Ca Chelators and Membrane Transport: "Geometric" Considerations

Ronald F. Abercrombie[a,*] and James E. Moore[b]

Departments of Physiology[a] and Pediatrics[b], Emory University School of Medicine, Atlanta, GA, 30322, USA

Abstract: We examine theoretically the ability of diffusible cytoplasmic carriers to facilitate delivery of substrate to sites within biological membranes. Two processes are analyzed. In one, delivery of Ca to a *site* on a membrane surface is examined. As a contrasting example, chelator-supported diffusion to a uniform absorbing *surface* is considered.

INTRODUCTION

Intracellular Ca exerts control over a range of cellular responses. Maintaining a low basal intracellular Ca ion concentration provides the background for calcium signaling, and this requires transport *via* pumps into organelles and across the plasma membrane. We consider whether the presence of mobile intracellular calcium chelators aids the transport process and examine how "geometric" considerations enter the analysis by contrasting a regime of small transport sites to one in which absorption takes place at *any* location on a surface.

Calcium and diffusion to transport sites: possible role for chelated Ca ions. In its most basic representation, the Ca ion activity relates to the frequency of strikes that the ions make against a site. If we imagine a small entry site residing on the surface of a calcium pump, then the local calcium activity would be proportional to the frequency of Ca ion strikes against this site. The central question becomes the following: "If calcium is available on mobile chelators, could the local Ca ion activity at the site rise above that expected when no mobile chelators are present?" In other words, with a favorable local electrostatic or ionic environment around a binding site, would the *presence of calcium chelators* influence the frequency with which these ions strike the site?

To formulate this question we follow a treatment of Decker and Levitt [1], originally derived for hydrogen ion flux through pores, which finds its basis in a treatise of Smoluchowski [2]. In the development of these equations, it is generally assumed that the target absorbs each ion that strikes it, providing a mathematical boundary condition of zero ion concentration at the target surface. A zero ion concentration may seem a stringent physical constraint at the entry site of Ca ions. However, an equivalent assumption which contains the same mathematical boundary condition is that each Ca ion may strike this site only once and that an approach and strike of one ion, followed by its return to the medium, would not interfere with that of another. This assumption likely becomes more realistic in the low calcium concentration limit. We therefore consider unidirectional flux toward a site, without the second-order complications of ion-ion interference, and thus we seek a quantitative description of diffusion-limited strike frequency that would apply at the low concentration limit.

*Address correspondence to this author at the Department of Physiology, Emory University School of Medicine, Atlanta, GA, 30322, USA; Tel: (404) 727-7425; Fax: (404) 727-2648; E-mail: Ronald.abercrombie@emory.edu

MATHEMATICS OF DIFFUSION TO DISCRETE SITES

The diffusion of calcium ions of concentration $[Ca]$ surrounding the surface of a "dome" representing a calcium entry site (a spatially defined target) must satisfy the diffusion-reaction equation, given here in spherical coordinates:

$$\frac{\partial [Ca]}{\partial t} = D_{Ca}\frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial [Ca]}{\partial r}\right) + F \tag{1}$$

We define F as the net feed rate from the *buffered* pool. This term represents an excess delivery (above diffusion) of Ca ions which dissociate from a nearby buffer. $F = 0$ for the case of simple diffusion without a calcium chelator. D_{Ca} is the *free* calcium diffusion coefficient; r is the distance from the center of a dome representing the pump's calcium inlet site; and t is time. We give solutions of this equation for the steady state, $\frac{\partial [Ca]}{\partial t} = 0$, thus representing an average behavior over time.

For a uniform concentration of chelator $[B]_b$ and chelated Ca $[BCa]_b$ and a fixed calcium concentration $[Ca]_b$ in the bulk solution, the term F in equation (1), representing the extra delivery to the site *via* the chelator (net feed rate), may be derived from $F = k_{off}[BCa]_b - k_{on}[B]_b \cdot [Ca]$ by substituting the equilibrium expression for k_{on} within the bulk solution as $k_{on} = \dfrac{k_{off}[BCa]_b}{[B]_b[Ca]_b}$,

where k_{off} is the rate constant of Ca-chelator dissociation and k_{on} is that of association.

F would then be represented in the following form:

$$F = k_{off}[BCa]_b\left(\frac{[Ca]_b - [Ca]}{[Ca\ ]_b}\right) \tag{2}$$

In biological solutions, k_{off} typically would be in the range of $1 - 100$ s^{-1} for chelators such as BAPTA or EGTA at neutral pH. The terms $[BCa]_b$, the buffered calcium concentration, and $[B]_b$, the free buffer concentration, represent these values in bulk solution as well as near the site. These concentrations would be in the millimolar range, and nearly uniform around the site if the local dwell time of the Ca-chelator were much less than the reciprocal of the off-rate constant, $1/k_{off}$ (~ 0.01 to 1 s). A dwell time, Δt_d, given by $\Delta t_d = (\Delta x)^2/D_B \approx 10^{-9}$ s for values of Δx in the nm range, and D_B

$\sim 10^{-5}$ cm^{2}s^{-1}, ordinarily would satisfy this requirement. (D_B represents the *chelator* diffusion coefficient.)

