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Branched Reaction Mechanism for the Na/K Pump as an Alternative Explanation for a Nonmonotonic Current *vs.* Membrane Potential Response

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Summary. Nonmonotonic velocity *vs.* membrane potential curves are often taken as evidence that two steps involve charge movement through the electric field. However, a branched reaction scheme in which only one step involves charge movement per cycle can lead to a nonmonotonic response. A similar case occurs in enzyme kinetics: nonmonotonic velocity *vs.* substrate curves are often taken as evidence for two different substrate-binding sites. However, a branched reaction scheme in which only one substrate binds per complete cycle can lead to a nonmonotonic response (*see* Segel, I.H. 1975. Enzyme Kinetics. pp. 657–659. John Wiley & Sons, New York). Some analytical constraints on the relative sizes of the rate constants of a branched reaction mechanism that give rise to nonmonotonic responses are derived. There are two necessary conditions. (*i*) The rate of at least one step in the branched pathway must be less than the rate of the step after the branch. (*ii*) The rate of the pathway in which S binds first must be slower than the rate of the other pathway. Analogous cases give rise to nonmonotonic current *vs.* membrane potential curves. A branched mechanism for the Na/K pump provides an alternative explanation for a nonmonotonic pump current *vs.* membrane potential relationship.

Key Words voltage dependence · charge movement · current-voltage characteristic · membrane transport kinetics · branched reaction mechanisms · ion pumps

Introduction

One method of classifying enzyme reactions is by the number of substrate molecules that are bound by the protein. Similarly, one method of classifying transport reactions that result in net charge movement is by the number of steps that are affected by membrane potential. Recently the current *vs.* membrane potential response curve (I *vs.* E_m) for the Na/K pump has been extensively studied (reviewed by DeWeer, Gadsby & Rakowski, 1988). In cardiac cells and squid giant axons, the response is monotonic. (Gadsby, Kimura & Noma, 1985; Rakowski, Gadsby & DeWeer, 1989). However, under some (Lafaire & Schwarz, 1986; Schweigert, Lafaire & Schwarz, 1988; Rakowski, Vasilets & Schwarz, 1990) but not all (Rakowski & Paxson, 1988) conditions the I *vs.* E_m curve in oocytes is nonmonotonic. Do these results necessarily imply a structural difference between the cardiac and axon Na/K pump and the oocyte Na/K pump? The cardiac and axon pump would appear to have only one voltage-dependent step; perhaps there are two negative charges in the transport pocket which balance the two K ions but not the three Na ions. In contrast the nonmonotonic I–V curve in oocytes is usually taken as evidence of two membrane potential dependent steps per cycle (Lafaire & Schwarz, 1986). This could occur if the transport pocket were charged both when three Na ions and when two K ions are being transported, e.g., net charge of empty pocket is -1. Then the Na translocation step would result in the movement of the equivalent of two positive charges through the electric field and the K translocation step would result in the movement of the equivalent of 1 positive charge through the field. Changes of membrane potential that increase the rate of Na efflux would decrease the rate of K influx. If the rates of the Na translocation efflux step and the K translocation influx step were equal and rate limiting at $E_m = 0$, then an increase in E_m would slow down the K steps and thus slow the overall reaction. A decrease in E_m would slow down the Na steps and thus slow the overall reaction. This would result in a nonmonotonic response to E_m. Thus models with two membrane potential sensitive steps can account for a nonmonotonic I *vs.* E_m response.

Are two membrane potential sensitive steps *required* to account for the nonmonotonic response? If so, then the model for the oocyte nonmonotonic response seems distinct from the model for the cardiac and axon Na/K pump monotonic response. Unbranched models with only a single membrane potential dependent step generate monotonic responses; detailed analysis of such models has been presented by Hansen et al. (1981) and by Läuger

(1984).[1] Models with two voltage-dependent steps can appear monotonic over a limited range of membrane potential, but, in theory, the response will be nonmonotonic over a very large potential range. An alternative model that also accounts for the nonmonotonic response of the oocyte is presented in this paper. In this other model only one step is membrane potential sensitive, but this step occurs in random order with another step of the cycle. In this branched mechanism changing one rate constant converts the nonmonotonic response to a monotonic response. This model offers a simple explanation for the different I *vs.* E_m responses in oocytes and cardiac cells.

