Biochimica et Biophysica Acta, 466 (1977) 57—67

BBA 77647

THE EFFECT OF CALCIUM ION TRANSPORT ATPase UPON THE PASSIVE CALCIUM ION PERMEABILITY OF PHOSPHOLIPID VESICLES

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(Received September 9th, 1976)

Summary

The uptake and release of Ca^{2+} by sarcoplasmic reticulum fragments and reconstituted ATPase vesicles was measured by a stopped-flow fluorescence method using chlortetracycline as Ca^{2+} indicator.

Incorporation of the Ca^{2+} transport ATPase into phospholipid bilayers of widely different fatty acid composition increases their passive permeability to Ca^{2+} by several orders of magnitude. Therefore in addition to participating in active Ca^{2+} transport, the (Mg^{2+} + Ca^{2+})-activated ATPase also forms hydrophilic channels across the membrane. The relative insensitivity of the permeability effect of ATPase to changes in the fatty acid composition of the membrane is in accord with the suggestion that the Ca^{2+} channels arise by protein-protein interaction between four ATPase molecules. The reversible formation of these channels may have physiological significance in the rapid Ca^{2+} release from the sarcoplasmic reticulum during activation of muscle.

Introduction

Incorporation of the Ca^{2+} transport ATPase of sarcoplasmic reticulum into phospholipid vesicles increases their passive permeability to Ca^{2+}, sucrose, choline, sodium, chloride and sulfate to levels similar to or higher than that of sarcoplasmic reticulum membranes [1—4]. These observations provided the first clear indication that in addition to its generally accepted role in active Ca^{2+} transport, the (Ca^{2+} + Mg^{2+})-activated ATPase also contributes to the regulation of the passive permeability properties of the membrane.

* The work was performed by R.L. Jilka in partial fulfillment of the requirments for the Ph.D. degree.

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The methods used in the earlier work for the measurement of the rate of Ca^{2+} release involved manual mixing, thus the time resolution was limited to several seconds. This prevented accurate assessment of the initial rate of Ca^{2+} release.

In the meantime we adapted the observations of Caswell [5,6] to the development of a rapid mixing stopped-flow method based on the use of chlortetracycline [1] as fluorescent Ca^{2+} indicator. This technique permits the analysis of the early phase of Ca^{2+} transport and release from native and artificial membranes under relatively simple conditions, on a time scale ranging from a few milliseconds to several seconds.

These observations extend our earlier findings to artificial membranes of widely different fatty acid composition and permit a more quantitative description of the effect of Ca^{2+} transport ATPase upon the permeability of phospholipid vesicles in terms of the apparent first order rate constants of the early phases of Ca^{2+} uptake and release.

Experimental procedures

Rapid kinetic measurement

Rapid kinetic measurements were carried out on an Aminco-Morrow stopped-flow apparatus linked with a Shimadzu QV-50 spectrophotometer, a rapid kinetic amplifier and a Tektronix 545B oscilloscope. A Hanovia high pressure Xenon arc lamp (150 W) served as a light source for excitation (400 nm). Fluorescence emission was measured at 90° to the incident beam through a combination of three filters which passed light above 415, 455, and 485 nm respectively and reduced the intensity of scattered exciting light below detectable levels.

For Ca^{2+} uptake measurements syringe A was usually filled with microsomes (1 mg protein/ml) or reconstituted ATPase vesicles (0.5 mg protein/ml) in 0.1 M imidazole pH 7.4 containing 10^{-4} M chlortetracycline; syringe B contained 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline with ATP, Mg^{2+}, (or Mn) Ca^{2+} and EGTA added in concentrations indicated in the legends.

The rate of Ca^{2+} release was measured after active Ca^{2+} accumulation or passive equilibration of the vesicles with $CaCl_2$ (syringe A), by rapidly diluting the vesicle suspension with a solution of 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline and 50 mM EGTA (syringe B) and following the decrease in fluorescence intensity. The effect of A23187 on the Ca^{2+} release was studied in a similar manner by adding the ionophore to the solution in syringe B. Time resolution of the oscilloscope was varied between 0.1 s/cm to 16 s/cm and the traces were recorded with a Polaroid oscilloscope camera. Most measurements were performed at 25°C. At lower or higher temperatures the reaction chamber of the rapid mixing apparatus was thermostatically controlled and the solutions were brought to the required temperature prior to mixing.

