Ca^{2+} uptake in SR vesicular dispersions at various temperatures. *A*, Ca^{2+} uptake in SR vesicular dispersions initiated by laser photolysis of caged ATP. Caged ADP, no nucleotide, or no SR served as controls. The curves were processed by the absorbance amplifier system and recorded on the chart recorder. *B*, Ca^{2+} uptake in SR vesicular dispersions initiated by lamp photolysis of caged ATP. Caged ADP, no nucleotide, or no SR served as controls. (---, flash interval.) The curves were processed by the spectrophotometer system and recorded on the chart recorder. SR vesicular dispersions consisted of 82 μl 40 mM Tris maleate, 8 mM $MgCl_2$, 120 mM KCl, 100 μM arsenazo III, 200 μM glutathione, 2 mg SR protein/ml, 50 μM $CaCl_2$ in addition to the [Ca^{2+}] provided by the SR vesicles, and 2.3 mM caged ATP for laser experiments, or 1 mM caged ATP for lamp experiments (~270 ms flash), pH 7.0.

Ca^{2+} uptake kinetics of SR membrane vesicular dispersions initiated by the photolytic release of ATP induced by use of the Hg arc lamp and by the frequency-doubled ruby laser are presented in Fig. 2 for a temperature range of −2° to 26°C. The results show the effect of the change in temperature on the kinetics, i.e., a slowing of the Ca^{2+} uptake rate with decrease in temperature. In the 7-s time interval shown, only the slow phase of Ca^{2+} uptake was evident at 26°C and only the fast phase was apparent at −2°C, but both phases were observed at the intermediate temperatures in the laser experiments. The controls using caged ADP, or no nucleotide, or no SR are also represented. With laser photolysis the controls showed no decrease in absorbance. With lamp photolysis there was a slight absorbance change in the control experiments.

A total amount of Ca^{2+} uptake of 60 ± 6 nmol Ca^{2+}/mg ATPase was observed in th e slow phase with use of the laser, 120 ± 12 nmol Ca^{2+}/mg ATPase with the lamp (after the flash). The slow phase mathematically followed

TABLE I
KINETIC PARAMETERS OF Ca^{2+} TRANSPORT IN SR VESICULAR DISPERSIONS

T	Specific rate constant	Initial rate*	Half-life	Enzyme activity
°C	s^{-1}	$\frac{nmol\ Ca^{2+}}{mg\ ATPase \cdot s}$	*s*	$\frac{2\ mol\ Ca^{2+}}{mol\ E{\sim}P \cdot s}$
Laser experiments				
25.0–26.0	0.60	42.	1.1	3.5
12.0–13.5	0.11	8.8	6.3	0.73
6.8–8.0	0.053	3.3	13.	0.28
−2.0 to −0.5	0.020	0.77	35.	0.064
Lamp experiments				
19.6–21.2	0.30	28.	2.3	2.3
7.8	0.057	9.3	12.	0.78
1.8–2.5	0.035	4.4	20.	0.37

The enzyme activity is based on the maximum of ~6 (nmols E~P)/(mg ATPase). Each temperature is represented by 8–10 measurements, with a standard deviation of ±15% in laser experiments with both detector systems and by 6–8 measurements with a standard deviation of ±20% in lamp experiments with the spectrophotometer system. Different SR preparations are involved. SR vesicular dispersions consisted of 82 μl 40 mM Tris maleate, 8 mM $MgCl_2$, 120 mM KCl, 100 μM arsenazo III, 200 μM glutathione, 2 mg SR protein/ml, 50 μM $CaCl_2$ in addition to the [Ca^{2+}] provided by the SR vesicles, and 2.3 mM caged ATP for laser experiments, or 1 mM caged ATP for lamp experiments (~ 270 ms flash), pH 7.0.

*The initial rate is expressed with respect to enzyme concentration.

