Equilibrium and Kinetic Studies of Calcium Transport and ATPase Activity in Sarcoplasmic Reticulum *

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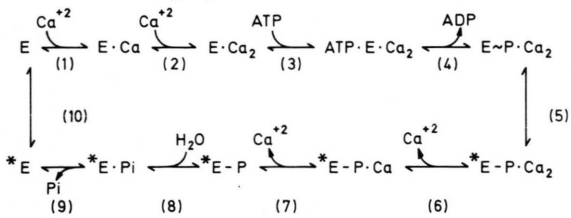
A number of equilibrium and kinetic measurements are presented to characterize the partial reactions of the ATPase and transport cycle in sarcoplasmic reticulum vesicles. The cycle begins with calcium and nucleotide binding on sites available on the outer surface of the vesicles. A phosphorylated enzyme intermediate is then formed, and the calcium sites are subjected to a change in their orientation and their affinity for calcium. It is shown that steps involved in calcium release on the inner side of the vesicles are rate limiting for the cycle, and are followed by hydrolytic cleavage of the intermediate with release of inorganic phosphate and recycling of the enzyme.

Introduction

The calcium pump associated with the ATPase enzyme of sarcoplasmic reticulum (SR) was first discovered by Hasselbach and Makinose [1, 2], and Ebashi and Lipman [3], and characterized by steady state experimentation [4 – 6]. Subsequent findings on enzyme phosphorylation with ATP [7, 8], reversal of the pump [9 – 12], and phosphorylation of the enzyme with Pi [13] led to formulation of the following scheme which is derived from the original paper by Carvalho, *et al.* [14]:



In our laboratory, we are using both equilibrium and kinetic experimentation to study the partial reactions of the catalytic and transport cycle.

Methods

SR vesicles were prepared from rabbit leg muscle as previously described [15]. Calcium binding was measured by equilibration in chromatography columns [16] and steady state ATPase activity was followed by determination of Pi [17]. Rapid quench experiments were carried out with a Dionex D133 multimixing apparatus [18] or with a mixing device operating with syringes driven by a stepping motor [19]. In rapid kinetic experiments, phosphoenzyme and Pi production were measured using γ-[^{32}P]ATP [18, 20]. Radioactive ^{45}Ca tracer was used for measurements of active transport [20].

Results and Discussion

The first partial reaction of the ATPase cycle may be considered to be the binding of calcium to the activating (high affinity) sites of the enzyme. We find that the maximal number of these sites is 8 nanomoles per mg of SR protein, and that binding occurs in a cooperative manner [16] and is pH dependent (Fig. 1). At neutral pH, the apparent binding constant is 2.3×10^6 M^{-1}. However, fitting the experimental binding curve requires a mechanism including interaction of sites, and a binding affinity shift of at least two orders of magnitude (from $1.2 \pm 0.3 \times 10^5$ M^{-1} to about 5×10^7 M^{-1}) as a consequence of initial binding. We also find that binding of one calcium releases one proton [21] from a pro-

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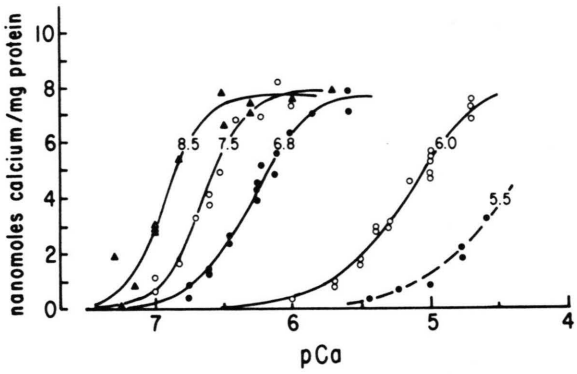

Fig. 1. Effect of pH on the Ca^{2+} concentration dependance of calcium binding [22]. The measurements were carried out following equilibration by column chromatography. Buffer consisted of 10 mM Tris (pH 8.5), Hepes (pH 7.5), Mops (pH 6.8), or Mes (pH 6.1 and 5.5). In addition to buffer, the medium contained 10 mM $MgCl_2$, 80 mM KCl, 45 μM $^{45}CaCl_2$, and various concentrations of EGTA. The passive binding measured here was identical in the presence or in the absence of Ca^{2+} ionophores. Temperature was 25 °C.

