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Steady state of an ATP-driven calcium pump: Limitations on kinetic and thermodynamic parameters

(active transport/bioenergetics/enzyme kinetics/membranes)

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ABSTRACT **A numerical analysis was carried out to explore limitations on kinetic and thermodynamic parameters for an ATP-driven Ca pump. A conventional pump reaction cycle was employed, with a transport stoichiometry of two Ca ions per cycle. Rigid requirements were imposed to represent the needs of physiological function, defined as the ability to maintain the cytoplasmic Ca concentration below 10^{-7} M against a 3 mM concentration on the opposite side of the membrane. Realistic physical limits were placed on the magnitudes of rate constants for individual reaction steps. Reversibility under laboratory conditions was assumed. The results show that these requirements can be satisfied simultaneously only if the equilibrium constant for binding Ca from the cytoplasmic (uptake) side of the membrane is much larger than the binding constant on the discharge side. More generally, the results demonstrate that limitations on rate constants make it possible for the pump to maintain an adequate rate only if steady-state levels of kinetically important (slowly reacting) reaction intermediates do not become too disparate. Experimental data for the sarcoplasmic reticulum calcium pump support these theoretical conclusions.**

This paper reports the results of a theoretical analysis of the steady state of an ATP-driven Ca pump. A conventional reaction scheme was adopted. Rate constants were allowed to vary within reasonable limits, and all possible combinations of rate constants were tested with the aid of a computer program to determine what limitations are imposed by the need to satisfy the requirements of physiological function. A prime motivation for the work was to focus on a question raised by Jencks (1). Jencks has proposed a set of formal rules for a reaction cycle capable of coupling ATP hydrolysis to active transport and has pointed out that these rules do not require differing binding affinities for the transported ion on the uptake and discharge side of the membrane. However, he suggests that kinetic considerations make differing affinities likely, and the results will be seen to provide strong support for this suggestion. The results also relate to another dictum of Jencks (1), which suggests that reaction intermediates of a pump reaction cycle should be present at roughly comparable concentrations if an adequate steady rate is to be maintained. It will be seen that this principle is applicable only to intermediates that undergo inherently slow reactions in the reaction cycle.

The analysis is applicable to any pump that is used to maintain the cytoplasmic concentration of Ca ions ($[Ca]_{cyto}$) at 10^{-7} M or below (2) by active transport of Ca to the extracellular medium or to an intracellular segregated compartment, such as the sarcoplasmic reticulum. (The subscript "out" will be used to refer to either situation.) A reaction cycle with a stoichiometry of two Ca ions transported per ATP hydrolyzed is assumed because the molar free energy available from ATP hydrolysis under physiological conditions (3) is more than adequate for the transport of two ions against the prevailing concentration gradient—i.e., $[Ca]_{out}$ in the millimolar range and $[Ca]_{cyto} < 10^{-7}$ M. The 2:1 stoichiometry is the established stoichiometry of the Ca pump of muscle sarcoplasmic reticulum (4). Initial studies of a plasma membrane ATP-driven Ca pump report a 1:1 stoichiometry, but kinetic data in the same paper suggest that 2:1 may be more likely (5). The analysis in the present paper neglects the possible electrical contribution to the work of transport that can arise from a transmembrane potential, because theoretical questions pertaining to the effect of a potential on kinetic parameters have not yet been adequately discussed in the literature. (For the sarcoplasmic reticulum and red cell plasma membrane Ca pumps, the electrical contribution would in any case have to be insignificant.)

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REACTION SCHEME AND LIMITS ON RATE CONSTANTS

Fig. 1 shows the reaction scheme adopted for the analysis, which is a modified version of the "minimal" reaction sequence for an ATP-driven Ca pump given by de Meis and Vianna (6). E and E′ designate two different conformational states of the protein, with Ca binding sites facing the cytoplasm of the cell in state E and facing the opposite side of the membrane in state E′. The phosphorylated forms of E and E′ have quite different chemical reactivities, and this is indicated by use of different symbols for the phosphoenzyme bonds. The scheme conforms to the general rules for vectorial transport systems proposed by Jencks (1).