Branched models have been suggested previously for the Na/K pump. The branches involve product dissociation steps and nonessential substrate binding. For example, K release can occur before or after the binding of ATP to a regulatory site (Moczydlowski & Fortes, 1981). Also ATP can bind before or after phosphate release (Sachs, 1988). There is no *a priori* reason to exclude the possibility that a voltage-dependent step could be part of a branched mechanism.[2] If a branched mechanism is the reason for a nonmonotonic I *vs.* E_m response then a search to identify two voltage-dependent steps would be fruitless.

One feature of many proposed models that explain a nonmonotonic velocity (v) *vs.* [S] response is that there are at least two binding sites for S. For example, the Cl dependence of Cl/Cl exchange in red cells is nonmonotonic; this has led to the suggestion of a substrate site and a modifier site for chloride (for example, *see* discussion in Gunn et al., 1989). However, single substrate enzyme models that include a branch can also give rise to nonmonotonic responses. An early model was proposed by King (1956) of random ordered addition in a bisubstrate reaction. When the binding steps did not equilibrate rapidly, certain choices of rate constants led to a nonmonotonic v *vs.* S curve. Ferdinand (1966) derived some of the constraints on the phenomonological parameters required to achieve a nonmonotonic curve (*see* discussion by Segel, 1975). Botts (1957) considered several other classes of branched, single substrate mechanisms and characterized the variety of responses that may occur. In addition, some of the constraints on the relationship between the individual rate required in order to obtain a nonmonotonic response were derived. More recently, Sanders (1986) has examined the possible responses of random ordered binding cotransport systems. In this paper, some explicit constraints on the rate constants that lead to a nonmonotonic response for a branched substrate binding model are derived. These constraints form the basis for the analysis of a branched membrane potential model. Since these constraints involve a limited number of rate constants, it is easily shown when a change of the rate of a single rate constant converts a nonmonotonic response to a monotonic response.

A preliminary report of this work has been presented (Milanick, 1987).

Theory

The rate equation for a model which includes a branch for the binding of substrate is simpler than a model which includes a branch for the membrane potential sensitive step. The former model has been analyzed extensively (Segel, 1975, and references therein; Sanders, 1986). The analysis of branched substrate models will be presented first and then the branched membrane potential model will be discussed. The goal of the analysis is to determine some of the constraints on the choice of rate constants that result in a nonmonotonic response for branched models.

v *vs.* S

A branched reaction scheme is illustrated in Fig. 1*A*. For convenience step 5 is assumed to be irreversible. In this model S can bind to the one substrate site on

[1] For a electroneutral exchange of X^* and X that involves voltage-dependent steps, a nonmonotonic v *vs.* E_m curve is very likely. The X^* efflux half-cycle and X influx half-cycle must both include a voltage-dependent step. Since the effect of E_m on the influx half-cycle is the opposite as on the efflux half-cycle, the overall cycle may have a nonmonotonic dependence on E_m (Eisner & Lederer, 1985).

[2] One can imagine that every reaction has a number of branches under some conditions. Thermodynamic considerations require that each pathway of a reaction have the same overall equilibrium constant. The reaction proceeds along all of the pathways and a finite amount of each of the intermediates is formed. From a kinetic point of view, one of the pathways may predominate so that the reaction appears to have a particular or preferred order. Also, the amount of some intermediates may be negligible. Nevertheless, some of the product comes from these other pathways because of finite fluctuations in the conformational and energy states of the molecules, consistent with the thermodynamic viewpoint. (*See* Hearon et al., 1959; footnote 3 in Milanick & Gunn, 1982.) This notion of branches can be considered as an extreme case of the dynamic motion of proteins or the wobble of channels (*cf.* the accounts of Fröhlich, 1984; Läuger, 1985).

