"Adaptive" Behavior of Ligand-Gated Ion Channels: Constraints by Thermodynamics

Michael D. Stern
Division of Cardiology, Johns Hopkins Medical Institutions, Baltimore, Maryland 21205 USA

ABSTRACT The calcium-induced calcium release channel of the cardiac sarcoplasmic reticulum has been reported to inactivate in a novel manner (termed "adaptation"), which permits reactivation by exposure to successively higher concentrations of calcium. I examined the limitations placed by thermodynamics on the possible kinetic mechanisms for such behavior. The mechanism suggested by Györke and Fill, in which the affinity of a calcium-binding site decreases during adaptation, is not thermodynamically feasible for a passive system, but requires an external input of free energy. Possible sources of such energy are 1) metabolic energy, which is excluded by the fact that adaptation was observed in isolated channels in the absence of ATP, or 2) coupling of ion permeation to gating, for which there is currently no evidence. I derived a general limit on the thermodynamic feasibility of a sequence of channel activations and adaptations, irrespective of channel kinetics, from the requirement that the free energy must decrease during the spontaneous evolution of the system from the state existing immediately after a step increase in $[Ca^{2+}]$ to the state of maximum open probability that follows. The opening of the channel must involve an increase in free energy, which must be compensated by the free energy released by the incremental binding of calcium. This requirement leads to a complicated system of inequalities, which was simplified and manipulated algebraically into the form of a linear programming problem. Numerical solution of this problem showed that the sequence of adaptations of the SR channel observed by Györke and Fill requires the presence of at least 10 calcium-binding sites on the channel if it is to occur in the absence of exogenous sources of free energy. This indicates either that a large number of calcium-binding sites participate in the regulation of the SR calcium release channel, or that the existing data are significantly flawed with respect to the low open probability in the resting state, the importance of "calcium spike" artifacts from flash photolysis, or both.

INTRODUCTION

Excitation-contraction coupling in the heart is mediated by a process of calcium-induced calcium release. Central to this process is the sarcoplasmic reticulum (SR) calcium release channel or "ryanodine receptor" (RyR), which gates the release of SR calcium in response to trigger calcium entering the cell during the action potential. There is a paradox involved in this process, in that the quantity of calcium released from the SR is smoothly graded as a function of the amount of trigger calcium, even though the released calcium might be expected to feed back and trigger the release of more calcium regeneratively. There are a variety of approaches to explaining this paradox (Stern, 1992), but all depend on understanding the kinetics of the RyR, and, in particular, whether it inactivates.

Györke and Fill (1993) presented evidence for a novel form of inactivation of the RyR that they term "adaptation." Single RyR channels incorporated into lipid bilayers, when exposed to a jump in *cis* (cytosolic face) calcium concentration, displayed a rapid increase in open probability, which then declined over several seconds. Unlike a conventional inactivation process, however, a further increment in bathing calcium was able to elicit a second transient period of high open probability. This process could be repeated several times.

The adaptation phenomenon remains controversial because it has only been demonstrated with calcium steps produced by photolysis of caged calcium using nanosecond laser pulses. This produces an overshoot of $[Ca^{2+}]$ lasting microseconds because the calcium released from the photolyzed chelator (DM-Nitrophen in the experiments of Györke and Fill) takes a finite time to reequilibrate with the remaining calcium buffers. This brief overshoot is difficult to measure, but calculations indicate that it may be as large as several orders of magnitude (Lamb et al., 1994). Such an overshoot could produce a transient activation of the channel, causing the artifactual appearance of "adaptation" as the channel relaxed to its steady-state response. However, the adaptive response of the RyR channel remains consistent, despite expected variations in the size of the overshoot under different conditions, and no "adaptation" is seen when calcium-activated K channels are studied in the same apparatus (M. Fill, private communication). Because of the potential importance of adaptation to the mechanism of calcium-induced calcium release in the heart (and probably in brain and skeletal muscle as well), it is worthwhile to consider what kinetic schemes could account for such behavior. In this paper, I will not propose any explicit kinetic scheme for adaptation, but rather will deduce certain major constraints that thermodynamics places on the possible form of any such model.

Received for publication 6 January 1995 and in final form 26 January 1996.

Address reprint requests to Dr. Michael D. Stern, 2 Avers Court, Reisterstown, MD 21136. Tel.: 410-252-4361; Fax: 410-252-3865; E-mail: mstern@welchlink.welch.jhu.edu.

0006-3495/96/05/2100/10 $2.00

In their original paper, Györke and Fill suggested a mechanism in which calcium binds to an activating site whose affinity then decreases during adaptation, causing the calcium to dissociate, thus making the site available for triggering by a further increase in calcium. A scheme of this kind appears in Fig. 1. The vertical positions of the states in this diagram were chosen to suggest their relative free energies. The relative free energies of states, which differ in their number of bound calcium ions, depend on the calcium concentration of the solution; the drawing shows the situation in a relatively low free calcium solution. In the absence of an exogenous source of free energy, the low-affinity open state must have a lower free energy than the high-affinity open state, because the transition proceeds spontaneously in the direction of the downward-slanting arrow. This relationship is independent of $[Ca^{2+}]$ because both open states have bound calcium. On the other hand, the change in free energy upon binding of calcium (length of vertical arrows) must, by definition, be larger for the low-affinity states than for the high-affinity states. The low-affinity adapted state (the state reached after dissociation of calcium from the binding site) must therefore lie in the lowest free energy position, as shown. But this implies that it, rather than the high-affinity closed state, would be the resting state of the channel in a low calcium solution. This suggests that schemes of this kind, in which a calcium-binding site is made "reusable" by dissociating the calcium after adaptation, can only work if an exogenous source of free energy is provided to drive irreversible and/or cyclic processes.

