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A QUENCH-FLOW KINETIC INVESTIGATION OF CALCIUM ION ACCUMULATION BY ISOLATED CARDIAC SARCOPLASMIC RETICULUM

DEPENDENCE OF INITIAL VELOCITY ON FREE CALCIUM ION CONCENTRATION AND INFLUENCE OF PREINCUBATION WITH A PROTEIN KINASE, MgATP, AND CYCLIC AMP*

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SUMMARY

Ca^{2+} accumulation at pH 6.8 by isolated rabbit heart microsomes derived chiefly from sarcoplasmic reticulum was investigated by a quench-flow technique. The reaction was terminated at preset times by addition to the reaction mixture of an equal volume of 10 to 50 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid buffered at pH 6.0. The initial velocity of Ca^{2+} accumulation by microsomal preparations exhibiting a steady state Ca^{2+} accumulation of 25.6 nmol Ca^{2+}/mg increased from 3.67 to 33.4 nmol Ca^{2+}/mg · s as the free Ca^{2+} concentration was raised from 0.2 to 18.9 μM. Preincubation of the cardiac microsomes with a partly purified soluble cardiac cyclic AMP-dependent protein kinase, MgATP, and cyclic AMP lead to a significant increase in the initial Ca^{2+} accumulation rate. The amounts of Ca^{2+} that were found to accumulate in the first 200 ms of the reaction are comparable to the quantities of the ion that according to literature data need to be removed from the myofilaments and the myoplasm for induction of relaxation of the myocardial fibers.

INTRODUCTION

It is believed that in mammalian myocardium the ability of the sarcoplasmic reticulum to accumulate Ca^{2+} provides the principal means for effecting relaxation [1, 2]. Studies of the Ca^{2+} accumulation process in isolated sarcoplasmic reticulum have in the past concentrated on the relatively slow linear oxalate-supported uptake of

Abbreviations: Cyclic AMP: adenosine 3′,5′-cyclic monophosphate; protein kinase: adenosine 3′-5′-cyclic monophosphate-dependent protein kinase; EGTA: ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

the ion, at the neglect of the fast short-lasting initial phase. In fact, this phase, which presumably is the one most pertinent to the relaxation process, has not yet been studied at low, physiologically relevant free Ca^{2+} concentrations, because the method hitherto employed, stopped-flow spectrophotometry with the chelometric dye murexide as Ca^{2+} indicator [3–7, 27], lacks the required calcium sensitivity.

In the present communication a method is presented that allows the estimation of initial rates of Ca^{2+} accumulation by isolated cardiac sarcoplasmic reticulum over the whole range of myoplasmic free Ca^{2+} concentrations encountered during the contraction-relaxation cycle. The data obtained are consistent with the concept of the predominant role of the sarcoplasmic reticulum in the relaxation of heart muscle. It is also shown that cyclic AMP assissted phosphorylation of sarcoplasmic reticulum protein is associated with an acceleration of the rapid initial phase of Ca^{2+} accumulation.

METHODS AND MATERIALS

Microsomes derived chiefly from sarcoplasmic reticulum were isolated from rabbit hearts according to the method of Harigaya and Schwartz [3, 27]. Soluble cyclic AMP-dependent cardiac protein kinase was purified from calf heart extracts by DEAE-cellulose chromatography [8]. The procedures for the preparation of the microsomes, for their phosphorylation by the partly purified protein kinase, and for the assay of Ca^{2+} accumulation by millipore filtration are described or referred to in another publication [9]. $^{45}CaCl_2$ (specific activity 10–40 Ci/g Ca) was purchased from the Radiochemical Centre Amersham. For the source of the other reagents see reference [9].

Free Ca^{2+} concentrations were calculated by equations similar to those derived by Katz et al. [10] (see also ref. 9).

