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Quantitative Determination of the Calcium Involved in the Regulation of the Ca^{2+}-ATPase Activity in Sarcoplasmic Reticulum Vesicles

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Abstract

The dependence of the Ca^{2+}-ATPase activity of sarcoplasmic reticulum vesicles upon the intravesicular concentration of calcium accumulated after active uptake was studied. The internal calcium concentration was modified by addition of the ionophore A23187 at the steady state of accumulation. About half of the calcium accumulated could be released at low ionophore concentration without any concomitant activation of the Ca^{2+}-ATPase. This population of calcium might consist of calcium free in the lumen of the vesicles or bound to the bilayer at sites which do not interact with the ATPase activity. At higher concentrations of ionophore (above 1.75 nmol A23187/mg protein) the release of calcium activated this enzyme. This phenomenon was independent of the extravesicular calcium concentration and might be explained by assuming second species of calcium ions bound to the inner side of the membrane and in close functional interaction with the Ca^{2+}-ATPase.

Key Words: Ca^{2+}-ATPase; internal calcium regulation; ionophore A23187; sarcoplasmic reticulum vesicles.

Introduction

The decrease of the concentration of ionized calcium in the cytoplasm which induces the relaxation process in skeletal muscle depends essentially on the function of the Ca^{2+} transport system present in the sarcoplasmic reticulum (for review see Hasselbach, 1964; Tada *et al.*, 1978; Berman, 1982). *In vitro*

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preparations of vesicular sarcoplasmic reticulum (SR)[4] are able to accumulate calcium actively by the action of a Ca^{2+}–Mg^{2+}-ATPase embedded in the lipid bilayer (Hasselbach, 1974). The translocation of calcium has been determined to be coupled to the splitting of ATP with a stoichiometry of 2 Ca^{2+} pumped in per ATP hydrolyzed (Bréthes *et al.*, 1979; Yamamoto *et al.*, 1979; Berman, 1982). The amount of calcium accumulated inside the vesicles reaches a steady-state level determined by the balance between the active influx and a passive efflux. The rate of ATP hydrolysis at the steady state of accumulation is generally lower than the initial rate (i.e., the rate measured during the first seconds of the Ca^{2+} uptake). SR vesicles treated with a calcium ionophore display a higher ATPase activity than untreated membranes (Scarpa *et al.*, 1972; Gerdes and Møller, 1983). These observations have suggested that the ATP consumption is controlled by the concentration of calcium inside the vesicles.

The analysis of the mechanism of the ATPase regulation, the study of the thermodynamics of the calcium uptake process, and the determination of the parameters controlling the calcium release require a quantitative knowledge of the state of the calcium in the vesicular lumen at the steady state of accumulation (i.e., free, bound, and/or in interaction with the ATPase molecules). The aim of this work was to study the dependence of the Ca^{2+}-ATPase activity on the intravesicular concentration of calcium. For this, the amount of internal calcium was modified by addition, at the steady state of accumulation, of different amounts of the calcium ionophore A23187.

Materials and Methods

Sarcoplasmic reticulum vesicles (SR) were prepared as described previously (Arrio *et al.*, 1974) and stored at about 20 mg/ml in 5 mM K-Pipes, pH 6.8, and 100 mM KCl at − 18°C. SDS polyacrylamide gel electrophoresis and Coomassie Blue staining showed that the Ca^{2+}-ATPase (110,000 daltons) represented 80 to 90% of the total protein content (Tenu *et al.*, 1974). This composition was similar to that obtained by Malan *et al.* (1975) and indicated that the preparation was chemically similar to the "light" SR described by Meissner *et al.* (1973). It has also been shown that 8% of the vesicular material is of an inside-out orientation and inactive for calcium transport (Chevallier *et al.*, 1977).

Calcium uptake and ATPase activities were simultaneously measured using potentiometric methods in a medium containing 5 mM K-Pipes, pH 6.8,

[4] Abbreviations: SR, sarcoplasmic reticulum vesicles; Pipes, 1, 4-piperazinediethanesulfonic acid; $C_{12}E_8$, dodecyloctaoxyethylene glycol monoether; EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Ca_i, internal total calcium concentration.