There are several possible sources of free energy that must be considered. Metabolic energy might drive irreversible channel reactions in vivo. However, the lipid bilayer experiments of Györke and Fill were conducted in the absence of ATP. This was done to avoid the binding of Mg^{2+} (a cofactor of ATP) to DM-nitrophen, but it fortuitously excluded any requirement for ATP-derived energy in the adaptation process. In addition, although ATP is known to be a powerful modulator of RyR gating, it has not been shown to be hydrolyzed by the channel.

Ion permeation

The flux of ions permeating the channel down their electrochemical gradient could provide a large source of free energy to drive reactions. This is easier to visualize in the case of a carrier than of a channel, but it has, in fact, been observed in the *Torpedo* chloride channel (Richard and Miller, 1990). If ion permeation energy played a crucial role in adaptation, then one would expect that adaptation would not occur at the reversal potential, when the electrochemical potential of the permeating ion is the same on both sides of the channel. Channel gating cannot, of course, be measured in the absence of permeating ion flow, but one would expect that, if adaptation were strongly coupled to permeation, RyR gating kinetics would be very sensitive to the direction and magnitude of current flow, and to the nature and concentration of the permeant ion. This has not been reported, although it has not been searched for carefully.

Dissipative calcium binding and dissociation

The process of raising and lowering $[Ca^{2+}]$, with the consequent binding and dissociation of calcium from binding sites, is an irreversible process in which energy, supplied by the external system (which varies $[Ca^{2+}]$), is dissipated. It cannot, therefore, be taken for granted that equilibrium thermodynamic arguments can fully characterize the possible behavior of such a system.

I will demonstrate below that, even for a nonequilibrium system, it is possible to generalize the themodynamic argument given above to an arbitrary kinetic model, leading to a requirement for a large number of calcium-binding sites to fit the data of Györke and Fill. The essence of the argument is relatively simple. The open probability of the channel is very low when it has adapted to resting steady-state $[Ca^{2+}]$. This implies that the free energy of the open state must lie above the mean free energy of the channel molecule by an amount that is large compared to kT. The bathing calcium is then stepped to a higher value. The subsequent evolution of the channel passes through a state of substantial peak open probability PK_i. Because this evolution proceeds spontaneously, the second law of thermodynamics requires that the free energy of the system must decrease, which requires that the free energy needed to promote the channel to the open state must be less than that released by the incremental binding of calcium.

The situation is shown schematically in Fig. 2, which is built around actual data taken from Györke and Fill (1993). The bathing $[Ca^{2+}]$ is stepped up repeatedly, as shown in the bottom row of the figure (the rise occurs within microseconds, but appears slow because of the response time of the calcium electrode used to monitor it). After each step,

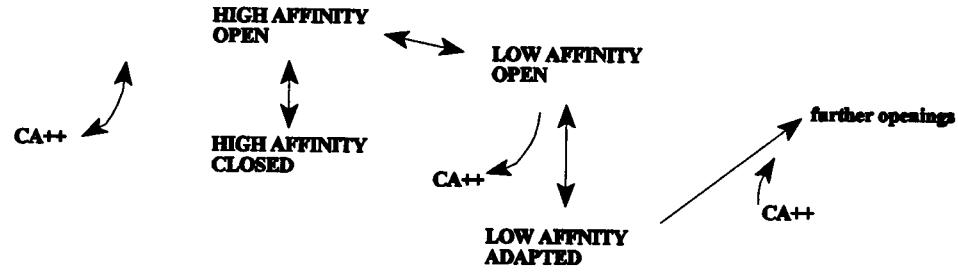

FIGURE 1 A possible kinetic scheme embodying the suggestion of Györke and Fill that adaptation is produced by a conformational change that reduces the affinity of a calcium-binding site. The vertical position of the states reflects their relative free energies (see text).

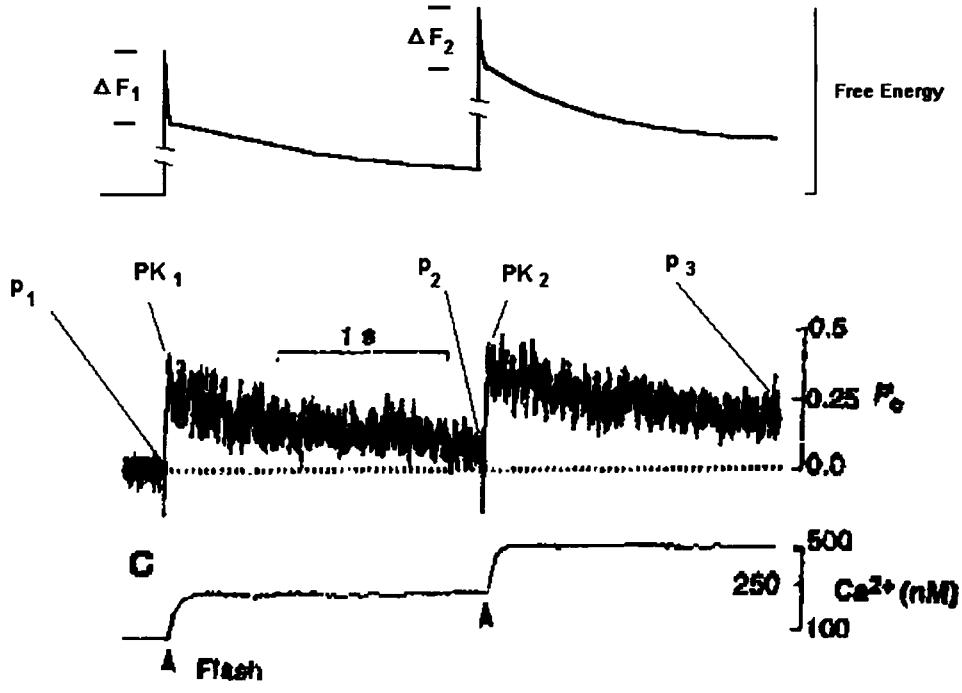

FIGURE 2 The sequence of events during two sequential calcium steps applied to a single sarcoplasmic reticulum calcium release channel in a lipid bilayer. The bottom two rows, taken from the data of Györke and Fill, show the free calcium of the bathing medium and the ensemble averaged open probability of the channel. The top row is a sketch of the free energy changes of the system that must underlie the spontaneous occurrence of the process. The equilibrium and peak open probabilities are labeled with the variables that represent them in the theoretical derivation given in the text.

