

THEORETICAL STUDY OF THE VOLTAGE AND CONCENTRATION DEPENDENCE OF THE ANOMALOUS MOLE FRACTION EFFECT IN SINGLE CALCIUM CHANNELS

New Insights Into the Characterization of Multi-Ion Channels

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ABSTRACT Several recent independent studies on macroscopic Ca currents have demonstrated the anomalous mole fraction effect in mixtures of Ca and Ba at concentrations of 10 mM or less. Recently, Hess and Tsien (1984; *Nature* 309) proposed a dual binding site model, based upon Eyring rate theory, to account for this effect in L-type cardiac Ca channels. This model predicts that the anomalous mole fraction effect can be accounted for solely in terms of open single channel permeation properties; it was able to adequately reproduce the effect for macroscopic Ca currents recorded in 10 mM solutions. However, the electrochemical gradients under which single Ca channel current recordings are routinely made with the patch clamp technique vary dramatically from those used for macroscopic Ca currents. To properly assess the general validity of the Hess and Tsien model at the single Ca channel level, the effects of both large electrical potentials and elevated divalent concentrations must be understood. Computer simulations were therefore carried out using the original parameters used by Hess and Tsien under conditions designed to mimic those used in patch clamp studies. The permeation behavior generated by this model is quite complex. In particular, hyperpolarization and increased divalent concentration combine to reduce and ultimately abolish the anomalous mole fraction effect. It may therefore be very difficult to observe the anomalous mole fraction effect at the single Ca channel level; the dual-site model displays a relationship between current and mole fraction generally associated with a single-site model under the conditions frequently employed to resolve single Ca channel activity. Nonetheless, analysis of such monotonic mole fraction behavior can still be used as a test for the general validity of the dual-site model. Apparent K_m s for Ca and Ba can be extracted from such monotonic behavior, and may not only be functions of membrane potential but may also depend upon the total divalent cation concentration. This is a unique prediction which is incompatible with the simple single-site model. Our analysis provides (a) a possible resolution for the apparent discrepancies presently existing in the experimental literature regarding the existence of the anomalous mole fraction effect at the single Ca channel level, (b) a mechanistic description of previously unexplained observations on the voltage-dependence of the anomalous mole fraction effect, and (c) a useful theoretical framework for future experimentation designed to test the general validity of the dual binding site model of the Ca channel.

INTRODUCTION

Here we theoretically demonstrate the predicted effects of both (a) membrane potential and (b) total divalent cation concentration on the anomalous mole fraction effect in single-calcium channels using the dual binding site model originally proposed by Hess and Tsien (1984). Our rationale for doing so has been the recent appearance in the

literature of apparently conflicting data concerning the existence of the anomalous mole fraction effect at the single Ca channel level, and therefore the appropriateness of the Hess and Tsien (1984) model in describing ion permeation through these channels.

Several recent independent voltage clamp studies on whole cell macroscopic calcium currents in both muscle (frog striated: Almers and McCleskey, 1984; guinea pig ventricle: Hess and Tsien, 1984; McDonald et al., 1986), and neuronal (molluscan neurons: Byerly et al., 1985) tissues have demonstrated the anomalous mole fraction effect. These studies showed, in various external solutions

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containing a constant total divalent cation (Ca and Ba) concentration of 10 mM or less, that the peak size of the current was not a simple monotonically increasing function of the Ba mole fraction, but instead initially declined and displayed a clear minimum before subsequently increasing as the Ba mole fraction increased. In particular, in their work on macroscopic whole cell Ca currents in single guinea pig ventricular myocytes, Hess and Tsien (1984) addressed the issue of whether the anomalous mole fraction effect was due solely to open-channel permeation properties, or was also mediated by alterations in channel gating properties. Applying nonstationary noise analysis (c.f., Sigworth, 1980) to currents recorded in mixtures of Ca and Ba (total concentration 10 mM), they concluded that the anomalous mole fraction effect was due solely to channel permeation properties. Using Eyring rate theory formalism (Eyring et al., 1949; Hille and Schwarz, 1978) they modeled the cardiac L-type Ca channel as a symmetrical, single-file, two ion-binding site pore (for similar proposed models see: Almers and McCleskey, 1984; Byerly et al., 1985; Iijima et al., 1986) and were able to adequately reproduce the anomalous mole fraction behavior of the whole cell currents they observed. However, this study did not directly demonstrate the effect at the single-channel level.

Two recent patch clamp studies have experimentally tested for the existence of the anomalous mole fraction effect at the level of the single-calcium channel, and have yielded apparently conflicting results. In an elegant series of experiments on *Aplysia* neurons, Chesnoy-Marchais (1985) was able to directly demonstrate the anomalous mole fraction effect at the single-channel level using mixtures of Ca and Ba at a total concentration of 60 mM. In addition, Chesnoy-Marchais (1985) phenomenologically demonstrated that the effect was only observable in a rather narrow, depolarized range of potentials (0 to +10 mV); at more hyperpolarized potentials (-20 to -80 mV) the single-channel currents increased in a simple monotonic fashion with the Ba mole fraction. While Chesnoy-Marchais' (1985) results clearly demonstrated that there was a membrane potential range where the anomalous mole fraction effect existed, no explanation for the mechanism underlying the potential dependence of the effect was proposed. Subsequently, Yue and Marban (1987) recorded single Ca channel activity in guinea pig ventricular myocytes using mixtures of Ca and Ba at a total concentration of 110 mM. Using single channel conductance (measured over an unspecified potential range) as a measure of the effect, they observed a simple monotonically increasing conductance-mole fraction relationship with increasing Ba mole fraction. Yue and Marban (1987) therefore proposed that either (a) the anomalous mole fraction effect observed by Hess and Tsien (1984) might be mediated by factors other than permeation alone, and/or (b) their dual binding site model might require marked revision.