Under physiological conditions, there will be selection pressure to maintain the rate of the process, and thus any mutations in transport protein structure that allow the concentration of transport intermediate on a kinetically unfavored branch pathway to increase or allows a reaction that uncouples the cycle will not be favored. However, no such pressure will exist at nonphysiological conditions. Thus it is possible, if not likely, that at high substrate concentrations or under other unusual conditions, branched pathways will occur since there would be no selection pressure against the branch.

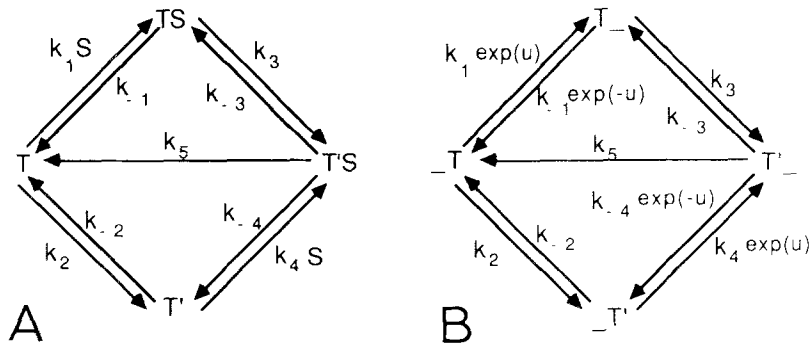

Fig. 1. Branched reaction schemes. (*A*): Branched reaction for the binding of S. S can bind to the transporter when the transporter is in the T or T' state. (*B*): Branched reaction for membrane potential dependent steps. The membrane potential dependent conformational change $\_T$ to $T\_$ can occur before or after the conformational change from T to T'

Table 1.

$v = (iS^2 + jS)/(k + lS^2 + mS)$

where

$i = k_1k_3k_4k_5$

$m = k_1k_{-2}k_{-4} + k_{-1}k_{-3}k_4 + k_{-1}k_4k_5 + k_1k_3k_{-4} + k_3k_4k_5 + k_2k_4(k_{-1} + k_{-3} + k_3) + k_1k_{-2}(k_{-3} + k_5 + k_3)$

$j = k_5[k_2k_4(k_{-1} + k_3) + k_1k_{-2}k_3]$

$l = k_1k_4(k_{-3} + k_5 + k_3)$

$k = k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-4} + k_{-1}k_{-2}k_{-5} + k_{-1}k_2k_3 + k_{-1}k_2k_{-4} + k_{-1}k_2k_5 + k_{-2}k_3k_{-4} + k_{-2}k_3k_5 + k_2k_3k_{-4} + k_2k_3k_5$

The rate constants can be in any set of consistent units.

the protein before or after the change from T to T'. T to T' could represent the binding of a different substrate and then this is the standard bisubstrate reaction with random order.

The steady-state rate equation for this model is given in Table 1 (*see* Segel, 1975). The v *vs.* S curve will be nonmonotonic (pass through a maximum) if

$$im < jl \tag{1}$$

(Botts, 1957; Ferdinand, 1966; Segel, 1975). This constraint can be restated as

$$k_3(k_{-1}k_{-3}k_4 + k_{-1}k_4k_5 + k_1k_3k_{-4} + k_3k_4k_5) < k_2k_4k_5(k_{-1} + k_3). \tag{2}$$

This inequality forms the basis for the following analysis.