the open probability of the channel increases from its equilibrium value p_i to a peak value PK_i, and then falls again to the new equilibrium value p_{i+1}. The free energy changes associated with this process are sketched in the top row. Each time $[Ca^{2+}]$ is increased, the free energy of the system increases to a new value $F_{max,i}$ as a result of the change in free energy of the calcium solution, the channel having not yet responded. This energy derives ultimately from the energy invested in the system by the laser flash. Then, as the open probability of the channel spontaneously increases, the free energy of the whole system must decrease by an amount ΔF_i. On the other hand, the free energy $F_{max,i} - \Delta F_i$ of the peak open state must, by definition, be larger than the minimum possible free energy $F_{min,i}$ of all states with open probability PK_i. This latter value can be calculated from the standard free energies of the channel states, without reference to the actual kinetics of the channel. Then, a fortiori, $F_{max,i} > F_{min,i}$. For the process in the figure to be realizable for a channel with a given set of states, it must be possible to find a set of standard free energies for those states such that this inequality is satisfied for each of the calcium steps, subject also to the constraint that the equilibrium open probabilities at each of the specified bathing calcium concentrations should, in fact, be the observed p_i. As I shall show below, for a given set of (experimentally observed) open probabilities, this realizability condition can only be satisfied if the number of calcium-binding sites on the channel is sufficiently large. This is logical, because the binding of calcium to these sites must provide the free energy required to open the channel.

The calculation required to demonstrate this is quite long and will only be presented in outline here. The starting point is the expression for the free energy F of a dilute solution of channel molecules, the total concentration of which, a, is assumed to be very small, together with free calcium:

$$F = \sum f_k(c_k) + f_{Ca}(c_{Ca}) \tag{1}$$

$$f(x) = x\left(RT \log\left(\frac{x}{C_0 e}\right) + \mu_x\right), \tag{2}$$

where the sum is taken over all possible states of the channel (each of which constitutes a species in the "solution"), c_x stands for the molar concentration of species x (for example $c_k = aP_k$, where k represents any state of the channel and P_k is the ensemble-average probability that the a channel is in state k). The subscripts on the f's in Eq. 1 indicate that they are to be evaluated (from Eq. 2) using the standard free energy μ_k of the species in question. The procedure is then as follows:

1. The equilibrium concentrations $c_{k,i}$ of the kth species in the ith calcium solution ($[Ca^{2+}] = c_{Ca,i}$) are determined by minimizing the free energy F, subject to the constraints of conservation of total calcium and total number of channels. This is done by the method of Lagrange multipliers.

2. The free energy $F_{max,i}$ immediately after a calcium jump is found by inserting these equilibrium concentrations into Eq. 1, together with the new value of free calcium.

3. The free energy $F_{min,i}$ is the minimum possible value of the free energy at the time of peak open probability. It is determined by varying the c_k, starting from the configuration found in step 2, so as to minimize F subject to the constraints of conservation of total calcium (in the new solution), conservation of total channels, and open proba-

bility PK_i. For the general case this can be quite messy, because the new value of $[Ca^{2+}]$ after rebinding of calcium to the channels must be computed. Because, however, it is assumed that channels are sparse compared to the number of calcium ions in the solution, the computation can be carried out to first order in a. This is equivalent to ignoring the change of $[Ca^{2+}]$, δ_{Ca}, which results from rebinding, when computing the minimizing values of c_k, which is done, again, using Lagrange multipliers. The value of δ_{Ca}, which is proportional to a, is then determined as the difference between the old and new concentrations of bound calcium. Equation 1 is expanded in a Taylor series to first order in δ_{Ca} (about the ambient calcium concentration $c_{Ca,i+1}$) to calculate $F_{min,i}$ to first order in a.

4. The difference $F_{max,i} - F_{min,i}$, which is proportional to a in this approximation, is required to be more than zero for the process to proceed spontaneously, for each specified calcium jump i. The spontaneous evolution of the system, with a decrease in free energy, may proceed by any change in the state populations, subject to the constraints that the number of channels is constant, calcium is removed from the solution as required to supply the bound calcium in the various states of the channel, and the open probability reaches the experimentally observed value. The difference $F_{max,i} - F_{min,i}$ is an upper bound on the possible decrease of free energy along such a path; if it is negative, there can be no spontaneous reaction path that satisfies these constraints.

This would seem to be an impossible program for a completely arbitrary channel. However, the problem can be simplified by noting that the required thermodynamic states of the system are, at each step, those that minimize F, subject to some set of constraints. One partitions the states of the channel into groups of states with the same number of bound calcium ions. The minimization can then be carried out in two stages. In the first stage, one minimizes with respect to variations of population within a group, subject to the constraint that the total population of the group remains constant (call it a_i). In the second stage, one minimizes with respect to variations in a_i, subject to the constraint that $\Sigma a_i = a$, conservation of total calcium, and, when carrying out step 3, fixed open probability PK_i. If the open states are grouped separately, then the minimizations in the first stage are independent and do not affect the total calcium balance (because the amount of bound calcium is the same for all states in a group) or the total open probability. Therefore, one can carry out the intra-group minimizations "in advance," i.e., assume that all of the states within a group are in mutual equilibrium. The minimization of the free energy of a group, by the method of Lagrange multipliers, leads to

$$c_{ij} = \frac{a_i e^{-(\mu_{ij}/RT)}}{Z_i} \tag{3}$$

$$Z_i \equiv \sum_j e^{-(\mu_{ij}/RT)}, \tag{4}$$

where the sum is taken over all the states in group i. As one might expect, this takes the form of an equilibrium canonical ensemble (partition function Z_i), for the group of states i, which are in equilibrium with each other under conditions that minimize the free energy.

