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Stoichiometries of calcium and strontium transport coupled to ATP and acetyl phosphate hydrolysis by skeletal sarcoplasmic reticulum

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The stoichiometries of Ca^{2+} and of Sr^{2+} transport by the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum have been previously reported to be 2 and 1, respectively, when determined by flux ratio methods (Mermier, P. and Hasselbach, W. (1976) Eur. J. Biochem. 69, 79–86; Holguin, J.A. (1986) Arch. Biochem. Biophys. 251, 9–16). We have measured transport of Ca^{2+} and Sr^{2+} by the pulsed pH-stat method, when supported by ATP or the pseudo-substrate acetyl phosphate (AcP). The stoichiometry of ATP-supported Ca^{2+} transport, Ca^{2+}/ATP, was pH dependent and varied from 2.0 at pH 6.5 to 1.0 at pH 8.0. Sr^{2+}/ATP ratios showed a similar pH dependence and were approx. 7–18% lower. Ca^{2+}/AcP ratios showed little pH dependence and varied from 2.0 to 1.7 in the pH range 6.5 to 8.0. Sr^{2+}/AcP ratios were 17–34% lower, with maximum differences at the pH extremes. Ruthenium red, which blocks calcium efflux from calcium release channels, increased measured stoichiometries by less than 10%. It is concluded that the transport of both Ca^{2+} and Sr^{2+}, when supported by either ATP or a pseudo-substrate, have similar stoichiometries and occurs via identical mechanisms. The relatively low Sr^{2+} transport ratios have been related to uncoupled reverse flux through the Ca^{2+}-ATPase cation transport channel. Subintegral M^{2+}/substrate ratios appear to be an intrinsic feature of active transport by the Ca^{2+} pump of skeletal muscle sarcoplasmic reticulum.

Introduction

Skeletal muscle sarcoplasmic reticulum (SR) membranes are involved in excitation-contraction coupling. Following release of stored Ca^{2+} through the release channel, which initiates muscle contraction, activation of the Ca^{2+}-ATPase results in relaxation due to active Ca^{2+} transport against a concentration gradient, which utilizes the free energy of hydrolysis of ATP. The SR Ca^{2+} pump has been intensively studied as a model energy transducing system, mainly due to its ready experimental access, SR membranes form tightly sealed vesicles in vitro that allow transport processes, including stoichiometry, to be readily studied. The catalytic cycle, which has been described in terms of at least eight intermediate species, is completely reversible. A preformed Ca^{2+} gradient can be utilized to synthesize ATP under suitable conditions (for reviews, see Refs. 1–4).

The monomeric Ca^{2+}-ATPase appears to be able to carry out all of the reactions of the membranous enzyme in both forward and reverse directions [5–7]. The monomer possesses two cation binding sites, one nucleotide binding site, and a phosphorylation site. These structural features readily account for the observed transport stoichiometry that approaches an integral value of 2.0 for Ca^{2+}/ATP under optimal conditions, where transport is presumed to be tightly coupled to ATP hydrolysis. However, under conditions of variable pH, temperature and low external (cytosolic) Ca^{2+} (< 0.5 μM), less than theoretical transport stoichiometries occur that cannot readily be explained on the basis of increased passive membrane permeability [8].

Abbreviations: Ca^{2+}-ATPase, Ca^{2+}- and Mg^{2+}-activated adenosine 5′-triphosphatase (EC 3.6.1.38); SR, sarcoplasmic reticulum; A23187, calcium specific ionophore; AcPase, acetylphosphatase activity; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.

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The question of variable stoichiometry is of considerable interest with respect to the mechanisms of intramolecular energy transfer during active transport [1,2].

