ESTIMATION OF INTRACELLULAR [Ca^{2+}] BY NONLINEAR INDICATORS

A Quantitative Analysis

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ABSTRACT When spatial gradients of intracellular free [Ca^{2+}] are present, intracellular calcium indicators that have a nonlinear response to [Ca^{2+}] may yield an estimate of [Ca^{2+}] that differs from the spatial average [Ca^{2+}]. We present two rules that provide (*a*) general criteria to distinguish those classes of indicators that will yield an overestimate of spatial average [Ca^{2+}] from those that will yield an underestimate, and (*b*) limits on the extent to which spatial average [Ca^{2+}] might be over- or underestimated. These rules are used to interpret quantitatively the aequorin luminescence signals obtained from cardiac ventricular myocardium.

INTRODUCTION

The changes in intracellular free [Ca^{2+}] ([Ca^{2+}]) that accompany many physiological phenomena (1) are complex; [Ca^{2+}] varies, often rapidly and inhomogeneously, throughout the cell (2–7). Full understanding of the role of [Ca^{2+}] in these phenomena would therefore require precise knowledge of the spatial distribution of [Ca^{2+}] throughout the cytoplasm. In certain cases, it is now possible to obtain spatial images of intracellular [Ca^{2+}] (4, 8), but the most common situation at present is to estimate [Ca^{2+}] from spatially averaged calcium indicator signals, especially when fast time response is required. Unfortunately, when spatial inhomogeneities of [Ca^{2+}] are present, the estimate of [Ca^{2+}] derived from spatially averaged signals may differ considerably from spatial average [Ca^{2+}] if the indicator used has a nonlinear response to [Ca^{2+}] (3). The lack of a firm theoretical basis for understanding the nature and extent of this problem for nonlinear indicators of various types has limited severely the utility of intracellular calcium indicators; quantitative interpretation of such signals has been either acknowledged to be uncertain (9), or done on the assumption that gradients of intracellular [Ca^{2+}] are negligible (10).

Here we develop a rigorous theoretical framework for the use of nonlinear calcium indicators to estimate spatial average [Ca^{2+}] from spatially averaged indicator signals. Our analysis is developed in terms of the most widely used calcium indicator, aequorin, but it is general, and applicable with slight formalistic modification to many other indicators as well.

THEORETICAL RESULTS

Definitions and Assumptions

Throughout our analysis, we assume the following (*a*) There are no delays between changes in indicator output and changes in intracellular [Ca^{2+}]. (*b*) The calcium indicator is uniformly distributed in the cytoplasm. The extent to which these assumptions apply, or can be made to apply after suitable compensation of signals, has been evaluated previously for various calcium indicators (11).

Define $\underline{C}$ as the spatial average [Ca^{2+}] so that

$$\underline{C} = (1/V_T) \iiint_P C(x, y, z)\, dx\, dy\, dz, \quad (1)$$

where the integration is with respect to volume; P is the volume region of the cytoplasm; P has volume V_T; and $C(x, y, z)$ is a function whose value is equal to the [Ca^{2+}] at the spatial coordinates x, y, and z.

The luminescence measured from the entire preparation, L, is given by

$$L = \iiint_P l[C(x, y, z)]\, dx\, dy\, dz, \quad (2)$$

where l is a function whose value is the luminescence derived from a unit volume of cytoplasm in which the [Ca^{2+}] is given by $C(x, y, z)$.

To account for variations between preparations (e.g., amount and activity of aequorin, optical absorption properties), L is normalized by the maximal luminescence (L_{max}), which would be measured if cytoplasmic [Ca^{2+}] were to be raised instantly to saturating levels. In practice, L_{max} is derived from the time integral of L when the preparation is lysed in saturating [Ca^{2+}] (12). From Eq. 2, the normalized luminescence (L/L_{max}) can be expressed

$$L/L_{max} = (1/V_T) \iiint_P l_n[C(x, y, z)]\, dx\, dy\, dz, \qquad (3)$$

where $l_n[C(x, y, z)] = l[C(x, y, z)]/(L_{max}/V_T)$. $l_n[C(x, y, z)]$ is the luminescence derived from a unit volume of cytoplasm normalized by the maximum luminescence for that unit volume.

