Invariance of Stoichiometry of the Sarcoplasmic Reticulum Calcium Pump at Physiological Calcium Concentrations – a Reevaluation

Wilhelm Hasselbach and Andrea Migala

Max-Planck-Institut für Medizinische Forschung, Abteilung Physiologie, Jahnstraße 29, D-6900 Heidelberg, Bundesrepublik Deutschland

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The decline of the transport ratio of the sarcoplasmic calcium pump observed in a recent study (A. Gafni and P. D. Boyer, Proc. Natl. Acad. Sci. USA **82,** 89–101 [1985]) results from the retardation of calcium oxalate precipitation at low calcium/protein ratios. The prevailing high internal calcium level supports a rapid calcium backflux and a compensatory ATP hydrolysis during net calcium uptake which reduces the transport ratio. Yet, the determined calcium backflux does not fully account for the decline of the transport ratio. A supposed modulation of the stoichiometry of the pump by external calcium (0.1 μM) is at variance with results of previous studies showing a constant transport ratio of two in the same calcium concentration range.

Introduction

The analysis of the kinetic and energetic properties of the sarcoplasmic reticulum calcium transport system has been considerably facilitated by the exceptional properties of its isolated membrane fragments to form tightly sealed vesicles. These preparations allow to measure simultaneously active calcium transport and the accompanying reaction of the transport enzyme with ATP or other energy donors [1, 2]. It is well established that under appropriate conditions at saturating calcium concentrations, pH 7, and 20 °C the uptake of two calcium ions is connected with the splitting of one molecule of ATP. This ratio was first determined in oxalate containing solutions in which calcium transport leads to the formation of calcium precipitates in the lumen of the vesicles [3–5]. Yet the presence of oxalate is not an essential prerequisite for the functioning of the pump with a transport ratio of two. When calcium uptake and ATP splitting are measured during very short time intervals in the absence of oxalate, the system operates with the same transport ratio [6]. A transport ratio of two also determines the stoichiometry of ATP synthesis by the pump when it is forced to run backwards [7].

The magnitude as well as the invariance of the transport ratio has great implications for the mechanism and the energetics of the calcium transport system as discussed by Tanford [8]. The question can be raised whether we are dealing with a single reaction cycle or if sub-cycles exist in which the pump operates at subsaturating calcium concentrations with a reduced stoichiometry. A reduced stoichiometry could allow the pump to create and to maintain higher ratios of internal calcium/external calcium than if it would operate with a constant coupling ratio of two. The calcium concentration ratios as they exist in the living muscle and as they are produced by isolated sarcoplasmic reticulum vesicles in vitro could be established by the pump working with a transport ratio of two [9]. The energy of ATP would be completely used for the creation of these ratios resulting in an efficiency of the pump of app. one. This would mean that the system operates near its thermodynamic equilibrium.

Materials and Methods

Conventionally prepared light sarcoplasmic reticulum vesicles [4] were incubated in media containing 2 mM ATP, 5 mM $MgCl_2$, 5 mM oxalate, 20 mM histidine, pH 7.0, 40 mM KCl and 0.1 mM $^{45}CaCl_2$ at 20 °C. The reaction was started by Ca^{2+} addition. Aliquots were taken to terminate the reaction by filtration (Sartorius nitrocellulose filters 0.45 μm) for monitoring the time course of total Ca^{2+} uptake. To monitor the occurrence of Ca^{2+} oxalate crystals during Ca^{2+} accumulation, the membranes were dissolved with Triton X-100, final concentration 0.1%, to release the unprecipitated Ca^{2+}. The remaining crys-

Abbreviations: EGTA, ethyleneglycol bis(2-aminoethyl)-N,N;N′,N′-tetraacetic acid.

Reprint requests to Prof. Dr. W. Hasselbach.