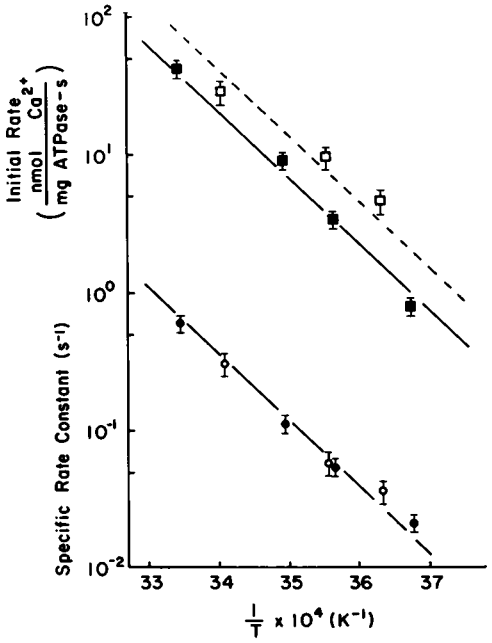

FIGURE 3 Arrhenius plot of the specific rate constants (•, ○) and the initial rates (■, □) of Ca^{2+} transport in SR vesicular dispersions. Each temperature is represented by 8–10 measurements, with a standard deviation of ±15% in laser experiments (•, ■) with both detection systems; by 6–8 measurements, with a standard deviation of ± 20% in lamp experiments (○, □) with the spectrophotometer system. Different SR preparations are involved.

first-order kinetics. Table I lists the values of the specific rate constant, initial rate (expressed with respect to the enzyme concentration), half-life, and enzyme activity. "Enzyme activity" as used here represents the initial rate in terms of the Ca^{2+}-ATPase transport function, with units of 2 mol Ca^{2+} per mol E~P per second, where E~P is the maximum phosphorylated enzyme formed, ~6 nmol/mg ATPase, as previously determined by a quenched-flow technique (9, 20).[1] Each temperature is represented by 8–10 measurements with a standard deviation of ±15% in laser experiments with both detector systems and by 6–8 measurements with a standard deviation of ±20% in lamp experiments with the spectrophotometer detector system, even using different SR preparations.

Arrhenius plots of the specific rate constants and initial rates are shown in Fig. 3. Data from both lamp and laser photolysis experiments are presented. The activation energy, E_a, determined from the slope of the specific rate constant vs. reciprocal absolute temperature plot, is 22 ± 1 kcal mol^{-1}.

Ca^{2+} uptake kinetics for SR membranes in hydrated, oriented multilayers are shown in Fig. 4 for the temperature range of −1.2° to 21°C with laser photolysis of caged ATP, and for the temperature 7.0°C with a ls Hg arc lamp flash. The slowing of the rate of Ca^{2+} uptake with decrease in temperature is evident. In the 7 s time interval shown, only the slow phase of Ca^{2+} uptake was seen at 21°C and only the fast phase was apparent at −1.2°C, but both