100 mM KCl, 1 mM ATP, 5 mM $MgCl_2$, and between 20 and 200 μM $CaCl_2$ at 20°C. Usually reactions were started by the addition of SR (0.2 mg/ml final concentration). The accumulation of calcium was determined indirectly by recording the extravesicular free calcium concentration with a Ca^{2+} electrode (F-2112 Ca Radiometer) on a pHm 84 (Radiometer) pH meter connected to a data acquisition system (Kontron PSI80D computer equipped with a 12-bit Analog/Digital interface). Before each experiment, the Ca^{2+} electrode was calibrated and data stored in the computer for further treatment. The time response of the Ca^{2+} electrode ranged between 1 and 5 sec. This time response was fast enough for recording a slow Ca^{2+} release induced by low concentrations of ionophore used in the study.

The signal from the electrode was recorded every 200 msec and treated by a compiled BASIC program to calculate in real time the intravesicular amount of calcium per mg protein. This parameter was converted into an analog signal recorded on a chart recorder. The presence of ionophore A23187 in the medium (up to 20 μM) did not affect the response of the Ca^{2+} electrode. No adsorption of A23187 on the electrode could be detected in the range of concentrations used throughout this work.

The ATPase activity was measured through the liberation of H^+ during ATP hydrolysis by the pHstat method (GK2421C pH electrode; pHm 84 pH meter; TTT60 titrator and ABU80 autoburette Radiometer). Under the experimental conditions the hydrolysis of 1 mol of ATP corresponded to the liberation of 0.52 $\pm$ 0.02 proton equivalent as determined by parallel comparison of the rates of liberation of H^+ and P_i. It has to be noted that this ratio was constant at all times of the experiment (initial phase of calcium uptake, steady state of accumulation and after A23187 addition).

Protein concentration was determined by the method of Lowry *et al.* (1951) using BSA as a standard.

Ionophore A23187 was purchased from Boehringer; stock solution was prepared at 5 mM in ethanol/DMSO (50/50) solution. ATP was purchased from Calbiochem. All other reagents were analytical grade from Merk.

Results and Discussion

SR vesicles actively accumulated calcium against its concentration gradient with concomitant ATP hydrolysis (Fig. 1, curve a). Under the conditions described in Fig. 1, the accumulation of calcium reached a steady-state level of about 65–75 nmol Ca^{2+} per mg protein. The ATPase activity decreased progressively during the accumulation process from an initial rate of 1.45 $\pm$ 0.15 μmol ATP hydrolyzed/mg protein/min to 0.72 $\pm$ 0.12 μmol ATP hydrolyzed/mg protein/min at the steady-state level of accumulation. A

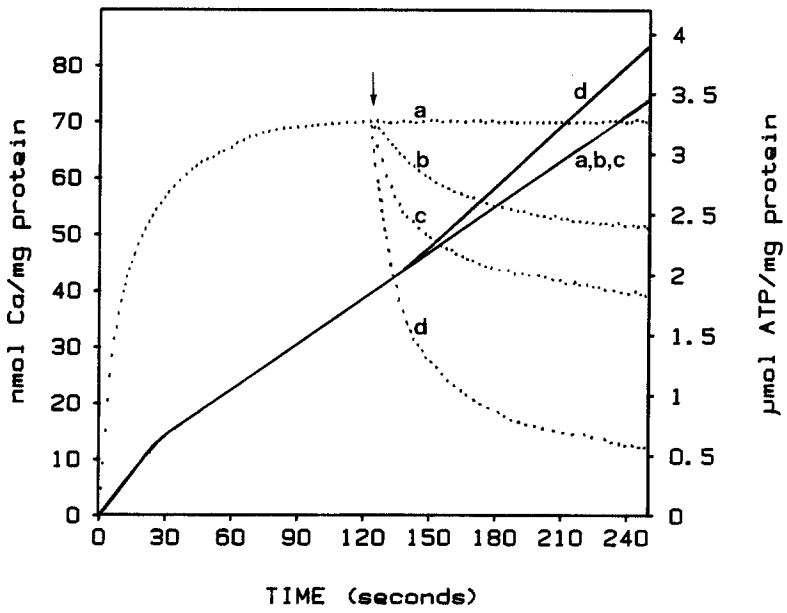

Fig. 1. Efflux of calcium and ATPase activation induced by A23187. Kinetics of calcium uptake (dotted curves) and ATP hydrolysis (solid curves) were simultaneously measured as described in Materials and Methods. Experimental conditions: 5 mM K-Pipes, pH 6.8, with 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, and 50 μM $CaCl_2$ at 20°C. The reaction was started by addition of SR (0.2 mg/ml final concentration). Ionophore addition: (a) no addition; (b) 0.5 nmol/mg protein; (c) 1 nmol/mg protein; (d) 5 nmol/mg protein.

transition from a faster to a slower rate was particularly noticeable at about 50–55 nmol Ca^{2+} per mg protein (Fig. 1) and may be explained by the saturation of calcium sites inside the vesicles (Ikemoto, 1976) and the inhibition of catalytic steps of the ATPase (Shigekawa, 1978; Tada *et al.*, 1978).