The minimum free energy of the group is then found to be $F_i = f_i(a_i)$, where f_i signifies the function defined in Eq. 2, evaluated using the standard free energy $\mu_i = RT \log(Z_i)$. In other words, the equilibrium ensemble of states in one group that is found in the first stage of the global minimization is thermodynamically equivalent to a single state with standard free energy μ_i. This means that one can represent any channel by a channel that has $n + 1$ closed states (with $0, \ldots n$ calcium ions bound, where n is the maximum number of calcium ions bound in any closed state of the original channel) and one open state (with m calcium ions bound). The assumption is made here that all open states have the same number of bound calcium ions; this is not essential to the method, but markedly simplifies the computation, which is very large, even with this simplification.

One is now in a position to carry out the calculation outlined in steps 1–4 above. This leads to an expression for $F_{max,i} - F_{min,i} > 0$ that fills several pages and will not be reproduced here. Fortunately, it is possible to simplify this appreciably by a large amount of algebra (partial factorizations, use of logarithmic identities, and discarding positive factors). This was done using the computer algebra language Macsyma (Macsyma Corp., Arlington, MA). In addition one has the constraints that $c_{o,k} = ap_k$, i.e., the equilibrium (i.e., adapted) open probability at each calcium level must be the observed value. For each calcium jump, the two constraint equations corresponding to the calcium levels before and after the jump can be reduced to one equation by eliminating μ_o, the standard free energy of the open state, between them, i.e., the free energy of the open state is determined, relative to other states, by the requirement of low resting open probability, as discussed above. The resulting single constraint equation (for each calcium jump k) is then used to simplify further the inequality $F_{max,k} - F_{min,k} > 0$, ultimately reducing it to a linear inequality.

The final result of these manipulations is a pair of systems of constraints:

$$\sum_{j=0}^{n} Q_{kj} r_j > 0 \tag{5}$$

$$\sum_{j=0}^{n} W_{kj} r_j = 0, \tag{6}$$

where the variables r_j are defined as

$$r_j \equiv \left(\frac{c_{ca,1}}{c_0}\right)^j e^{-(\mu_j/RT)}, \tag{7}$$

and the coefficients are defined as

$$Q_{kj} \equiv -\left(\frac{c_{ca,k}}{c_{ca,1}}\right)^{j}\left(\log\left(\frac{c_{ca,k+1}}{c_{ca,k}}\right)((PK_k - p_k)m + j(p_k - 1))\right.$$

$$- (PK_k - 1)\log\left(\frac{p_k(c_{ca,k+1}/c_{ca,k})^{m}(p_{k+1} - 1)}{(PK_k - 1)p_{k+1}}\right) \quad (8)$$

$$\left. - PK_k \log\left(\frac{PK_k}{p_k}\right)\right)$$

$$W_{kj} \equiv \frac{(p_k - 1)c^{j}_{ca,k+1}p_{k+1} - \dfrac{p_k c^{m}_{ca,k+1}(p_{k+1} - 1)}{c^{m-j}_{ca,k}}}{c^{j}_{ca,1}}. \quad (9)$$

To recapitulate, I have shown that, for any channel scheme, for the observed sequence of activations and adaptations to be thermodynamically allowed, the inequalities 7 and Eqs. 8 must be satisfied, where n, the upper limit of the summations, is the maximum number of bound calcium ions in any closed state, and m is the number of calcium ions bound in the open state. Max(n, m) is therefore the number of calcium-binding sites on the channel. The kinetics of a particular channel scheme are identified here by the standard free energies μ_j of the closed states (actually effective values for groups of closed states with j bound calcium ions). Therefore, for the observed adaptation to be possible for a given number of calcium-binding sites, there must exist some set of μ_j for which Eqs. 5 and 6 are satisfied. By Eq. 7, this is equivalent to saying that there must exist a set of r_j for which Eqs. 5 and 6 are satisfied. Because μ_j may be chosen arbitrarily, the only constraint on the choice of r_j, by Eq. 7, is

$$r_j \geq 0, \quad (10)$$

the choice $r_j = 0$ corresponding to placing the free energy of the state so high that it is energetically inaccessible (in other words, removing the state j from the system). Finally, note that the inequalities 5 are definite, linear, homogeneous inequalities. Therefore, if a set of r_j can be found that satisfy Eqs. 5 and 6, they can always be scaled up by a positive factor so as to make the smallest value of Eq. 5 equal to 1. Therefore, so far as the existence of solutions goes, Eq. 5 is equivalent to

$$\sum_{j=0}^{n} Q_{kj} r_j \geq 1. \quad (11)$$

The constraints 6, 10, and 11 form a standard problem in the field of linear programming. A Fortran program was written to determine the existence of solutions for 6, 10, 11 using the Simplex algorithm of linear programming, adapted from *Numerical Recipes* (Press et al., 1990). The numerical data were taken from the experiment shown in Fig. 2. Calcium levels were 100, 200, and 500 nM. The peak open probabilities PK_i were 0.3 and 0.4. The steady-state (adapted) open probabilities p_2 and p_3 were 0.06 and 0.2, respectively. The resting (adapted?) open probability in the starting solution with 100 nM free calcium is not distinguishable from zero in the figure. Openings of the channel in this condition are very rare, amounting to an open probability $p_1 < 10^{-4}$ (M. Fill, private communication). The existence of solutions to Eqs. 6, 10, and 11 is shown in Fig. 3. No solutions exist for small numbers of calcium-binding sites. This is mainly due to the small open probability of the channel in 100 nM free calcium, which implies that the open state must lie at a free energy substantially above the resting free energy of the molecule, in units of kT. For $p_1 = 10^{-4}$, there must be at least 10 calcium-binding sites; for $p_1 = 10^{-3}$ this is relaxed to 7. Note also that the number of calcium ions that can be bound in a closed state must equal or exceed the number bound in the open state for a solution to exist. This is logical, because the channel must be able to "adapt" into a closed state even while the bathing calcium remains elevated. This is further evidence of the nonreusability of calcium-binding sites in a thermodynamically passive system.