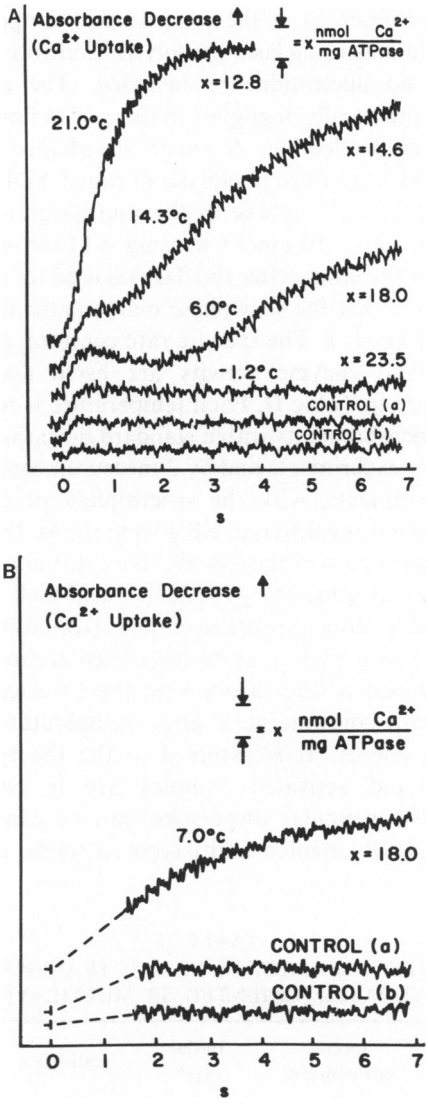

FIGURE 4 Typical experimental curves for Ca^{2+} uptake in hydrated, oriented SR multilayers at various temperatures. *A*, Ca^{2+} uptake in SR multilayers initiated by laser photolysis of caged ATP. Caged ADP (*a*) or no nucleotide (*b*) served as controls. *B*, Ca^{2+} uptake in SR multilayers initiated by lamp photolysis of caged ATP. Caged ADP (*a*) or no nucleotide (*b*) served as controls. (---, flash interval). The curves were processed by the spectrophotometer system and recorded on the chart recorder. Oriented SR membrane multilayers were made by sedimenting 1 mg SR protein onto Mylar from a 2 ml solution of 40 mM Tris maleate, 8 mM $MgCl_2$, 120 mM KCl, 50 μM arsenazo III, 1.75 mM glutathione, 25 μM $CaCl_2$ in addition to the [Ca^{2+}] provided by the SR vesicles, pH 7.0. After the supernatant was removed and the multilayers were suspended over a saturated $ZnSO_4 \cdot 6H_2O$ solution for <1 h, 130 nmol caged ATP were added in the dark to the multilayer surface and allowed to diffuse within the multilayers. The multilayers were partially dehydrated overnight using a saturated $ZnSO_4 \cdot 6H_2O$ solution.

[1]Because the ATPase is ~67% of the membrane protein, ~6 nmol E~P/mg ATPase would correspond to ~4 nmol E~P/mg protein. Others have obtained similar results for the maximum phosphorylated enzyme formed. Inesi and coworkers (21) report 3.5–4.0 nmol E~P/mg protein, and DuPont (22) claims 3 nmol E~P mg protein.

phases are observed at the intermediate temperatures in the experiments using laser photolysis. Controls with caged ADP or no nucleotide are included. The absorbance change is practically negligible in the case of flash photolysis with no nucleotide. A small absorbance change is observed with the flash photolysis of caged ADP.

The total Ca^{2+} uptake in the multilayer in the slow phase was 100 ± 10 nmol Ca^{2+}/mg ATPase when either the laser or the lamp (after the flash) is used for photolysis. It was found that the slow phase mathematically followed first-order kinetics. The specific rate constant, initial rate, half-life, and enzyme activity are listed according to temperature in Table II. Each temperature is represented by 9–11 measurements with a standard deviation of ±30% in the laser experiments and by 2 measurements in the Hg lamp experiments, with the spectrophotometer detector system, even using different SR preparations. In Fig. 5 are the Arrhenius plots of the specific rate constants and initial rates. The activation energy is 22 ± 1 kcal mol^{-1}.

Thermodynamic properties of the activated complex for the slow kinetic phase may be calculated according to the absolute reaction rate theory with the knowledge of the specific rate constant at a given temperature and the activation energy. It is assumed in the theory that the reactants and activated complex are in equilibrium. Because SR vesicular dispersions can be considered as liquids and SR oriented multilayers as solids, $\Delta H^* \cong E_a$

TABLE II
KINETIC PARAMETERS OF Ca^{2+} TRANSPORT IN HYDRATED, ORIENTED SR MULTILAYERS

T	Specific rate constant	Initial rate*	Half-life	Enzyme activity
°C	s^{-1}	$\frac{nmol\ Ca^{2+}}{mg\ ATPase \cdot s}$	s	$\frac{2\ mol\ Ca^{2+}}{mol\ E\sim P \cdot s}$
Laser experiments				
21.0	1.2	110	0.58	9.2
14.3	0.29	31	2.4	2.6
6.0–7.5	0.23	26	3.0	2.2
−1.2–0.9	0.04	5.1	17	0.42
Lamp experiments				
7.0	0.37	37	1.9	3.1

The enzyme activity is based on the maximum of ~6 (nmol E~P)/(mg ATPase). Each temperature is represented by 9–11 measurements, with a standard deviation of ±30% in laser experiments and by 2 measurements in lamp experiments. The spectrophotometer system was used. Different SR preparations are involved. Oriented SR membrane multilayers were made by sedimenting 1 mg SR protein onto Mylar from a 2 ml solution of 40 mM Tris maleate, 8 mM $MgCl_2$, 120 mM KCl, 50 μM arsenazo III, 1.75 mM glutathione, 25 μM $CaCl_2$ in addition to the [Ca^{2+}] provided by the SR vesicles, pH 7.0. After the supernatant was removed and the multilayers were suspended over a saturated $ZnSO_4 \cdot 6\ H_2O$ solution for < 1 h, 130 nmol caged ATP was added in the dark to the multilayer surface and allowed to diffuse within the multilayers. The multilayers were partially dehydrated overnight using a saturated $ZnSO_4 \cdot 6H_2O$ solution.