Why Errors in the Estimate of Spatial Average [Ca^{2+}] Can Occur

An estimate of spatial average intracellular [Ca^{2+}] is obtained by referring the normalized luminescence (L/L_{max}) to a calibration curve, which is an in vitro determination of l_n. An error in the estimate of $\underline{C}$ may occur in the case of nonlinear calibration curves because a given spatial average [Ca^{2+}] can be associated with a multitude of L/L_{max} values (see Eqs. 1 and 3). From Eq. 3, it is clear that the estimate of spatial average [Ca^{2+}] will be accurate only when the following approximation holds

$$(1/V_T) \iiint_P l_n[C(x, y, z)]\, dx\, dy\, dz \sim l_n(\underline{C}). \qquad (4)$$

Of course, Eq. 4 will always hold if l_n is a linear function of $C(x, y, z)$. In the case of nonlinear indicators of [Ca^{2+}], Eq. 4 will not hold in general unless $C(x, y, z)$ is constant and therefore equal to spatial average [Ca^{2+}], $\underline{C}$. Two rules are presented that describe how this approximation breaks down for particular $l_n[C(x, y, z)]$ and $C(x, y, z)$.

Criteria for Determining Whether Overestimation or Underestimation of Spatial Average [Ca^{2+}] Will Occur

Fig. 1 *A* shows a plot of L/L_{max} vs. spatial average [Ca^{2+}] ($\underline{C}$) for the case when the calibration curve has an upward curvature [more precisely, when $d^2l_n(C)/dC^2 > 0$] throughout the range of [Ca^{2+}] in the preparation. The solid curve is the calibration curve (l_n plotted as a function of $\underline{C}$). Because of the upward curvature of the calibration curve, it turns out that a given value of spatial average [Ca^{2+}] ($\underline{C}'$) can be associated with any number of L/L_{max} values that are greater than or equal to the value expected from the calibration curve (i.e., L/L_{max} values reside on the dashed line on or above the solid calibration curve). This is true for any particular choice of $\underline{C}'$, so that the locus of possible L/L_{max} values plotted vs. spatial average [Ca^{2+}] would be the entire shaded region in the figure.

From Fig. 1 *A*, then, it is clear that by projecting a measured L/L_{max} value through the solid calibration curve, one obtains the largest possible spatial average [Ca^{2+}] that could give rise to such an L/L_{max}. In other words, when the calibration curve has an upward curvature, one obtains an upper limit estimate of spatial average [Ca^{2+}].

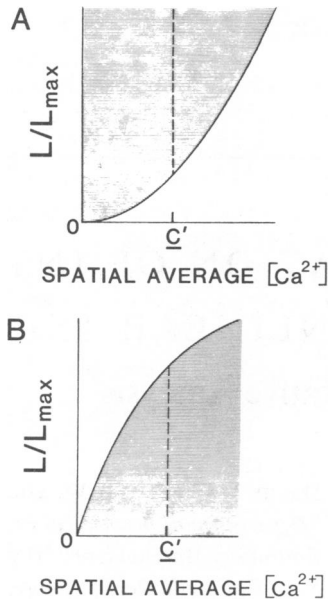

FIGURE 1. How overestimates (*A*) and underestimates (*B*) of spatial average [Ca^{2+}] can occur. Solid curves are hypothetical calibration curves (l_n). Dashed lines are the range of normalized luminescence (L/L_{max}) values that could arise from [Ca^{2+}] distributions with spatial average [Ca^{2+}] = $\underline{C}'$. Shaded regions represent all possible relationships between normalized luminescence (L/L_{max}) and spatial average [Ca^{2+}]. *A*: case when $d^2l_n(C)/dC^2 > 0$. *B*: case when $d^2l_n(C)/dC^2 < 0$.

Fig. 1 *B* depicts the situation when the calibration curve has a downward curvature [$d^2l_n(C)/dC^2 < 0$]. An entirely analogous line of reasoning would show that a lower-limit estimate of spatial average [Ca^{2+}] would be obtained in this case. We summarize these points in the following rule.

Rule I. If the calibration curve has an upward curvature [$d^2l_n(C)/dC^2 > 0$] for all [Ca^{2+}] in the preparation, then an upper-limit estimate of spatial average [Ca^{2+}] will be calculated by referring L/L_{max} through the calibration curve. Conversely, if the calibration curve has a downward curvature [$d^2l_n(C)/dC^2 < 0$] for all [Ca^{2+}] in the preparation, then a lower-limit estimate of spatial average [Ca^{2+}] will be obtained. If the calibration curve has both upward and downward curvature over the range of [Ca^{2+}] in the preparation, then either an over- or underestimate of spatial average [Ca^{2+}] may result, depending upon the particular spatial distribution of [Ca^{2+}]. A proof of this rule is outlined in the Appendix.