It is important to attempt to reconcile these apparently conflicting observations, particularly those obtained on the cardiac L-type channels. As has been pointed out by Hille (1984), the permeation properties of multi-ionic channels can be very complex; to fully understand their permeation properties requires experimentation over a wide range of potentials and concentrations. However, to date very little attention has been addressed to the effects of either membrane potential or absolute total divalent concentration on the behavior of the anomalous mole fraction effect in Ca channels. In order to experimentally evaluate the general applicability of the Hess and Tsien (1984) model at the single Ca channel level using the anomalous mole fraction effect as a criterion, the predicted effects of these two parameters must be understood. Such an understanding is particularly important for patch clamp studies on single Ca channels, since both (a) large membrane potential gradients and (b) large divalent cation concentrations are routinely used to help resolve single-channel current events. The simulations presented here demonstrate the combined effects of (a) varying the membrane potential over the range of 0 to -100 mV and (b) increasing the divalent cation concentration from 10 to 110 mM on the behavior of the anomalous mole fraction effect. We have focused our analysis to these restricted ranges so as to be of greatest general predictive value for experimentation, since it is over these ranges that most single-channel experiments are conducted and compared with macroscopic results. Finally, by careful examination of mole fraction experiments, we have proposed a new test to identify the multi-ionic nature of the channel that is independent of the assumptions of Eyring rate theory.

A preliminary report of this work has been previously presented in abstract form (Campbell, D. L., R. L. Rasmusson, and H. C. Strauss, submitted for publication).

METHODS

General Background

The Ca channel permeation model of Hess and Tsien (1984) accounts for high divalent selectivity by hypothesizing that the channel contains two intrachannel binding sites that possess a high affinity for divalent cations (e.g., affinity for Ca similar to that displayed by various Ca-binding proteins such as troponin-C and calmodulin; cf., Robertson et al., 1981; Johnson et al., 1986; Lansman et al., 1986). Hence, selectivity arises from cationic affinity (i.e., specific binding) as opposed to cationic rejection (Tsien et al., 1986; Hille, 1975b, 1984). Therefore, at millimolar divalent concentrations the channel is nearly always occupied by at least one divalent cation (usually a Ca ion). To overcome the blocking effects which would be produced by divalent occupancy at such high affinity sites, cations bound within the channel are postulated to electrostatically repel each other. Electrostatic repulsion effectively reduces the affinity of the opposite intrachannel site. Double occupancy is therefore a prerequisite for conduction: when the channel is singly occupied by a divalent cation it is effectively blocked. In addition, because the intrachannel sites have a higher affinity for Ca than for Ba, a single Ca ion is able to more effectively repel Ba out of the channel than the reverse situation. According to the Hess and Tsien (1984) model it is the opposing

interaction between relative binding site affinity and electrostatic repulsion that produces the anomalous mole fraction effect: under conditions where the effect occurs the channel should spend (a) relatively less time being doubly occupied and (b) relatively more time being singly occupied by Ca then it does under conditions where the effect is reduced or absent.

Model Channel and Quantitative Methods

The calcium channel was modeled using the original formalism and parameters proposed by Hess and Tsien (1984), i.e., as a single-file pore displaying an energy profile consisting of three energy barriers and two energy wells, as shown in Fig. 1 (cf., Hille and Schwarz, 1978). It was assumed that (a) the channel was symmetric, (b) the electrical field due to a potential applied across the membrane was linearly distributed across the channel with respect to distances between membrane energy peaks and wells, and (c) the distances between barrier heights and wells were equally distributed across the membrane. The nine possible states and allowed transitions between them are shown diagrammatically by the directed scheme of Fig. 1 b. Only one ionic transition at a time was allowed into an unoccupied well. Transitions whereby one ion binding to an already occupied site lead to the propulsion of other ions through the channel (i.e., a knock on scheme; cf., Hodgkin and Keynes, 1955; Hille, 1984) were not allowed.

The values for the various free energy differences for both Ca and Ba ions were estimated from Fig. 4 b given in Hess and Tsien (1984). The barrier heights for ion permeation in the absence of an applied electrical field were therefore set at 4, -7.5, and 4 RT for Ca, and 4, 1, and 4 RT for Ba, while well depths were set at -15 RT for Ca and -11 RT for Ba. Other details of the model are given in Fig. 1.

The free energy changes used to calculate the respective rate constants for transitions to and from the various channel states (Fig. 1 b) consisted of the sum of (a) the purely chemical energy change given by the difference between well depth and barrier height in the absence of an electric field, (b) the energy change experienced by a charge moving through an applied electrical field (given by $zV/6$, where z is the valence of the ion, V is the transmembrane potential, and $1/6$ is the fractional

electrical distance between a well and a peak), and (c) the energy change due to the electrostatic repulsive force experienced between two ions in the channel (given by $z_1 z_2 \ln(F)$, where F is the so-called repulsion factor, which for divalent-divalent interactions is set at $F = 81$; Hess and Tsien, 1984). Rate constants were calculated using Eyring rate theory (rate = $kT/h \exp(-\Delta G)$, where k , T , h , and ΔG have their usual thermodynamic meaning) as in other treatments of ionic channel permeation (Eyring et al., 1949; Hille, 1975a; Hille and Schwarz, 1978; Begenisich and Cahalan, 1980, a and b).

Simulations to evaluate the equilibrium current flow through the model calcium channel were performed using a modification of a program kindly supplied by Dr. Gerry Oxford. This program used a matrix inversion method (Begenisich and Cahalan, 1980 a,b) to solve the nine simultaneous differential equations of the occupancy probabilities for the nine possible states of the model. The simulation software was converted to Turbo-87 Pascal by Dr. Joseph Hill. To extract estimates of apparent $K_{0.5}$ for both Ca and Ba (see Results), a least-squares Marquardt fitting routine was employed. This program was written in Basic by R. Rasmusson. Both simulations and fitting routines were run on an IBM AT.