The Ca^{2+}-ATPase is relatively non-specific with respect to its substrate and energy source. The nucleotides ATP, ITP and GTP support transport in the skeletal muscle enzyme. Cardiac Ca^{2+}-ATPase appears to be relatively more specific for ATP. Van Winkle et al. [9] have reported Ca^{2+}-independent GTPase that is not linked to Ca^{2+} transport, suggesting that independent pathways may be involved. Orugusu et al. [10] have recently demonstrated GTP-dependent Ca^{2+}-uptake and E-P formation. However, rates of transport were approx. 10% of those for Ca^{2+}, and Ca^{2+}-independent GTPase was relatively high. Thus there appear to be differences in substrate handling between skeletal and cardiac SR. In addition to nucleotides, the pseudo-substrates, acetyl phosphate, *p*-nitrophenyl phosphate, 2,4-dinitrophenyl phosphate and furylacryloyl phosphate are effective energy sources. The ratios of Ca^{2+} transported per mole of pseudo-substrate have been reported to vary, depending on pH, and the nature of the substrate [11].

The Ca^{2+}-ATPase actively transports Sr^{2+} ions. Sr^{2+} binds with high affinity and positive cooperativity to the Ca^{2+}-binding sites [12]. However, the characteristics of Sr^{2+} transport differ from those of Ca^{2+}. More Sr^{2+} is accumulated in SR vesicles in the absence of a precipitable anion [13]. This may be related to the fact that unlike Ca^{2+}, binding of Sr^{2+} at millimolar concentrations to internally orientated low-affinity cation sites does not inhibit turnover of the pump. Furthermore, a preformed gradient of Sr^{2+} cannot be coupled to ATP synthesis [14]. Since the pathway for dissipation of the Sr^{2+} gradient appears to be via the Ca^{2+}-ATPase itself, vectorial and scalar reactions of the reverse Sr^{2+}-ATP cycle are, in effect, uncoupled.

The possibility that the Ca^{2+} pump may bind and transport one Ca^{2+} ion per phosphorylation/dephosphorylation cycle has been considered [15,16]. Johnson et al. [16] have calculated that the coexistence of 2 : 1 and 1 : 1 cycles would not confer any thermodynamic advantages to the system at high Ca^{2+} gradients. The reports that the transport of Sr^{2+} has a 1 : 1 stoichiometry [12,13] are therefore of considerable interest, especially since the high affinity binding sites on the Ca^{2+}-ATPase for both of these cations appear to be identical. In these reported studies, Sr^{2+}/ATP ratios were determined from the ratios of fluxes of Sr^{2+} and of ATP hydrolysis.

We have developed Ca^{2+}-stat and pH-stat methods for determining transport stoichiometries in SR vesicles by pulsed additions of ATP or Ca^{2+} [8,17]. In this study we have adapted the pulsed pH-stat method to measure the stoichiometry of Ca^{2+} and Sr^{2+} transport with ATP and with the pseudo-substrate, acetyl phosphate. The results, in contrast to previous suggestions, indicate that the transport stoichiometries for both of these cations are identical and non-integral under in vitro conditions. A preliminary account of this work has been presented [18].

Materials and Methods

Sarcoplasmic reticulum vesicles were isolated from rabbit back and hind limb white muscle, according to the method of Eletr and Inesi [19]. This preparation is relatively free of the 400-kDa ryanodine binding protein that confers high Ca^{2+} conductance to heavy (cisternal-junctional SR) membranes [20]. The stock suspension of membrane vesicles (8–14 mg/ml) was stored at −70°C in 10 mM imidazole, pH 7.4, and 0.3 M sucrose. Protein concentration was determined by the method of Lowry et al. [21], using bovine serum albumin as standard. The Ca^{2+}-ATPase was found to constitute > 85% of total protein, as determined by SDS-PAGE, followed by staining with Coomassie blue. Maximum E-P levels from P_i, determined at pH 6.0 in the presence of 30% dimethyl sulfoxide [22], were in the range 5.0 to 5.5 nmol/mg, which represents 50–55% purity of the ATPase with respect to catalytic sites.