There are well-established upper limits on the magnitude of rate constants for individual steps of enzymic reactions (7) and these limits have to be made part of the analysis if physically meaningless solutions of the rate equations are to be avoided. One severe restriction, for a pump designated to operate at a significant rate when $[Ca]_{cyto}$ is as low as 10^{-7} M, is on the rate constant for the binding of Ca to E. This rate constant cannot be larger than the rate constant for a diffusion-controlled reaction—about 10^8 to 10^9 $M^{-1}sec^{-1}$. I have accordingly imposed an upper limit of 10^9 $M^{-1}sec^{-1}$ on k_{23} (note that $k_{12} = 2k_{23}$). A similar restriction applies to the rate constants for binding of Ca to E′, but the situation here is quite different because $[Ca]_{out}$ is large (assumed value 3 mM, see below). If the rate constant for binding of Ca to the E′ sites is at all close to the value for a diffusion-controlled reaction, the actual rate of binding at $[Ca]_{out} = 3$ mM must be very much larger than the overall turnover rate of the pump. The rate of dissociation of Ca from these sites is also unlikely to represent a rate-limiting step in the transport cycle (the results in effect show that it cannot be), and this leads to the conclusion that Ca binding to the sites of E′ can without loss of generality be considered to be a rapid equilibrium process, as has been done in the scheme of Fig. 1.

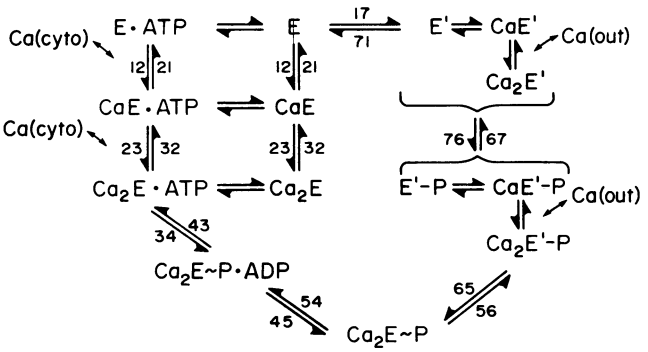

FIG. 1. Minimal reaction scheme. The numbers refer to the numbers assigned to rate constants k_{ij} for individual steps. Rate constants for steps in which something is bound to the protein are bimolecular—e.g., k_{12} is the rate constant for the reaction Ca(cyto) + E → CaE. The two Ca binding sites on E are assumed to be identical and independent, and there is assumed to be no interaction between these sites and the binding site for ATP. In algebraic terms, this is expressed by the relations $k_{12} = 2k_{23}$ and $k_{32} = 2k_{21}$ and by the identity of these rate constants for binding of Ca to E and to E·ATP. Unnumbered steps were treated as rapid equilibria. See text for other details.

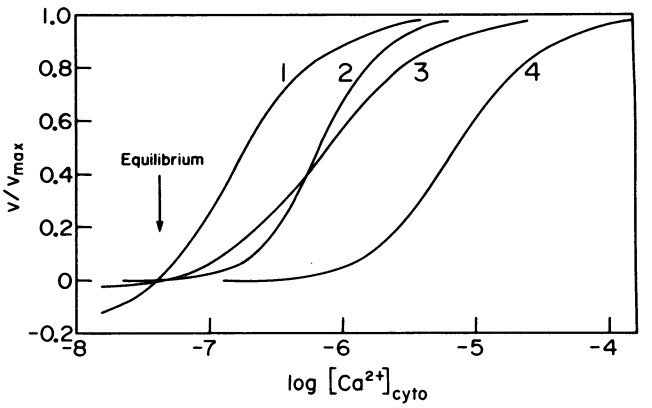

FIG. 2. Typical computer-generated rate curves. Values for V_{max} range from 35 to 62 cycles sec^{-1}. Hill plot slopes at the midpoint are 1.34, 1.81, 1.05, and 1.29, respectively, for curves 1, 2, 3, and 4. Curve 4 is not acceptable because physiological function requires a midpoint for the rate curve at $[Ca]_{cyto} < 10^{-6}$ M.