For consistency and ease of presentation, the following terminology has been used: "0" refers to an empty intrachannel site, "D" to either a Ca or a Ba cation, and subscripts "1" and "2" to intrachannel binding sites 1 and 2, respectively. All solution concentrations (unless otherwise noted) refer to the total divalent (Ca + Ba) concentration on side 1. All mole fractions are referenced with respect to Ba. Membrane potential was defined to be in the direction such that hyperpolarization corresponded to side 2 being at a more hyperpolarized potential than side 1. Thus, in the more conventional intracellular terminology, side 1 would correspond to extracellular, side 2 to intracellular, and membrane potential to the intracellular potential. All simulations presented were carried out under asymmetric conditions, i.e., only the total Ca and Ba concentrations on side 1 were altered; side 2 Ca concentration was set at 0.1 μ M to mimic the conditions of a cell-attached patch recording. Current flow due to monovalent cations has not been included (cf., Hess and Tsien, 1984).

It is important to emphasize that in the simulations presented we have not attempted to quantitatively match any particular set of experimental results by exhaustive testing for optimization of the various model parameters. Instead, it is our major goal to demonstrate important qualitative trends which would be generated by the Hess and Tsien (1984) model under conditions usually employed in studies of Ca channels, trends which up to now have been either overlooked or unappreciated. Perturbations of specific parameters affected the quantitative aspects of the model results, but the qualitative nature of the predictions presented here still hold (simulations not shown).

RESULTS

General Model Predictions: Effects of Membrane Potential and Divalent Cation Concentration

The effect of membrane potential (range 0 to -100 mV) on the open calcium channel current as a function of the Ba mole fraction in a 10 mM solution is presented in Fig. 2. Absolute current amplitudes are given in Fig. 2 A, while in Fig. 2 B the currents have been normalized with respect to those generated at 0.0 Ba mole fraction at each potential. This normalization procedure was adopted to better illustrate the important overall trends, which would otherwise tend to be obscured due to the small size of the currents in the more depolarized range of potentials. At 0 mV the inward single channel current displays a clear minimum in the Ba mole fraction range of 0.8-0.9. However, with

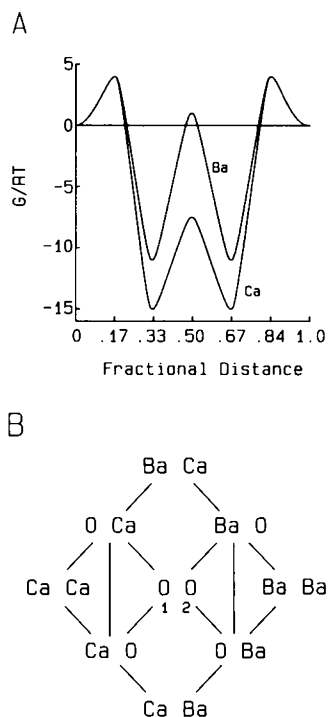


FIGURE 1 (A) Free energy profiles for a single Ca or Ba ion in the absence of both an applied electric field and other ions within the channel. (Abscissa) fractional electrical distance, d , across the channel. (Ordinate) potential energy divided by RT. The channel is symmetric, with energy peaks and wells equally separated by $1/6 d$. (B) State diagram for the nine possible occupancy states of the model channel. Intrachannel sites 1 and 2 are denoted by subscripts 1 and 2, an empty site by 0, a filled site by either Ca or Ba, and transitions between the states by the lines.

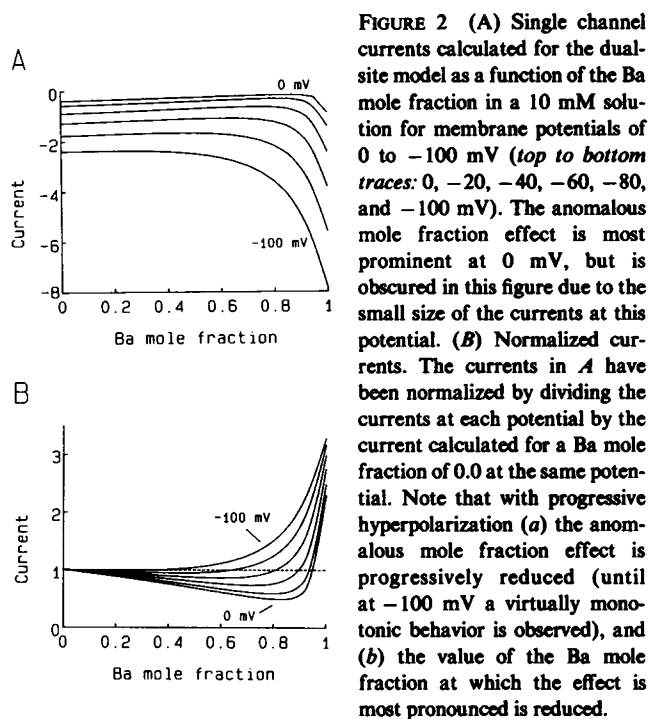


FIGURE 2 (A) Single channel currents calculated for the dual-site model as a function of the Ba mole fraction in a 10 mM solution for membrane potentials of 0 to -100 mV (top to bottom traces: 0, -20, -40, -60, -80, and -100 mV). The anomalous mole fraction effect is most prominent at 0 mV, but is obscured in this figure due to the small size of the currents at this potential. (B) Normalized currents. The currents in A have been normalized by dividing the currents at each potential by the current calculated for a Ba mole fraction of 0.0 at the same potential. Note that with progressive hyperpolarization (a) the anomalous mole fraction effect is progressively reduced (until at -100 mV a virtually monotonic behavior is observed), and (b) the value of the Ba mole fraction at which the effect is most pronounced is reduced.

increasingly hyperpolarized potentials there is a progressive decline in both (a) the magnitude of the anomalous mole fraction effect and (b) the value of the Ba mole fraction at which the effect is most pronounced. Eventually, at -100 mV the open channel current becomes a virtual monotonically increasing function of the Ba mole fraction. Similar effects are produced in both 60 and 110 mM solutions. This predicted potential-dependence of the anomalous mole fraction effect is qualitatively in very good agreement with results obtained by Chesnoy-Marchais (1985) for single Ca channels in *Aplysia* neurons in mixtures of Ca and Ba at a total concentration of 60 mM (see Fig. 10).