Coupling ratios for Ca^{2+} and for Sr^{2+} were determined by the Ca^{2+}-pulse pH-stat method, as described by Meltzer and Berman [17]. In this method, hydrolysis of substrate is continuously monitored, by following the release of H^+ by the pH-stat technique. The burst of H^+ release, following pulsed addition of either Ca^{2+} or Sr^{2+}, was used to calculate M^{2+}/substrate ratios. The apparatus comprised a Radiometer ABU 80 Autoburette, a PHM 82 pH meter, a TTT60 Titrator, a type G2040 glass electrode and a type K4040 calomel electrode. Titrant was approx. 20 mM CO_2-free NaOH, which was standardised against 0.1 M HCl (BDH). Solutions were equilibrated with, and the reaction conducted, under CO_2-free N_2. The standard reaction mixture, 3 ml, contained 100 mM KCl, 5 mM $MgCl_2$ and 0.3 mg/ml SR vesicles. All experiments were carried out at 25°C. The stoichiometry of H^+ release during ATP hydrolysis was determined in reaction medium, to which was added 4% (w/w) of the ionophore A23187, and 50 μM $CaCl_2$. H^+ release, following addition of MgATP or AcP was measured in the pH range 6.50 to 8.0. H^+ release during AcP hydrolysis was measured when catalysed by alkaline phosphatase, 60 μg/ml. The reaction medium for transport contained 5 mM potassium oxalate and, in addition, either 5 mM MgATP or 2 mM AcP. Transport stoichiometries were determined in the pH range 6.5 to 8.0. The reaction was started by addition of SR vesicles, 0.3 mg/ml. The initial steady state rate was established over 3–5 min, following which pulses of Ca^{2+} or Sr^{2+} were added that gave rise to bursts of up to 2 μmol of

H^+. The amounts of M^{2+} added varied from 2 to 0.8 μmol at pH 6.5 and 8.0, respectively. The amount of H^+ liberated during active transport of M^{2+} was calculated by extrapolation of steady state rates, before and after its addition, to the mid-point of the burst of H^+ release. Results were expressed as the means of 3–5 determinations. Variability of measurements for Ca/ATP at pH 7.25, expressed as variance, was < 5% throughout the pH range measured.

Ruthenium red, ATP ('Vanadate free') and acetyl phosphate (Li, K salt), and alkaline phosphatase (from *Escherichia coli*) were obtained from Sigma (U.S.A.). ATP was shown to be > 99% Pure by HPLC and was standardized from its optical absorbance at 260 nm, using $E_{259} = 15.4$ [23]. Acetyl phosphate was standardized by the method of Lipmann and Tuttle [24], using succinic anhydride as standard. Fresh solutions of AcP (50–100 mM) were assayed as 75%-80% pure by this method. Analar $CaCl_2$ was from BDH (U.K.). $SrCl_2$ (> 98.5%) was purchased from Kanto Chemicals (Tokyo). Solutions of $CaCl_2$ and $SrCl_2$ (100 mM) were standardized by chloride titration (Radiometer, Copenhagen). The calcium specific ionophore, A23187, was purchased from Calbiochem (U.S.A.).