Details of the preparation of phospholipid vesicles, reconstituted ATPase vesicles, and skeletal muscle microsomes were reported previously [4,7] together with the techniques used for measurement of ATPase activity and Ca^{2+} transport.

Material

Synthetic preparations of phospholipids were obtained from the following sources: dioleyl phosphatidylcholine from Applied Science Laboratories, State College, Pa. or from Miles Labs., Kankakee, Ill; dipalmitoyl phosphatidylcholine, Sigma Chemical Company, St. Louis, Mo.; dimyristoyl phosphatidylcholine, Calbiochem., San Diego, Calif. Asolectin was obtained from Associated Concentrates and was purified before use as described earlier [4]; chlortetracycline was supplied by Sigma Chemical Co., St. Louis, Mo.

Results

The rate of Ca^{2+} uptake by fragmented sarcoplasmic reticulum

Chlortetracycline emits intense fluorescence upon binding Ca^{2+} or Mg^{2+} in a hydrophobic environment. This property was utilized by Caswell [5,6] to follow the migration of Ca^{2+} across mitochondrial and sarcoplasmic reticulum membranes. Active accumulation of Ca^{2+} in the vesicle interior from media of low Ca^{2+} concentration produces an increase in the fluorescence of chlortetracycline due to binding of accumulated Ca^{2+} to the membrane. The technique is

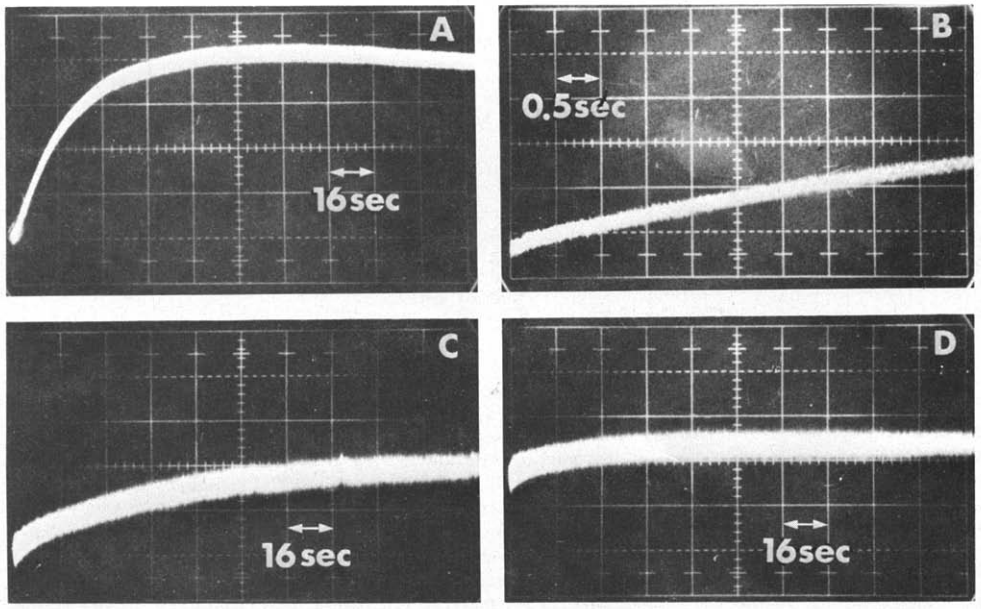


Fig. 1. Fluorescence changes during Ca^{2+} uptake by sarcoplasmic reticulum and reconstituted ATPase vesicles. A and B. Microsomes. The reaction was performed at 25°C by mixing microsomes (1 mg/ml) in 0.1 M imidazole pH 7.4 and 10^{-4} M chlortetracycline (syringe A) with a solution (syringe B) that contained 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline, 10^{-4} M $MgCl_2$, 1.8 mM $CaCl_2$, 2 mM EGTA and 1 mM ATP. Oscilloscope setting: A. 1 V/division 16 s/division; B. 0.5 V/division and 0.5 s/division. C. Reconstituted Asolectin-ATPase vesicles. Syringe A: 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline and 0.5 mg enzyme protein/ml. Syringe B: 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline, 10^{-4} M $MgCl_2$, 10^{-4} M $CaCl_2$ and 5 mM ATP. 25°C; oscilliscope 0.1 V/division, 16 s/division. D. Reconstituted dipalmitoyl phosphatidylcholine ATPase vesicles. Conditions are the same as under (C) but the reaction was performed at 37°C.

readily adaptable for rapid kinetic studies in a stopped-flow fluorometer and provides a simple technique for the measurement of Ca^{2+} uptake at close to physiological Ca^{2+} concentrations, which are too low for work with murexide [8–10].