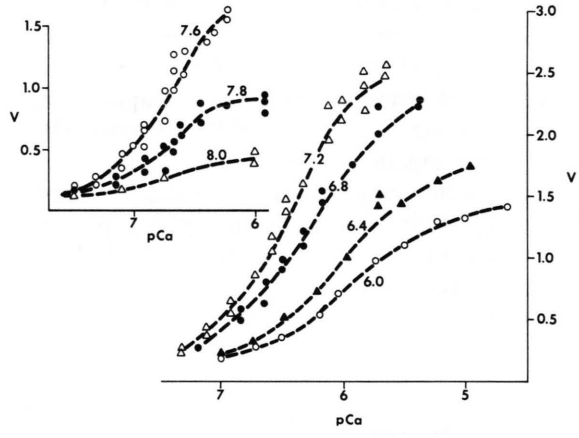

Fig. 2. Effect of pH on the Ca^{2+} concentration dependence of steady state ATPase activity. ATP hydrolysis was measured by determination of Pi. The reaction mixture contained 10 mM Tris-maleate adjusted to the proper pH, 80 mM KCl, 10 mM $MgCl_2$, 50 M $CaCl_2$, EGTA as required to obtain the proper pCa at different pH levels, and 10 μM A23187 to render the vesicles "leaky" to Ca^{2+}. Temperature was 25 °C.

tein residue that participates as a ligand for calcium and dissociates H^+ with a pK 7.3 [22].

In steady state experiments, a similar but not identical relationship is found for the Ca^{2+} concentration dependence of ATPase activity as a function of pH (Fig. 2). It is clear, nevertheless, that the calcium binding studied in equilibrium conditions represents the behaviour of the calcium sites involved in enzyme activation and transport function.

Addition of ATP to enzyme activated with calcium is rapidly followed by formation of the phosphorylated enzyme intermediate with a rate constant of $80-150\ s^{-1}$ [18, 23]. The maximal level of phosphoenzyme found in the presence of saturating ATP is 4 nanomoles per mg of SR protein (Fig. 3).

Formation of the phosphoenzyme is followed by rapid translocation (Fig. 3) of 2 mol of calcium per mole of phosphoenzyme, from the outer surface of the membrane into a position which is not accessible to equilibration with the medium [24, 25]. This position may correspond to the "occluded" state of Dupont [26] and Takakuwa and Kanazawa [27]. Finally, following a rate limiting step, hydrolytic cleavage of the phosphorylated enzyme intermediate permits the enzyme to recycle and proceed into steady state activity (Fig. 3).

A basic requirement for the vectorial character of active transport is that the calcium sites undergo a change in orientation and a reduction in affinity ($E\sim P\cdot Ca_2 \rightleftharpoons {}^*E-P\cdot Ca_2$ in the reaction scheme) to