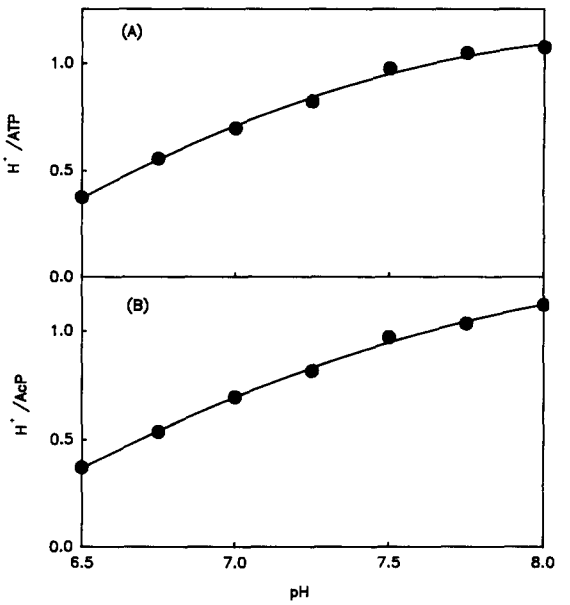

Fig. 1. pH dependence of H^+/substrate ratios. The reaction medium, 3.0 ml, at 25°C, contained 100 mM KCl, 5 mM $MgCl_2$, 50 $CaCl_2$ and 4% (w/w) calcium ionophore, A23187. In (A) 0.9 mg SR protein and 3 μmol ATP were added. The line is the best fit to the binomial least-squares regression, $H^+/ATP = -11.336 + 2.879 \times pH - 0.166 \times pH^2$. In (B) 2 μmol acetyl phosphate and 60 μg/ml alkaline phosphatase were added. The line is the regression, $H^+/AcP = -10.820 + 2.711 \times pH - 0.152 \times pH^2$.

Results

Proton release per mole of substrate hydrolysed at various pH values was determined by addition of known aliquots of ATP to a suspension of SR vesicles in the presence of saturating [Ca^{2+}] and calcium ionophore. Liberation of H^+ was measured during hydrolysis of AcP by alkaline phosphatase. The results for the pH range 6.5 to 8.0 are shown in Fig. 1. H^+ release for both ATP and AcP varied from 0.37 to 1.10 mol H^+ per mol of substrate hydrolysed.

The stoichiometries of cation (M^{2+}) transport, coupled to ATP or acetyl hydrolysis, were determined over the pH range 6.5 to 8.0. A direct comparison between Ca^{2+} and Sr^{2+} was possible under identical conditions, using the same SR vesicles. A typical pH-stat profile, with ATP as substrate, is shown in Fig. 2. An initial steady-state rate of hydrolysis, in the presence of oxalate and absence of added Ca^{2+}, corresponded to basal rates of ATPase activity. This rate was only slightly (< 10%) decreased by the addition of 0.2 mM EGTA, indicating that endogenous Ca^{2+}, present in the preparation of SR vesicles and contaminating reagents, is decreased by active transport to levels of free Ca^{2+} that are well below the K_m for Ca^{2+}-activated hydrolysis.

Addition of a pulse of Ca^{2+} or Sr^{2+} resulted in immediate activation of ATP hydrolysis. Following sequestration of M^{2+} into vesicles as insoluble oxalates, hydrolysis switched to a linear rate, which was similar to the initial 'basal' rate. It is significant that the 'switch off', following depletion of added-M^{2+}, is sharper in the case of Ca^{2+} than of Sr^{2+}. This is explained by their relative affinities for the divalent cation binding sites on the Ca^{2+}-ATPase. $K_{0.5}$ for Sr^{2+} is 10–14-fold higher than for Ca^{2+} [10]. The sequence of addition, either $Ca^{2+}/Sr^{2+}/Ca^{2+}$ or $Sr^{2+}/Ca^{2+}/Sr^{2+}$, had no obvious effects on the calculated stoichiometries.

The pH dependence of ATP-dependent transport stoichiometries is shown in Fig. 3. Coupling ratios were

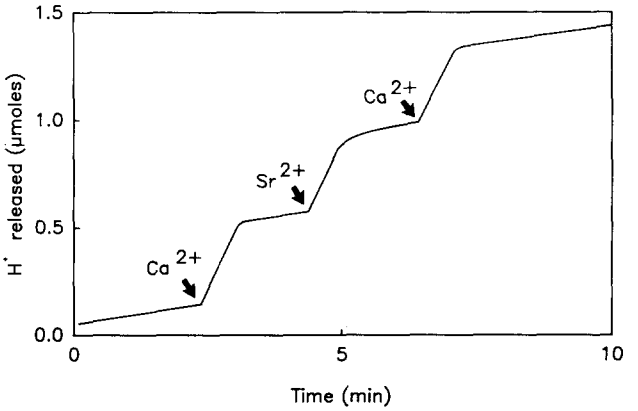

Fig. 2. Typical titration curve for the determination of the coupling ratio of divalent cation transport to substrate hydrolysis. The reaction medium, 3.0 ml, contained 0.9 mg SR protein, 100 mM KCl, 5 mM $MgCl_2$, 5 mM $K_2C_2O_4$, 5 mM ATP, and was maintained at pH 7.25 by pH-stat titration. $CaCl_2$ or $SrCl_2$, 0.5 μmol, were added where indicated.