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tals were removed by filtration (Sartorius Millipore nitrocellulose filters 0.22 μm and 0.1 μm) from the solution. This procedure relies on the fact that the uptake media is highly supersaturated in Ca^{2+} oxalate and hence crystals once formed do not redissolve [4]. Calcium fluxes during net calcium uptake were measured after the vesicles were preloaded with small amounts of radio-active calcium under the conditions described above. The vesicles contained 31±4 nmol calcium/mg, ±SE determined by atomic absorption spectroscopy. Calcium uptake was started by the addition of 100 nmol/ml cold calcium, and the change in time of radioactivity in the external medium was measured. In some experiments oxalate was omitted and added shortly before the addition of cold calcium. The unidirectional fluxes were calculated from the initial change in radioactivity (15 s) and the separately measured net calcium uptake. Radioactivity was measured by liquid scintillation counting.

Results and Discussion

The problem of pump stoichiometry has mostly been addressed in studies confined to the initial period of calcium uptake. In contrast to the results of the reported findings, supporting a constant transport ratio of two, in a number of more recent studies transport ratios smaller than two have been observed under various conditions [9, 10]. Obviously, smaller ratios can result from membrane fractions containing calcium releasing channels [12, 13], from fractions containing leaky vesicles or from the presence of non-vesicular membrane fragments. It is well established that the sarcoplasmic reticulum membranes become leaky at alkaline pH and elevated temperature. True uncoupling of the pump has been inferred from the observation that under such conditions the passive calcium flux is only modestly increased and thus can hardly account for the decline of the transport ratio. Yet this conclusion is not stringent because when, after calcium loading, the passive permeability is measured, the permeability of solely those vesicles is monitored which were able to retain calcium while those vesicles which had become highly permeable, do not contribute to calcium efflux. It is the enzymatic activity of these highly permeable vesicles which reduces the transport ratio during the initial phase of calcium uptake. Since we must assume that in the usual preparations of sarcoplasmic reticulum vesicles some unsealed membrane fragments are present, the observed calcium/ATP ratio of 2 must, therefore, be considered as an upper limit.

As mentioned above, for considerations concerning the performance of the pump it is important to know whether the initial transport ratio is maintained or if it declines when the calcium level in the medium falls and the calcium binding sites of the pump become desaturated. This problem has been approached by simultaneously measuring the rate of calcium uptake and the calcium-dependent ATP splitting in solutions containing 5 mM ATP, 5 mM magnesium, 5 mM oxalate, 0.125 mM calcium and no or 0.2 mM EGTA [4, 5, 14]. From the analytically determined total calcium concentrations in the medium, the free calcium concentration was calculated by using the stability constant of calcium EGTA given by Schwarzenbach [15]. At the applied concentrations of 0.2 mM EGTA, the complexing agent does not interfere with the activity or the stability of the sarcoplasmic reticulum preparation. It was observed that for ATP and all other tested nucleoside triphosphates the calcium transport ratio declined from its upper value of two to values below one when the level of ionized calcium fell from 7×10^{-8} M to 2×10^{-8} M. The question as to whether we were dealing with a true change in stoichiometry could not unambiguously be answered because it proved difficult to establish the small activity of the calcium-dependent ATP hydrolysis at low calcium levels sufficiently accurately as the small difference between total enzymatic activity measured in the presence of calcium and the basal activity measured in the presence of EGTA. These experiments were resumed by Weber *et al.* [16] who showed that, if at low concentrations of ATP the basal ATPase is largely turned off, transport ratios of two down to calcium concentrations of even 3×10^{-8} M could be observed. Calcium concentrations in the range of $3-7 \times 10^{-8}$ M correspond to the calcium level observed in resting muscles [17]. The transport ratio should finally decline from two to zero if at further falling calcium concentrations in the medium the attainment of a chemical equilibrium becomes significantly affected by passive leakage.

Most recently, the coupling between calcium transport and calcium-dependent ATPase was reinvestigated by Gafni and Boyer [18]. The authors measured the burst of ATP splitting which is started by the addition of calcium to the medium and which terminates when the calcium concentration in the

medium had declined to non-activating levels as shown by Hasselbach and Makinose [3]. The results were evaluated by relating the size of the ATP burst to the amount of calcium which had been added to the assay. This method yields neither the calcium transport ratio at saturating nor at unsaturating calcium levels but a complex mixture of both values. The authors performed their studies at different calcium/protein ratios by changing the protein concentration in the assays and found

1) that the specific activities of calcium transport and calcium-dependent ATP hydrolysis substantially decline when the protein concentration is increased and

2) that the calcium transport ratio declines from a value of two observed at low protein concentrations to a value of one at high protein concentrations.