The effects of progressively increasing the total divalent cation concentration on the anomalous mole fraction effect are presented in Fig. 3. Normalized currents as a function of the Ba mole fraction in 10, 60, and 110 mM solutions and at potentials of 0, -40, and -80 mV are illustrated. At any fixed membrane potential, raising the divalent concentration decreases the magnitude of the anomalous mole fraction effect. Ultimately, at high divalent concentrations the effect is abolished and a monotonically increasing mole fraction dependence of the open channel current is produced. This concentration-dependent abolition of the anomalous mole fraction effect predicted by the Hess and Tsien (1984) model is qualitatively consistent with the experimentally observed monotonic mole fraction behavior recently reported by Yue and Marban (1987) for guinea pig ventricular L-type Ca channels in mixtures of 110 mM total Ca and Ba.

The open channel current-voltage (I-V) relationships in 10 and 60 mM solutions and for Ba mole fractions of 0.0

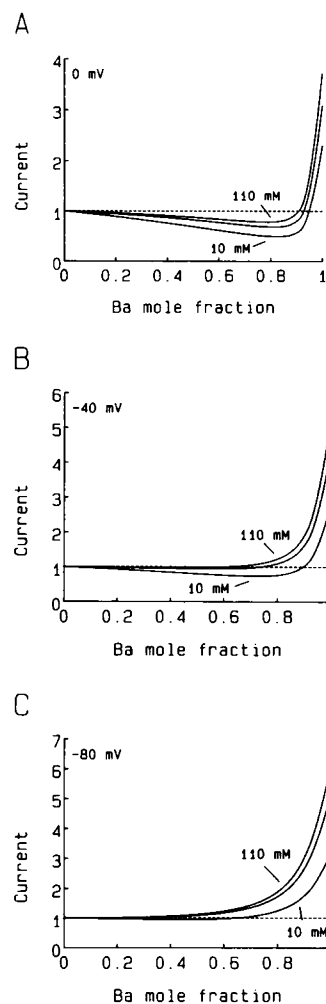


FIGURE 3 Concentration dependence of the anomalous mole fraction effect. Normalized currents (as in Fig. 2 B) as a function of the Ba mole fraction in 10, 60, and 110 mM solutions at three different potentials (A, 0 mV; B, -40 mV, and C, -80 mV). Note that at any given potential and any fixed value of Ba mole fraction increasing divalent concentration reduces the anomalous mole fraction effect.

and 0.8 are presented in Fig. 4. For both 10 and 60 mM solutions the I-V curves for the two different mole fractions crossover. The anomalous mole fraction effect will only be observed at potentials more depolarized than the respective crossover potentials. The voltage sensitivity of the effect is therefore critically dependent upon the nonlinear nature of the open channel I-V relationship under bi-ionic conditions.

The results presented in Figs. 2-4 therefore illustrate two important trends in the observable behavior of the anomalous mole fraction effect for the dual binding site Ca channel model. First, in solutions of fixed total divalent cation concentration, the effect will only be evident in a limited range of depolarized potentials. This range will be determined by the crossover potential for the given conditions. At potentials hyperpolarized to the crossover potential the dependence of the open channel current on the Ba mole fraction will approach that predicted for a simple single binding site model (c.f., Hagiwara, 1975; Hille, 1975b; Hagiwara and Byerly, 1981; Hess and Tsien, 1984). Second, at any fixed mole fraction, raising the total divalent cation concentration will produce a depolarizing shift in the crossover potential. In the limit of high divalent

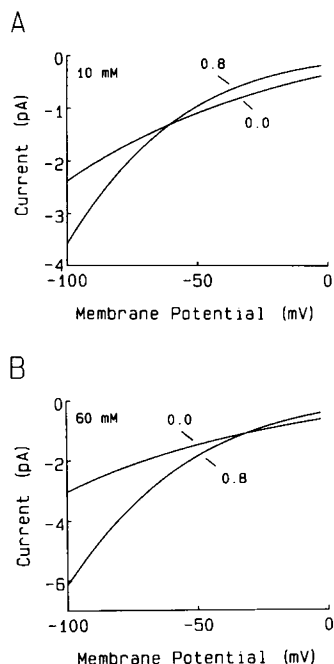


FIGURE 4 Open channel current-voltage (I-V) relationships calculated in 10 and 60 mM solutions for Ba mole fractions of 0.0 and 0.8. Note that (a) the I-V curves for the two different mole fractions crossover, and (b) an increase in total divalent concentration causes this crossover to occur at less hyperpolarized potentials. The anomalous decrease in current is only manifested depolarized to the crossover potential.

concentration the behavior of the open channel current on the Ba mole fraction at any fixed potential will again approach that predicted for a single binding site model. In combination, these two factors will interact to limit both the magnitude of the anomalous mole fraction effect and the potential range at which it will be observable.

Mechanistic Interpretation: Voltage and Concentration Dependence of Open Channel State Occupancy Probabilities

To conceptually illustrate how membrane potential and total divalent cation concentration should combine to produce the effects illustrated in Figs. 2–4, Fig. 5 shows a highly simplified schematic designed to illustrate the effects of membrane potential and concentration on the occupancy probabilities of the channel. In this figure, *A* corresponds to the channel being singly occupied at either site 1 or site 2 ($D_1, 0_2$ or $0_1, D_2$), *B* to the channel being singly occupied exclusively at site 2 ($0_1, D_2$), and *C* to the channel being doubly occupied (D_1, D_2).

In the absence of a membrane potential field and in a low divalent cation concentration but relatively high Ba mole fraction (e.g., 0.7–0.9) solution the occupancy probabilities shown in Fig. 5*A* ($D_1, 0_2$ or $0_1, D_2$) would be favored. The channel is singly occupied most of the time by a Ca ion bound to either site 1 or site 2. Entering Ba ions are rapidly ejected from the channel due to electrostatic repulsion by the more tightly bound Ca. The anomalous mole fraction effect is therefore pronounced under these conditions.