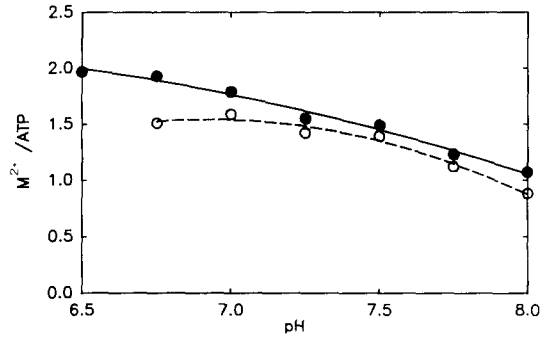

Fig. 3. pH dependence of coupling ratios of Ca^{2+} (●———●) and Sr^{2+} (○———○) transport to ATP hydrolysis. Conditions were as described for Fig. 2.

maximal at pH 6.50, where Ca^{2+}/ATP approached 2.0, and declined to approx. 1.0 at pH 8.0. Similar pH dependence has been previously shown, using a Ca^{2+}-stat method [8]. Sr^{2+} coupling ratios, Sr^{2+}/ATP, were approx. 7–18% lower over the pH range 6.75 to 8.0. Values for Sr^{2+}/ATP are not reported at pH 6.50 values, since the lowered Sr^{2+} affinity rendered the titration end point less certain. Differences between Ca^{2+} and Sr^{2+} coupling ratios, taken over the measured pH range, were not significant.

The stoichiometries for Ca^{2+} and for Sr^{2+} transport, using acetyl phosphate as energy source, are shown in Fig. 4. In contrast to ATP-dependent transport, Ca^{2+}/AcP ratios were in the range 1.7 to 2.0, and relatively independent of pH between pH 6.5 and 8.00. Sr^{2+}/AcP ratios were 17–34% lower than for Ca^{2+}/AcP. This difference was significant ($P < 0.02$).

The present experiments thus yielded similar values for the coupling ratios of Ca^{2+} and Sr^{2+} and for ATP and AcP, which support widely differing rates of pump turnover. Lowered stoichiometries at alkaline pH values could be due to efflux of translocated M^{2+}. The Ca^{2+} efflux channel has in fact been shown to be stimulated at alkaline pH [25]. Ruthenium red, a polyvalent organic cationic dye, has been shown to be a potent blocking agent for the Ca^{2+} efflux channel [26]. The stoichiometries for ATP-supported M^{2+} transport were therefore determined in the presence of the dye. Ruthenium red, 20 μM, had no effect on Ca^{2+}/ATP or Sr^{2+}/ATP ratios at pH 6.75. The calcium channel blocker did increase these ratios by 7–8% at pH 7.75.

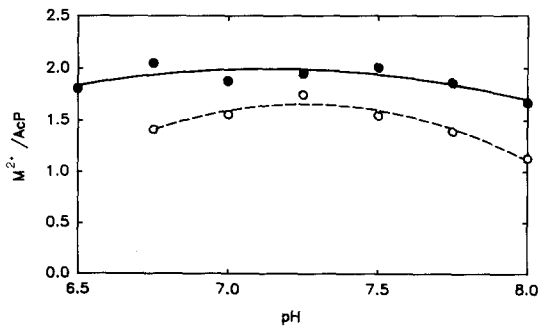

Fig. 4. pH dependence of coupling ratios of Ca^{2+} (●———●) and Sr^{2+} (○———○) transport to acetyl phosphate hydrolysis. Conditions were as described in Fig. 3, except that 2 mM acetyl phosphate was used instead of ATP as substrate.