Monotonic

Rapid Equilibrium (Slow Product Release)

It is well known that if the steps of the branch equilibrate rapidly compared to the rate of step 5, then the response is always monotonic (Segel, 1975). This statement is easily proved by examining inequality 2. If k_5 is much smaller than *all* the other rate constants, the terms $k_{-1}k_3k_4k_5$ and $k_3k_3k_4k_5$ are negligible compared to other terms on the left-hand side. Thus the requirement for a nonmonotonic response becomes

$$k_{-1}k_3k_{-3}k_4 + k_1k_3k_3k_{-4} < k_2k_4k_5(k_{-1} + k_3). \tag{3}$$

By assumption k_5 is less than all the other rate constants so this inequality is not true. Thus the response is monotonic for a rapid equilibrium branch mechanism.

The NonSubstrate-Binding Steps

When $k_3 > k_2$, then the response is always monotonic. This can be confirmed by examining inequality 2. If both sides of the inequality are divided by $k_{-1}k_2k_{-3}k_4$ the inequality can be rearranged to be of the form

$$k_3/k_2A + k_3/k_2Y < Y \tag{4}$$

where A and Y are combinations of rate constants and always positive. If $k_3 > k_2$ this inequality cannot be true. In this case the response is monotonic.

Summary

Sufficient conditions for a monotonic response are that k_5 be small or that $k_3 > k_2$. Thus, necessary conditions for a nonmonotonic response are that k_5 cannot be small and that $k_3 < k_2$.

Nonmonotonic

k_5

From the above analysis the response will be nonmonotonic if k_5 is larger than all other rate constants and $k_3 < k_2$. However, a nonmonotonic response

will also occur if k_5 is smaller than many of the other rate constants. For example, a nonmonotonic curve can be obtained when $k_3 = k_{-3} = k_5$ and all the other rate constants are larger.[3] Inequality 2 provides the complete constraint on the size of k_5.

Forward vs. Reverse Rate Constants

Suppose that the reverse rates are slow compared to the forward rates. This assumption includes the requirement that the product $k_{-1}k_5$ be small. Any value of k_5 consistent with these assumptions will result in a nonmonotonic response if $k_3 < k_2$. Segel (1975, pp. 460–461) provides an intuitive explanation of how this case results in a nonmonotonic response.

$k_3 = 0$

When $k_3 = 0$, then the *v vs.* S curve is nonmonotonic. This is because if $k_3 = 0$, then $i = 0$, but j and l are non-zero (*see* Table 1). This case is essentially the same kinetic scheme as competitive substrate inhibition as discussed by Segel (1975, p. 819). The $k_3 = 0$ case can be considered a special case of the branched mechanism since it meets the requirements that k_5 be larger than a least one step of the branch ($k_5 > 0 = k_3$) and $0 = k_3 < k_2$. Thus a "false start" mechanism can also result in a nonmonotonic response.

Converting Nonmonotonic to Monotonic Responses

Both a two-site model and a branched mechanism can give rise to nonmonotonic responses. An inhibitor that acts only at the second site (the substrate site that causes inhibition) provides evidence for a two substrate-site model and against a branched model. However, the observation that an agent converts a nonmonotonic response to a monotonic response is not sufficient evidence that the agent binds to the second site; an agent that modifies only one rate constant of a branched mechanism can convert a nonmonotonic response to a monotonic response. For example, an increase in k_3 or a decrease of k_5 or k_2 can lead to a monotonic response. In addition, a site-directed mutation that eliminates a nonmonotonic response (removes substrate inhibition) is consistent with (*i*) the removal of the modifier-binding site, or (*ii*) a shift in one of the rate constants of a branched mechanism.

[3] For example, $k_1 = k_2 = k_4 = k_{-1} = k_{-2} = k_{-4} = 30$ and $k_3 = k_{-3} = k_5 = 0.3$.