Limits on the Extent to which Spatial Average [Ca^{2+}] Can Be Over- or Underestimated

Limits can be set on the extent to which spatial average [Ca^{2+}] is over- or underestimated, provided that maximum

and minimum bounds on the $[Ca^{2+}]$ in the preparation are known.

Fig. 2 *A* shows the relationship between L/L_{max} and spatial average $[Ca^{2+}]$ for a calibration curve with an upward curvature, provided that $[Ca^{2+}]$ in the preparation is bounded: $C_{min} \leq [Ca^{2+}] \leq C_{max}$. From rule I, it is known that the solid calibration curve gives the largest possible spatial average $[Ca^{2+}]$ that could yield a given L/L_{max}. It can be shown that there is a simple relationship between L/L_{max} and the smallest possible spatial average $[Ca^{2+}]$ ($\underline{C}_{lim}$) that could yield that L/L_{max}: it is the dashed line (Fig. 2 *A* and Eq. 5) that connects the points on the calibration curve at C_{min} and C_{max}

$$L/L_{max} = \frac{l_n(C_{max}) - l_n(C_{min})}{C_{max} - C_{min}} (\underline{C}_{lim} - C_{min}) + l_n(C_{min}). \quad (5)$$

Hence, the shaded region in between the dashed and solid curves in Fig. 2 *A* defines the range of all possible combinations of spatial average $[Ca^{2+}]$ and L/L_{max}, provided that $C_{min} \leq [Ca^{2+}] \leq C_{max}$. Fig. 2 *B* summarizes the situation when the calibration curve has a downward curvature. In this case, the solid calibration curve describes the relation between L/L_{max} and the smallest spatial average $[Ca^{2+}]$ that could give rise to that L/L_{max}, while the dashed line (also described by Eq. 5) provides the relationship between L/L_{max} and the largest spatial average $[Ca^{2+}]$ ($\underline{C}_{lim}$) that could be associated with a given L/L_{max}. To summarize, we have another rule.

Rule II. If a calibration curve has an upward curvature $[d^2l_n(C)/dC^2 > 0]$ for all $[Ca^{2+}]$ in the preparation, and if $C_{min} \leq [Ca^{2+}] \leq C_{max}$, then the smallest possible spatial average $[Ca^{2+}]$ that could be associated with a given L/L_{max} is $\underline{C}_{lim}$ as defined in Eq. 5. If a calibration curve has a downward curvature $[d^2l_n(C)/dC^2 < 0]$, and $C_{min} \leq [Ca^{2+}] \leq C_{max}$, then the largest possible spatial average $[Ca^{2+}]$ for a given L/L_{max} is also given by $\underline{C}_{lim}$ in Eq. 5. A proof of this rule is also outlined in the Appendix.

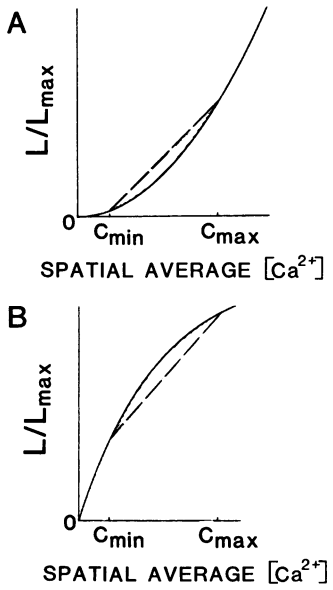

FIGURE 2. Limits on the error in the estimate of spatial average $[Ca^{2+}]$. Solid curves are hypothetical calibration curves (l_n). Dashed lines (Eq. 5) provide limits on the extent to which spatial average $[Ca^{2+}]$ can be underestimated (*A*) or overestimated (*B*). Shaded regions represent all possible relationships between L/L_{max} and spatial average $[Ca^{2+}]$ when $[Ca^{2+}]$ in the preparation is bounded by C_{min} and C_{max}. *A*: case when $d^2l_n(C)/dC^2 > 0$. *B*: case when $d^2l_n(C)/dC^2 < 0$.