Taking into account a value of about 0.2 μmol ATP hydrolyzed per min and per mg protein for the basal ATPase activity (measured in the presence of an excess of EGTA—not shown) and a stoichiometry of 2 calcium pumped in per ATP hydrolyzed, the active influx and passive efflux rates at the steady state of accumulation can be calculated to be about 1 μmol Ca^{2+}/mg protein/min.

The addition of the ionophore at the steady state induced an efflux of calcium and, depending on the amount of A23187 added, an activation of the Ca^{2+}-ATPase (Fig. 1). For low concentrations of ionophore (below 1.75 nmol of A23187 per mg of protein, i.e., 1 mol of ionophore for 4–5 mol of ATPase), a new steady state of accumulated calcium was reached and the ATPase activity remained unchanged. For higher ionophore concentrations the rate of efflux and the amount of calcium released were greater (Fig. 1).

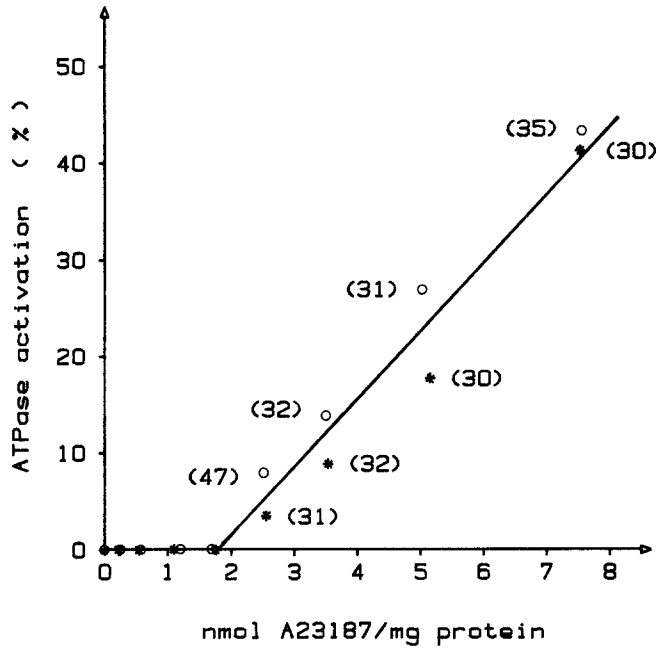

Fig. 2. Activation of the Ca^{2+}-ATPase activity as a function of A23187 concentration. The experimental conditions were the same as described in Fig. 1 except that two different concentrations of external calcium were used: (*) 50 μM and (○) 100 μM. At the steady state of accumulation, the $[Ca^{2+}]_{ex}$ was 27 and 70 μM respectively. The ionophore was added when the steady state of accumulation was reached (2 min after the beginning of the reaction). The percentage of activation was obtained by the ratio of the ATPase activity measured before and after the addition of ionophore. The average value of the ATP hydrolysis at the steady state of accumulation was 0.6 μmol ATP per min per mg protein. The numbers in the parentheses are the internal amount of calcium (in nmol Ca^{2+} per mg protein) at which the ATPase activation was apparent.

An activation of the enzyme became apparent when the amount of calcium remaining trapped inside the vesicles reached about 32–35 nmol Ca^{2+} per mg of protein (Fig. 1). This corresponded to a total internal calcium concentration (Ca_i) of 6.4 to 8.7 mM assuming a vesicular volume of 4 to 5 μl per mg of protein (Arrio *et al.*, 1977). Moreover, the increase of the ATPase activity was linearly related to the concentration of ionophore when the concentration was higher than 1.75 nmol of A23187 per mg of protein (Fig. 2). The threshold of activation was the same for all the concentrations of A23187 tested (from 1.75 to 7.5 nmol A23187 per mg of protein) and was also independent of the ionized external calcium concentration at the steady state (Fig. 2).