Four of the steps of the scheme of Fig. 1 represent chemical transformations of substrate (phosphoenzyme formation) or protein conformational changes. First-order-reaction rate constants for such processes are always relatively small (7), usually $<10^4$ sec^{-1}. A conservative upper limit of 3×10^4 sec^{-1} has accordingly been imposed on the forward and reverse rate constants for these steps.

The following additional assumptions were made. The binding of ATP to E (with or without bound Ca) was treated as a rapid equilibrium because the physiological steady-state concentration of ATP is large. It was decided arbitrarily not to investigate the effect of varying the parameters involved in the phosphorylation of E' by P_i. The sequence $E' \rightleftharpoons E'\cdot P_i \rightleftharpoons E'$-P has been treated as a single step in Fig. 1, and the rate constants for that step are pseudounimolecular rate constants, k_{76} implicitly containing the concentration $[P_i]$. All rate and equilibrium constants are in principle functions of pH and of the concentration of Mg and K ions, and the values used are intended to apply to the physiological levels of these substances. Likewise, concentration terms such as [ATP] refer to the equilibrium mixture of different ionization states and complexes such as MgATP, as they exist in the physiological milieu.

PHYSIOLOGICAL STEADY STATE

Cytoplasmic concentrations of ATP, ADP, and inorganic phosphate (P_i) are maintained at constant levels (at least in the resting state) by metabolic processes that operate faster than the pump. The following values have been assumed (8) and treated as invariants: [ATP] = $[P_i]$ = 5 mM; [ADP] = 0.04 mM. A constant value has been used also for the extracytoplasmic Ca concentration, $[Ca]_{out}$ = 3 mM. The sarcoplasmic reticulum Ca pump, immediately after muscle contraction, may work against a lower $[Ca]_{out}$, but its physiological effectiveness in maintaining a low value of $[Ca]_{cyto}$ in the resting state is determined by its ability to operate at a reasonable rate when the concentration in the sarcoplasmic reticulum lumen is in the millimolar range.

With all other concentration terms fixed, physiological function is completely described by a plot of the reaction rate (V) as a function of $[Ca]_{cyto}$, as illustrated by Fig. 2. Each curve is effectively specified by only three kinetic parameters: the maximal velocity (V_{max}) attainable at high $[Ca]_{cyto}$; the midpoint of the curve, *often called* the "apparent K_m," which will be designated here as $[Ca]_{1/2}$; and the degree of cooperativity with respect to $[Ca]_{cyto}$, conveniently expressed in terms of the slope of a kinetic Hill plot. The number of variable rate and equilibrium constants in the scheme of Fig. 1 is much larger than three, and therefore many different combinations of these parameters can be expected to lead to essentially indistinguishable rate curves.

Two conditions specify whether a rate curve is "acceptable" for its intended physiological function. (*i*) The pump must operate at an adequately high rate at high values of $[Ca]_{cyto}$, which imposes a lower limit on V_{max}. All ion pumps studied so far have been found to have a maximal turnover rate under optimal laboratory conditions of the order of 100 sec^{-1}, indicating the existence of one or more "difficult" steps in any real pump mechanism, and this creates an upper limit on V_{max} in addition to the physiologically required lower limit. (*ii*) The physiological steady-state concentration of cytoplasmic Ca is the concentration at which the pump reaction exactly neutralizes the inward movement of Ca resulting from passive leaks and other dissipative processes. A pump designed to maintain $[Ca]_{cyto}$ below 10^{-7} M must therefore maintain a reasonable level of activity at very low values of $[Ca]_{cyto}$ in the vicinity of 10^{-7} M. When combined with the upper limit on V_{max} and with reasonable estimates of leak rates, this leads to $[Ca]_{1/2} < 10^{-6}$ M as a reasonable criterion for an acceptable rate curve. Curves 1–3 in Fig. 2 satisfy this condition, but curve 4 does not. The experimentally observed $[Ca]_{1/2}$ is $<10^{-6}$ M both for the sarcoplasmic reticulum Ca pump and the red cell plasma membrane Ca pump (4, 5).