A representiative trace of the increase in fluorescence intensity during ATP dependent Ca^{2+} uptake by white skeletal muscle microsomes is shown in Fig. 1A and B at two time resolutions. The Ca^{2+} uptake approaches steady state after about 30 s of incubation in agreement with earlier observations [10] without indication of the rapid initial ATP dependent Ca^{2+} binding suggested by Ohnishi and Ebashi [8]. ATP dependent Ca^{2+} uptake also occurs with reconstituted ATPase vesicles containing Asolectin (Fig. 1C) or dipalmitoyl phosphatidylcholine (Fig. 1D) as the principal phospholipids, but the rate and extent of this process in much less than with microsomes.

The rate of Ca^{2+} uptake and its dependence on free (Ca^{2+}) (Fig. 2) or ATP concentration (Fig. 3) are in essential agreement with data in the literature obtained either by measurement of ^{45}Ca distribution [11–13] or by the murexide technique [10].

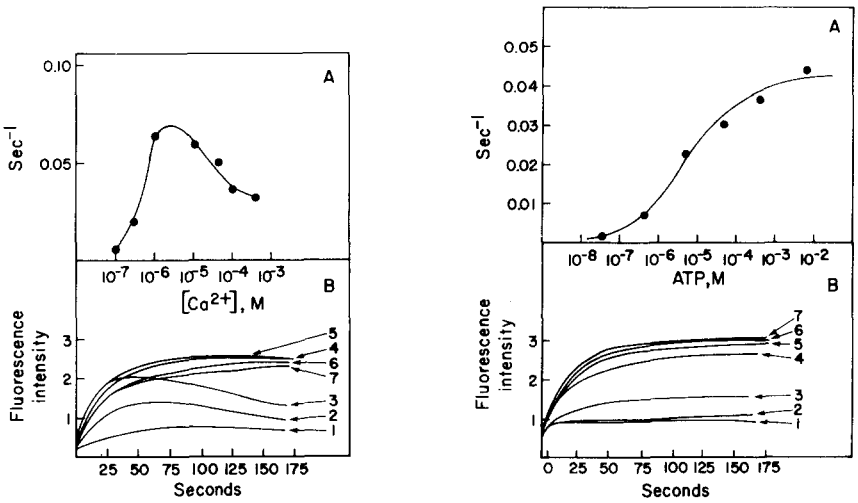


Fig. 2. Effect of [Ca^{2+}] concentration upon the rate of Ca^{2+} uptake. Syringe A: 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline and 1 mg microsomal protein/ml. Syringe B: 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline, 10^{-4} $MgCl_2$, 1 mM ATP, 2 mM EGTA with sufficient $CaCl_2$ added to produce free [Ca^{2+}] concentrations of 10^{-7} M (1) $3 \cdot 10^{-7}$M (2) 10^{-6} M (3) 10^{-5}M (4) $5 \cdot 10^{-5}$ M (5) 10^{-4} M (6) $5 \cdot 10^{-4}$ M (7). Temperature, 25°C. The rate constants of Ca^{2+} uptake (upper) were derived from the first order plots of the initial phases of the fluorescence changes indicated in the lower figure. At the higher Ca^{2+} concentrations an ATP independent rapid increase in fluorescence intensity was observed which was essentially complete within the mixing time. The traces do not include this component of the fluorescence change.