The activation of the ATPase was not due to an increase of the external calcium concentration after the action of the ionophore since the external

Table I. Influence of the External Concentration of Calcium on the Steady-State Level of Accumulation and on to the Rate of Calcium Release Induced by A23187[a]

$[Ca^{2+}]^b$ (μM)	$[Ca^{2+}]^{\square}_{ex}$ (μM)	Ca_i° (nmol/mg protein)	V^{+}_{efflux} (nmol/mg protein/min)
20	3	34	45
50	27	60	113
70	41	61	162
100	70	67	188
200	170	78	212

[a]The assay conditions were the same as those described in Fig. 1 except that the calcium concentration was varied between 20 and 200 μM.

[b]External concentration of calcium added at the beginning of the experiment; (□) external concentration of calcium at the steady-state level of accumulation; (○) calcium accumulated inside the vesicles at the steady state of accumulation; (+) rate of calcium release upon addition of 1.75 nmol A23187 per mg protein (0.35 μM final concentration). This concentration of ionophore did not activate the ATPase activity (Fig. 2). The ATPase activity measured at the steady state of accumulation was not significantly different for the different calcium concentrations used (from 0.75 μmol ATP hydrolyzed/mg protein/min for 20 μM external calcium to 0.6 μmol ATP hydrolyzed/mg protein/min for 200 μM external calcium).

calcium was always in large excess compared to the activation constant of the enzyme (0.5–1 μM; Yamamoto *et al.*, 1979) (Table I). Furthermore, arguments indicated that a direct effect of the ionophore on the enzyme activity was nonexistent. The activation of the ATPase did not show a saturation curve in the presence of various concentrations of ionophore (Fig. 2). Moreover, any activation of the activity of the ATPase by the ionophore was observed when the enzyme was solubilized either by Triton X-100 or $C_{12}E_8$. Those results indicate that the level of calcium inside the vesicles was the only factor responsible for the activation of the enzyme in presence of ionophore.

This calcium threshold of 32–35 nmol Ca^{2+} per mg of protein indicates the existence of two distinct population or "pools" of calcium inside the vesicles differing in their effect on the kinetics of Ca^{2+}-ATPase activity. When the amount of internal calcium was below 30–35 nmol Ca^{2+}/mg protein, the feedback inhibition of the ATPase by the accumulated cation was reversed. We assume that this amount of calcium represents a pool of calcium localized at the inner side of the membrane in close interaction with the enzyme, controlling its activity. Additional accumulated calcium might represent a different population, free in the lumen of the SR vesicles and/or bound to inner sites not interacting with the ATPase. This latter pool represented half of the calcium accumulated and was released primarily by the addition of ionophore and should be in equilibrium with the calcium controlling the ATPase activity.

The action of the ionophore on the membrane was a function of its concentration. Below 1.75 nmol per mg of protein, the ionophore acted as a

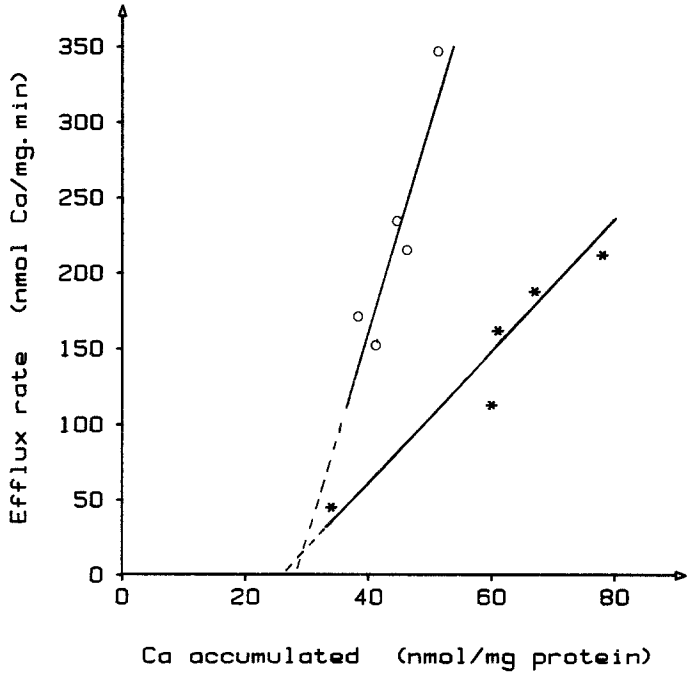

Fig. 3. Rate of calcium release induced by A23187 as a function of the amount of accumulated calcium. The different steady states of accumulation were obtained by varying the external calcium concentration. The experimental conditions are the same as those described in Table I except that two concentrations of $MgCl_2$ were used: (○) 2.5 mM; (*) 5 mM. The rate of calcium release was measured after addition of A23187 (1.75 nmol/mg protein) at the steady state of accumulation.