Quench-flow kinetic measurements were performed in a DURRUM MODEL D-134 multimixing apparatus at a room temperature of about 22 °C. Syringe A contained microsomes (0.4–1.6 mg protein/ml) and syringe B contained 50 or 100 μM $^{45}CaCl_2$ (specific activity 8–32 Ci/mol), 10 mM Tris-ATP, and 0-0.59 mM EGTA. The solutions in both syringes were buffered with 40 mM histidine-HCl, pH 6.8 or 7.2, containing 120 mM KCl and 5 mM $MgCl_2$. The quenching solution in syringe C contained 120 mM KCl, 40 mM histidine-HCl, and in proportion to the total Ca^{2+} concentration 10 to 50 mM EGTA. The pH of this solution was 6.0 when Ca^{2+} accumulation was measured at pH 6.8 and it was 5.8 when Ca^{2+} accumulation was measured at pH 7.2. The mixing ratio of syringes A, B, and C was 1 : 1 : 2; the total volume was 0.5 ml. A sample of 0.4 ml of the mixed solutions was immediately pipetted onto a Millipore filter of type GS (0.22 μm pore size) or type HA (0.45 μm pore size) and membrane vesicles were collected there by suction filtration. The radioactivity trapped on the filter was counted by liquid scintillation spectrometry, using a Nuclear Chicago Mark II spectrometer.

The reaction times of the DURRUM apparatus were checked spectrophotometrically following the reaction of Fe^{3+} with CNS^-. The quenching time was found to be less than 5 ms.

For measurements of Ca^{2+} accumulation at time intervals greater than 10 s the manual mixing technique was used. Simultaneous measurements were routinely

carried out in the absence of ATP and the values obtained were subtracted from those for total Ca^{2+} accumulation.

The kinetic data were fitted by Spline third order functions [11], employing an ES 1010 computer, and the initial velocity of Ca^{2+} accumulation was estimated as the first derivative of the smoothed curves at time zero.

The significance of the data was judged by Student's "t" test [12].

RESULTS

EGTA has been shown not to penetrate into the vesicles of isolated skeletal muscle sarcoplasmic reticulum [13] and Ca^{2+} outflow from Ca^{2+} loaded vesicles is slow, when outside Ca^{2+} is complexed at neutral pH in the presence of MgATP [14]. In a series of preliminary experiments, in which the manual mixing technique was used, Ca^{2+} accumulation by cardiac sarcoplasmic reticulum was stopped at different times by adding an equal volume of the quenching solution to a reaction mixture consisting of 0.2–0.9 mg of microsomal protein/ml in 120 mM KCl, 5 mM Tris-ATP, 5 mM $MgCl_2$ and 40 mM histidine-HCl of pH 6.8 (standard medium). A pH value of about 6.37 results after mixing of the two solutions (combined medium). No loss of Ca^{2+} from the vesicles occurred within 30 s after mixing (Table I). A slow accumulation of Ca^{2+} was observed when unloaded vesicles were incubated in the combined medium. The amount of Ca^{2+} which is accumulated under these conditions in 30 s was not greater than 1 per cent of the total Ca^{2+} accumulation capacity at a free Ca^{2+} ion concentration of 1.0 μM (Table I). Similar results were obtained at other Ca^{2+} concentrations and when reaction mixtures buffered at pH 7.2 were stopped with a quenching solution buffered at pH 5.8.

The total time that was required in the flow experiments immediately after quenching for the transfer of the quenched reaction mixture to the filtration apparatus

TABLE I

STOPPAGE OF Ca^{2+} ACCUMULATION BY EGTA

Means ±S.D. from 4 experiments. The manual mixing technique was used in these experiments. The total concentrations of Ca^{2+} and EGTA in the standard medium were 50 μM and 64.8 μM, respectively. The EGTA concentration in the quenching solution was 10 mM. For further explanations see text.

Condition	Ca^{2+} accumulation at $[Ca^{2+}]_{free} = 1$ μM (nmol/mg protein)	
	30 s	5 min
Ca^{2+} accumulation in standard medium. The reaction was stopped by millipore filtration	11.5±1.14	19.4±0.99
Ca^{2+} accumulation in standard medium. The reaction was terminated by addition of an equal volume of stop solution and vesicles were collected by filtration within 30 s	12.5±1.34	20.3±1.31
Ca^{2+} accumulation in combined medium. The reaction was stopped by millipore filtration	0.22±0.17	1.61±0.73

and for filtration did not exceed 10 s. Changes in Ca^{2+} distribution between sarcoplasmic reticulum vesicles and medium following addition of EGTA are therefore expected to be even smaller than those reported in Table I. Complex formation between EGTA and Ca^{2+} is a fast process, which is complete in less than 30 ms [15]. Since Ca^{2+} accumulation by cardiac microsomes can be effectively terminated by EGTA without significant changes in Ca^{2+} distribution during the following 10 s, it is now possible with the present technique to measure Ca^{2+} accumulation at physiologically relevant Ca^{2+} concentrations in time intervals of the order of 100 ms.