Holding both the mole fraction and divalent cation concentration constant, hyperpolarization favors the occupancy state shown in Fig. 5*B* ($0_1, D_2$) over those in Fig. 5*A*

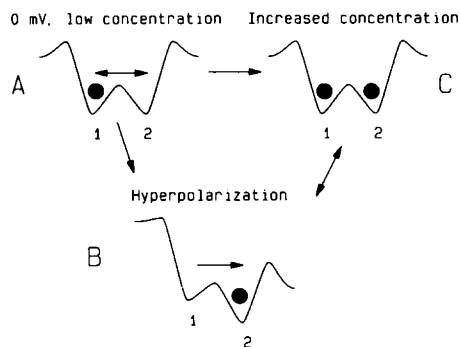


FIGURE 5 Schematic illustration of charge movement through the model dual-site Ca channel when it is always occupied by at least one divalent cation. Predicted effects of increased hyperpolarization and increased concentration. (A) No membrane potential is applied across the channel, and solution 1 is a low concentration but high Ba mole fraction (e.g., 0.7–0.9) solution. Under such conditions the channel is occupied most of the time by a single Ca ion at either site 1 or site 2, and is therefore blocked. (B) Hyperpolarization favors increased occupancy at site 2. This state presents an empty single binding site 1 to incoming Ca and Ba ions a greater percentage of the time; the dual-site channel therefore begins to behave like a single-site channel. (C) Increased concentration causes the channel to be doubly-occupied a greater percentage of the time, regardless of the mole fraction; the dual-site channel therefore again begins to behave like a single-site channel. Cyclic movement through these states causes conduction without ever leaving the channel unoccupied.

(either $D_1, 0_2$ or $0_1, D_2$) by increasing the rate constant for transition from site 1 to site 2 and decreasing the rate constant for the reverse transition. With increasing hyperpolarization, (a) external Ca and Ba ions encounter an empty site 1 a greater percentage of the time, and (b) the relative binding affinity for Ca versus Ba becomes increasingly less important as the $z\Delta V$ factor begins to dominate the free energy term for exit from the channel. Competitive binding for the single external site 1 therefore becomes the rate-limiting step in the conduction process, causing the dual binding site channel to behave as a single site channel. Abolition of the anomalous mole fraction effect with increasingly hyperpolarized membrane potentials is thus a direct consequence of any dual ion, single-file system in which the first ion to enter is tightly bound to but relatively mobile within the channel.

Holding both the mole fraction and membrane potential constant, increasing total divalent cation concentration should promote double channel occupancy (D_1, D_2 ; Fig. 5*C*) through a simple mass action effect. Even though the mole fraction is kept constant, increasing divalent concentration causes the channel to spend more time in the doubly occupied state; thus, the channel should again behave more and more like a single competitive binding site.

Fig. 6 demonstrates the model mechanisms underlying the potential-dependence of the anomalous mole fraction effect by showing the probability occupancy states affecting permeation in a 10 mM solution of Ba mole fraction 0.8. Only those states that have a probability of >0.01 are shown. The curves show the summed probability of the

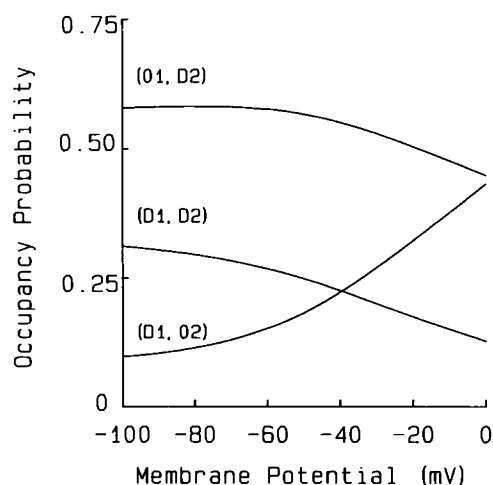


FIGURE 6 Potential-dependence of occupancy probability of the major states affecting the conduction process in a 10 mM solution of Ba mole fraction 0.8. Only those states which have an occupancy probability of >0.01 are illustrated. Progressive hyperpolarization reduces the occupancy probability of states $(D_1, 0_2)$, while it increases the occupancy probability of states $(0_1, D_2)$ and (D_1, D_2) .

channel being singly occupied at site 1 ($D_1, 0_2$), doubly occupied (D_1, D_2), and singly occupied at site 2 ($0_1, D_2$). With hyperpolarization, the probability of single occupancy at site 1 ($D_1, 0_2$) decreases, while the probability of single occupancy at site 2 ($0_1, D_2$) increases. This in turn leads to an increase in the effective availability of site 1 to incoming ions, and a concomitant increase in the probability of the channel being in the doubly occupied state (D_1, D_2). It is this shift in open channel occupancy probability towards the $(0_1, D_2)$ and (D_1, D_2) states that leads to the reduction and ultimate disappearance of the anomalous mole fraction effect.

Model results on the effects of increasing divalent concentration on occupancy probability of the major channel states affecting permeation in solutions of Ba mole fraction 0.8 and at a membrane potential of -40 mV are presented in Fig. 7. Fig. 7.A illustrates that increasing the total divalent concentration simultaneously (a) decreases the probability of the channel being singly occupied by a Ca ion, and (b) increases the probability of the channel being doubly occupied. The channel therefore spends less time in blocked states and more time in conducting states as the divalent concentration is increased, even though the mole fraction is kept constant. To further illustrate this trend toward single site (monotonic) behavior with increasing concentration, Fig. 7.B shows the ratio of the probability of site 1 being occupied by a Ca to the probability of the same site being occupied by a Ba (same conditions as in Fig. 7.A). With increasing concentration, the probability of site 1 being occupied by a Ca relative to Ba declines; at very high divalent concentrations this ratio approaches a limiting value (i.e., that predicted for a saturated, equilibrium single-site condition). It is the combined shift in open channel probability away from singly occupied (blocked)