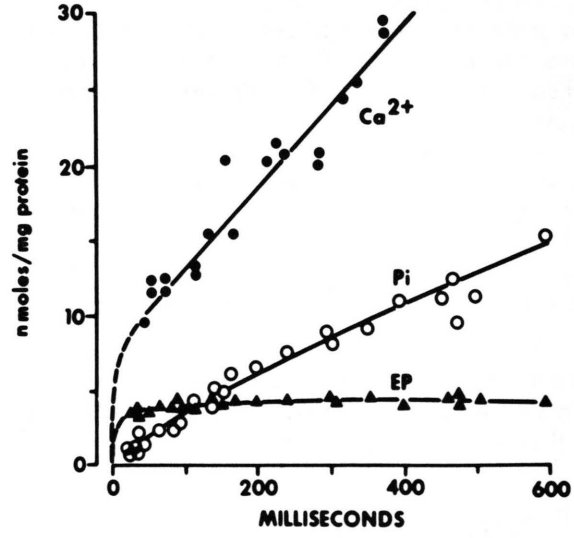

Fig. 3. Utilization of ATP for calcium transport in SR vesicles. Time resolution of the initial reaction was obtained by rapid quench methods [20]. It is clear the phosphoenzyme formation is an early event, accompanied by transfer of two moles calcium per mole of phosphoenzyme from the outer surface of the vesicles to a location that is not accessible to the EGTA used as a quencher. Steady-state Pi release and calcium transport follow the initial burst of enzyme phosphorylation and calcium transfer. Temperature was 25 °C.

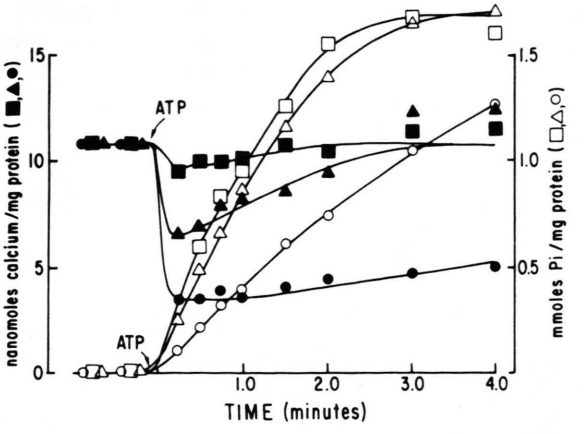

Fig. 4. Calcium bound to high affinity sites of purified ATPase is released when the binding sites are converted to a low affinity state following a pulse of ATP. Detection of net release, however, is facilitated by inhibition of phosphoenzyme hydrolysis with dimethylsulfoxide [22]. Calcium binding was measured by radioactive tracer and filtration. ATP hydrolysis was determined as ^{32}Pi release from γ-[^{32}P]ATP. The reaction mixture consisted of 20 mM Mes (pH 6.1), 80 mM KCl, 5 mM $MgCl_2$, 50 μM $^{45}CaCl_2$, 10 mM [^{3}H]glucose, and 0.5 mg of ATPase/ml. At zero time, 1.0 mM ATP was added. Temperature was 25 °C. Dimethylsulfoxide concentrations were 0% (■, □), 20% (▲, △), and 30% (●, ○).

allow release of calcium into the lumen of the vesicles before the enzyme is hydrolyzed and a new cycle begins. It is apparent that these steps preceding calcium release are rate limiting for completion of the cycle since most of the phosphoenzyme retains bound calcium. In fact, following release from the low affinity state sites, the phosphoenzyme undergoes rapid hydrolysis and recycles to a state permitting binding of more calcium on the outer surface of the vesicles. For this reason, *net* calcium release from the enzyme upon phosphorylation can be observed with *leaky* vesicles only if hydrolytic cleavage of the phosphoenzyme following release is delayed by suitable experimental manipulations (Fig. 4).

These experiments indicate that the partial reactions following enzyme phosphorylation and preceding hydrolytic cleavage are rate limiting. When the experimental system consists of native vesicles permitting net calcium uptake associated with ATPase activity, a rate limitation is imposed by the accumulation of calcium in an "inner compartment" (reactions (6) and (7)), and consequent buildup of the phosphoenzyme species containing bound calcium (*E−P · Ca, *E−P · Ca_2, and E ~ P · Ca_2 in the reaction scheme). These species undergo a very slow decay as demonstrated in double quench experiments in which SR vesicles are first allowed to react with ATP in the presence of calcium to form phosphoenzyme, and then further enzyme phosphorylation is interrupted by Ca^{2+} chelation with EGTA. Phosphoenzyme decay is then monitored by acid quenching at serial times.