*The initial rate is expressed with respect to enzyme concentration.

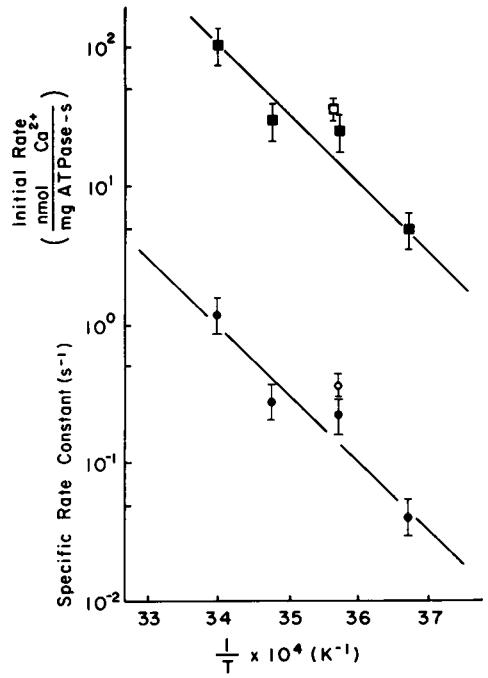

FIGURE 5 Arrhenius plot of the specific rate constants (●, O) and the initial rates (■, □) of Ca^{2+} transport in hydrated, oriented SR multilayers. Each temperature is represented by 9–11 measurements, with a standard deviation of ±30% in laser experiments (●, ■), and by 2 measurements in lamp experiments (O, □). The spectrophotometer system was used. Different SR preparations are involved.

where ΔH^* is the enthalpy or heat absorbed by the activated complex. ΔH^* for both SR dispersions and multilayers is therefore 22 ± 1 kcal mol^{-1}. The free energy of activation, ΔG^*, is found to be ~18 kcal mol^{-1} for SR dispersions and ~17 kcal mol^{-1} for SR multilayers. The entropy of the activated complex is ~16 cal deg^{-1} mol^{-1} and ~18 cal deg^{-1} mol^{-1} for SR dispersions and multilayers, respectively.

DISCUSSION

Major Points

The determination of the functional properties of the membrane protein in hydrated, oriented multilayers, in addition to vesicular dispersions, of membranes such as the isolated SR, affords the opportunity to use a greater variety of techniques in the study of related structural changes. Static and dynamic structures of the SR membrane may be obtained directly with x-ray diffraction, which requires the use of hydrated, oriented multilayers to obtain sufficient detail. Lamellar x-ray diffraction gave information concerning the membrane profile structure, i.e., the structure projected onto the normal to the membrane plane, and equatorial diffraction was used to study the in-plane membrane structure. As hydrated, oriented multilayers, the SR vesicles are flattened and stacked such that the ave rage orientation of the membrane planes is parallel to the substrate on which the multilayer is formed.

Because the SR vesicles comprise most of the multilayer volume so that the water spaces are extremely small, compared with dispersions, it is difficult to have excess substrate relative to the enzyme, and there is the possibility that the ability of the SR membrane to transport Ca^{2+} could be altered. Therefore, it was important to investigate the kinetics of the Ca^{2+} transport process in oriented SR membrane multilayers. Experiments with SR membrane vesicular dispersions were helpful for determining useful relative reactant concentration conditions for the multilayers and at the same time served as a reference with regard to the active Ca^{2+} transport properties of SR membranes for comparison with these properties in the multilayers.

The Ca^{2+} transport process is similar for SR membranes in both vesicular dispersions and oriented multilayers. At least two kinetic phases of Ca^{2+} uptake are observed in the laser photolysis experiments: (*a*) a fast phase that occurs simultaneously with E~P formation (9, 20, 23, 24) and may represent Ca^{2+} "occlusion" due to its Ca^{2+}-ionophore insensitivity (9, 20, 22, 23) and (*b*) a slow phase that is attributed to Ca^{2+} translocation across the membrane profile due to its Ca^{2+}-ionophore sensitivity (9, 20, 25, 26). There is a somewhat greater lag between the fast and slow kinetic phases of Ca^{2+} uptake observed in the multilayers, compared with the dispersions.