The average calcium concentration profile $[Ca]$ (a function of r) may be obtained by solving the diffusion-reaction equation (1) with the appropriate boundary conditions: in the bulk, $[Ca] = [Ca]_b$; and, at the reaction site, $[Ca] = 0$ (used to estimate unidirectional flux or strike frequency). Assuming that $F = 0$, the steady-state solution of equation (1) is:

$$\frac{[Ca]_b - [Ca]}{[Ca]_b} = \frac{r_o}{r} , (r \geq r_o) \qquad (3)$$

where r_o is the radius of a dome representing the Ca site.

Assuming that a Ca buffer is present, with F defined by equation (2), the steady-state solution for $[Ca]$ from equation (1) is:

$$\frac{[Ca]_b - [Ca]}{[Ca]_b} = \frac{r_o}{r} \exp\left(\frac{r_o - r}{\lambda}\right), (r \geq r_o) \qquad (4)$$

with the constant λ explicitly defined in equation (8). That this expression solves the differential equation (1) under the definition of F given in equation (2) can be confirmed by substitution of $[Ca]$ from equation (4) into equation (1).

Taking the simpler case of no Ca chelators, i.e., $F = 0$, with the $[Ca]$ profile given as in equation (3), and assuming the site to be represented by a hemispherical dome of radius r_o, the standard expression can be derived for the steady-state diffusion-limited strike frequency v. Flux is determined from the Ca diffusion coefficient and the slope of the concentration profile and is converted to particles per second, v, by multiplying the target area and Avogadro's number.

$$v = D_{Ca} \cdot 2\pi r_o^2 N \frac{\partial [Ca]}{\partial r}\bigg|_{r=r_o} \qquad (5)$$

or

$$v = D_{Ca} \cdot [Ca]_b \cdot 2\pi r_o N , \qquad (6)$$

where N is Avogadro's number, r_o is in cm, D_{Ca} is the free Ca diffusion coefficient (cm^{2}s^{-1}), and $[Ca]_b$ is in units of moles cm^{-3}.

In the presence of a Ca-releasing chelator, the expression similarly derived (from equation 4) for the frequency of strikes becomes (see, for example, [1])

$$v = D_{Ca} \cdot [Ca]_b \cdot 2\pi r_o N (1 + r_o / \lambda) , \qquad (7)$$

with λ given by

$$\lambda = \sqrt{\frac{[Ca]_b \cdot D_{Ca}}{[BCa]_b \cdot k_{off}}} \qquad (8)$$

$[BCa]_b$ must have the same units as $[Ca]_b$ and λ has units of cm, if D_{Ca} has units of cm^{2}s^{-1}.

Equations (6) and (7) are central to this discussion and follow directly from equations (3) and (4), respectively. These results should be valid as long as no interference takes place among striking and exiting Ca ions at the site, each approaching Ca ion strikes the site only once, and a uniform concentration of chelated Ca surrounds the site. If these conditions do not prevail, then (7) may represent a lower limit as discussed later. Equations (7) and (8) thus provide a basis for estimating the effects of the variables $[Ca]_b$, k_{off}, D_{Ca}, $[BCa]_b$, and r_o on Ca^{2+} delivery to a hypothetical target. They illustrate that a Ca ion striking the site may come from one of two possible sources: a free Ca ion in solution, or a calcium ion that is released from a chelator molecule as it migrates sufficiently close to the site.

As shown in equation (7), the relative size of the second component, or the chelator contribution, is given by the ratio of the capture site radius r_o compared to the parameter λ. For the case of Ca transport, λ would be defined in a region about which we have *limited knowledge*. If we use the known values of the free Ca diffusion coeffieient D_{Ca} ($\sim 10^{-5}$cm^{2}s^{-1}), k_{off} in free solution for chelators such as EGTA or BAPTA (~ 1s^{-1} -100s^{-1}), and typical values for $[BCa]_b$ ($\sim 10^{-3}$M) and $[Ca]_b$ ($\sim 10^{-7}$M), then the parameter λ would be in the $\sim 30 - 300$ nm range, larger than any reasonable estimate of a target size r_o (~ 0.1 -1 nm). Thus, there would be little significant contribution of Ca ions released by chelators onto a small site, assuming that these reside in free solution of biological media. If Ca chelators were to contribute to transport, then either they must have very fast k_{off} rates or an unusual microscopic environment must prevail near the capture site. Thus, (a) the Ca off-rate, k_{off}, could be much greater at the site, (b) the diffusion coefficient of free calcium, D_{Ca}, could be much lower than in bulk solution, (c) the effective concentration of chelated calcium at the site, $[BCa]$, could be greater than in bulk solution, or perhaps (d) the size of the capture site, r_o, could be larger than we would predict. If r_o were large compared to λ (for example if buffers are present in large concentrations or have a high k_{off}), then according to equation (7), chelators *would* aid solute movement to the capture site. A capture site for Ca transport, r_o, is expected to be small ($\sim$nm), however, and buffer facilitation of transport is *not* expected, assuming free solution values for $[Ca]_b$, D_{Ca}, $[BCa]_b$, and values for k_{off} of ~ 1s^{-1} – 100s^{-1}.