Cooperativity with respect to $[Ca]_{cyto}$ is also a physiologically helpful property if transient increases in $[Ca]_{cyto}$ above its steady-state value are expected to be encountered, as would be true for a muscle cell. The response of pump velocity to increasing $[Ca]_{cyto}$ is steeper (as in curve 2 of Fig. 2) when the degree of cooperativity is high. However, cooperativity was not treated as an essential factor in the present analysis: both high and low values for the Hill coefficient were considered acceptable.

All ATP-driven pumps are reversible (9) and can be used to synthesize ATP under appropriate laboratory conditions—i.e., [ATP] = $[Ca]_{cyto}$ = 0 and high values of [ADP], $[P_i]$, and $[Ca]_{out}$. A reasonable reverse rate under these conditions was one of the requirements imposed on the analysis. (Whether it is theoretically possible to design an ATP-driven pump with one very slow reverse step, and still to satisfy all other conditions, was not

tested.) The reversibility of the pump under laboratory conditions does not imply that a significant reverse reaction rate ($-V$ in Fig. 2) can be attained at physiological levels of ATP, ADP, P_i, and external Ca, when $[Ca]_{cyto}$ drops below its equilibrium value. In fact, $dV/d[Ca]_{cyto}$ cannot change abruptly at the equilibrium point in Fig. 2 ($V = 0$), so that, as the figure shows, negative rates are significantly large only for curves with very low $[Ca]_{1/2}$.

METHOD OF CALCULATION

The differential equations that define the steady state according to the scheme of Fig. 1 can be written down on inspection. The simplest way to obtain a numerical solution for a given set of kinetic parameters is to use the matrix method for simultaneous linear equations (10). Alternatively, a closed form equation can be derived by use of standard procedures of enzyme kinetics (11–13). A closed form equation was used here because it is more convenient when a large number of calculations is to be carried out. To simplify the equation, the binding of ADP to $Ca_2E \sim P$ was treated as a rapid equilibrium process. A sufficient number of solutions was checked by the matrix method to provide assurance that no error had been made in the derivation of the closed form equation. The matrix method was also used to test the effect of the assumption of rapid equilibrium for ADP binding, and it demonstrated that this assumption has no significant influence on the results if it can be assumed that the rate constant for ADP binding is reasonably close to the value for a diffusion-controlled bimolecular reaction.

The kinetic parameters of Fig. 1 are not all independent because the equilibrium condition for the overall reaction has to be satisfied. As shown previously (3), $([Ca]_{out}/[Ca]_{cyto})^2$ is of the order of 5×10^9 at equilibrium, which leads to the relation

$$\frac{K^2_{Ca(cyto)}K_{34}K_{56}K_{ATP}[ATP]}{K^2_{Ca(out)}K_{17}K_{76}K_{ADP}[ADP]} = 5 \times 10^9, \qquad [1]$$

in which $K_{ij} = k_{ij}/k_{ji}$. K_{ATP} is the binding constant for ATP to the E state, which has the same value for binding to E, CaE, and Ca_2E because of the assumed absence of interaction between the binding sites for Ca and ATP. K_{ADP} is the binding constant for ADP, equal to k_{54}/k_{45}. $K_{Ca(cyto)}$ and $K_{Ca(out)}$ are the intrinsic binding constants for Ca to the E and E′ states, respectively. (The successive binding constants for two independent identical sites—e.g., the successive constants for $E \rightleftharpoons ECa \rightleftharpoons ECa_2$—are equal to $2K_{Ca}$ and $K_{Ca}/2$, respectively.) For binding to the sites on E, $k_{12} = 2k_{23}$, $k_{32} = 2k_{21}$, and $K_{Ca(cyto)} = k_{23}/k_{21}$. As noted before, [ATP] and [ADP] are taken as fixed at their physiological levels and the physiological level of $[P_i]$ is implicitly contained within K_{76}.

