BOUNDS ON THE ESTIMATION OF CALCIUM BY AEQUORIN LUMINESCENCE IN THE PRESENCE OF INHOMOGENEITY

MICHAEL D. STERN
Division of Cardiology, Johns Hopkins Medical Institutions, Baltimore, Maryland 21205; Cardiovascular Section, Gerontology Research Center, National Institute of Aging, National Institutes of Health, Baltimore, Maryland 21224

ABSTRACT The photoprotein aequorin, which emits light as a nonlinear function of calcium concentration, is often used to measure intracellular calcium. In the presence of inhomogeneities or fluctuations of calcium concentration, the nonlinearity results in discrepancies between mean calcium concentration estimated from average aequorin light and the true mean. It is usually assumed that the error is an overestimation, but in the presence of large calcium fluctuations, errors of either direction are possible. Here we show that for aequorin to overestimate the mean calcium, the point in the calcium-light plane representing the true mean calcium and measured mean aequorin light must lie in the convex envelope of that segment of the aequorin response curve that lies between the minimum and maximum values of fluctuating calcium, and must lie above the curve. By explicitly constructing this region, we derive a quartic equation that gives the largest measured calcium for which aequorin can be assumed to give an overestimate, as a function of the maximum calcium fluctuation. In particular, if calcium fluctuations do not exceed 1 mM, aequorin measurements below 7.25 μM may be assumed to overestimate the true mean calcium.

The intensity of luminescence of the photoprotein aequorin is a markedly nonlinear function of the calcium concentration. It is widely recognized that, as a result, estimation of intracellular calcium by the luminescence of injected aequorin will be in error if there is spatial or temporal inhomogeneity in the intracellular distribution of calcium. It is generally assumed that, because the aequorin light curve is convex in the range of calcium concentrations normally encountered in the cell interior, any such error will be an overestimate of the true mean calcium concentration. It is intuitively clear that, since the aequorin response curve is sigmoidal rather than convex, the presence of large fluctuations of calcium, even if present in a small fraction of the cell, or for a small fraction of the time, might render this assumption falacious.

Recent evidence indicates that in heart muscle, in apparently steady states, there may be large oscillations in myoplasmic calcium. The peak calcium in these oscillations has been variously estimated (1–3) to lie between 1 and 40 μM, which approaches the level at which the aequorin-light curve has its inflection point. It is therefore appropriate to determine, in a relatively formal manner, the conditions under which the apparent calcium estimated from mean aequorin light can be assumed to be an upper limit for the true mean calcium. Here we analyze this problem by assuming that calcium concentration sampled by aequorin is statistically distributed within a finite range, bounded by upper and lower limits. Such limits might be found, for example, by arguing that the local concentration of calcium around the sarcoplasmic reticulum during release could not exceed the source concentration within that organelle. Our mathematical task will be to show that, using these limits on the range of calcium fluctuations, we can determine a safe upper limit on the measured calcium (i.e., the calcium calculated from the mean aequorin light by means of the aequorin calibration curve) such that, if the measured calcium falls below the safe limit, we may take it to be an overestimate of true mean calcium.

We approach the problem in several steps. We first consider what possible combinations of true mean calcium and mean aequorin light could arise from possible distributions of calcium concentration within the limiting range. We show that, regardless of the details of the calcium distribution, all such combinations lie within a certain convex region in the calcium-light plane. For any given calcium distribution, it is clear that the aequorin light will overestimate true mean calcium if the corresponding point in the plane lies above the aequorin calibration curve. In the second step we show how to geometrically construct the

Please address correspondence to Dr. Stern at the Gerontology Research Center.

region consisting of all possible combinations of calcium and light that lie above the calibration curve and could arise from a calcium distribution lying within the assumed range of fluctuation. From the geometrical construction, it will become clear that there exists a safe value of mean calcium, below which all realizable combinations of calcium and light lie above the calibration curve. In the third step we abstract from the geometrical picture an analytical equation for the safe value, which we solve numerically.