The time dependence of the amount of Ca^{2+} taken up by cardiac microsomal vesicles at different free Ca^{2+} concentrations as measured with the quench-flow method is shown in Fig. 1. In this experiment Ca^{2+} concentrations varied over two orders of magnitude within the range thought to exist in the myoplasm during the cardiac contraction cycle [17]. The experiment was repeated with six different preparations. The relationship between the rate curves at the various free Ca^{2+} concentrations indicated was the same for different preparations, although there was considerable variation in the absolute amounts of Ca^{2+} accumulated. The accumulation process was non-linear with respect to time even in the first 200 ms [7]. Initial velocities were therefore calculated from curves fitted to the experimental points by Spline functions (see "Methods and Materials"). At free Ca^{2+} concentrations of 0.2, 0.5, 2.0, 4.22, 9.45, and 18.9 μM initial rate values of 3.67, 5.27, 9.01, 9.26, 18.7, and 33.4 nmol Ca^{2+}/mg protein per s were calculated from the data in Fig. 1. Ca^{2+} accu-

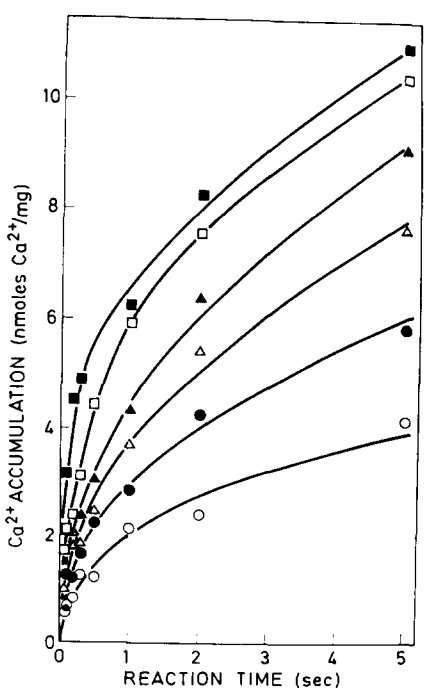

Fig. 1. Time course of Ca^{2+} accumulation by isolated cardiac sarcoplasmic reticulum at 22 °C and pH 6.8. Free Ca^{2+} concentrations were 0.2 μM (-○-), 0.5 μM (-●-), 2.0 μM (-△-), 4.22 μM (-▲-), 9.45 μM (-□-), 18.9 μM (-■-). For experimental details see Methods and Materials.

mulation at steady state, i.e. the amounts of Ca^{2+} taken up by the vesicles at the above free Ca^{2+} concentrations in 5 min [16], amounted to 10.7, 17.4, 20.0, 23.3, and 28.2 nmoles Ca^{2+}/mg protein, respectively.

Initial Ca^{2+} accumulation rate and steady state Ca^{2+} accumulation are not proportional to each other at all free Ca^{2+} ion concentrations investigated. This becomes apparent, when both functions were examined in the double reciprocal plot. As reported by other authors [16], the steady state value, denoted here also as Ca^{2+} accumulation capacity, obeys a linear dependence in the Ca^{2+} concentration range 0.2–18.9 μM. The maximal amount of Ca^{2+} accumulated by the vesicles is estimated to be 25.6 nmol Ca^{2+}/mg. The $K_{0.5}$ value for Ca^{2+} accumulative capacity, which equals the ambient Ca^{2+} concentration in the medium at which after achievement of a dynamic equilibrium between Ca^{2+} accumulation and Ca^{2+} release by the vesicles the latter were filled to half their capacity, amounts to about 0.28 μM.