Fig. 3. Effect of ATP concentration upon the rate of Ca^{2+} uptake. Final concentrations in the reaction mixture were: 0.1 M imidazole pH 7.4, 0.1 mM chlortetracycline, 0.05 mM $CaCl_2$, 0.05 mM $MgCl_2$, 0.5 mg microsomal protein per ml and ATP in concentrations indicated on the abscissa temperature; 25°C. Lower figure contains the traces of fluorescence intensities obtained at the following ATP concentrations: 1, no ATP; 2, $5 \cdot 10^{-8}$ M; 3, $5 \cdot 10^{-7}$ M; 4, $5 \cdot 10^{-6}$ M; 5, $5 \cdot 10^{-5}$ M; 6, $5 \cdot 10^{-4}$ M; 7, $5 \cdot 10^{-3}$ M. In the upper figure the rate constants derived from the first order plots of fluorescence changes are plotted as the function of free ATP concentration. The change in fluorescence intensity caused by Ca^{2+} in the absence of ATP is not included in the traces.

The half-maximal rate of Ca^{2+} uptake is reached at about $5 \cdot 10^{-7}$ M $[Ca^{2+}]$ with a marked inhibition at $[Ca^{2+}]$ concentrations higher than 10^{-4} M. This is probably attributable to the inhibitory effect of high internal $[Ca^{2+}]$ concentration upon the Ca^{2+} transport ATPase [14].

At free $[Ca^{2+}]$ concentrations of 10^{-7}—10^{-6} M the initial rise in fluorescence intensity indicating binding of Ca^{2+} to the membrane is followed after about 30 s by a gradual decrease, which may be due to the reversal of Ca^{2+} pump as a result of ADP accumulation. A similar phenomenon was also observed using the murexide technique [15].

The concentration of ATP providing half maximal rate of fluorescence change is about $5 \cdot 10^{-6}$—10^{-5} M, i.e. only slightly higher than the values reported in the literature [12,16,17].

These observations give confidence that the method provides reasonable information about the rate of Ca^{2+} uptake under a wide range of $[Ca^{2+}]$ and ATP concentrations. Interestingly the increase in fluorescence intensity during Ca^{2+} uptake is only slightly influenced by the presence of 2.5 mM oxalate in the medium. As the fluorescence intensity depends only upon the concentration of membrane bound Ca^{2+}, the method cannot be used to determine the total amount of Ca^{2+} taken up by microsomes.

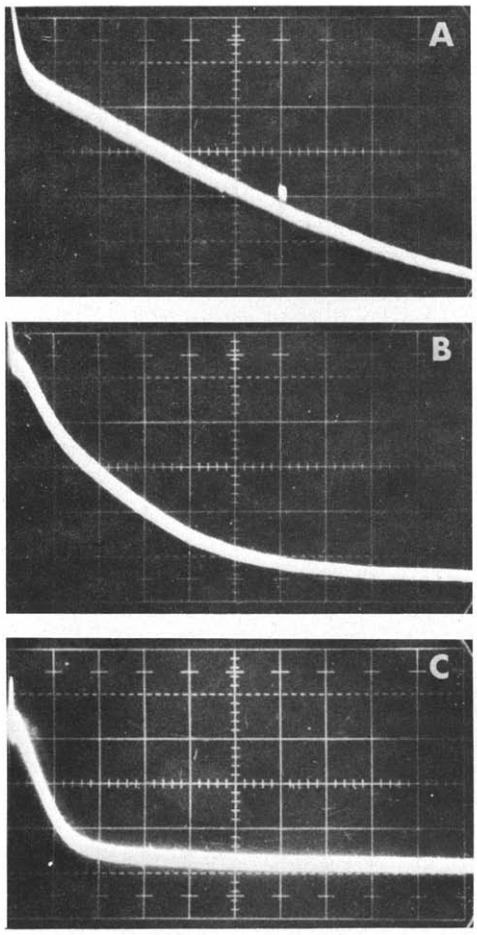
The rate of Ca^{2+} release from microsomes

The Ca^{2+} accumulated by microsomes is rapidly released upon the addition of EGTA [18] or the Ca^{2+} ionophore A23187 [19].

The correlation between the rate of Ca^{2+} release and the amount of Ca^{2+} contained by the microsomes was studied in the following manner. Microsomes were loaded with Ca^{2+} at 25°C at a series of medium Ca^{2+} concentrations either by ATP dependent active Ca^{2+} transport or by passive equilibration with Ca^{2+} in the absence of ATP. After steady state was reached, the release of Ca^{2+} was initiated in the rapid mixing apparatus by the addition of EGTA to a final concentration of 25 mM. Representative traces obtained at 4, 25 and 37°C are shown in Fig. 4. The release of Ca^{2+} occurs in three kinetically distinct phases. The initial rapid phase may represent the dissociation of Ca^{2+} bound to the outside surface of the membrane. This is followed by an intermediate and a slow phase of fluorescence change which presumably reflect the release of Ca^{2+} from the interior of sarcoplasmic reticulum vesicles. The rate of this Ca^{2+} release clearly increases with increasing temperature.