Discussion

Previous studies on Sr^{2+} transport have reported that the coupling ratio, Sr^{2+}/ATP, is 1.0, i.e. one Sr^{2+} ion is transported per turnover of the Ca^{2+}-ATPase, in contrast to the generally accepted coupling ratio of 2 for Ca^{2+} transport. This implies that the mechanisms for coupled transport of Ca^{2+} and of Sr^{2+} are different. Since both cations appear to bind with high affinity and positive cooperativity ($n_H(Ca^{2+}) = 1.6$ to 3.5 and $n_H(Sr^{2+}) = 1.06$ to 1.83) [12] to two independent sites on the Ca^{2+}-ATPase, and occupation of both sites is necessary for phosphorylation, this would imply that only one of the bound Sr^{2+} ions is transported following formation of $Sr_2 \cdot$ E-P. Previous studies [12,13], indicating coupling ratios for Sr^{2+} of one, were based on independent measurements of flux rates for Ca^{2+} transport and ATP hydrolysis. Errors may thus occur from the two independent measurements. Mermier and Hasselbach [13] obtained values for Ca^{2+}/ATP and Sr^{2+}/ATP of 1.9 and 1.1, respectively, at pH 7.45 in the presence of oxalate. Comparable data from the present study were 1.6 and 1.5. Mermier and Hasselbach also reported that transport ratios for both ions approximate one in the absence of oxalate. Under the latter conditions, the kinetics of uptake of both ions was complex. Measured lower coupling ratios may also be due to a population of unsealed or leaky vesicles. The present study, in contrast, using the Ca^{2+}-pulse pH-stat method, showed that Ca^{2+} and Sr^{2+} are transported with similar stoichiometries, with coupling ratios that approach 2. The advantages of this method are that calibration depends solely on standardisation of ATP and divalent cation solutions, and that direct comparison between their coupling ratios is possible in a single assay. The pH-stat method was also shown here to be suitable for determining acetyl phosphate dependent coupling ratios, and of being a convenient and reliable method for continuous monitoring of acetylphosphatase activity. Previously, the Lippman and Tuttle method [24] has been used to monitor the decrease in substrate concentration, which is not ideal, owing to the insensitivity of this technique.

Coupling ratios for AcP-dependent Ca^{2+} transport are similar to those with ATP. Bodley and Jencks [27] have concluded that the mechanism of catalysis is identical for ATP and all pseudo-substrates of the Ca^{2+}-ATPase, including acetyl phosphate, although the turnover rate for the latter is 50-fold less than that for ATP, predominantly due to decreased phosphorylation

rate. In contrast to ATP, AcP reacts with the enzyme in the absence of Ca^{2+}, and undergoes hydrolysis that is not coupled to transport [28]. Rossi et al. [11] have reported a marked pH dependence of Ca^{2+} transport and of coupling ratios with *p*-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate.

Uncoupled reverse efflux of Sr^{2+} presumably is a major factor in the observed lower coupling ratios for Sr^{2+} in the forward direction. Differences in pH sensitivity for transport are not immediately obvious. Alkaline conditions have been previously shown to result in uncoupling in both skeletal [8,11] and cardiac [9] SR vesicles. This has been explained by the pH dependence of efflux via ryanodine-sensitive Ca^{2+} release channels. Low coupling ratios at pH 8.0 were virtually unaffected by ruthenium red (this study), which blocks efflux via these channels. The observed pump stoichiometry at alkaline pH may be a manifestation of intrinsic uncoupling or 'slippage' of the pump. It is possible, for example, that a pathway for Ca^{2+} efflux via the ATPase itself, uncoupled to ATP synthesis, may be favoured under alkaline conditions.

Coupling ratios for Sr^{2+} transport, linked either to ATP or AcP hydrolysis, are lower than the corresponding values for Ca^{2+} transport at all pH values. These do not however approach the previous suggestion for 1 : 1 stoichiometry for Sr^{2+}/ATP. The observed differences may be related to differing affinities of these cations for the transport sites, as illustrated in Fig. 2. We have previously shown that measured coupling ratios are decreased at low $[Ca^{2+}]_{out}$ in the submicromolar range, suggesting that the degree of saturation of transport sites is correlated with efficiency of coupling of the pump. The present experimental conditions are such that transport sites are saturated during most of the period of pulsed uptake ensuring maximal observed coupling ratios. However, in the case of Sr^{2+}, due to its lower affinity, transport sites are unoccupied for a significant period during the assay and may account for the lower observed Sr^{2+}/ATP and Sr^{2+}/AcP ratios. Differences between Ca^{2+} and Sr^{2+} transport, with ATP as substrate, are small and it is therefore concluded that the mechanisms of binding and transport of the two cations are essentially similar in the forward direction of the pump.