DISCUSSION

The adaptation of the ryanodine receptor described by Györke and Fill represents a novel type of behavior for an ion channel. Its possible functional consequences are considerable. Adaptation would endow the channel with a kind of rate-sensing capability. In its ideal form, an adaptive channel would fully adapt to any steady concentration of the ligand, and then respond sensitively to changes in the concentration of that ligand, even if the change were small in relation to the steady background level. In other words, the ideal adaptive channel would behave like an analog differentiator. There are many potential uses for such a device in cell physiology. The work of Fabiato (1985) provides evidence that the calcium-induced calcium release (CICR) mechanism of the cardiac sarcoplasmic reticulum is sensitive to the rate of calcium application, and a rate-sensitive calcium release channel might assist in explaining the paradoxical stability of CICR in the intact cell despite the positive feedback it creates (Stern, 1992). In general, there are many situations in which signaling occurs via a messenger ligand whose background concentration may be unknown a priori. Teleologically, nature might be expected to make good use of a molecular differentiator.

The analysis in this paper shows that there are serious limits to the extent to which a passive molecular device such as a channel can act like an ideal differentiator for a chemical signal. At the molecular level, everything is in constant thermal motion. For a resting channel to remain closed despite thermal fluctuations, there must be a free energy barrier preventing its opening; this may be pictured as a spring holding the channel closed. Any signal that opens the channel must provide the energy to surmount this barrier. The free energy available from an increase in the concen-

n=1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
m=1														
2														
3														
4														
5														
6														
7						*	*	*	*	*	*	*	*	*
8							*	*	*	*	*	*	*	*
9								*	*	*	*	*	*	*
10									+*	+*	+*	+*	+*	+*
11										+*	+*	+*	+*	+*
12											+*	+*	+*	+*
13												+*	+*	+*
14													+*	+*
15														+*

FIGURE 3 Solutions to Eqs. 6, 10, and 11 for various combinations of n, the maximum number of calcium ions bound in closed states (*across*), and m, the number of calcium ions bound to the open state(s) (*down*). The plus signs indicate the existence of a solution when $p_1 = 10^{-4}$, and the asterisks show allowed solutions when this is relaxed to $p_1 = 10^{-3}$.

tration of a ligand against an already large background concentration is small unless a large number of binding sites for that ligand are present. The number of binding sites required depends inversely on the ratio of the ligand concentration change to the background concentration. Therefore it is not possible for a passive molecular device with a reasonable number of ligand binding sites to serve as an ideal differentiator, detecting small changes in ligand concentration against a much larger background. As the calculations above show, even the sensing of relatively moderate concentration changes (about twofold) by the ryanodine receptor requires a large number of calcium-binding sites. The ryanodine receptor is a very large molecule, a homotetramer of large peptides. It is not implausible that there could be many calcium-binding sites on such a structure, and in fact, studies using cloned hybrid proteins have suggested the presence of as many as five calcium-binding sites per peptide on the skeletal muscle ryanodine receptor (Chen and MacLennan, 1994). The active involvement of so many calcium-binding sites in the gating of the channel implies the existence of a large number of states. This is also suggested by the single-channel records of Györke and Fill, which show channel adaptation occurring in a gradual manner, even in a single sweep. The presence of many states that are significant to the gating behavior of the channel will make it difficult to validate explicit kinetic models of the channel.

Györke and Fill have suggested that the so-called quantal release phenomenon exhibited by IP3-gated calcium release channels (which are homologous to ryanodine receptors) may actually be single-channel adaptation. Photoaffinity labeling studies have suggested the presence of a single type of IP3 binding site (Mourey et al., 1993), and rapid mixing kinetic studies in permeabilized cells have suggested the cooperative involvement of four identical sites (one on each peptide) in the rapid opening of the channel, although other, slower binding sites may exist and might be involved in adaptation (Meyer et al., 1990). Whether these results are thermodynamically compatible with adaptation depends on how low the "adapted" open probability is at each steady level of IP3; studies of the adaptation phenomenon in single IP3 channels are needed. It is interesting to note that many other ligand-gated channels are coupled to a G-protein cascade, which provides active amplification, freeing them from the need to obtain the free energy for signal transduction from the ligand binding itself.

The advantage of the thermodynamic method of analysis is that it is very general. The only assumption made is that the adapted states reached long after each calcium jump are equilibrium states. In addition, the calculation assumed that all open states carry the same amount of bound calcium. This is probably not a fundamental restriction; the analysis could be carried out using separate groups of open and closed states at each calcium binding stoichiometry. However, this would substantially complicate an already large algebraic problem, and it is not clear whether the final simplifications that result in a linear system of inequalities would exist, because these depend on substituting the equilibrium open probability constraints in the free energy inequality to eliminate logarithmic terms. In view of the requirement that large numbers of calcium-binding sites be occupied in the open state, it seems unlikely that the addition of open states with smaller numbers of bound calcium

ions would qualitatively change the thermodynamic results, although such states might be kinetically important.

The calculation presented above was carried out using a mixture of thermodynamic and kinetic concepts, and relied on the use of the expression for the free energy of an ideal solution, and the application of that free energy in the slowly varying nonequilibrium states that describe the evolution of the channel after each perturbation. The need to justify this procedure may be avoided if the result is regarded as a theorem about kinetic models rather than actual physical channels. The calculation relied upon the assumption that the ideal free energy (Eqs. 1 and 2) is nonincreasing during the free evolution of the system. As shown in the Appendix, for any standard chemical kinetic model based on mass action, which obeys the principle of detailed balance expected of a thermodynamically passive system, it is possible to choose a set of "standard free energies" such that the expression for the ideal free energy is nonincreasing. Then the theorem may be restated as follows: No mass action kinetic model that satisfies the detailed balance requirements that the product of equilibrium constants around any closed cycle of states is unity, and the net binding of calcium around any such cycle is zero, can fit the data of Györke and Fill, unless it allows for the binding of at least 10 calcium ions. This is a mathematical property of kinetic models; whether it is considered to be related to the second law of thermodynamics becomes a matter of taste. The application of this theorem to actual physical channels depends on how well they can be represented by mass action kinetics. It is left to the judgment of the reader whether it is likely that nonideal effects would give rise to a situation in which a physical channel that binds, say, two calcium ions would behave in a manner that can only be fit by a kinetic model with more than 10 binding sites.