Microsomes were loaded with Ca^{2+} at medium Ca^{2+} concentrations ranging from 10^{-7} to $5 \cdot 10^{-4}$ M by ATP dependent pumping and at $5 \cdot 10^{-4}$ M to 10^{-2} M by passive equilibration. The Ca^{2+} release was initiated by lowering the medium Ca^{2+} concentration to less than 10^{-8} M with the addition of 25 mM EGTA.

The relationship between medium $[Ca^{2+}]$ concentration during loading and the rate of Ca^{2+} release at 25°C is shown in Fig. 5. The initial rate of Ca^{2+} release, determined from first order plots of the change in fluorescence intensity after the addition of EGTA, increases with increasing concentration of medium $[Ca^{2+}]$ during loading to about $5 \cdot 10^{-5}$ M, followed by a steep decline at higher Ca^{2+} concentration. As the medium Ca^{2+} concentration during release remains below 10^{-7} M throughout, this decline in rate probably reflects the effect of Ca^{2+} on the permeability of the membrane either during Ca^{2+} uptake or Ca^{2+} release.



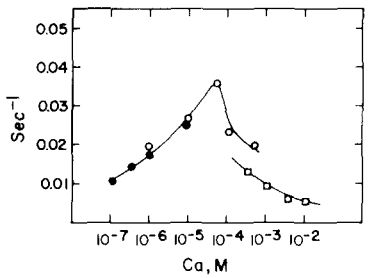


Fig. 4. Representative traces of the fluorescence changes connected with Ca^{2+} release from microsomes. Microsomes (1 mg protein/ml) equilibrated with 1 mM $CaCl_2$ in a medium of 0.1 M imidazole pH 7.4 and 10^{-4} M chlortetracycline were mixed in rapid mixing apparatus with equal volume of a solution of 0.1 M *imidazole*, 10^{-4} *M chlortetracycline and 50 mM* EGTA. *Temperature*; 4°C (A), 25°C (B), or 37°C (C). The rate of change in fluorescence intensity was followed at an oscilloscope setting of 0.5 V/division and 16 s/division.

Fig. 5. *The rate of* Ca^{2+} *release from microsomes after active or passive loading with* Ca^{2+} *at 25°C. Microsomes* (1 mg/ml) were actively loaded (○) with Ca^{2+} by incubation for 1 min in a medium of 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline, 0.05 mM $MgCl_2$, 2.5 mM ATP, and $CaCl_2$ in concentrations indicated on the abscissa. The Ca^{2+} release was initiated by the addition of an equal volume of 0.1 M imidazole pH 7.4, 10^{-4} M chlortetracycline and 50 mM EGTA. The changes in fluorescence intensity were followed at 25°C at 0.5 V/division sensitivity with 16 s/division time resolution. In other experiments (●) 1 mM EGTA was also included in the reaction mixture during Ca^{2+} uptake and the concentration of $CaCl_2$ was adjusted to give free [Ca^{2+}] concentrations indicated on the abscissa. For passive loading with Ca^{2+} (□) microsomes were incubated overnight in a medium of 0.1 M imidazole, 10^{-4} M chlortetracycline and $CaCl_2$ at concentrations indicated on the abscissa at 4°C. Release of Ca^{2+} was initiated as described above. The apparent first order rate constants were calculated from first order plots of fluorescence traces.