Previous studies have shown that Ca^{2+} and Sr^{2+} are handled differently during pump reversal. In particular, higher intravesicular concentrations in the absence of oxalate [13], and the fact that dissipation of a preformed gradient of Sr^{2+} is not coupled to ATP synthesis, indicate that internally orientated, low-affinity transport sites discriminate between the two cations. It would appear therefore that the cation binding sites on the high-affinity E_1 form of the ATPase do not differentiate between Ca^{2+} and Sr^{2+}, whereas the low-affinity site(s) ($K_d \cong 1$ mM), characteristic of the E_2 conformation, show a marked selectivity, with preference for the physiological divalent cation.

The observed subintegral coupling ratios, as low as 1.2 at pH 8.0 (Fig. 3), appear to represent a true reflection of the stoichiometry of divalent cation transport. Simonides and van Hardeveld [29] have confirmed similar values for rat skeletal muscle SR. The general assumption has previously been that experimentally observed suboptimal coupling ratios are due to passive leakage via undefined pathways through the membrane, or efflux through the physiological calcium release channel. This channel is located in terminal and junctional regions of SR. The preparation of SR vesicles used in the present studies, as described by Eletr and Inesi [19], appears to be relatively free of release channel-containing vesicles [20]. Furthermore, Ruthenium red, which is a potent inhibitor of calcium release channels, did not restore coupling ratios to the theoretical value of 2.0. The fact that the observed coupling ratios for ATP- and AcP-dependent transport are comparable is also evidence against a role for passive leaks or release channels as an explanation for non-integral stoichiometry. Pump turnover with AcP, which is 50-fold slower than with ATP [27], is expected to accentuate the effects of passive leaks. Coupling ratios for AcP were in fact higher than those for ATP at alkaline pH (1.6 versus 1.0 at pH 8.0).

Non-integral coupling ratios could conceivably be explained by a varying proportion of pump units operating with either 2 : 1 or 1 : 1 stoichiometry. This appears to be unlikely, since the binding of two Ca^{2+} ions is required for phosphorylation, and in the process 2 Ca^{2+} ions are tightly occluded. Coupling ratios as low as 0.2 have been measured at $[Ca^{2+}]_{out}$ levels well below $K_{0.5(Ca^{2+})}$ [17] implying that a continuous range of coupling ratios is possible.

It would appear therefore that non-integral stoichiometry is an intrinsic property of Ca^{2+} transport in isolated SR. Its mechanism is, however, controversial. This laboratory [1,8,30] and that of Boyer [15] have proposed that the Ca^{2+}-ATPase may exhibit variable stoichiometry or irreversible intramolecular uncoupling due to 'slippage' of the pump. Inesi and De Meis [20] have recently studied the factors that limit ATP-dependent filling of isolated SR vesicles. They have concluded that at high lumenal $[Ca^{2+}]$, and in the presence of ADP, slippage of Ca^{2+} through a channel within the ATPase results in true uncoupling of the pump.

It is of interest that the conditions under which transport was measured in this study, with pulsed additions of Ca^{2+}, are similar to the physiological situation that exists in vivo. Ca^{2+} release from terminal SR cisternae results in a pulsed increase of Ca^{2+} that activates the turnover of the ATPase. In the resting state skeletal muscle fibre cytosolic Ca^{2+} levels are in the range ($\cong 0.05$ μM) that has been shown to result in

phenomenologically uncoupled turnover of the Ca^{2+}-ATPase [8], which may be of physiological significance.

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