In analysis of the slow kinetic phase, the specific rate constant and initial rate were slightly larger for the multilayers, compared with the dispersions. This was true apart from the calcium calibrations since the specific rate constant is independent of concentration. In both forms, multilayer and dispersion, the amount of Ca^{2+} uptake during the slow phase per milligram ATPase was similar. The activation energy was identical in each case, an indication that the same reaction, Ca^{2+} translocation across the membrane profile, is occurring. Likewise, the thermodynamic parameters, enthalpy, free energy, and entropy of the activated complex were comparable.

The availability of the technique of flash photolysis of caged ATP combined with Ca^{2+} detection through the use of a metallochromic indicator has made it possible to study the kinetics of the Ca^{2+} transport process in hydrated, oriented multilayers of isolated SR membranes for the first time. Knowledge of the kinetics of the Ca^{2+} transport process in the oriented multilayers of SR membranes is essential to the study of possible associated structural changes of the Ca^{2+}-ATPase molecule through x-ray diffraction measurements. Because Ca^{2+} uptake initiated by the flash photolysis of caged ATP has been examined over the broad temperature range of below 0°C to room temperature, dynamic x-ray diffraction experiments with flash photolysis of caged ATP may be performed either with (*a*) time resolution or (*b*) low temperature, to trap an intermediate within the experimental time window. We note that this work provides the more general basis for similar structural and functional studies of chemically activated membrane proteins in ordered condensed phases utilizing the flash photolysis of appropriate caged compounds and the spectroscopic detection of their functional processes.

The time-resolved x-ray experiment, in which a structural change in the SR membrane profile, initiated by flash photolysis of caged ATP, was measured by Blasie et al. (3, 4), can now be correlated with the kinetics of the Ca^{2+} transport process. The low concentration of caged ATP and the experimental time intervals (200 ms UV flash and 200–500 ms x-ray exposure times) utilized, when compared with the kinetics of the Ca^{2+} transport process, indicate that the observed structural change in the Ca^{2+}-ATPase occurring within the first 200–500 ms following the UV flash was associated with the fast kinetic phase of Ca^{2+} uptake (which is thought to be Ca^{2+} "occlusion" due to its insensitivity to a Ca^{2+}-ionophore [9, 20, 22, 23) and the formation of E~P (9, 20, 23, 24) (which occurs simultaneously with Ca^{2+} "occlusion").

Experimental Details

The total amount of Ca^{2+} associated with the slow phase of Ca^{2+} uptake in SR dispersions initiated with laser photolysis, 60 ± 6 nmol Ca^{2+}/mg ATPase, and the photolytic conversion of caged ATP to ATP of ~2% or ~35 nmol ATP/mg ATPase, confirmed the ratio of ~2:1 mol/mol for Ca^{2+} transported across the membrane profile to hydrolyzed ATP. This value of the mole ratio (~2:1) is reasonable because only a small Ca^{2+} concentration gradient is formed under these conditions due to the small ATP to ATPase ratio (~3) as evidenced by the similarity of the Ca^{2+} uptake kinetics with subsequent laser flashes.

However, with the oriented multilayer it is not possible to measure the amount of ATP released upon photolysis. With an assumed ratio of ~2:1 for Ca^{2+} transported across the membrane profile to hydrolyzed ATP and with a maximum uptake of 100 ± 10 nmol Ca^{2+}/mg ATPase in oriented multilayers during the slow phase, the indicated photolytic conversion of caged ATP to ATP is roughly 26% or ~50 nmol ATP/mg ATPase. The ~26% photolytic release of ATP is reasonable, since three consecutive flashes generally produced similar Ca^{2+} uptake curves and a greater photolytic conversion efficiency of caged ATP to ATP is expected in the multilayer as compared with the dispersion because the absorbance conditions were better optimized (i.e., caged ATP concentration relative to the optical path length).

The main advantage to adding the nucleotide by photolysis as compared with the typical direct addition methods, is that photolysis of a caged compound provides a rapid release of the compound (caged ATP has a dark reaction rate constant of ~220 s^{-1} at pH 7.0 and 22°C [12]) if an appropriate light source, such as a laser, is used. Thus, the Ca^{2+} transport cycles of the ensemble of Ca^{2+}-ATPase molecules are synchronized. Further, the photolysis technique eliminates any dilution effects caused by simple addition. Dilution effects are especially critical

when dealing with a small volume or an extremely short path length which can occur with dispersions and always occur with multilayers. Also, simple addition of ATP to the surface of an oriented multilayer can destroy the orientation of the membrane and alter the multilayer water content, possibly creating a partial dispersion.