A curvilinear plot is obtained for the initial Ca^{2+} accumulation velocity. The non-linearity in the double-reciprocal plot indicates a kinetic heterogeneity of the system and prohibits the calculation of a simple Michaelis-Menten constant under these conditions. Deviation from linearity becomes apparent only when a wide range of Ca^{2+} concentrations is considered. It was not detected when Ca^{2+} concentrations

TABLE II

ENHANCEMENT OF Ca^{2+} ACCUMULATION BY CARDIAC MICROSOMES FOLLOWING INCUBATION OF THE MICROSOMAL MEMBRANES WITH A PARTLY PURIFIED CYCLIC AMP-DEPENDENT PROTEIN KINASE AND CYCLIC AMP

Rabbit heart microsomes, 0.4–0.8 mg protein per ml solution containing 120 mM KCl, 5 mM $MgCl_2$, 5 mM Tris-ATP, and 40 mM histidine-HCl buffer of pH 6.8, were incubated for 3 min at 25 °C in the presence (phosphorylated membranes) and absence (control membranes) of 5 μM cyclic AMP and 0.2 mg/ml of partly purified soluble calf heart protein kinase having a specific activity of 12.0 nmol of inorganic phosphate/mg · min with histone as substrate [9]. The microsomal membranes were subsequently washed with 50 mM KCl/20 mM Tris-HCl solution of pH 6.8 [9, 18]. The amount of Ca^{2+} accumulated by control and phosphorylated membranes in 300 ms was measured by the quench-flow technique at pH 7.2 and at total concentrations of Ca^{2+} and EGTA of 50 μM and 127 μM, respectively. The quantities of Ca^{2+} accumulated at steady state were 8.0±2.3 and 7.93±2.0 nmol/mg, respectively, for control and phosphorylated membranes. Ca^{2+} uptake in the presence of 2.5 μM Tris-oxalate amounted to 2.23±1.24 and 9.06±3.75 nmoles/mg · min for control and phosphorylated membranes, respectively.

Preparation No	Amount of Ca^{2+} accumulated in 300 ms (nmol/mg protein)	
	Control membranes	Phosphorylated membranes
1	0.36	0.72
2	0.32	0.70
3	0.77	1.19
4	0.93	1.17
5	0.79	0.88
6	0.90	1.01
7	0.82	1.26
8	0.75	1.24
Mean ±S.D.	0.70±0.23	1.02±0.23

were varied from 0.2 to 4.22 μM. Unlike Ca^{2+} accumulation capacity, the initial velocity displays no saturation with regard to free Ca^{2+} within the range of the Ca^{2+} concentrations investigated. A similar striking difference in the dependence of Ca^{2+} accumulation rate and steady state on p*Ca* has been noted by Weber et. al. [13] for isolated skeletal muscle sarcoplasmic reticulum, although their manual mixing technique did not allow them to measure accumulation rates earlier than 4 s after the start of the reaction.

In previous investigations from this and other laboratories [9, 18, 19] oxalate-promoted Ca^{2+} uptake was found to be accelerated after phosphorylation of a microsomal protein by a cyclic AMP-dependent protein kinase. Using the quench-flow method a small but reproducible increase in Ca^{2+} accumulation could be demonstrated following preincubation of the microsomal membranes in the presence of a partly purified protein kinase, MgATP, and cyclic AMP (Table II). This preincubation, which had been shown to result in the incorporation of about 2 nmol of inorganic phosphate per mg of membrane protein [9], leads to significant stimulation ($P < 0.05$) of the initial Ca^{2+} accumulation. As has been shown before [9, 18, 19], steady state Ca^{2+} accumulation by isolated cardiac sarcoplasmic reticulum is not changed by such preincubation of the membranes.