Table 2.

$$(A + Be^{x} + Ce^{2x})/(D + Ee^{x} + Fe^{2x} + Ge^{-x} + He^{-2x})$$

where

$$A = k_{-1}k_2k_4k_5$$
$$B = k_2k_3k_4k_5 + k_1k_{-2}k_3k_5$$
$$C = k_1k_3k_4k_5$$
$$D = k_1k_{-2}k_{-4} + k_{-1}k_{-3}k_4 + k_{-1}k_4k_5 + k_{-1}k_2k_4 + k_1k_3k_{-4} + k_{-2}k_3k_5 + k_2k_3k_5$$
$$E = k_1k_{-2}k_{-3} + k_1k_{-2}k_5 + k_1k_{-2}k_3 + k_2k_{-3}k_4 + k_3k_4k_5 + k_2k_3k_4$$
$$F = k_1k_4(k_{-3} + k_5 + k_3)$$
$$G = k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-5} + k_{-1}k_2k_3 + k_{-1}k_2k_5 + k_{-2}k_3k_{-4} + k_2k_3k_{-4}$$
$$H = k_{-1}k_{-2}k_{-4} + k_{-1}k_2k_{-4}$$

Summary

In order to obtain a nonmonotonic response with a single substrate system there must be a branch in the reaction mechanism. For the case illustrated in Fig. 1*B* two additional requirements are (*i*) at least one step in the branched pathway is less than the rate of the step after the branch, and (*ii*) the pathway in which S binds first is slower than the other pathway. These criteria are necessary but not sufficient. Two particular cases that do lead to a nonmonotonic response are (*i*) k_5 is faster than all other steps and $k_3 < k_2$, and (*ii*) the reverse rates are slow and $k_3 < k_2$. Analogous criteria were derived by Botts (1957) for a branched model involving a nonessential activator.

Membrane Potential

A nonmonotonic *v vs.* E_m curve is often interpreted to imply at least two voltage-dependent steps (Lafaire & Schwarz, 1986). However, a branched membrane potential model (Fig. 1*B*) can also generate a nonmonotonic *v vs.* E_m curve. The Eyring/Läuger formalism (Läuger, 1985) is convenient for including the effect of E_m on the rate constants: $k_+ = k_+' \exp(x)$ with $x = \mu/2 = FE_m/2RT$ and for a reverse reaction, $k_- = k_-' \exp(x)$. One important difference between the effect of membrane potential and of substrate concentration is that E_m affects both forward and reverse rates between two intermediates whereas [S] only affects the forward rate between two intermediates. The kinetic equation for the model shown in Fig. 1*B* was derived using the King-Altman method (Segel, 1975) and is given in Table 2. If the reverse rates of the voltage-dependent step are slow so that the terms $G \exp(-x)$ and $H \exp(-2x)$ are small, then the equation can be re-

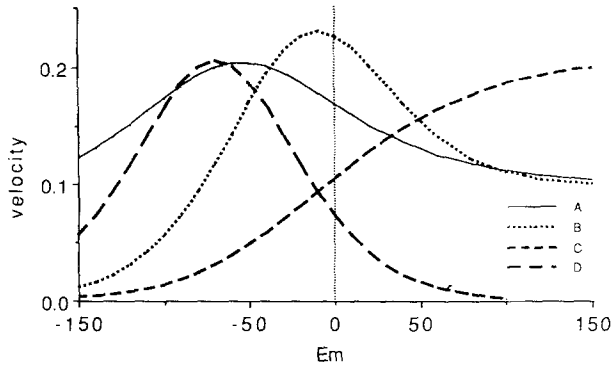

Fig. 2. The effect of membrane potential on velocity for different choices of rate constants. Steps 1 and 4 are membrane potential dependent steps. k_5 is fast. Curve *A*: Reverse steps are slow. k_3 is slow. The rate constants are $k_1 = 1$, $k_2 = 1$, $k_3 = 0.1$, $k_4 = 1$, and $k_5 = 10$. The ratio of forward to reverse rate constants is 100. Curve *B*: Reverse steps are not slow. k_3 is slow. The rate constants are the same as in *A* except that the ratio of forward to reverse rate constants is 1. Curve *C*: k_3 is not slow. The increase in k_3 has converted the response from nonmonotonic to monotonic. The rate constants are the same as in *B* except that $k_3 = 1$. The velocity is four times greater than the value plotted. Curve *D*: $k_3 = 0$. All the other rate constants same as *B*