The Ca^{2+} uptake experiments with dispersions and multilayers are not rate limited by (*a*) the release of ATP from caged ATP, (*b*) the diffusion of the released ATP to the neighboring ATPase, or (*c*) the on and off reaction of Ca^{2+} and arsenazo III (14). The kinetics of the slow phase of Ca^{2+} uptake in SR dispersions initiated by flash photolysis of caged ATP were similar for experiments using the laser and the lamp (flash ~270 ms and analysis after ~580 ms). Fig. 3 shows the two data sets together on a plot of the specific rate constants and initial rates vs. reciprocal absolute temperature. The kinetics of the slow phase of Ca^{2+} uptake by SR multilayers at ~7°C using the laser vs. the lamp (flash 1 s and analysis after 1 s) are shown to be comparable in Table II and Fig. 5.

Reproducible results were obtained with SR multilayer preparations as well as with dispersions. In the dispersion data the observed deviation was greater with the lamp than with the laser, as a result of an unavoidable variation in focusing and in flash time. For multilayers the deviation in the data was greater with the laser than for dispersions, due to an inherent variability in the shape of the laser beam and to the geometry of the experimental setup (9). Even with a constant laser energy, the shape of the laser beam can vary considerably as it passes through different portions of the KDP crystal. Because only a section of each multilayer was monitored, any variation in beam shape was critical.

The controls associated with SR dispersion experiments involve flash photolysis of caged ADP, or photolysis in the absence of nucleotide or SR. No change in absorbance was observed upon laser photolysis, indicating that energy dissipation during photolysis and the photolysis reaction had no effect. With lamp photolysis the controls showed a slight absorbance change that could be due to some bleaching of the dye arsenazo III. In experiments with oriented SR membrane multilayers, the controls consisted of (*a*) flash photolysis of caged ADP and (*b*) photolysis in the absence of nucleotide. The absorbance change upon photolysis in the absence of the nucleotide is practically negligible, representing very little bleaching of the dye. Flash photolysis of caged ADP results in a small absorbance change, possibly due to divalent cation binding to the released nucleotide.

CONCLUSION

The ATP-initiated Ca^{2+} transport process was studied for SR membranes in vesicular dispersions and hydrated, oriented multilayers over the temperature range of $-2°$ to 26°C, with the use of flash photolysis of caged ATP and an extravesicular metallochromic Ca^{2+} indicator. At least two phases of Ca^{2+} uptake were observed: a fast phase that occurs simultaneously with E~P formation (9, 20, 23, 24) and may be Ca^{2+} "occlusion" (9, 20, 22, 23) and a slow phase attributed to Ca^{2+} translocation across the membrane profile (9, 20, 25, 26). The Ca^{2+} transport process appeared to be similar for the dispersions and multilayers except for a slightly greater lag between the fast and slow phases in the case of the multilayers. Also, Ca^{2+} translocation occurred at a somewhat faster rate in the multilayers than in the dispersions. The activation energy for the slow kinetic phase of Ca^{2+} uptake in the dispersions and the multilayers was identical, thus indicating the slow phase was the same process, Ca^{2+} translocation, in both forms of SR.

Use of a Hg arc lamp or frequency-doubled ruby laser for photolysis of the caged compound proved to be equally suitable for initiating the Ca^{2+} transport process. The laser, however, afforded the advantages of time resolution and synchronization of the Ca^{2+} transport cycles of the ensemble of the Ca^{2+}-ATPase molecules.

Knowledge of the kinetics of the Ca^{2+} transport process in vesicular dispersions and hydrated, oriented multilayers of the SR membrane, provides important information for designing and interpreting many types of dynamic structural study of the Ca^{2+}-ATPase. Dynamic structural experiments may be performed either (*a*) with time resolution or (*b*) with low temperature to trap an intermediate within the observation time window. This kinetic information concerning SR membrane multilayers has enabled one ATP-induced structural change in the SR membrane profile already established directly by time-resolved x-ray diffraction utilizing flash photolysis of caged ATP, to be related to a particular aspect of the active Ca^{2+} transport process of the SR membrane.

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