classical permeabilizing agent releasing the accumulated calcium. Above this value, A23187 exhibited an uncoupler effect on the activity of the ATPase and induced the suppression of the inhibition by discharging the regulator sites of the enzyme.

Different calcium content inside the vesicles could be obtained at the steady-state level of the active accumulation by varying the concentration of the external calcium (Table I). The influence of the calcium concentration inside the vesicles on the rate of the calcium efflux induced by an ionophore concentration of 1.75 nmol per mg of protein was studied (Table I). The selected concentration of ionophore was under the level which induced an activation of the enzyme (Fig. 2) and thus allowed us to analyze the efflux with an opposite active uptake constant. As expected for an ionophoric action, the rate of the efflux was linearly dependent on the level of the internal calcium (for a Ca_i ranging between 34 to 78 nmol Ca^{2+} per mg of protein) (Fig. 3).

When the magnesium concentration was lowered from 5 to 2.5 mM in the external medium, the same effect was noticed; however, for the same Ca_i the rate of efflux increased (Fig. 3). This behavior could be explained by the assumption (Pfeiffer *et al.*, 1978) that the ionophore A23187 has about the same binding affinity for both calcium and magnesium but transports calcium faster than magnesium. Therefore, the ionophore might induce an influx of magnesium toward its concentration gradient. These results, then, might easily be explained in terms of a competition between calcium and magnesium for the ionophore molecules.

Also interesting was the fact that the extrapolation of these two straight lines to zero efflux gave the value of about 30 nmol Ca^{2+} per mg protein inside the vesicles. This value might correspond to the amount of calcium bound inside the vesicles and was in excellent agreement with the value of 32–35 nmol Ca^{2+} per mg of protein determined as controlling the ATPase activity (Fig. 2).

Conclusion

By addition of various amounts of A23187, at least two different populations of calcium could be demonstrated by their differential effects on the activation of the Ca^{2+}-ATPase. Moreover, the presence of these two pools was also evident in studying the rate of release of calcium induced by a low concentration of ionophore. The data demonstrated that about half of the calcium accumulated at the steady state could be released through an ionophore-driven efflux without concomitant Ca^{2+}-ATPase activation.

If this population is considered free calcium in the vesicular lumen, an ionic gradient of calcium between the internal and external compartment between 120 : 1 to 300 : 1 was built up during the uptake and actively maintained during the steady state. This value is much higher than previously reported (for review, see Sandow, 1970). This high concentration of free calcium determined inside the vesicles after active loading is an important point for understanding the fast phenomenon of the physiological release for the cellular contraction. Under this high calcium gradient, after electrical stimulations the membranous calcium channels will then drive and increase rapidly the cytoplasmic calcium in the area of the contractile proteins.

The existence of two distinct pools of calcium was also evident from release studies using the ionophore X537A after passive loading of SR vesicles (Vale and Carvalho, 1975). The authors concluded that about half of the cation was bound to the external side of the membrane and that most of the cation inside the vesicles was free and not bound to the inner side of the bilayer. This difference from our interpretations might arise from the fact

that their data were obtained after passive loading experiments and not after calcium uptake.

The second population of calcium was able to regulate the Ca^{2+}-ATPase activity when an equivalent of 4 to 5 mol Ca^{2+} were bound per mole of enzyme. This pool might consist of slowly exchangeable calcium (Diamond *et al.*, 1980) and might be localized on the inner side of the vesicles and involved in the catalytic cycle of the ATPase (Dupont, 1980). However, the precise nature of the internal binding sites could not be determined in our study. This population of calcium might be directly bound to the ATPase, but calcium sequestering proteins such as calsequestrin or the M55 acidic binding protein (McLennan and Holland, 1975) inside the vesicles could also be involved in this pool and participate in the regulation of the ATPase.

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