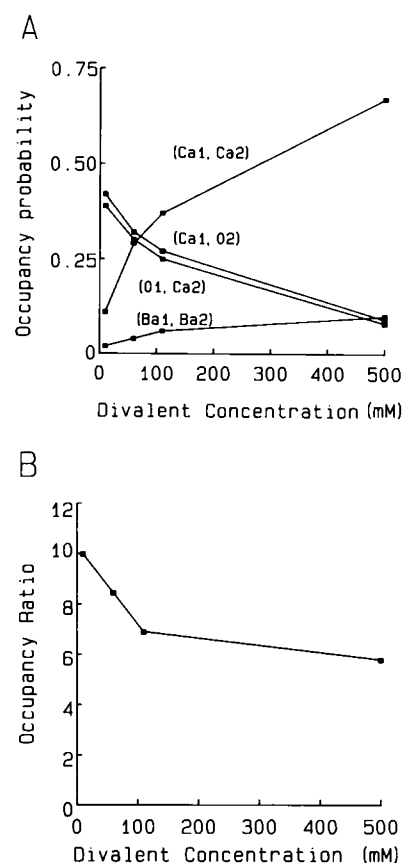


FIGURE 7 Concentration-dependence of occupancy probability of the major states affecting the conduction process. The effects of increasing divalent concentration for solutions of Ba mole fraction 0.8 at a membrane potential of -40 mV are illustrated. Only those states that have an occupancy probability of >0.01 are illustrated. Even though the mole fraction is held constant, increasing divalent concentration simultaneously reduces the probability of single occupancy while increasing the probability of double occupancy. (B). The effect of increasing divalent concentration on the ratio of site 1 occupancy by Ca to site 1 occupancy by Ba at -40 mV and Ba mole fraction 0.8. As divalent concentration increases, the occupancy of site 1 by Ba relative to Ca increases, effectively reducing the amount of time the channel is blocked by a single Ca ion. Saturation of the channel by high concentrations of divalent cations ultimately leads to abolition of the anomalous mole fraction effect.

states and towards doubly occupied (conducting) states with increasing divalent concentration that leads to the reduction and ultimate disappearance of the anomalous mole fraction effect.

DISCUSSION

Experimental Implications

The model results which we have presented contain several important and testable implications for the design of protocols to test for the multi-ionic nature of calcium channels and the applicability of the dual binding site model proposed by Hess and Tsien (1984) using the anomalous mole fraction effect. As demonstrated, both

hyperpolarization and increased divalent cation concentration can combine to favor channel states that cause the dual site channel to behave progressively more and more like a simple single-site channel (i.e., hyperpolarization favors O_1, D_2 , increased concentration favors D_1, D_2). Therefore, according to this model, it would be very difficult to observe the anomalous mole fraction effect at the single-channel level using conditions which presently are routinely employed in patch clamp studies on cardiac calcium channels. Additionally, the nonlinear open channel I-V relationships predicted under bi-ionic conditions could conceivably obscure the effect even more when chord conductances are used. This can be appreciated by reference to Fig. 8, which shows the open channel I-V relationship previously presented in Fig. 4 for a 10-mM solution overlayed with the corresponding slope conductances for each curve: the anomalous mole fraction effect, when measured as single channel conductance, is present over an even smaller potential range than when it is measured by absolute single channel current amplitude.

Our results therefore suggest that to rigorously test for the existence of the anomalous mole fraction effect at the single cardiac Ca channel level will require using both lower divalent cation concentrations in the patch pipette and more depolarized potentials than are routinely used in present studies (e.g., possibly values closer to those used by Chesnoy-Marchais (1985) for *Aplysia* neuronal Ca channels). Whether both the single channel currents and, more importantly, the very small changes predicted to occur in them will be resolvable under such conditions awaits experimental testing.

High divalent concentrations and relatively large mem-

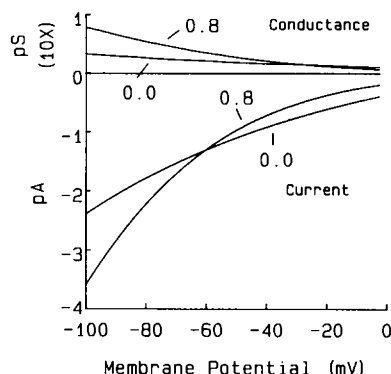


FIGURE 8 Open channel current-voltage relationship (lower panel; same data as in Figure 4.A) and corresponding open channel slope conductance (upper panel) in 10 mM solutions of Ba mole fraction 0.0 and 0.8. Slope conductance was calculated over successive 5 mV potential increments. The corresponding crossover potential is more depolarized for the open channel slope conductance relationship than it is for the open channel I-V relationship. The range of potentials over which the anomalous mole fraction effect will be observable using slope conductance as a measure is considerably more narrow than the range of potentials where the effect can be observed using single channel current amplitudes under identical conditions.

brane potentials may have to be used in order to reliably resolve the current carried through low conductance single calcium channels. Can the validity of the dual binding site model be tested at the single channel level if all that can be observed is a monotonic dependence of current on the Ba mole fraction? Hess and Tsien (1984; see also Hille, 1975b, 1984) pointed out that a simple one binding site model of the Ca channel (see Hagiwara, 1975; Hagiwara and Byerly, 1981, for reviews) always produces a monotonic dependence of the channel current on the Ba mole fraction, regardless of the K_m values chosen. A simple rearrangement of the equation describing current flow through such a single competitive binding site channel (see Fig. 2 of Hess and Tsien, 1984) gives the following:

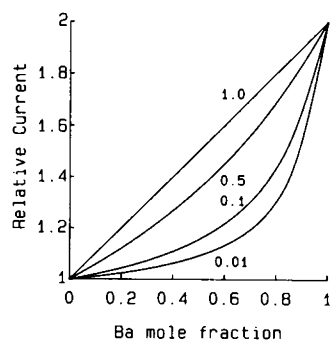
$$I_{\text{Total}} = \frac{I_{\text{Ca,max}} [\text{Ca}]_0 + I_{\text{Ba,max}} [\text{Ba}]_0 (K_{\text{mCa}}/K_{\text{mBa}})}{[\text{Ca}]_0 + K_{\text{mCa}} + [\text{Ba}]_0 (K_{\text{mCa}}/K_{\text{mBa}})} \quad (1)$$