	A	*B*	*C*	*D*
k_1	1	1	1	1
k_2	1	1	1	1
k_3	0.1	0.1	1	0
k_4	1	1	1	1
k_5	10	10	10	10
ratio	100	1	1	1

duced to

$$(A + Be^x + Ce^{2x})/(D + Ee^x + Fe^{2x}) \qquad (5)$$

which is similar to that of Eq. (1) in Table 1.[4] The above analysis provides a convenient starting point for analyzing the branched membrane potential model.

If k_5 is not small and if $k_2 > k_3$, the *v vs.* E_m response can be nonmonotonic (Fig. 2, curve *A*). One can relax the requirement that the reverse steps be slow (Fig. 2, curve *B*). After systematic simulations no conditions have been found in which the *v vs.* E_m plot is nonmonotonic (for $E_m = -100$ to $+100$ mV) unless $k_3 < k_2$.

Discussion

The constraints on the rate constants of branched kinetic schemes for responses that are nonmonotonic have been derived. One critical requirement is that the step following the branch is faster than at least one step of the branch, i.e., the steps in the branch do not rapidly equilibrate. Also the preferred pathway at low substrate concentrations must be faster than at high substrate concentrations (*cf.* Segel, 1975).

[4] The analysis of Botts (1957) explicitly considers the effect of a constant term in the numerator on the response to S.

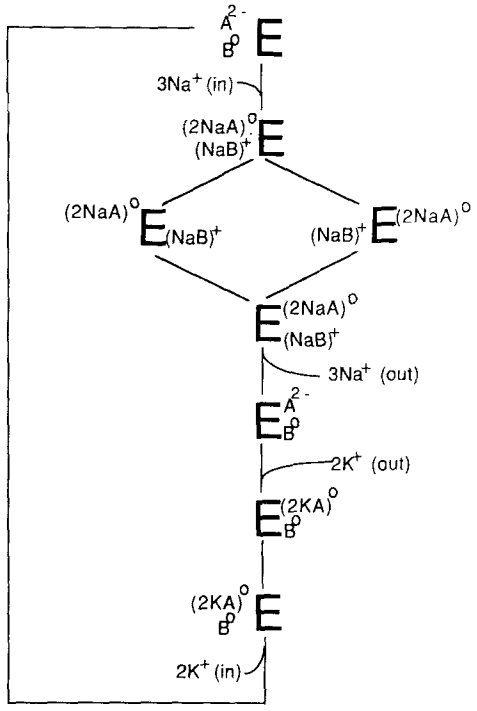

Fig. 3. A possible branched mechanism for the Na/K pump. There are two pockets in the pump, A and B. The empty A pocket has two negative charges and the empty B pocket has no net charge. When the A pocket is loaded with two Na or two K ions, the translocation of the ions does not involve net charge movements. In contrast, when the B pocket is loaded with one Na ion, the translocation does involve net charge movement; so, this is the one potential-dependent step in the cycle. For convenience the location of A or B on the left side of *E* indicates that the pocket has access to the intracellular solution and on the right side indicates that the pocket has access to the extracellular solution. Thus the translocation of a pocket A from inside to outside is denoted as AB*E* to B*E*A

Nonmonotonic *I vs.* E_m Curves

The *v vs.* E_m curve for the Na/K pump in oocytes may be nonmonotonic (Lafaire & Schwarz, 1986; Schweigert et al., 1988; Rakowski et al., 1990). This has been interpreted to imply that there are two voltage-dependent conformational changes in the pump cycle, e.g., the Na translocation step and the K translocation or binding step. A branched reaction scheme can also give rise to a nonmonotonic *v vs.* E_m curve. The problem is to propose conformational changes that could be involved in the branch.