In these experiments, two populations of phosphoenzyme may be distinguished kinetically [28]. In Figs. 5A and 5B, it may be seen that approximately 30% of the phosphoenzyme decays rapidly, and the remaining 70% slowly. In the light of these and other experiments, we attribute the two populations of the enzyme to E ~ P · Ca_2 and *E−P · Ca_2, which coexist at significant concentrations when saturated with calcium. The fast component must be due to decay of the *E−P · Ca species, and the slow component to transformation of E ~ P · Ca_2 to *E−P · Ca_2. The ratio of the two phosphoenzyme species in this pseudo-equilibrium (*e.g.* phosphorylation much faster than hydrolysis; therefore, nearly all the enzyme units remain phosphorylated in steady state conditions) is approximately 0.5.

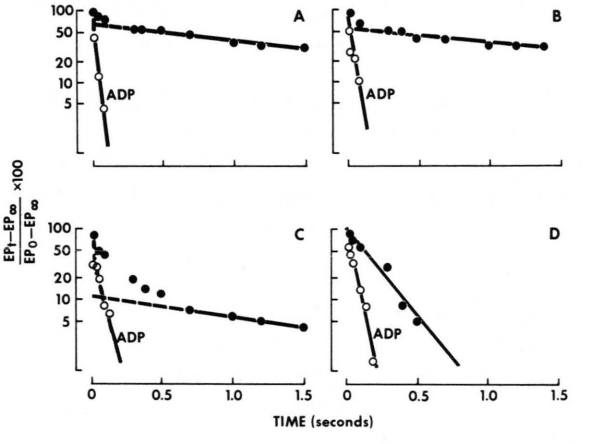

Fig. 5. Decay of phosphoenzyme formed from ATP in vesicles sustaining net calcium uptake (A and B), and in vesicles rendered permeable with the ionophore X537A (C and D). SR vesicles were first incubated with 25 μM [^{32}P]ATP in a medium containing 20 mM Tris-maleate (pH 6.8), 80 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, 0.66 mM phosphoenolpyruvate and 60 micrograms pyruvate kinase/ml. After 500 (A and C) or 20 (B and D) milliseconds, the phosphorylation reaction was quenched with an equal volume of the same medium, except for the replacement of $CaCl_2$ by 15 mM EGTA, and, when indicated, 3 mM ADP. A second quenching with PCA and Pi was then carried out at serial time intervals. Temperature was 25 °C.

A diphasic decay is observed even when calcium accumulation is allowed to proceed for only a few milliseconds. However, if net Ca^{2+} accumulation is prevented by using "leaky" vesicles, a much greater fraction of the phosphoenzyme exhibits a rapid decay (Figs. 5C and 5D). In conditions of minimal Ca^{2+} accumulation, the entire population of phosphoenzyme decays monotonically (Fig. 5D) with an apparent rate constant of $8-9\ s^{-1}$. This number is consistent with the turnover of the enzyme in the absence of back inhibition. This is found to be approximately 2 μmol/min/mg protein in steady state experiments (25 °C) with leaky vesicles. A similar figure is obtained by multiplying the apparent rate constant of phosphoenzyme decay ($8-9\ s^{-1}$) by the maximal number of phosphorylated enzyme units (4 nmol per mg protein).

The kinetic constant of $*E-P \cdot Ca_2$ decay, however, is still slower than the true rate constant for hydrolytic cleavage of the phosphoenzyme (*E−P in reaction 8 of the scheme). This constant can be obtained by first phosphorylating the enzyme with $^{32}P-Pi$ in the absence of calcium [13], then diluting the medium with non-radioactive Pi, and following phosphoenzyme decay by acid quenching at serial time intervals (Fig. 6A). In the presence of saturating Mg^{2+} and K^+ (pH 6.8), the rate constant for hydrolytic cleavage of the phosphoenzyme is $60\ s^{-1}$ (25 °C).