The authors try to explain the decline of both activities by proposing that at high protein concentrations the release of calcium from calcium oxalate would become rate limiting. The drop in the specific rate of ATP hydrolysis from 2 to 0.4 μmol/mg·min is related to a concomitant drop of the free calcium concentration from 4 μM to 0.1–0.15 μM. The authors do not specify whether the calcium oxalate (crystals?) under consideration is located in the solution outside the vesicles or in the vesicular lumen. The presence of calcium oxalate crystals in the medium is unlikely because calcium oxalate has a high tendency to form supersaturated solutions [4]. Under the prevailing conditions, 80% of the calcium present in the medium should precipitate spontaneously. The assumed reduction of the free calcium concentration at high protein concentrations is at variance with the fact that the solution supersaturated in calcium oxalate functions as a calcium buffer. At all protein concentrations calcium uptake has to start at the same free calcium concentration and it should decline not before 80% of the calcium present has been accumulated by the vesicles. The more important problem concerns the second finding, namely the drop of the transport ratio at high protein concentrations. Evidently, this effect can be expected if calcium passively leaks out of the vesicles and ATP hydrolysis has to maintain a steady state of calcium distribution (see above). The authors reject this possibility on two grounds:

1) Following the complete uptake of calcium, the ATPase falls back to the rate of the calcium-independent basal ATPase.

2) The rate of passive calcium flux from calcium oxalate loaded vesicles is too small to account for the observed effect [19, 20].

Both arguments, however, are not conclusive. The observations referred to concern properties of the system after calcium uptake has terminated. The ratio of calcium/ATP, however, is determined by properties controlling the system during the uptake period. During this time interval considerable differences must be expected to exist in the state of calcium inside the vesicles depending on the concentration of protein in the system, as demonstrated by the results described in Fig. 1. Calcium oxalate crystals are only formed after the internal calcium concentration has reached a critical value. This value is reached with relatively little delay at high calcium/protein ratios (3 μmol Ca^{2+}/mg protein, *cf.* Fig. 2 [18]) and the stored calcium oxalate crystals are nearly completely retained by the 0.1 μm filters after membrane lysis (Fig. 1A). On the other hand, at high protein concentrations in the system, the mean maximal load only amounts to 0.3 μmol Ca^{2+}/mg protein giving rise to small crystals which after lysis mostly pass through the filters (Fig. 1B). Since small crystals are more soluble than larger ones, the prevailing level of ionized calcium inside the vesicles is higher at high than at low protein/calcium ratios [21]. As a result of the high internal calcium level, at high protein/calcium ratios ATP splitting and calcium uptake are inhibited. For an explanation of the simultaneous decline of the transport ratio under these conditions, additional assumptions have to be made: Either

1) high internal calcium supports a rapid calcium backflux into the medium, or

2) internal calcium more strongly inhibits calcium uptake than the calcium-dependent ATP hydrolysis.

As to the first alternative, additional ATP had to be hydrolysed to compensate calcium backflux during and after termination of calcium uptake. Yet no calcium-dependent ATP hydrolysis could be detected after the cessation of calcium uptake indicating that at least in this state, calcium backflux is not enhanced. Furthermore, the rates of passive calcium efflux and calcium-calcium exchange were found to be similarly small at high and low protein/calcium ratios when measured subsequently to calcium loading. However, the properties of the system in this state need not reflect its properties prevailing during calcium uptake. We therefore have determined the efflux of calcium from vesicles preloaded with a small