When the single binding site equation is rewritten in this form, it can be appreciated that the behavior of the total current as a function of the Ba mole fraction will be governed largely by the ratio of the respective K_m s for Ca and Ba. The effect of the K_m ratio ($K_{\text{mCa}}/K_{\text{mBa}}$) on the behavior of the single-site model is illustrated in Fig. 9.A, which shows the relative current as a function of the Ba mole fraction for ($K_{\text{mCa}}/K_{\text{mBa}}$) ratios of 1.0, 0.5, 0.1, and 0.01. In this figure the currents have been normalized such that the maximum relative Ba current is twice the maximum relative Ca current (c.f., Fig. 2 in Hess and Tsien, 1984). A progressive reduction of the K_m ratio from 1.0 to 0.01 increases the concavity of the current-mole fraction relationship.

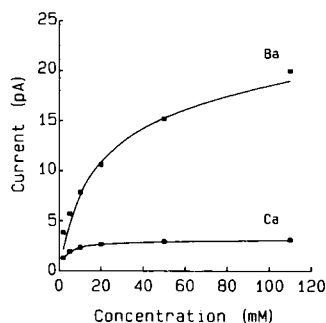
The K_m ratio ($K_{\text{mCa}}/K_{\text{mBa}}$) that can be extracted from mole fraction experiments by fitting Eq. (1) to observed currents has an interesting and direct application for testing for the existence of the dual-site model under conditions where monotonic mole fraction behavior is observed. As has been experimentally demonstrated by Hess, Lansman and Tsien (1986), under asymmetric mono-ionic conditions (i.e., only Ca or Ba present in the patch pipette) current through the cardiac L-type Ca channel has a tendency to display saturation phenomena with increasing Ca or Ba concentrations. Under such mono-ionic conditions, these investigators fit the concentration dependence of single channel Ca and Ba currents (recorded at 0 mV) with simple Michaelis-Menten functions. Under asymmetric mono-ionic conditions the dual-site model approximates saturation behavior with increasing Ca or Ba concentrations, allowing for estimation of apparent K_m s using the single-site Eq. (1) (c.f., Hagiwara and Byerly, 1981; Hess, Lansman, and Tsien, 1986; Tsien, et al., 1987). These apparent mono-ionic K_m s will be functions of membrane potential.

Fig. 9.B presents results generated by the dual-site model for the mono-ionic cases of Ca and Ba at -100 mV (side 1 Ca was varied from 2 to 110 mM), overlayed with a

A



B



C

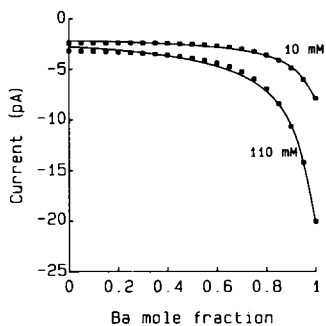


FIGURE 9 (A). Schematic illustration of the effect of reducing the relative ratio (K_{mCa}/K_{mBa}) from 1.0 to 0.5, 0.1 and 0.01 on the total current generated by the single-site equation. The currents have been adjusted such that the relative maximum Ba current is twice the relative maximum Ca current (c.f. Figure 2 in Hess and Tsien, 1984). (B). The behavior of the dual-site model channel under mono-ionic conditions for both Ca and Ba at -100 mV (side 1 concentration was varied from 2 to 110 mM). The solid line is the least-squares, best-fit curve of the single-site equation to the data. The apparent K_m ratio was (K_{mCa}/K_{mBa}) = 164.84×10^{-3} . (C). Least-squares, best-fit curves of the single-site equation (solid curves) to currents generated by the dual-site model as a function of the Ba mole fraction (points) in 10 mM and 110 mM solutions at -100 mV. Extracted K_m ratios were: 10 mM, (K_{mCa}/K_{mBa}) = 29.59×10^{-3} ; 110 mM, (K_{mCa}/K_{mBa}) = 66.67×10^{-3} .

representative least-squares, best-fit curve of the data to the single-site equation. Table I (part A) presents summarized K_m s derived from a complete least-squares, best-fit analysis to the single-site equation of currents generated by the dual-site model under mono-ionic conditions for both Ca and Ba at membrane potentials ranging from -40 to -100 mV. Under such mono-ionic conditions the dual-site model predicts that the relative ratio (K_{mCa}/K_{mBa}) decreases with increasing hyperpolarization.

The single-site equation can again be used to fit the currents and extract least-squares estimates of K_m ratios for Ca and Ba under bi-ionic conditions where the dual-site model is displaying simple monotonic-dependency upon the Ba mole fraction. Such fits for both 10 and 110 mM solutions at -100 mV are presented in Fig. 9.C. Part B of Table I summarizes results of a least-squares K_m ratio analysis of currents generated as a function of the Ba mole fraction in 10, 60, and 110 mM solutions. (For complete-

TABLE I

A Mono-ionic Conditions

V_m (mV)	K_{mCa} (mM)	K_{mBa} (mM)	(K_{mCa}/K_{mBa}) ($\times 10^{-3}$)
-40	4.9	23.6	207.63
-60	3.8	21.0	180.95
-80	3.3	19.3	170.98
-100	3.0	18.2	164.84

B Bi-ionic Conditions (Mole Fraction Estimates)

Concentration	10 mM	60 mM	110 mM
V_m (mV)	(K_{mCa}/K_{mBa}) ($\times 10^{-3}$)	(K_{mCa}/K_{mBa}) ($\times 10^{-3}$)	(K_{mCa}/K_{mBa}) ($\times 10^{-3}$)
-40	0.38	12.97	20.35
-60	7.10	23.81	32.15
-80	16.31	40.65	48.08
-100	29.59	60.61	66.67