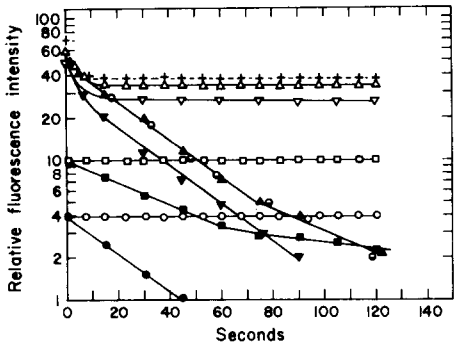


Fig. 6. The permeability of Asolectin vesicles to Ca^{2+}. Asoleictin vesicles (0.5 μmol P/min) prepared as described earlier [4] were equilibrated for 2 min in a medium of 0.1 M imidazole pH 7.4 and 10^{-4} M chlortetracycline with Ca^{2+} concentrations of 10^{-5} M (○, ●) 10^{-4} M (□, ■), $3 \cdot 10^{-4}$ M (▽, ▼) 10^{-3} M (◇, ▲) and $3 \cdot 10^{-3}$M (+, ⬬) by sonication with a Branson sonifer at 60 W in a dry ice ethanol bath at about 25—30°C. Ca^{2+} release was initiated by mixing the vesicle suspension in rapid mixing apparatus at 25°C with equal volume of 0.1 M imidazole 10^{-4} M chlortetracycline and 50 mM EGTA (○, □, ▽, △, +). In parallel exepriments the release medium also contained 10^{-6} M A23187 in addition to the components listed above (●, ■, ▼, △, ⬬).

The first order rate constant of Ca^{2+} release in the presence of 25 mM EGTA is of similar magnitude as the rate of ATP mediated active Ca^{2+} uptake. The rate of Ca^{2+} release markedly increased in the presence of 10^{-6} M A23187 or by raising the temperature to 37°C.

The Ca^{2+} permeability of phospholipid bilayers and reconstituted ATPase vesicles

Asolectin vesicles were equilibrated with 0.01—3 mM $CaCl_2$ by ultrasonic treatment and the Ca^{2+} release was measured after the addition of 25 mM EGTA in the rapid mixing apparatus (Fig. 6). After a rapid initial decrease in fluorescence intensity, presumably due to the removal of Ca^{2+} from the outer surface of vesicles, the fluorescence remained constant indicating that essentially no further Ca^{2+} release occurred during a 2-min observation period. The steady levels of fluorescence intensity are roughly proportional to the intravesicular Ca^{2+} concentration, in agreement with earlier findings [7]. On addition of 10^{-6} M A23187 together with EGTA, the rate of Ca^{2+} release increased, yielding a first order rate constant of about 0.03 s^{-1}, which is close to the rate of Ca^{2+} release from microsomes in the absence of ionophore.

The Ca^{2+} permeability of purified ATPase vesicles and reconstituted ATPase preparations containing asolectin or dioleyl phosphatidylcholine is orders of magnitude greater than that of phospholipid vesicles (Fig. 7). At 4°C (Fig. 7C) after a rapid initial decline of fluorescence intensity the Ca^{2+} release from ATPase vesicles approaches first order kinetics with apparent rate constants in the range of 0.012—0.015 s^{-1}. The rate of Ca^{2+} release from phospholipid vesicles is less than 10^{-5} s^{-1}. The rapid initial drop in fluorescence intensity presumably reflects the dissociation of Ca^{2+} from external binding sites in the presence of EGTA. At 25 and 37°C the rate of Ca^{2+} release from ATPase vesicles increases sharply without detectable change in the rate of Ca^{2+} release from phospholipid vesicles.