The analytical procedure consisted of a computer program in which variable parameters were each assigned a range of possible values, subject to the equilibrium condition of Eq. 1. All possible combinations of the parameters were used to generate rate curves, and results that did not satisfy the physiological requirements (V_{max} and $[Ca]_{1/2}$) or the reversibility condition were rejected. To keep the computer program within finite bounds, lower limits were assigned to all k_{ij}, so as to avoid combinations of k_{ij} values that would in any case be rejected by the requirement for minimal forward and reverse rates. Isomerization rate constants were each assigned five possible values, equally spaced logarithmically between the lower limit and the upper limit discussed earlier. $K_{Ca(out)}$ was also assigned five possible values, corresponding to degrees of saturation of 0.01, 0.10, 0.50, 0.90, and 0.99, respectively, for the Ca binding sites in state E′ at physiological $[Ca]_{out}$. K_{ADP} was assigned three possible values, again chosen to correspond to equilibrium degrees of saturation of the binding sites from below to above 0.5. K_{ATP} was also assigned three possible values. It is an experimentally known fact (4, 14) that K_{ATP} is very large (of the order of 10^6 M^{-1}), and this knowledge suggested a higher range of values for K_{ATP} than would otherwise have been chosen. (Even the lowest value employed corresponds to 97.5% saturation of the binding sites under physiological conditions.)

Because exploration of limits on $K_{Ca(cyto)}$ and $K_{Ca(out)}$ was a prime motivation for this study, additional comments concerning these binding constants are appropriate. It was intended to place no direct upper or lower limit on $K_{Ca(out)}$, and the range of values for $K_{Ca(out)}$ would have been extended if the results had indicated a need to do so. In fact, not a single acceptable solution to the rate equations was obtained with the highest assigned value, undoubtedly because a high level of saturation of the Ca binding sites of E′ would prevent attainment of a minimal value for V_{max}. Likewise, only 3% of otherwise acceptable results had $K_{Ca(out)}$ at its lowest assigned value, presumably a reflection of the reversibility condition. Thus, no need to extend the range was found. In the case of $K_{Ca(cyto)}$ there is an unavoidable upper limit because of the physical limitation on the rate constant for binding of Ca. With $k_{23} \leq 1 \times 10^9$ $M^{-1}sec^{-1}$ and the minimal value of 20 sec^{-1} imposed on k_{21}, $K_{Ca(cyto)}$ has to be $\leq 5 \times 10^7$ M^{-1}. However, no lower limit was imposed. The rate constant k_{23} was not assigned fixed values but was calculated (after all other variable parameters had been assigned) on the basis of the equilibrium condition, Eq. 1. This permitted values of k_{23} far below the upper limit and correspondingly low values of $K_{Ca(cyto)}$.

To conclude this section, it should be noted that exploratory calculations were made to test the effect of variations in the assigned limits on numerical values. The calculations showed that variations by a factor of 2 or 3 could not have significantly affected the conclusions. Likewise, increasing the *number* of choices for variable parameters (keeping more or less the same upper and lower limits) would have increased the absolute *number* of acceptable results (and thereby the time of computation) but would have had no other effect.

RESULTS

The assigned values for variable parameters led to nearly 2×10^7 possible combinations. About 98% were rejected by the absolute rate requirements (e.g., high values for all of the k_{ij} are incompatible with the upper limit on V_{max}), but the most important factor proved to be the physiological need to have $[Ca]_{1/2} < 10^{-6}$ M. Only 7,833 solutions (0.045% of the total) satisfied this requirement. Extending the calculations showed that there would have been 64,233 otherwise acceptable results with $[Ca]_{1/2}$ between 10^{-6} and 10^{-5} M, 113,117 results with $[Ca]_{1/2}$ between 10^{-5} and 10^{-4} M, and at least an equal number with even higher values of $[Ca]_{1/2}$.