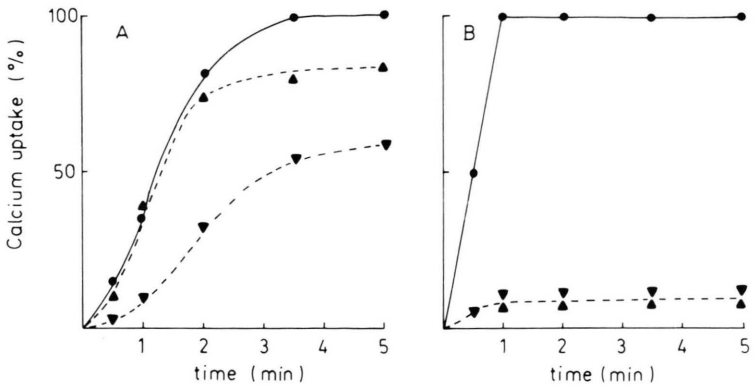

Fig. 1. Dependence on the protein/Ca^{2+} ratio of Ca^{2+} oxalate precipitation during Ca^{2+} accumulation by sarcoplasmic reticulum vesicles.

The composition of the uptake medium is given in Materials and Methods. At the low protein/Ca^{2+} ratio of 0.03 mg/100 nmol (A) the uptake phase lasts app. 3 min and can easily be measured (●———●). In contrast, at the high protein/Ca^{2+} ratio of 0.3 mg/100 nmol (B), Ca^{2+} uptake terminates in app. 1 min (●———●). Hence, the directly observed uptake period corresponds to the period of ATP extra splitting monitored by Gafni and Boyer [18]. At the low protein/Ca^{2+} ratio (A) crystal formation lags behind Ca^{2+} uptake (▼) and after 5 min only 55% of the stored Ca^{2+} are retained by the 0.22 μm (▼) filters after membrane lysis. Filters with a pore size of 0.1 μm (▲) retained all Ca^{2+} taken up by the vesicles during the initial uptake period. After 2 min 20% of the stored Ca^{2+} are released by Triton X-100. At the high protein/Ca^{2+} ratio (B) maximally as little as 10% of the stored Ca^{2+} are retained by 0.22 and 0.1 μm filters after Triton X-100 lysis (▼, ▲). Hence, larger Ca^{2+} oxalate crystals are formed at low than at high protein/Ca^{2+} ratios. As a consequence of the dependence of the solubility product of Ca^{2+} oxalate on the size of the crystals, higher concentrations of ionized Ca^{2+} prevail at high than at low protein/Ca^{2+} ratios inside the vesicles [21]. The conventionally used solubility product for Ca^{2+} oxalate is a limiting value and applies only to solutions in contact with comparably large crystals. The consequences of the high internal Ca^{2+} concentrations at high protein/Ca^{2+} ratios are discussed in the text.

quantity of radio-active calcium during subsequent loading. Considerable differences in the initial efflux rates were observed between high and low protein/calcium ratios. At low protein/calcium ratios, calcium efflux proceeded slowly (15±4 nmol/mg·min; ±SE, $n = 6$; 20 °C) as previously reported [22]. At high protein/calcium ratios, however, calcium influx induces a quite rapid calcium efflux (80±9 nmol/mg·min, ±SE, $n = 6$). This calcium influx induced calcium efflux might be related to the calcium turnover observed under steady state conditions in the absence of calcium precipitating ions [23]. Under the prevailing uptake conditions at high protein/calcium ratios, net calcium uptake is about 20% smaller than calcium influx. This would only partially account for the reduced transport ratio. Hence, the second explanation for the effect of internal calcium, a preferential inhibition of calcium, uptake also must be considered. However, like an enhanced passive calcium backflux a reduction of the coupling ratio at high internal calcium concentrations cannot result in an improvement of the sarcoplasmic reticulum calcium pump capability (*cf.* [24]). The reduction of the stoichiometry of the pump at high protein ratios evidently is not fully understood. A strong argument against the physiological importance of a modulation of stoichiometry by external calcium must be seen in the fact that the pump, operating with a coupling ratio of two, can maintain the physiological calcium concentration ratio of 10,000 with little energy dissipation [9].

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