DISCUSSION

We illustrate the utility of these rules by interpreting quantitatively aequorin luminescence signals obtained from cardiac ventricular tissue (Fig. 3). Normalized luminescence (L/L_{max}) from a contracting muscle is shown in Fig. 3 *A*. In Fig. 3 *B*, the solid curve is the aequorin calibration curve (l_n), and the dashed line is Eq. 5 with $C_{min} = 0.05$ μM (< resting $[Ca^{2+}] \sim 0.2$ μM) and $C_{max} = 5$ μM (a reasonable assumption for cardiac muscle). Since $d^2l_n(C)/dC^2 > 0$ for $[Ca^{2+}] < 50$ μM, we know from rule I that the largest possible spatial average $[Ca^{2+}]$ is obtained (solid trace, Fig. 3 *C*) by referring L/L_{max} (Fig. 3 *A*) through the calibration curve (solid curve, Fig. 3 *B*). From rule II, we obtain the smallest possible spatial average $[Ca^{2+}]$ (dashed trace, Fig. 3 *C*) by referring L/L_{max} through the dashed plot of Eq. 5 (Fig. 3 *B*). The upper-limit estimate of spatial average $[Ca^{2+}]$ will equal spatial average $[Ca^{2+}]$ if $[Ca^{2+}]$ is spatially uniform; the lower-limit estimate of spatial average $[Ca^{2+}]$ will equal spatial average $[Ca^{2+}]$ if $[Ca^{2+}]$ is everywhere polarized at either C_{min} or C_{max}. In cardiac muscle, the true spatial average $[Ca^{2+}]$ might be near the lower-limit estimate during calcium release from sarcoplasmic reticulum, and approach the upper-limit estimate of spatial average

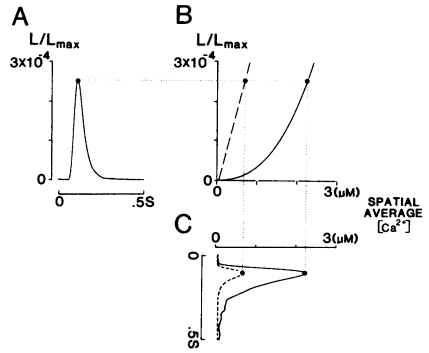

FIGURE 3. Quantitative interpretation of intracellular $[Ca^{2+}]$ transients in cardiac muscle. *A*: Normalized luminescence (L/L_{max}) from a twitching ferret papillary muscle into which aequorin had been microinjected. The muscle was bathed in a solution composed of NaCl, 92 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $NaHCO_3$, 20 mM; Na_2HPO_4, 1 mM; $NaC_2H_3O_2$, 20 mM; $CaCl_2$, 1.5 mM; and glucose, 10 mM, equilibrated with 95% O_2 and 5% CO_2 at 30°C. Stimulation rate was 0.5 Hz. Signal obtained by averaging 100 twitches, and filtering at 20 Hz. *B*: Relation between L/L_{max} and spatial average $[Ca^{2+}]$. Solid curve is an in vitro aequorin calibration curve ($[Mg^{2+}] = 2$ mM; pH 7.2, 30°C). Dashed line is Eq. 5 with $C_{min} = 0.05$ μM and $C_{max} = 5$ μM. *C*: Upper-limit (solid trace) and lower-limit (dashed trace) estimates of spatial average $[Ca^{2+}]$.

$[Ca^{2+}]$ during the later phases of the calcium transient as $[Ca^{2+}]$ gradients dissipate.

The theoretical results developed here can be readily adapted to other types of calcium indicators. In the case of fluorescent indicators such as quin2, the normalized fluorescence F/F_{max} (13) can be substituted for L/L_{max}, and f_n substituted for l_n, where f_n is the fluorescence derived from a unit volume of cytoplasm / (F_{max}/V_T). In the case of metallochromic indicators, three substitutions could be made: (*a*) $-\log(I_o/I_i)$ can be substituted for L/L_{max}, where I_i and I_o are the incident and transmitted beam intensities, respectively, (*b*) a_n should replace f_n, where a_n is absorbance per beam pathlength, and (*c*) integration should be with respect to beam pathlength instead of volume.

If estimates of $[Ca^{2+}]$ are derived from the ratio of two spatially averaged, calcium-dependent signals (as is done with the fluorescent indicator, fura2), it becomes extremely difficult to predict the potential errors in the estimate of spatial average $[Ca^{2+}]$. Our results do not apply directly to this case because the problem is fundamentally different; analysis of the ratio of two expressions of the form given in Eq. 2 is required.