Least-squares estimates of (K_{mCa}/K_{mBa}) ratios for the dual-site model under mono-ionic versus bi-ionic (i.e., varying Ba mole fraction) conditions. (A) Currents under mono-ionic conditions were generated using the dual-site model with only Ca or Ba present and by varying the side 1 concentration from 2 to 110 mM (2, 5, 10, 20, 50, and 110 mM). K_m s for a single-binding site model were then estimated using a least-squares fitting routine. (B) K_m s were obtained by least-squares fitting of the single-site equation to currents generated by the dual-site model as a function of the Ba mole fraction at the indicated potentials and concentrations (see Figs. 9.B-C).

ness, the results of fitting currents generated in 10 mM solutions are included; however, monotonic behavior in this case was only approached at -100 mV). When K_m s for Ca and Ba are extracted from the monotonic curves predicted under bi-ionic mole fraction conditions, the extracted K_m ratio is different from that predicted under mono-ionic conditions. First, at any fixed concentration, the K_m ratio increases with increasing hyperpolarization. Second, at any fixed membrane potential, the extracted K_m ratios will be functions of the total concentration; increasing concentration causes the extracted K_m ratios to converge.

These predicted trends in the behavior of the K_m ratio under mono-ionic as opposed to bi-ionic conditions provide a test for the dual-binding site model at the single channel level, even though it is only possible to observe monotonic mole fraction behavior. To summarize, the Hess and Tsien (1984) model predicts that: (a) the K_m ratio extracted under mono-ionic conditions at a given fixed potential will be different from those extracted under bi-ionic conditions at the same potential; (b) at a fixed concentration, the K_m ratio extracted from experimental mole fraction data will increase with hyperpolarization, while the ratio will decrease in the mono-ionic case; and (c) at a fixed potential, the bi-ionic K_m ratio will be concentration-dependent and will converge with increasing concentration (e.g., 60 to 90 to 110 mM). This latter effect provides an elegant test of the dual-site model, since it is incompatible with the predictions of a single-site model.

Fig. 10 presents an experimental test of prediction (b) (i.e., increasing apparent K_m ratio with hyperpolarization) using the published data of Chesnoy-Marchais (1985) on single *Aplysia* neuronal Ca channels. Using only the data points where monotonic mole-fraction behavior was observed in these channels, the single-site equation was fit to the data and the apparent K_m s extracted. The data of Chesnoy-Marchais (1985) is well fit by the single-site equation, and indicates that with increasing hyperpolarization the apparent K_m ratio is decreasing. With regard to prediction (c) (i.e., concentration-dependent apparent K_m ratios), to date we are unaware of any sufficiently complete experimental data where the concentration dependence of the mole fraction effect was studied in single Ca channels to allow us to extract the K_m ratios. Therefore, experimental verification (or rejection) of prediction (c) remains to be tested. While such experiments will be technically demanding and will require careful analysis, they should be feasible.

All of the simulation results which we have presented are, by necessity, model-dependent. It is for this very reason that we deliberately have not attempted to quantitatively match any particular set of experimental data, but instead have elected to illustrate general properties predicted by the model as a possible framework for future experimentation. However, it could be argued that one of the most significant shortcomings of the Hess and Tsien (1984) model, as well as many other recent models of ion permeation through membrane channels (e.g., Almers and McCleskey, 1984; Hille, 1975a; Begenisich and Cahalan, 1980a, b; Hille and Schwarz, 1978; Iijima et al., 1986), is that it is based upon the formalism of Eyring rate theory. The general validity of applying Eyring rate theory to descriptions of ion permeation through membrane channels has recently been questioned by some investigators (e.g., Cooper, Gates, and Eisenberg, 1988). In this regard, it is important to note that the proposed tests on the behavior of the relative (K_{mCa}/K_{mBa}) ratio for the dual-site

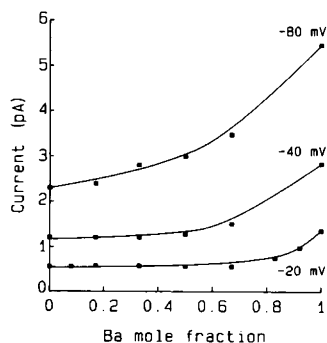


FIGURE 10 Least-squares, best-fit analysis of the single-site equation to the published data of Chesnoy-Marchais (1985) on single *Aplysia* neuronal Ca channels measured as a function of the Ba mole fraction in 60 mM total divalent patch pipette solutions at -20, -40, and -80 mV. Extracted K_m and relative ratio values were as follows: -20 mV, $K_{mCa} = 0.784$ mM, $K_{mBa} = 12.156$ mM, ratio = 64.5×10^{-3} ; -40 mV, $K_{mCa} = 0.97$ mM, $K_{mBa} = 10.105$ mM, ratio = 96.0×10^{-3} ; -80 mV, $K_{mCa} = 1.54$ mM, $K_{mBa} = 5.49$ mM, ratio = 280.5×10^{-3} . This convergence of K_m ratios with progressive hyperpolarization is consistent with a dual binding site model.

model under conditions where it is displaying simple monotonic behavior are particularly relevant, since the results predicted are totally independent of the underlying assumptions of Eyring rate theory.

In conclusion, the model results demonstrate that (a) the appearance of monotonically increasing single channel currents with Ba mole fraction and (b) the lack of an anomalous mole fraction effect at more hyperpolarized potentials are not inconsistent with the general predictions of the dual binding site model of Hess and Tsien (1984). Rather, such phenomena are actually predictive results which can be used to both test for the general applicability of the model and, if found to be applicable, to allow for more effective quantification of the various model parameters. These simulations therefore provide both a possible resolution of the apparent discrepancies presently existing regarding the existence of the anomalous mole fraction effect at the macroscopic as opposed to the single channel level, and a framework for future experimentation on the mechanisms of Ca channel permeation.

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