It must be emphasized that the validity of the analysis above depends strictly on the fact that calcium is stepped directly to each higher steady level without overshoot. The presence of any overshoot in $[Ca^{2+}]$, particularly one of several orders of magnitude, such as has been suggested to occur after photolysis of caged calcium, could, even if brief, provide the free energy to drive the channel transiently into the open state, invalidating the above arguments. This makes it imperative to prove that "adaptation" is not the result of such an overshoot. Taken the other way, the need for such large numbers of calcium-binding sites to explain the adaptation data might be taken as evidence favoring the possibility that adaptation is artifactual. Coupling of free energy fluxes from ion permeation to gating could also invalidate the argument. Richard and Miller (1990) have observed that steady-state transition rates between open and inactivated states of the *Torpedo* chloride channel depart from detailed balance, to an extent that depends on the transmembrane ion gradient. This indicates that free energy is coupled from permeation to gating to maintain nonequilibrium gating kinetics. This was a small effect that required careful searching. If coupling of free energy from permeation to gating were to make adaptation possible for an SR channel with a small number of calcium-binding sites, then it would have to supply a substantial fraction of the energy for opening the channel. In this case, one would expect that the entire phenomenon of channel opening and adaptation would depend markedly on the electrochemical gradient across the channel pore, tending to vanish altogether at the reversal potential. Such an effect should be conspicuous and ought to be sought experimentally. In the absence of evidence for such strong coupling of permeation to gating, one must assume that the qualitative features of passive gating should be observed.

The arguments presented in this paper indicate conditions under which adaptive gating might be thermodynamically allowed. They give no indication of how to construct a kinetic scheme to realize the phenomenon, or even any assurance that one exists. Recently, several explicit models have appeared in the literature that purport to reproduce the adaptation phenomenon. Cheng et al. (1995) have proposed a model in which each polypeptide in the homotetramer possesses two cooperative calcium-binding sites that are activating and one that is inactivating. The channel opens whenever the number of activating domains exceeds the number of inactivating domains. This model has a total of 12 calcium-binding sites, so it is within the thermodynamic limits computed above. The model is able to give a good qualitative reproduction of the data of Györke and Fill, although the predicted steady-state open probability in 100 nM calcium is too large (0.04). Sachs et al. (1995) have proposed a simple four-state model with one calcium-binding site. Adaptation is due to an allosteric transition that switches the calcium-binding site from activating (unoccupied = closed, occupied = open) to inactivating (unoccupied = open, occupied = closed). This clever mechanism can be made to produce complete adaptation, i.e., the steady-state open probability is independent of $[Ca^{2+}]$. In the example shown in their paper, the peak open probability after each calcium step is only about twice the steady-state open probability. This model can be solved analytically, which is easiest, by far, if it is assumed that the calcium-binding reactions are rapid, in which case the model reduces to a single, first-order, kinetic equation. In this case, it can be shown directly that, for any calcium step i,

$$\frac{PK_i}{p_i} < \frac{c_{ca,i+1}}{c_{ca,i}}, \tag{12}$$

where the meaning of the symbols is as before. In particular, a calcium step from 100 nM to 200 nM could, in the Sachs model, produce at most a twofold transient increase in open probability over the resting value, as compared to the observed increase of about a factor of 3000. This emphasizes an important point: although the thermodynamic analysis above is perfectly general, the need for a large number of calcium binding sites is a consequence of the specific values of the observed open probabilities. In particular, it is the combination of very low adapted open probability with relatively large transient open probability that requires a

large free energy input to achieve, and this, in turn, requires a large number of binding sites if the ratio of the $[Ca^{2+}]$ after and before the step is modest. This dependence on ratios is a consequence of the logarithmic dependence of free energy on concentration. The Sachs model is not, therefore, able to reproduce the observed data, so it does not offer a counter-example to the theorem. It is possible that, by replacing the single calcium-binding site in this model with a site binding multiple calcium ions in a highly cooperative manner, one might obtain a model that can more closely fit the observations, while still requiring only a small number of kinetically important states. This would be very valuable in modeling excitation-contraction coupling, in which the need to consider large numbers of states of each release channel introduces great complexity. Tang and Othmer (1994) have proposed a different four-state model, which they have embedded in a complete model of excitation-contraction coupling. In their paper, the behavior of this channel is displayed for large $[Ca^{2+}]$ steps. If their model is exercised with the actual $[Ca^{2+}]$ steps used by Györke and Fill, it performs very poorly, giving a large resting open probability (about 0.14 in 100 nM $[Ca^{2+}]$), and only a slight amount of adaptation after each step. This model therefore is also not in conflict with the thermodynamic predictions.

The requirement for a large number of calcium-binding sites depends strongly on the very small open probability that has been observed by Györke and Fill in 100 nM calcium. As a check on their observation, one may estimate the adapted open probability from the frequency of calcium sparks (Cheng et al., 1993) observed by confocal microscopy of intact cardiac myocytes loaded with the fluorescent probe fluo-3. If sparks are assumed to represent single openings of the calcium release channel, or regenerative events initiated by a single opening, then the observed frequency of sparks (~100/s/cell), together with an estimated open duration of 10 ms and an estimated number of 10^5 SR release channels per cell, would give an average open probability of 10^{-5} for the release channel in situ in a resting myocyte whose cytosolic $[Ca^{2+}]$ is roughly 100 nM. One must, therefore, take seriously the very low resting open probability observed by Györke and Fill, with its implication of a high degree of cooperativity in the activation of the channel by calcium.