Not surprisingly, selection of such a small fraction of the total number of possible results led to selective preferences for particular values of some of the assigned parameters or combinations thereof. Limitations on acceptable values for $K_{Ca(out)}$ have already been noted, and some measure of bias was evident for all parameters. To give just one kind of example, Table 1 shows that the ratios of forward and reverse rate constants for the same step (formally equal to the equilibrium constants $K_{ij} = k_{ij}/k_{ji}$) tended to be confined to fairly narrow limits. Highest and lowest values of K_{17}, K_{34}, K_{56}, and K_{76} (for 95% of the results) were found to differ by factors of 1.5×10^3 to 5×10^3. If all of the assigned k_{ij} values had been selected with equal probability, highest and lowest K_{ij} values would have differed by a factor of 2×10^5.

The observed limitations on the Ca binding constants were

Table 1. Ranges in K_{ij} representing 95% of all results with $[Ca]_{1/2} < 10^{-6}$ M

Constant	Range
$K_{Ca(cyto)}$	1.6×10^7–1.6×10^5
$K_{Ca(out)}$	340–30
K_{17}	120–0.080
K_{34}	250–0.050
K_{56}	25–0.0083
K_{76}	88–0.050

especially striking. The data given in Fig. 3 show that virtually all results with $[Ca]_{1/2} < 10^{-6}$ M have $K_{Ca(cyto)}$ within one order of magnitude of 10^6 M^{-1}. Similarly, 95% of all acceptable results make use of only two of the five assigned values for $K_{Ca(out)}$, corresponding to either 10% or 50% saturation of the binding sites in the E′ state. The observed close relation between $[Ca]_{1/2}$ and $K_{Ca(cyto)}$ was not confined to the physiologically acceptable results with $[Ca]_{1/2} < 10^{-6}$ M. For example, for one group of 1,400 results with $\log[Ca]_{1/2} = -4.5 \pm 0.1$, $\log K_{Ca(cyto)}$ was found to be in the range 4.5 ± 0.85 for 95% of the data. The explicit use of a stoichiometry of two Ca ions transported per reaction cycle is undoubtedly in part responsible for the fact that observed limits for Ca binding constants are narrower than for the other K_{ij} values listed in Table 1. Because of this stoichiometry, the Ca binding constants appear in Eq. 1 as K_{Ca}^2 and there is a corresponding kinetic duplication because $k_{12} = 2k_{23}$ and $k_{32} = 2k_{21}$. Variations in the Ca binding parameters are thus amplified and acceptable ranges become more restricted.

Another interesting aspect of the results was an interdependence between the acceptable kinetic parameters for different reaction steps, especially between steps that are adjacent in the reaction sequence. This interdependence can be explained on the basis of the fact that the net rate of all *individual* steps of a cyclic reaction sequence (where alternate pathways are not available) must at steady state be equal to the net *overall* rate (v)—i.e., for any reaction step, state $i \rightleftharpoons$ state j,

$$k_{ij}[\text{state } i] - k_{ji}[\text{state } j] = v. \qquad [2]$$

This relation manifests itself in two ways. (*i*) The equation requires $k_{ij}/k_{ji} > 1$, even when the two state probabilities are equal, and requires increasingly large values for k_{ij}/k_{ji} if kinetic parameters for other reaction steps are adjusted so as to cause an increase in the steady-state ratio [state j]/[state i]. (*ii*) The physical constraints on the k_{ij} values do not permit k_{ij}/k_{ji} to increase without limit, and this means that the state probability ratio must itself remain within a limited range. Propagation of this effect of Eq. 2 around the reaction cycle means that all state probabilities must remain at roughly comparable values (1). Conditions that lead to accumulation of most of the pump protein in a single state could not lead to an acceptable rate curve.