DIFFUSION OF MEMBRANE-PERMEANT SUBSTANCES TO A PLANAR SURFACE

The previous calculations considered three-dimensional diffusion toward small sites. As a comparison, we consider a calculation for diffusion to, and absorption at, any location on a planar surface. Though not an appropriate model for Ca transport, this might apply to substrates such as oxygen or carbon dioxide, which are capable of unrestricted crossing of a lipid membrane. We nevertheless consider the diffusible cytoplasmic carrier for the membrane-permeant substrate (oxyhemoglobin or bicarbonate) as a "chelator/carrier".

For absorption at any location on a planar membrane, the problem reduces to one dimension as expressed in equation (9):

$$\frac{\partial [C]}{\partial t} = D_C \frac{\partial^2 [C]}{\partial x^2} + F \qquad (9)$$

D_C and $[C]$ represent the diffusion coefficient and concentration of the membrane permeant (free) substrate and x is the distance from the surface. With F defined as before, the steady-state solution to equation (9) can be represented as

$$\frac{[C]}{[C]_b} = 1 - \exp(-x/\lambda), \tag{10}$$

where $[C]_b$ represents the bulk substrate concentration, and λ has its previous meaning. Here the introduction of the "feed" term "F" in equation (9) changes the solution from what would have been a linear one (with $F=0$) to an exponential form, equation (10).

From equations (10) and (8), and $v = N \cdot area \cdot D_C \cdot \dfrac{dC}{dx}$, the representation of strikes to an area of planar surface per second is given by $\dfrac{v}{area} = N\sqrt{D_C[C]_b \cdot [BC]_b k_{off}}$, which explicitly includes a chelator/carrier contribution in the term $[BC]_b$.

Another approach to providing a solution to equation (9) involves the explicit introduction of an unstirred layer and defining the distance over which a concentration gradient may exist close to the membrane surface. Here, the probability of a chelator contribution to the flux is related to the ratio of the size of the *unstirred layer* compared to λ (defined in equation 8).

In a third and simpler approach, the diffusion to a "planar-like" surface may be approximated by simply increasing the size of the target (r_o) in (7), corresponding to a larger region where the "chelated" molecules interact with a surface. Here, for example, the parameter r_o could represent the radius of a vesicle, the surface of which is highly permeable to the substrate, $[C]_b$. Thus the entire vesicle would constitute a target. Though the size of the parameter λ would depend on $[C]_b$, k_{off}, D_C, and $[BC]_b$ as given in an expression similar to that of equation (8), the size of "r_o" for this process becomes as large as the vesicle, increasing the likelihood of chelator facilitation of flux of membrane-permeant substrates according to (7).

MEASURED CHELATOR EFFECTS

Is there evidence that Ca chelators facilitate Ca transport? Even though this transport must act through relatively small transport sites, the answer is, perhaps surprisingly, yes. Effects of chelators on calcium transport have been reported in studies of both plasma membrane (PM) and endoplasmic reticulum (ER) type Ca pumps. Most of these studies were done measuring Ca flux with ^{45}Ca, and all were done using isolated membrane vesicles. For example, in 1979, Sarkadi, Shubert, and Gardos [3] demonstrated that Ca pumps of inside-out vesicles of erythrocyte membranes had a higher apparent Ca affinity when EGTA was present. In 1982, Berman [4] isolated vesicles of sarcoplasmic reticulum (SR) and, using calcium electrodes, detected SR calcium uptake stimulated by the presence of EGTA. He showed that the Ca pump of SR vesicles had a greater apparent Ca affinity when 50 μM EGTA was included in the uptake media. It is well known that calmodulin, an intracellular messenger that binds Ca, activates the PM Ca pump at a specific Ca-calmodulin receptor site ([5], [6]). Timmermans et al. [7] concluded that other calcium chelators (EGTA, calbindin-D28, and parvalbumin) increase the PM pump activity by another, fully distinct, mechanism: that of increasing the "free Ca in the immediate vicinity of the Ca pump". Enhancement of Ca flux by calbindin-D28 or EGTA was additive and independent of

that caused by calmodulin. Thus, EGTA and calbindin had actions that were different from the regulatory cofactor, calmodulin. Calbindin, the native vitamin-D dependent intracellular Ca-binding protein, helps to restore the body's calcium when Ca stores are depleted [8]. Chelator-assisted Ca delivery to plasma membrane pump inlet sites could be a mechanism of calbindin's action in maintaining a cell's and the organism's Ca homeostasis. In microsome (ER) preparations from brain, elevated Ca transport affinity was found in the presence of chelators, including EGTA, BAPTA, and BAPTA derivatives [9].