It is noteworthy that the maximal level of phosphoenzyme obtained by equilibration of the enzyme with Pi in the absence of Ca^{2+} (Fig. 6B) is 2.5 nmol/mg protein (pH 6.8, saturating Mg^{2+} and K^+). Therefore the equilibrium constant for the hydrolytic reaction with Pi in these conditions $\left(K=\frac{*E \cdot Pi}{*E-Pi}\right)$ is 0.6 to 2.2, depending on whether the total number of phosphorylation sites is considered to be 4 or 8 nmol/mg protein [29]. Correspondingly, the phosphorylation rate constant (k_{-8} in the reaction scheme) is $36-132\ s^{-1}$.

Since the rate constant for hydrolysis of the phosphoenzyme is significantly faster than the turnover in the absence of net Ca^{2+} accumulation, we infer that steps associated with Ca^{2+} release from $*E-P \cdot Ca_2$ are rate limiting in this case. In a previous description of the cooperative mechanism of calcium binding and enzyme activation, we have considered in greater detail the significance of structural changes associated with calcium binding and dissociation in SR ATPase [16]. It is of interest that this rate limitation can be removed when the ATPase is solubilized with the detergent $C_{12}E_8$. This again suggests that calcium dissociation is limited by structural constraints.

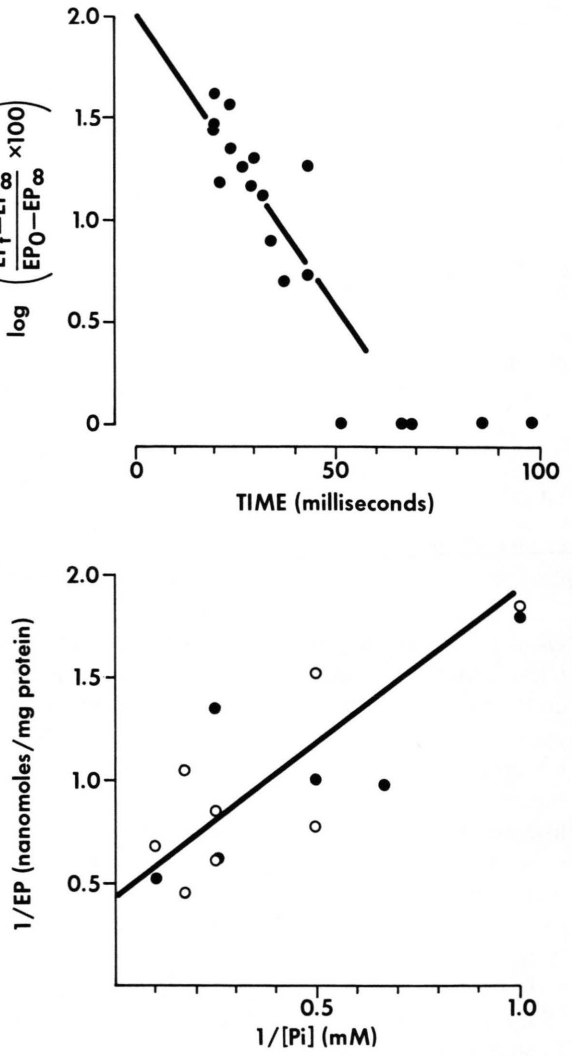

Fig. 6 A. Decay of phosphoenzyme formed from Pi in the absence of calcium. SR vesicles were first incubated with ^{32}Pi, 10 mM $MgCl_2$, and 0.5 mM EGTA at pH 6.0 for 30 seconds in order to obtain a high phosphoenzyme level. An equal volume of a medium containing a 12-fold excess of non-radioactive Pi, a neutral pH buffer, 80 mM KCl, and 10 mM $MgCl_2$ was then added using the Dionex multimixer. Decay of the phosphoenzyme was monitored after acid quenching at serial times. Temperature was 25 °C. B. Pi concentration dependence of enzyme phosphorylation in the absence of calcium in equilibrium conditions, saturating magnesium and neutral pH. The medium contained 20 mM Mops (pH 6.8), 80 mM KCl, 10 mM $MgCl_2$, and 0.5 mM EGTA.