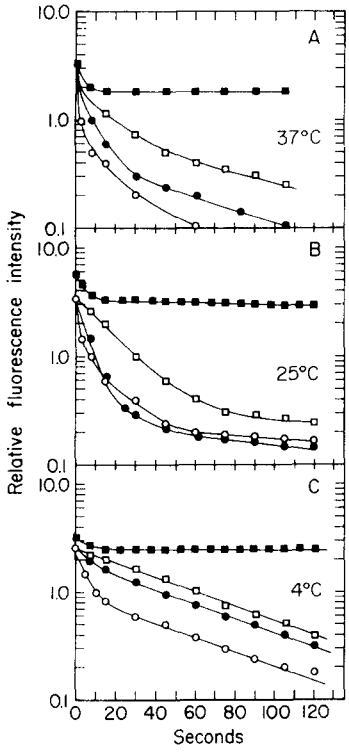


Fig. 7. The rate of Ca^{2+} release from Asolectin purified ATPase and reconstituted ATPase vesicles at different temperatures. The purified (Ca^{2+} + Mg^{2+})-activated ATPase was isolated according to MacLennan [23]. The reconstituted ATPase vesicles were prepared by mixing the purified ATPase with the phospholipids in a ratio of 1.71 μmol P/mg protein (Asolectin) or 2 μmol P/mg protein (dioleyl phosphatidylcholine) in a medium of 0.3 M KCl, 10 mM Tris pH 7.5, 0.02% NaN_3 and 1% cholate followed by dialysis against 0.3 M KCl, 10 mM Tris pH 7.5, and 0.02% NaN_3 and chromatography on Sepharose 4B. The vesicles suspensions were equialibrated with 1 mM $CaCl_2$, 0.1 M imidazole pH 7.4, and 10^{-4} M chlortetracycline and the Ca^{2+} release was initiated by rapid solution with an equal volume of 0.1 M imidazole, 10^{-4} M chlortetracycline and 50 mM EGTA at temperatures of 37°C (A), 25°C (B), and 4°C (C). The Ca^{2+} release from Asolectin vesiles and purified ATPase preparations was measured under identical conditions. Symbols: ■, Asolectin vesicles; □, reconstituted ATPase vesicles contaning Asolectin; ●, reconstituted ATPase vesicles contaning dioleyl phosphatidylcholine; ○, purified ATPase.

The rate constants calculated for the initial phase of Ca^{2+} release were 0.173, 0.138 and 0.043 s^{-1} at 25°C and 0.346, 0.231 and 0.14 at 37°C for the purified ATPase, dioleyl phosphatidylcholine-ATPase and Asolectin ATPase vesicles, respectively. The rates of Ca^{2+} release from ATPase vesicles containing diplamitoyl phosphatidylcholine or dimyristoyl phosphatidylcholine were only slightly slower (Fig. 8).

The Ca^{2+} permeability of reconstituted ATPase vesicles obtained in these experiments is close to that of sarcoplasmic reticulum membranes, which implies that the increase in the passive Ca^{2+} permeability caused by the incorporation of Ca^{2+} transport ATPase is less sensitive to changes in the fatty acid composition of membrane phospholipids than the active Ca^{2+} transport or ATPase activity [20].

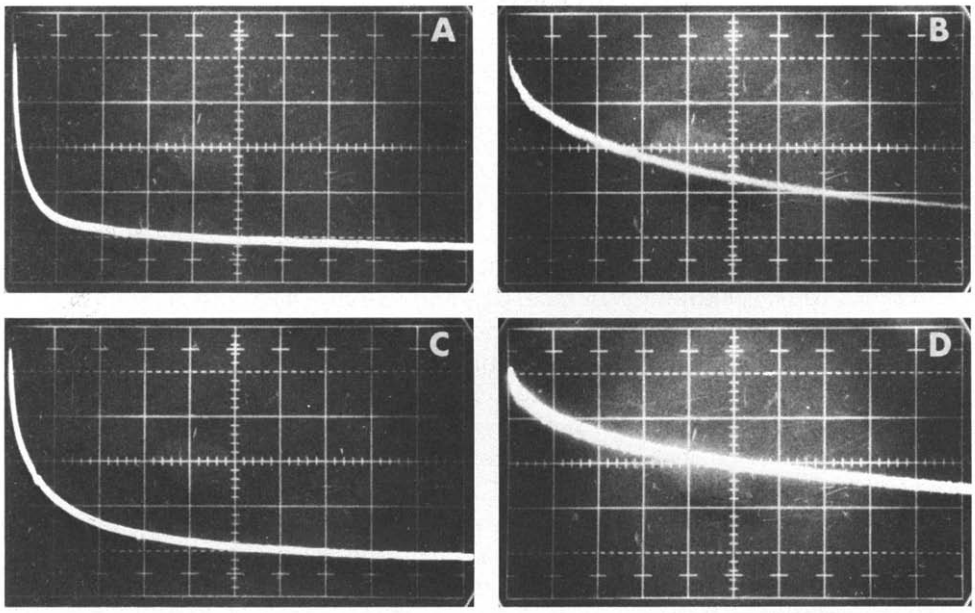


Fig. 8. The rate of Ca^{2+} release from reconstituted dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine vesicles. Reconstituted ATPase vesicles containing dipalmitoyl phosphatidylcholine (A, B) or dimyristoyl phosphatidylcholine (C, D) were prepared and the Ca^{2+} release was measured as described in Fig. 7. Temperature: 25°C; oscilloscope settings: A, 0.5 V/division, 16 s/division; B, 0.5 V/division; 1 V/division, 16 s/division; D, 1 V/division; 1 s/division.