All observed interdependence relations were consistent with this kind of explanation. For example, there were 318 acceptable results in which k_{56} had its highest allowed value (3×10^4 sec^{-1}). In 313 of these results, k_{65} was at or *above* its median value, and in all 318, k_{76} was at or *below* its median value. The bias in both cases serves to prevent too much of the protein from being tied up in the Ca_2E'-P $\rightleftharpoons$ CaE′-P $\rightleftharpoons$ E′-P equilibrium mixture. Another example is provided by Fig. 4, which shows that acceptable values for K_{17} depend strongly on K_{ATP}, clearly a response to the need to increase k_{17}/k_{71} as [E] is reduced relative to [E.ATP].

Cooperativity. The results obtained did not indicate any marked preference for kinetically cooperative or noncooperative velocity profiles as a function of $[Ca]_{1/2}$. The Hill coefficients for the four curves in Fig. 2 are representative in this regard. There was some clustering of values around 1.3, but high values, approaching 2.0, and low values, approaching 1.0, were not infrequent.

DISCUSSION

The most important result derived from these calculations is the severe restriction on the binding constants for Ca on either side of the membrane. It should be emphasized that this is not a *direct* consequence of limits placed on allowed values for the rate constants. These limits create only an upper bound for $K_{Ca(cyto)}$ and do not affect $K_{Ca(out)}$ at all. The observed result is also not directly attributable to thermodynamic constraints. Thermodynamics requires a very large equilibrium value for the ratio $[Ca]_{out}/[Ca]_{cyto}$, but the formal expression of this requirement (Eq. 1) does not demand $K_{Ca(cyto)} \gg K_{Ca(out)}$ because the other equilibrium constants could readily take on values (even

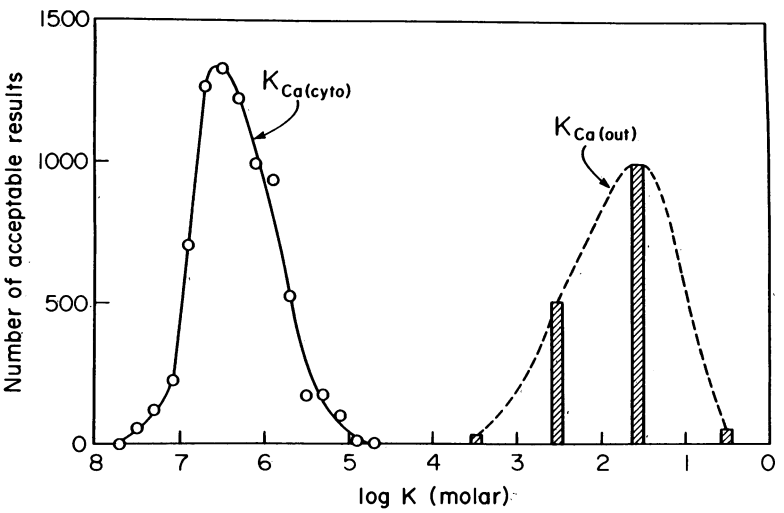

FIG. 3. Values of binding constants for Ca for all computed curves with $[Ca]_{1/2} < 10^{-6}$ M. The points for $K_{Ca(cyto)}$ represent *actual* numbers of acceptable values (of a total of 7,833) with $\log K_{Ca(cyto)}$ within ±0.05 of the value indicated on the abscissa. The analytical procedure allowed only five possible values for $K_{Ca(out)}$ and the bars represent *relative* numbers of solutions corresponding to each value. No acceptable solutions were obtained for the highest allowed value of $K_{Ca(out)}$. (Experimental points here and in Fig. 4 are not expected to generate perfectly smooth curves because most of the parameters that enter into the calculation were varied stepwise rather than continuously.)