Suppose the Na/K pump consists of two transport pockets (Fig. 3). Pocket A contains two nega-

tively charged residues. Thus when two K or two Na ions are bound pocket A has no net charge. Pocket B contains no net charge when empty, but contains a net positive charge when one Na ion is bound. The movement of pocket B with Na bound involves net charge movement through the electric field and thus is voltage dependent. In contrast, the movement of pocket A with Na bound or with K bound and of pocket B with no ion bound, does not involve net charge movement through the electric field and thus is voltage independent. The critical feature of this model is that the voltage-dependent step (movement of pocket B with Na bound) can occur before or after a voltage-independent step (movement of pocket A (loaded with two Na)). Some of these steps are slower than the subsequent step (dissociation of Na). For the sake of simplicity, the step following the branch is assumed irreversible (e.g., Na-out = 0).

How does this model generate a nonmonotonic *I vs.* E_m curve? Consider the situation with the rate constants used for Fig. 2, curve *A*. The reaction from *A/B* to */AB* is slower than the reactions from *AB/* to *A/B*, *AB/* to *B/A* and *B/A* to */AB*. Thus the left pathway from *AB/* to *B/A* to */AB* is the kinetically preferred pathway. When $E_m = 0$, the distribution of forms of the transporter is such that about as many transporters are in *B/A* as in *A/B*.

As the potential becomes positive from 0, the voltage-dependent reaction from *AB/* to *A/B* will be favored compared to the voltage-independent reaction *AB/* to *B/A*; thus, more molecules will be in the *A/B* conformation in the steady state than in the *B/A* conformation. But the reaction from *A/B* to */AB* is slow; so, the overall reaction rate is slowed.

As the potential becomes negative from 0, there will be a decrease in *A/B* and a step on the kinetically preferred pathway will also be slowed (*B/A* to */AB*). Thus the overall reaction rate is again slower, and the response is not monotonic.

The key feature for this response of a branched pathway model is that there are voltages ($V > 0$ in this case) where the predominant form (*A/B*) is not on the kinetically preferred pathway (*cf.* King, 1956; Segel, 1975).

In this model, it is straightforward to go from a nonmonotonic response to a monotonic response. For example if k_5 is slowed so that the branch steps equilibrate rapidly, the response will become monotonic. Alternatively an increase in k_3 will shift the curve from nonmonotonic to monotonic over the voltage range -100 to $+100$ mV. (Fig. 2, curve *C*).

Another different type of branched model is one in which the voltage-dependent Na translocation step can occur before or after ADP release. Furthermore, suppose that if this Na step occurs before ADP release, then the cycle cannot proceed ($k_3 = 0$). This would be analogous to the dead-end inhibition discussed above. This is another example of a branched mechanism for voltage-dependent steps that leads to a nonmonotonic response. (Fig. 2, curve *D*).

One way to determine if both the Na and the K steps are E_m dependent or if only the Na steps are E_m dependent is to measure the partial reactions that depend only upon Na or K. This has been done for the cardiac and renal pumps and the results suggest that the Na step is E_m dependent; the partial reactions that depend upon K are not affected by E_m (reviewed by DeWeer et al., 1988). Of course, if the E_m-dependent K step of these partial reactions was fast, one would also observe no effect of E_m on the overall rate until this fast step was sufficiently slow as to contribute to the rate-limiting reactions. It is also important to determine if the partial reactions are actually a part of the normal overall cycle. This appears to be true in dog kidney and human red cells (Sachs, 1986; Stein, 1986; Kaplan, 1985; Karlish et al., 1988) but has not been tested in the frog oocyte.

In summary, a nonmonotonic response does not necessarily eliminate models with a single substrate site or a single charge movement step. A branched mechanism is an alternative model to the more conventional models with multiple binding sites or multiple membrane potential dependent steps. Some of the constraints on the individual rate constants that are required in order to have a nonmonotonic response have been derived analytically. From these constraints it is straightforward to suggest how a nonmonotonic response could be converted to a monotonic response by the change of the rate of one step.

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