THE LOCAL ENVIRONMENT

What *might be* the local environment in which Ca and Ca chelators encounter Ca transport sites? Before the crystal structure of the SERCA pump was determined [10, 11], this question could not have been satisfactorily addressed. Most might have predicted Ca entry *via* the hydrophilic regions of the pump protein that extend into the cytoplasm. The location of the Ca entry site, at least according to one of these crystal structures, is within a region that would be partly surrounded by hydrophobic surfaces, i.e., a region that would likely be adjacent to, or perhaps partially submerged within, *the lipid bilayer*. (This may be seen in supplementary material given by Toyoshima and Nomura [11]; their **figure** is reproduced with permission). Thus, a possible pathway for Ca entry begins on the cytoplasmic surface near the phospholipid bilayer; the access path may skirt the lipid interface, and then follow onto an opening and Ca binding site deeper
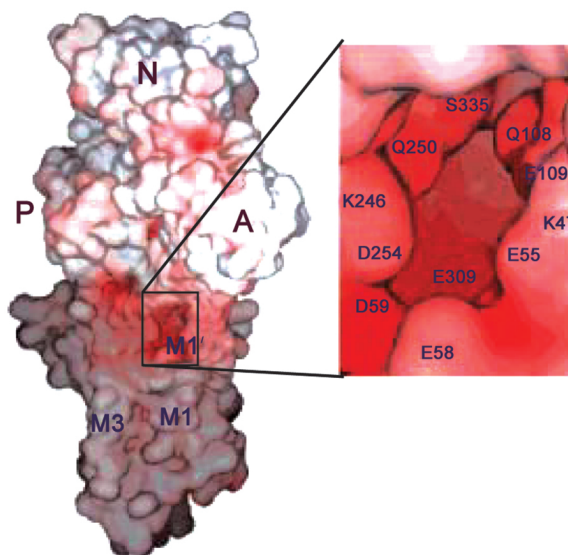
This Figure is taken with permission from the supplementary material of Toyoshima and Nomura [11]. A water-accessible channel leading to the Ca binding site within the protein is shown in the enlarged view of the inset to the right. Although the inlet channel itself has a predominantly negative charge (red in the color image) at its surface (to accommodate the positively charged calcium ion), this opening originates near the uncharged (hydrophobic) region of the molecule (grey in the color image), which would be embedded in the lipid of the bilayer membrane. "P", "N", and "A" domains are hydrophilic (cytoplasmic) domains and are generally positively charged (white); M1 and M3 represent portions of transmembrane alpha helices. The lettered symbols in the enlarged inset to the right represent identified amino acids of the inlet channel and are near the Ca binding site deeper within the pump protein.

within the pump's structure. It is conceivable that accessory molecules (such as Ca chelators) might ease Ca^{2+} delivery across a partially hydrophobic environment helping provide access to the Ca inlet site. Chelators could thus act as *local chaperones*. In such a unique local environment, the parameter λ would be smaller and the second term in equation (7), i.e., r_o/λ, larger than would be expected in free solution.

SUMMARY AND CONCLUSIONS

Two types of chelator effects appear at Ca pumps: a well-studied specific effect caused by calmodulin's interaction with a calmodulin receptor site on plasma membrane (PM) type pumps, and a less well-studied, and theoretically unexpected, non-specific effect caused by a number of different Ca chelators on both the PM and ER type pumps. The non-specific effect suggests the presence of an atypical microscopic environment surrounding the Ca inlet site that could alter a "chelator dwell time" or Ca off-rate.

For detecting the chelator effects that have been reported, experiments were always done using isolated membrane vesicles. If one accepts that *native* cellular Ca chelators function to facilitate Ca delivery to pump sites, it is not surprising that only when membranes have been stripped of their natural chelators by the isolation procedures of membrane preparation might one expect to identify a role of an exogenous chelator, as added synthetic or natural chelators replace those lost from the intact system. Ca delivery to its inlet site may take advantage of an interaction of low specificity between native Ca chelators and an unusual local environment at the intake site. This design could have been evolution's solution for two potentially conflicting requirements: maintaining a relatively low background of free intracellular calcium (for signaling purposes), but preserving the ability to rapidly transport large amounts of Ca across cellular membranes to quickly redistribute this ion.

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