The process of modeling channel adaptation is still in its infancy, and it is premature to debate the validity of particular explicit models. In view of the large number of states that are likely to be required, explicit modeling of calcium release channels will have to be undertaken very carefully, and with extensive experimental underpinning. If it is confirmed that the ryanodine receptor is truly able to switch repeatedly between the extremes of open probabilities suggested in the data of Györke and Fill in response to modest steps in $[Ca^{2+}]$, one must anticipate the participation of a substantial number of calcium-binding sites in its regulation.

APPENDIX: NONINCREASING PROPERTY OF THE "IDEAL FREE ENERGY" OF MASS ACTION KINETIC MODELS

The "thermodynamic" proof given in the text relies upon the use of an expression for the equilibrium free energy of an ideal solution and the application of that expression to a system that is in a slowly evolving, nonequilibrium state. We show here that it is not necessary to justify this procedure, if we assume instead that the system can be modeled as a solution reacting according to the differential equation of mass action kinetics, obeying the principle of detailed balance applicable to passive thermodynamic systems. From such a model, one can derive a set of standard free energies μ_i for the components, such that the ideal expression for the free energy of the system given in Eq. 1 is always nonincreasing during the free evolution of the concentrations of the components according to the differential equations of the kinetic model. This means that one can interpret the theorem proved in the text as a mathematical theorem regarding the possible solutions of the differential equations of such a kinetic model, without regard to the applicability of equilibrium thermodynamics to the physical system that is being represented.

Consider the ideal "free energy" F of the system given, by definition now, by Eqs. 1 and 2 in the text. The rate of change of F is given by

$$\frac{dF}{dt} = \sum_{i=1}^{N_c} \frac{\partial F}{\partial c_i} \frac{dc_i}{dt}, \tag{13}$$

where c_i are the molar concentrations of the components of the solution, whose evolution is governed by the differential equations of the kinetic model:

$$\frac{dc_i}{dt} = \sum_{i=1}^{N_r} (\nu_{ij}^- - \nu_{ij}^+) R_j, \tag{14}$$

in which ν^+_{ij} and ν^-_{ij} are the (positive) stoichiometric coefficients of the ith reactant on the left and right sides of the jth reaction, whose overall rate is R_j, and N_r is the total number of reactions in the model. The rate R_j of the jth reaction is given by

$$R_j = \left(\prod_{i=1}^{N_c} c_i^{\nu_{ij}^+} \right) k_{j+} - \left(\prod_{i=1}^{N_c} c_i^{\nu_{ij}^-} \right) k_{j-}, \tag{15}$$

where N_c is the total number of reactants in the system, and k_{j+} and k_{j-} are the forward and backward rate constants of the jth reaction. This assumes that the model obeys mass action kinetics in which the unidirectional rate of a reaction is proportional to the product of the concentrations of the reactants entering into it. This assumption, which is commonly used in constructing models for biochemical phenomena, is stronger than the assumption of the law of mass action for the equilibrium of a (bidirectional) reaction, which can be proved from statistical mechanics for the case of ideal (i.e., dilute) solutions (Landau and Lifshitz, 1958).

Combining Eqs. 14 and 15, and interchanging the order of summation, one finds

$$\frac{dF}{dt} = \sum_{j=1}^{N_r} \dot{f}_j, \tag{16}$$

where

$$\dot{f}_j = \left(\sum_{i=1}^{N_c} (\nu_{ij}^- - \nu_{ij}^+) \left(\log\left(\frac{c_i}{C_0} \right) + \mu_i \right) \right) \times \left(\left(\prod_{i=1}^{N_c} c_i^{\nu_{ij}^+} \right) k_{j+} - \left(\prod_{i=1}^{N_c} c_i^{\nu_{ij}^-} \right) k_{j-} \right) \tag{17}$$

is the rate of free energy change attributable to the action of the jth reaction. One is interested in the sign of this expression, which will not be altered by pulling out a positive factor P equal to the first term in the last summation in Eq. 17:

$$\dot{f}_j = -P\left(\sum_{i=1}^{N_c} \nu_{ij}\left(\log\left(\frac{c_i}{C_0}\right) + \mu_i\right)\right)\left(1 - \frac{\left(\prod_{i=1}^{N_c} (1/c_i^{\nu_{ij}})k_{j-}\right)}{k_{j+}}\right), \tag{18}$$

where $\nu_{ij} = \nu_{ij}^{+} - \nu_{ij}^{-}$ is the signed stoichiometric coefficient of the ith reactant in the jth reaction, as it would be written in a single-sided form.

We now make the assumption (to be justified later) that the ratio of forward and backward rate constants (i.e., the equilibrium constant) of a reaction is given by the expression that applies to an ideal solution in thermodynamic equilibrium (Landau and Lifshitz, 1958):

$$\frac{k_{j-}}{k_{j+}} = C_0^{\sum_{i=1}^{N_c} \nu_{ij}} \exp\left(-\sum_{i=1}^{N_c} \mu_i \nu_{ij}\right). \tag{19}$$

Using this and simple logarithmic identities, one finds

$$\dot{f}_j = -P\left(1 - \exp\left[\sum_{i=1}^{N_c} \nu_{ij}\left(\log\left(\frac{c_i}{C_0}\right) + \mu_i\right)\right]\right) \times \sum_{i=1}^{N_c} \nu_{ij}\left(\log\left(\frac{c_i}{C_0}\right) + \mu_i\right). \tag{20}$$

This can be made more compact by defining symbols q_j given by

$$\sum_{i=1}^{N_c} \nu_{ij}\left(\log\left(\frac{c_i}{C_0}\right) + \mu_i\right) \equiv q_j, \tag{21}$$

which puts (20) in the form

$$\dot{f}_j = -Pq_j(1 - e^{-q_j}). \tag{22}$$

Recalling that the factor P is positive, it is easily seen that the expression on the right-hand side of Eq. 22 can never be positive for real q_j. The ideal free energy F, the rate of change of which is given by Eq. 16, can never increase.