Discussion

The observations presented in this report permit the following conclusions.

Rapid mixing stopped flow measurements of the uptake and release of Ca^{2+} from fragmented sarcoplasmic reticulum vesicles using chlortetracycline as fluorescent indicator provide reliable estimates of the rates of these processes as compared with data obtained with the murexide [9,10] or filtration [13] techniques. A significant advantage of the chlortetracycline method is its simplicity and usefulness at physiological medium Ca^{2+} concentrations (10^{-5}—10^{-7} M), where the murexide method cannot by used. A major disadvantage is that the changes in fluorescence intensity may not be proportional to the amount of Ca^{2+} accumulated by the vesicles especially in the presence of Ca^{2+}-precipitating anions, and therefore it provides only an approximate rate of Ca^{2+} uptake but not the amount of Ca^{2+} accumulated.

Phospholipid vesicles prepared from Asolectin, dioleyl phosphatidylcholine, dipalmitoyl phosphatidylcholine, or dimyristoyl phosphatidylcholine do not release measurable amounts of Ca^{2+} during the usual time range (2—2.5 min) of these measurements. In contrast the rate of Ca^{2+} release from reconstituted ATPase vesicles containing the same phospholipids was comparable to or faster than the Ca^{2+} release from microsomes, indicating a major effect of the Ca^{2+} transport ATPase upon the passive Ca^{2+} permeability of phospholipid membranes. The rate of Ca^{2+} release is moderately influenced

by the nature of the lipid phase and was generally faster with dioleyl phosphatidylcholine than with dipalmitoyl phosphatidylcholine, or dimyristoyl phosphatidylcholine, as the principal phospholipids. The relatively small difference in the rate of passive Ca^{2+} release between reconstituted ATPase vesicles containing dioleyl phosphatidylcholine and dipalmitoyl phosphatidylcholine is in sharp contrast with the pronounced dependence of Ca^{2+} transport and ATPase activity upon the fatty acid composition of membrane lipids [20].

The increase in the passive permeability of the membrane induced by incorporation of the ATPase protein is not specific for Ca^{2+} but was also observed with respect to Na^+, choline$^+$, sucrose, Cl^-, and sulfate [4]. This suggests that in addition to the mechanism involved in active Ca^{2+} transport, the incorporation of the transport ATPase into phospholipid bilayers opens up larger hydrophilic channels which are not specific for Ca^{2+}. We suggested that the appearance of these channels may be related to the reversible association of several ATPase molecules into cylindrical polymers penetrating across the membrane, surrounding a hydrophilic center region [21,22].

The basis of this proposal was the finding that the number of intramembranous 85 Å particles seen by freeze-etch electron microscopy on native and reconstituted sarcoplasmic reticulum membranes was usually much less than the 40 Å surface particles observed after negative staining [4,21]. As both types of particles are features of the Ca^{2+} transport ATPase, these observations imply that the intramembranous particles arise by interaction of several ATPase molecules. The 40 Å surface particles frequently appear in "circular" arrays surrounding a densely-stained center region [4,21,23]. By image enhancement these arrays were resolved into symmetrical structures containing for subunits which probably represent an ATPase tetramer [22]. Recently independent evidence for the existence of ATPase oligomers was also obtained by ultracentrifuge [24] and fluorescence energy transfer experiments [22].

The ATPase oligomers are presumably stabilized by protein-protein interactions and if they constitute the structural basis of Ca^{2+} channels the relative insensitivity of the rate of Ca^{2+} release to changes in the fatty acid composition of the environment is not surprising.

Acknowledgements

The generous gift of A23187 by the Lilly Laboratories, Eli Lilly and Co., Indianapolis, Ind. is gratefully acknowledged. This work was supported by research grants AM 18117-08 from the National Institute of Arthritis, Metabolism and Digestive Diseases, USPHS, PCM 7600707 from the National Science Foundation and a grant in aid from the Missouri Heart Association Inc.

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