An overview of the effects of various experimental manipulations on the initial ATPase activity is show in Fig. 7. It is clear that the hydrolytic activity of native vesicles (Fig. 7A) is enhanced by rendering the membrane leaky by treatment with a divalent cation ionophore or with low concentrations of Triton X-100 (Fig. 7B). Yet, the phosphoenzyme levels remain nearly identical, indicating that, in either case, the rate of enzyme phosphorylation with ATP ($80-150\ s^{-1}$) is much higher than the effective rate of hydrolysis ($3-9\ s^{-1}$), and the entire population of enzyme units in the preparation remains phosphorylated.

A further increase in hydrolytic activity is obtained when the ATPase is solubilized (Fig. 7C). In this case, the phosphoenzyme level is somewhat lower, since the effective rate of hydrolysis ($50-60\ s^{-1}$) is significant with respect to the rate of enzyme phosphorylation with ATP.

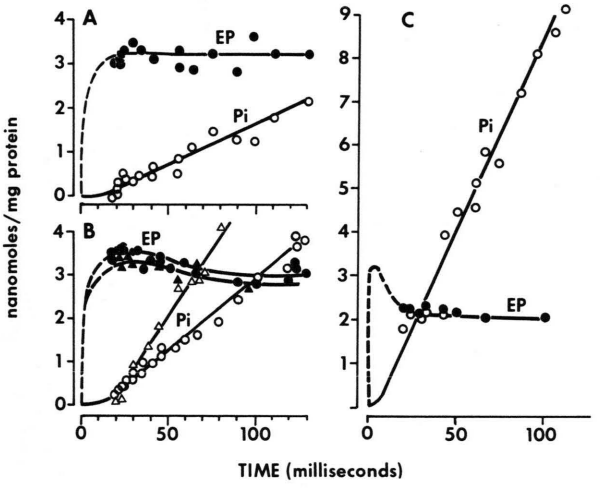

Fig. 7. Phosphoenzyme formation and Pi production following addition of ATP to native vesicles (A), vesicles rendered leaky with the ionophore X537A (▲, △) or 0.1% Triton X-100 (●, ○) (B), and ATPase solubilized with the detergent $C_{12}E_8$ (C). The experiments were carried out with a rapid mixing device, collecting several samples for each reaction time in order to obtain a large volume of quenched reaction mixture for analysis. The large volumes were necessary to offset the very low protein concentration used for these experiments. The medium contained 25–50 μg SR protein/ml, 80 mM KCl, 5 mM $MgCl_2$, 50 μM $CaCl_2$, and 20 mM Mops (pH 7.0) or Tris (pH 7.5, Fig. 7C). 20 μM X537A, 0.1% Triton X-100, or 1.0 mM $C_{12}E_8$ were present where indicated. The reaction was started by adding 10 μM [^{32}P]ATP and stopped by acid quenching at serial times. Temperature was 25 °C.