It was assumed above that the equilibrium constants of the reactions are given by the ideal thermodynamic expression in Eq. 19. But because the starting point here is a kinetic mathematical model rather than a physical system or a thermodynamic model, we are at liberty to choose the "standard free energies" that enter into Eq. 2 so as to satisfy this requirement. Rearranging Eq. 19 by taking logarithms of both sides yields

$$\sum_{i=1}^{N_c} \nu_{ij}\mu_i = \log\left(\frac{C_0^{\sum_{i=1}^{N_c} \nu_{ij}} k_{j+}}{k_{j-}}\right), \tag{23}$$

which is a set of linear equations for μ_i. Provided that these equations are consistent, one can always find at least one solution. The requirement for consistency is the algebraic statement of the requirement for the model of a passive system to obey detailed balance. This can be seen more clearly in the case of the system actually being considered, a channel that can be in multiple states, interacting with a solution of calcium ion. The reactions, in this case, are the transitions from one state of the channel to another, which may be accompanied by binding or release of one or more calcium ions. Each of these reactions give rise to one equation of Eq. 23, the left-hand side of which has two or three terms: a stoichiometric coefficient of +1 for the starting state, −1 for the final state, and, possibly, a coefficient for calcium ion. These equations can be directly solved by "walking" the state diagram as follows: 1) choose a value for the standard free energy of one of the states of the channel; 2) use an equation from Eq. 23 in which that state appears and solve for the standard free energy of the state to which the transition takes place; 3) continue "walking" from state to state, solving the relevant equation from Eq. 23 for the standard free energy of the state arrived at by each transition. Because the state diagram is connected, one will obtain, in this manner, values for all of the μ_i (the value for μ_{Ca} can actually be chosen arbitrarily). This scheme will result in a consistent solution to Eqs. 23, provided the sums of the logarithms of the equilibrium coefficients (i.e., the logarithm of the product of equilibrium coefficients) and the number of calcium ions bound are independent of the path taken to reach a particular state. This is equivalent to the statement that the product of equilibrium coefficients around a closed path through the state diagram equals 1, and the net cumulative binding of calcium around such a path is zero. These are statements of the usual notion of detailed balance, which is expected to apply to any passive kinetic model.

It has therefore been proved that, starting from a passive, mass action kinetic model, one can find a set of μ_i such that the "ideal free energy function" given in Eqs. 1 and 2 is always nonincreasing during the free evolution of the model. This is all that was required for the "thermodynamic" proof given in the text. Whether this function is a good approximation to the true thermodynamic free energy of an actual physical channel is a separate question that will not be dealt with here. A more interesting physical question is whether a passive molecular system that binds only a few calcium ions can appear to display reaction kinetics of high order in calcium. Such enhancement of cooperativity could be produced, for example, by positive feedback of calcium passing through the channel, but this makes use of the free energy of permeation and would not be applicable to the experiments of Györke and Fill, which were done with cesium as the current carrier. Obviously, such a hypothetical "enhanced cooperativity" system could not be represented by mass action kinetic equations. Whether statistical mechanics sets fundamental limits on such a nonideal phenomenon in a nonequilibrium system must be investigated.

The author would like to thank Michael Fill for helpful discussions regarding the data on adaptation, and Roger Traub, Don Coppersmith, and John Forrest of the IBM Thomas J. Watson Research Center for advice on the use of linear programming methods.

This work was supported in part by grant HL42050 of the National Heart Lung and Blood Institute.

REFERENCES

Chen, S. R., and D. H. MacLennan. 1994 Identification of calmodulin-, Ca(2+)-, and ruthenium red-binding domains in the Ca^{2+} release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 269:22698–22704.

Cheng, H., M. Fill, H. Valdivia, and W. J. Lederer. 1995. Models of Ca^{2+} release channel adaptation. *Science.* 267:2009–2010.

Cheng H., W. J. Lederer, and M. B. Cannell. 1993. Calcium sparks: elementary events underlying excitation-contraction. *Science.* 262: 740–4

Fabiato, A. 1985. Simulated calcium current can both cause calcium loading in and trigger the calcium release from the sarcoplasmic reticulum of a skinned cardiac Purkinje cell. *J. Gen. Physiol.* 85:291–320.

Györke, S., and M. Fill. 1993. Ryanodine receptor adaptation: control mechanism of Ca^{2+}-induced Ca^{2+} release in heart. *Science.* 260: 807–809.

Lamb, G. D., M. W. Fryer, and D. G. Stephenson. 1994. Ca(2+)-induced Ca^{2+} release in response to flash photolysis (letter; comment). *Science.* 263:986–988.

Landau, L. D., and E. M. Lifshitz. 1958. Statistical Physics. Pergamon Press, London.

Meyer, T., T. Wensel, and L. Stryer. 1990. Kinetics of calcium channel opening by inositol 1,4,5-trisphosphate. *Biochemistry.* 29:32–37.

Mourey, R. J., V. A. Estevez, J. F. Marecek, R. K. Barrow, G. D. Prestwich, and S. H. Snyder. 1993. Inositol 1,4,5-trisphosphate receptors: labeling the inositol 1,4,5-trisphosphate binding site with photoaffinity ligands. *Biochemistry.* 32:1719–1726.

Press, W. H., B. P. Flannery, S. A. Teukolsky, and W. T. Vetterling. 1990. Numerical Recipes: The Art of Scientific Computing. Cambridge University Press, Cambridge.

Richard, E. A., and C. Miller. 1990. Steady-state coupling of ion-channel conformations to a transmembrane ion gradient. *Science.* 247: 1208–1210.

Sachs, F., F. Qin, and P. Palade. 1995. Models of Ca^{2+} release channel adaptation. *Science.* 267:2010–2011.

Stern, M. D. 1992. Theory of excitation-contraction coupling in cardiac muscle. *Biophys J.* 63:497–517.

Tang, Y., and H. G. Othmer. 1994. A model of calcium dynamics in cardiac myocytes based on the kinetics of ryanodine-sensitive calcium channels. *Biophys. J.* 67:2223–2235.