APPENDIX

Proof of Rule I

Splitting the preparation into n equal-volume sites, with n large, Eqs. 1 and 3 become

$$L/L_{max} = \left[\sum_{i=1}^{n} l_n(C_i)\right]/n, \tag{A1a}$$

$$\underline{C} = \left(\sum_{i=1}^{n} C_i\right)/n, \tag{A1b}$$

where C_i is the $[Ca^{2+}]$ in the ith site.

Next, show that (*a*) if $d^2l_n(C)/dC^2$ is always > 0, then for a given spatial average $[Ca^{2+}] = \underline{C}$, $L/L_{max} \geq l_n(\underline{C})$, and (*b*) if $d^2l_n(C)/dC^2$ is always < 0, then for a given spatial average $[Ca^{2+}]$, $L/L_{max} \leq l_n(\underline{C})$. Proving these points will prove rule I as illustrated in Fig. 1.

Assume that the spatial average $[Ca^{2+}]$, $\underline{C}$, is given. Solving Eq. A1b for C_n and substituting into Eq. A1a:

$$L/L_{max} = \left\{\sum_{i=1}^{n-1} l_n(C_i) + l_n(n\underline{C} - C_1 - \ldots - C_{n-1})\right\}/n, \tag{A2}$$

where L/L_{max} is now a function of $C_1, \ldots, C_{n-1}$. Eq. A2 describes L/L_{max} for any spatial distribution of $[Ca^{2+}]$, given the constraint that spatial average $[Ca^{2+}]$ is equal to $\underline{C}$.

Taking the gradient of L/L_{max} (as expressed in Eq. A2) with respect to $C_1, \ldots, C_{n-1}$ and setting it equal to 0, we obtain

$$\begin{aligned}
\partial(L/L_{max})/\partial C_1 &= \{l_n'(C_1) - \\
&\quad l_n'(n\underline{C} - C_1 - \cdots - C_{n-1})\}/n = 0, \\
\partial(L/L_{max})/\partial C_2 &= \{l_n'(C_2) - \\
&\quad l_n'(n\underline{C} - C_1 - \cdots - C_{n-1})\}/n = 0, \\
&\vdots \\
\partial(L/L_{max})/\partial C_{n-1} &= \{l_n'(C_{n-1}) - \\
&\quad l_n'(n\underline{C} - C_1 - \cdots - C_{n-1})\}/n = 0,
\end{aligned} \tag{A3}$$

where $l_n'(X) = dl_n(C)/dC$ at $C = X$. From the assumption that $d^2l_n(C)/dC^2$ is either always >0 or always <0, it must be that $l_n'(C_i) = l_n'(C_j)$ if and only if $C_i = C_j$, so that Eq. A3 can only be satisfied when $C_1 = \cdots = C_n = \underline{C}$. Hence, the combination of values $C_1, \ldots, C_{n-1}$, where $C_1 = \cdots = C_n = \underline{C}$ is a unique critical point of L/L_{max}; that is, for a given spatial average $[Ca^{2+}]$, L/L_{max} is potentially at its absolute maximum or minimum value when $[Ca^{2+}]$ is spatially uniform.

Determine whether this L/L_{max} value is an absolute maximum or minimum by further determining the Hessian matrix ([H]) of L/L_{max} evaluated at this critical point (analogous to taking a second derivative of a single variable function, see reference 14). From Eq. A2, we have that

$$[H] = \begin{bmatrix} 2 & 1 & \cdots & 1 \\ 1 & 2 & \cdots & 1 \\ \vdots & \vdots & \ddots & \vdots \\ 1 & 1 & \cdots & 2 \end{bmatrix} \left.\frac{d^2l_n(C)}{dC^2}\right|_{C=\underline{C}}, \tag{A4}$$

where [H] is an $n-1 \times n-1$ matrix which is all 1s except for the major diagonal which is all 2s. It can be shown that [H] is positive definite if $d^2l_n(C)/dC^2 > 0$, and negative definite if $d^2l_n(C)/dC^2 < 0$ (14). This result and the result that $C_1, \ldots, C_{n-1}$ such that $C_1 = \cdots = C_{n-1} = C_n = \underline{C}$ constitutes a unique critical point of L/L_{max} prove that if $d^2l_n(C)/dC^2$ is always > 0, then, for a given spatial average $[Ca^{2+}]$, L/L_{max} takes on its absolute minimum value when $[Ca^{2+}]$ is spatially uniform. Conversely, these results also prove that if $d^2l_n(C)/dC^2$ is always <0, then, for a given spatial average $[Ca^{2+}]$, L/L_{max} manifests its absolute maximum value when $[Ca^{2+}]$ is spatially uniform. Plugging in $C_1, \ldots, C_n$ such that $C_1 = \cdots = C_n = \underline{C}$ into Eq. A1a, we see that the absolute maximum or minimum value of L/L_{max} is $l_n(\underline{C})$. Thus, points *a* and *b* above are proved.