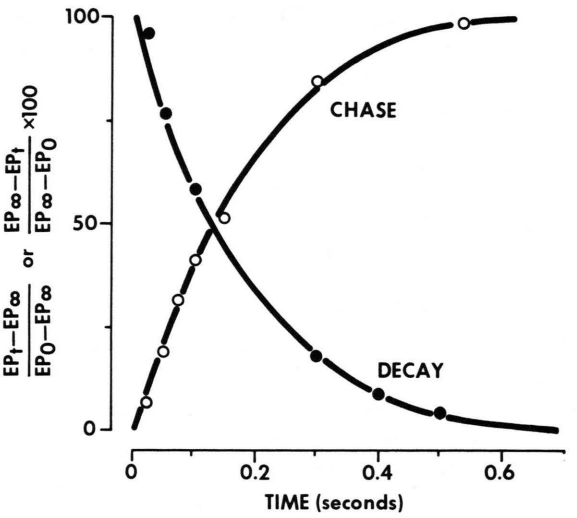

Fig. 8. Phosphoenzyme formation following addition of [^{32}P]ATP chase to phosphoenzyme formed with non-radioactive ATP. The phosphoenzyme was first formed by incubating leaky vessicles with 25 μM cold ATP, 20 mM Tris-maleate (pH 7.0), 80 mM KCl, 5 mM $MgCl_2$, and 20 μM X537A for 20 milliseconds. A pulse of [^{32}P]ATP was then added (without significantly changing the ATP concentration), and the reaction was quenched at serial times with acid. In a parallel experiment, the decay of the phosphoenzyme formed during the first 20 milliseconds was measured as for Fig. 5D.

Additional studies have been made of the partial reactions (reactions 9 and 10 in the scheme) related to recycling of the dephosphorylated enzyme to a form that can be activated by calcium to start a new cycle. It is known that the dissociation of Pi from the non-covalent E · Pi complex [23, 30], and the subsequent enzyme transformation before, or concurrently with, calcium binding to the activating sites [16, 31] proceeds with observable time constants under certain conditions. However, following the transient state, the enzyme seems to proceed without any measurable delay to further cycles following hydrolytic cleavage. This can be demonstrated by phosphorylating the enzyme in leaky vesicles, and then adding a pulse of radioactive γ-[^{32}P]ATP. It is shown in Fig. 8 that phosphorylation with the chase ATP proceeds with no delay when compared with the decay of the phosphoenzyme formed in the preliminary incubation (as in Fig. 5D).

A final point of interest is related to the reversal of the phosphorylation reaction with ATP (reaction (4)). In all cases (Fig. 5), a rapid disappearance of the phosphoenzyme is obtained when ADP is added

to the reaction medium. This phenomenon is at the limit of resolution of our instrument, but indicates that the first rapid component corresponds to reversal of reaction 4 ($k_{-4} > 200$ sec^{-1}).

Conclusions

Equilibrium and kinetic studies of the catalytic and transport cycle of the SR ATPase yield a number of equilibrium and rate constants for the partial reactions. Values derived from experimental measurements are listed in Table I. Some rate constants (not listed) can be approximated from known equilibrium constants assuming that the "on constant" is diffusion limited.

It is apparent that a basic feature of the mechanism for free energy transduction involves conversion of a phosphoenzyme species with high phosphorylation potential and tightly bound calcium, into a phosphoenzyme species with low phosphorylation potential and weakly bound calcium.

Another important feature of the ATPase activity and the transport cycle is the occurence of rather slow changes related to calcium binding and dissociation from the enzyme. Both these features may be shown to be rate limiting in different experimental conditions. On the other hand, the true rate constant for hydrolytic cleavage of the phosphorylated intermediate becomes rate limiting only when the ATPase is solubilized from its membrane matrix.

Table I. Listing of equilibrium and rate constants obtained from experimental studies on the partial reactions of the SR ATPase. The forward direction for all reactions is assumed to be that following ATP utilization. Only values pertinent to pH 6.8, 80 mM KCl, 5–10 mM $MgCl_2$, and 25 °C are listed. Some additional rate constants may be derived from known equilibrium constants (1), (2), (3), (6), (7) and (9) if the on constant is assumed to be diffusion limited.

Reaction	K	$k_{forward}$	$k_{reverse}$
(1), (2)	$K_{app} = 2.3 \times 10^6$ M^{-1}	–	–
(3)	$10^5 - 10^6$ M^{-1}	–	–
(4)	–	80–150 s^{-1}	> 200 s^{-1}
(5)	~0.5	–	–
(6), (7)	$K_{app} = > 10^{-3}$ M	–	–
(8)	0.6–2.2	60 s^{-1}	36–132 s^{-1}
(9)	$10^{-3} - 10^{-2}$ M	–	–
(10)	–	–	–
Overall turnover number (in the absence of net Ca^{2+} accumulation)		8–9 s^{-1}	

Acknowledgements

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