DISCUSSION

The quench-flow measurements described in the present communication made it possible to obtain kinetic data for the initial phase of Ca^{2+} accumulation by isolated cardiac sarcoplasmic reticulum at low, physiologically relevant free Ca^{2+} concentrations. The amounts of Ca^{2+} accumulated at different Ca^{2+} concentrations can now be compared with the quantities of Ca^{2+} which have to be removed from the myoplasm and the myofibrils to bring about relaxation of the contracted muscle. The amounts of Ca^{2+} required to achieve different levels of tension development in heart muscle have been estimated by determining the dependence of myofibrillar Ca^{2+} binding and isometric tension development upon the myoplasmic free Ca^{2+} concentration [17]. These amounts have to be removed for induction of relaxation in less than 200 ms in the myocardium of those mammalian species in which the cardiac relaxation period does not exceed this value [20, 21]. The efficiency of the heart sarcotubular system at 37 °C, on the other hand, can be calculated from (a) the quantities of Ca^{2+} accumulated by isolated sarcoplasmic reticulum at 22 °C within 200 ms (Fig. 1), (b) the sarcoplasmic reticulum content of heart muscle, which has been estimated to be 6.8 mg per g wet heart weight [2] and (c) the activation energy for rapid Ca^{2+} accumulation by sarcoplasmic reticulum, which has been estimated to be 6.4 kcal/mol [5]. As can be seen from the data in Table III, our isolated sarcoplasmic reticulum preparation is capable of accumulating Ca^{2+} rapidly enough to be assigned a predominant role in relaxation, particularly when tension development does not exceed the half maximal value. Although several assumptions are inherent in the comparison, it is conceivable that at maximal levels of cardiac tension activation, which are reached only in extraordinary situations, the properties of the sarcoplasmic reticulum are changed so as to allow faster accumulation. This occurs, for example, when myocardial cyclic AMP levels are raised by β-adrenergic catecholamines [18, 22] (see also Table II). It is also conceivable that other mechanisms for lowering the

TABLE III

COMPARISON OF THE AMOUNTS OF Ca^{2+} INVOLVED IN HEART MUSCLE TENSION DEVELOPMENT AND THE QUANTITIES OF Ca^{2+} ACCUMULATED BY CARDIAC SARCOPLASMIC RETICULUM IN 200 ms

The values of relative tension activation at various free Ca^{2+} concentrations and for the total amount of Ca^{2+} involved in this process are taken from the tables published by Solaro et al. [17] or are recalculated from their figures. The quantities of Ca^{2+} accumulated by the sarcotubular system per g wet heart weight at 37 °C were calculated by multiplying the amounts of Ca^{2+} taken up by isolated sarcoplasmic reticulum at 200 ms and at 22 °C (Fig. 1) with a factor of 11.4. This factor takes into account the content of 6.8 mg sarcoplasmic reticulum protein per g wet heart weight [2] and the activation energy of 6.4 kcal/mol for the accumulation process [5].

Concentration of free Ca^{2+} (μM)	Relative tension activation	Total Ca^{2+} to be removed from myoplasm and myofibrils for relaxation (nmol/g tissue wet wt.)	Ca^{2+} accumulated by sarcoplasmic reticulum in 200 ms (nmol/g tissue wet wt.)
0.2	0.03	9.2	10.2
0.5	0.05	11.8	14.2
2.0	0.5	22.4	20.6
4.22	0.88	31.7	25.2
9.45	0.97	50.6	31.4
18.9	$\approx$ 1.0	75.9	48.0

intracellular Ca^{2+} concentration, such as Ca^{2+} outward transport across the cell surface membrane, may be of greater importance under these conditions.

Comparisons between the quantities of Ca^{2+} to be removed from troponin and the myoplasm for induction of relaxation and the Ca^{2+} sequestered by isolated cardiac sarcoplasmic reticulum have hitherto been limited to conditions of high Ca^{2+} concentrations [4, 6]. In the present experiments the initial rate of Ca^{2+} accumulation by rabbit heart microsomes was estimated to be 33.4 nmol/mg · s at a free Ca^{2+} concentration of 18.9 μM, the highest Ca^{2+} concentration investigated. On the assumption that Ca^{2+} accumulation rates are proportional to total Ca^{2+} binding capacity [23] this value becomes comparable to an initial velocity of 130 nmol Ca^{2+}/mg · s, measured by McCollum et al. [4] with the murexide method at 30 μM Ca^{2+} in dog heart microsomes possessing a maximal binding capacity of 60 nmol Ca^{2+}/mg. For a Ca^{2+} concentration of 30 μM Nayler et al. [7] calculated a net Ca^{2+} accumulation rate of 8.3 nmol Ca^{2+}/mg · s from the amount of Ca^{2+} accumulated by rabbit heart microsomes in the first 250 ms of the reaction. Much lower Ca^{2+} accumulation rates were reported by Scarpa and Williamson [6] in a study in which the microsomes after incubation with ATP were mixed with a very small volume of $^{45}Ca^{2+}$-containing solution.