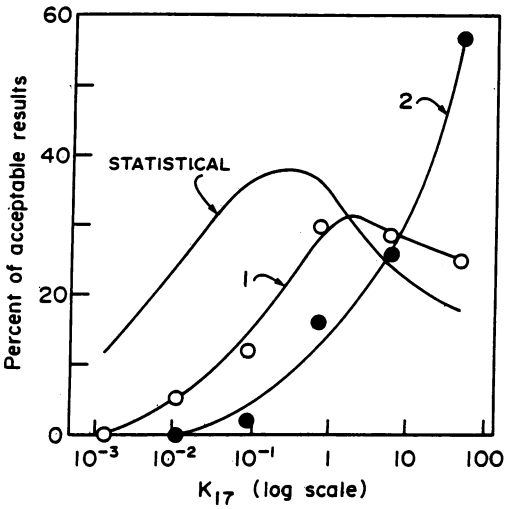

FIG. 4. Observed value for $K_{17} = k_{17}/k_{71}$ for results with $[Ca]_{1/2} < 10^{-6}$ M. Each point gives the number of results with log K_{17} within ±0.45 of the value indicated on the abscissa. Curve 1 represents data with $K_{ATP} = 8 \times 10^3$ M^{-1}, curve 2 data with $K_{ATP} = 8 \times 10^5$ M^{-1}. Combined data for all values of K_{ATP} would fall between these two curves. The curve labeled "statistical" is the distribution expected if all possible combinations of k_{17} and k_{71} were to appear among the results with equal probability.

within the assigned k_{ij} limits) that would satisfy the equation even with $K_{Ca(cyto)} = K_{Ca(out)}$. The result appears instead to reflect an inflexibility of the overall kinetics of the cycle that does not permit the kinetic parameter $[Ca]_{1/2}$ (apparent K_m) to become very different from the corresponding thermodynamic dissociation constant $1/K_{Ca(cyto)}$. Stated in this way, the result is not restricted to physiologically relevant conditions, but is applicable to all values of $[Ca]_{1/2}$.

Another important aspect of the data is the support they provide for the statement made by Jencks (1), that the concentrations of intermediate states of the transport protein should be roughly comparable at the physiological steady state if a reasonable turnover rate under these conditions is to be maintained. The limitation on Ca binding constants may be viewed as a special example of the same effect, because it serves to maintain filled and unfilled Ca binding sites at roughly equal levels under steady-state conditions. The principle involved here is perhaps intuitively obvious, given the finite range of values that rate constants can adopt, but purely formal analyses of free energy transduction often ignore the limits on k_{ij}, and it is useful to demonstrate by actual calculation that this can lead to unrealistic possibilities for reaction mechanisms. (Eq. 2 shows that this principle applies only to intermediates that undergo inherently slow reactions, with a modest upper limit on k_{ij} for conversion to at least one of the two adjacent intermediate states. It would not apply to transient states, reacting very rapidly in both directions, which might be included in a more elaborate scheme than that of Fig. 1. Steady-state levels of such states can of course be extremely small.)

The calculations of this paper explore limits on what is possible, and there was no intention to predict experimental data for any known system. However, experimental data for the sarcoplasmic reticulum Ca pump (the only system for which adequate data exist) are consistent with the results obtained here. For example, recent data by Inesi *et al.* (15, 16) yield $K_{Ca(cyto)} = 10^7$ to 10^8 M^{-1} and $K_{Ca(out)} = 300$ M^{-1}. Work from this laboratory (17) has recently shown by indirect means that $K_{17} \gg 1$. Given that K_{ATP} is large for this system, the result supports the conclusion from Fig. 4 that a high value of K_{ATP} should optimally be coupled with a high value of K_{17}.

It is worth noting in conclusion that the results of this study have some relevance to the mechanism of free energy transduction in active transport. The values for the Ca binding constants (both theoretical and experimental) support a structural model of the kind proposed some years ago by Jardetzky (18) and recently extended (19).

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