If $d^2l_n(C)/dC^2 > 0$ for some $[Ca^{2+}]$ within the preparation and $d^2l_n(C)/dC^2 < 0$ for other $[Ca^{2+}]$, then the regions with different curvatures can be considered separately, and the net result will reflect the volume-weighted average of the two regions. Thus, an overestimate or an underestimate of spatial average $[Ca^{2+}]$ will result, depending upon the particular spatial distribution of $[Ca^{2+}]$.

Proof of Rule II

First, show that there exist some spatial distributions of $[Ca^{2+}]$ where L/L_{max} and spatial average $[Ca^{2+}]$ are related as given in Eq. 5. If $[Ca^{2+}]$ is everywhere equal to either C_{min} or C_{max}, with fraction f of the cytoplasm at C_{max}, then spatial average $[Ca^{2+}]$ must be (from Eq. 1):

$$\underline{C} = f\,C_{max} + (1-f)\,C_{min}. \tag{A5}$$

The L/L_{max} that results from such a distribution must be (following Eq. 3)

$$L/L_{max} = f\,l_n(C_{max}) + (1-f)\,l_n(C_{min}). \tag{A6}$$

Solving Eq. A5 for f and substituting into Eq. A6, we recover Eq. 5, with $\underline{C} = \underline{C}_{lim}$.

Next, set $[Ca^{2+}]$ equal to either of two arbitrary values between C_{min} and C_{max}, and then show through the same logic that the entire region enclosed by Eq. 5 and l_n (shaded regions in Fig. 2) represent possible relations between L/L_{max} and spatial average $[Ca^{2+}]$ when $C_{min} \leq C \leq C_{max}$.

To complete the proof for the case when $d^2l_n(C)/dC^2 > 0$, show that, for a given L/L_{max}, spatial average $[Ca^{2+}]$ can never be $<\underline{C}_{lim}$ as defined in Eq. 5. Use a proof by contradiction.

Assume that for a given L/L_{max}, spatial average $[Ca^{2+}]$ ($\underline{C}$) can be $<\underline{C}_{lim}$ in Eq. 5. Then, from consideration of Fig. 2 *A* and Eq. 5, it must be

that

$$L/L_{max} > \frac{l_n(C_{max}) - l_n(C_{min})}{C_{max} - C_{min}} (\underline{C} - C_{min}) + l_n(C_{min}). \quad (A7)$$

Substituting into Eq. A7 for L/L_{max} from Eq. 3, and rearranging yields

$$\iiint_P \{[l_n(C_{max}) - l_n(C)](C - C_{min}) - [l_n(C) - l_n(C_{min})](C_{max} - C)\} \, dx \, dy \, dz < 0. \quad (A8)$$

The integrand in Eq. A8 is 0 where C is equal to either C_{max} or C_{min}; hence, taking the integral over a volume region P', comprised of all of region P in which C is not equal to either C_{max} or C_{min}, would not alter the value of the integral. Rewriting Eq. A8 with the integral evaluated over P', and dividing both sides of the resulting equation by $(C - C_{min})(C_{max} - C)$, we obtain

$$\iiint_{P'} \left\{ \frac{l_n(C_{max}) - l_n(C)}{C_{max} - C} - \frac{l_n(C) - l_n(C_{min})}{C - C_{min}} \right\} dx \, dy \, dz < 0. \quad (A9)$$

Yet, $d^2l_n(C)/dC^2 > 0$ by assumption, and $C_{min} < C < C_{max}$ in region P', so that by the mean value theorem of calculus (15), the integrand in Eq. A9 must always be >0. We have a contradiction since Eq. A9 cannot be true; hence, for a given L/L_{max}, spatial average $[Ca^{2+}]$ $(\underline{C})$ cannot be $<\underline{C}_{lim}$ in Eq. 5. To complete the proof for the case when $d^2l_n(C)/dC^2 < 0$, an entirely analogous line of reasoning would show that, for a given L/L_{max}, spatial average $[Ca^{2+}]$ $(\underline{C})$ cannot be $>C_{lim}$ in Eq. 5.

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