Initial Ca^{2+} accumulation is characterized by a rate much faster than that of the secondary phase of Ca^{2+} uptake, which proceeds linearly with respect to time [24]. This secondary phase has up to now been observed in isolated cardiac sarcoplasmic reticulum only in the presence of a Ca^{2+}-precipitating anion such as oxalate. Mermier and Hasselbach [25] measured it recently in skeletal muscle microsomes in

the absence of oxalate or phosphate. At free Ca^{2+} concentrations of 0.2, 0.5, and 2.0 μM, initial Ca^{2+} accumulation velocities of 3.67, 5.27, and 9.01 nmol/mg · s, respectively, were estimated with the quench-flow method for rabbit heart microsomes (Fig. 1). Linear Ca^{2+} uptake rates measured at these Ca^{2+} concentrations under similar conditions but in the presence of 2.5 mM oxalate amounted to only 0.055, 0.11, and 0.53 nmol/mg · s (Table 2 in ref. 9). Large differences between the initial rate of Ca^{2+} accumulation and the rate of the linear oxalate-promoted Ca^{2+} uptake have likewise been observed by Schwartz and coworkers [4, 24] in dog heart microsomes. In addition to the difference in the rate of the two processes, other dissimilarities have been found with respect to the optimal Mg^{2+} and H^+ concentrations, to nucleotide specificity, and to the effects of Ca^{2+} transport inhibitors [24, 25]. As the exact relationship between the two Ca^{2+} accumulation processes are still unknown, it is at present not permissible to draw conclusions from measurements of the slow linear Ca^{2+} uptake in the presence of oxalate about the fast process of initial Ca^{2+} accumulation, which in all likelihood is more representative of the physiological process of heart muscle relaxation than is the linear oxalate-supported uptake. In any event, the reported values for the rate of the linear oxalate-supported Ca^{2+} uptake by cardiac microsomal vesicles [2, 16, 24] are so low that they can account only for a small fraction of the calcium ions that have to be sequestered in heart muscle by the sarcoplasmic reticulum in the time available for induction of relaxation.

In view of the above considerations the acceleration of initial Ca^{2+} accumulation after preincubation of cardiac microsomes with protein kinase, MgATP, and cyclic AMP is of particular interest. In spite of the fact that, on the average, it amounted to only 46 per cent, it exceeds, reckoned in absolute terms of nmoles of Ca^{2+} accumulated per mg protein · s, the 4-fold enhancement of the rate of oxalate-promoted Ca^{2+} uptake by protein kinase and cyclic AMP which was measured in our laboratory in a time interval of minutes (Table II and ref. 9) as well as the 2 to 3-fold acceleration of this uptake that was observed by Tada et al. [18] at comparable free Ca^{2+} concentrations in dog heart microsomes. It should be pointed out that present conditions for the measurement of Ca^{2+} accumulation were those optimal for the stimulation of oxalate-promoted linear Ca^{2+} uptake due to microsomal protein phosphorylation by a cyclic AMP-dependent protein kinase [9]. A search for optimal conditions for the stimulation of initial accumulation rates was not undertaken. However, even the relatively small percentage increase in initial Ca^{2+} accumulation that was observed following incubation with protein kinase, MgATP, and cyclic AMP may at free myoplasmic Ca^{2+} concentrations between 0.5 and 2 μM have marked effects on tension decay (see ref. 17 and Table III).

The finding that the initial velocity of Ca^{2+} accumulation increased after preincubation of the microsomal membranes with protein kinase, MgATP, and cyclic AMP, whereas the accumulation capacity remained unchanged, raises the question, whether the velocity of Ca^{2+} release may increase, too, following such preincubation of the membranes. This point may be worth investigation, especially since Ca^{2+} fluxes in both directions are known to change with increasing filling of microsomal vesicles with Ca^{2+} [26].

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