The rate of light emission by a fixed quantity of aequorin in the presence of a calcium concentration C is given (4) by

$$\frac{L}{L_{max}} = \left(\frac{1 + kC}{1 + K + kC}\right)^3, \tag{1}$$

where L_{max} is the saturating rate of light emission and $K = 125$ and $k = 2.6\ \mu M^{-1}$. From now on we will assume that light intensity is normalized, so that $L_{max} = 1$. The function $L(C)$ is plotted in Fig. 1. It is a monotonic S-shaped function, with an inflection point at $C = 47\ \mu M$. If the calcium concentration in the region from which light is averaged has an arbitrary probability distribution, $P(C)$, with lower and upper limits, C_{min} and C_{max}, then the mean intensity will be

$$L_m = \int_{C_{min}}^{C_{max}} L(C)P(C)\,dC, \tag{2a}$$

while the true mean calcium will be

$$C_m = \int_{C_{min}}^{C_{max}} C\,P(C)\,dC. \tag{2b}$$

The normalization of the probability distribution is expressed by

$$\int_{C_{min}}^{C_{max}} P(C)\,dC = 1. \tag{2c}$$

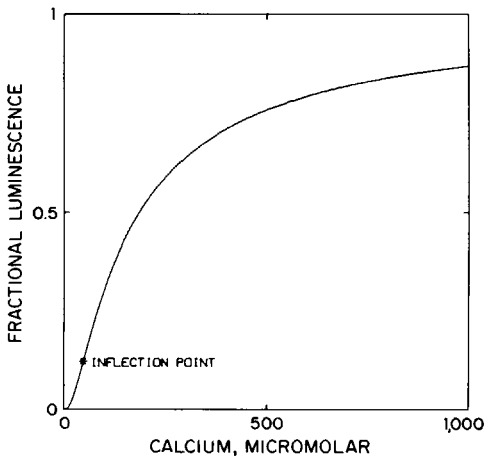

FIGURE 1 The nonlinear relation between normalized aequorin luminescence (ordinate) and calcium concentration (Eq. 1). In the low calcium region the relationship is convex, but over the large range of calcium plotted here it is sigmoidal, with an inflection point shown by the asterisk.

The apparent mean calcium concentration C_{ap} will be the value calculated from the mean aequorin light such that $L(C_{ap}) = L_m$. If, for a particular distribution, $P(C)$, we plot C_m vs. L_m as a point in the calcium-light plane of Fig. 1, then the condition for the apparent calcium to be an upper limit for the true mean calcium is simply that the point lie above the curve L. To determine conditions under which that will be the case, we determine the region CE, which is the locus of all possible points in the plane that represent possible values of (C_m, L_m) for any possible calcium distribution P. Let the range of fluctuation of calcium lie between C_{min} and C_{max}. Then we arrive at the following theorem.

THEOREM 1

The region CE is the convex envelope of the segment S of the curve L between C_{min} and C_{max}, i.e., the smallest closed convex set containing that segment of the curve.

Proof

First note that CE contains the segment S, since a distribution P concentrated on a single constant value C^o of calcium between C_{min} and C_{max} will give $L_m = L(C^o) = L(C_m)$. CE is a convex set since if a and b are positive numbers with $a + b = 1$, then if P_1 and P_2 are two distributions that give rise to the points (L_{m1}, C_{m1}) and (L_{m2}, C_{m2}) then $aP_1 + bP_2$ is a properly normalized probability distribution that gives rise to the point $(aL_{m1} + bL_{m2}, aC_{m1} + bC_{m2})$ which lies on the chord between the first two points, because of the linearity of Eqs. 2a, b. Therefore, we need to prove that CE is the smallest such convex set. Suppose that CC is any closed convex set containing the segment S. If $p = (L_m, C_m)$ is the point corresponding to a calcium distribution concentrated on a finite number of values of calcium C_k, $k = 1 \ldots N$ with probabilities p_k, then p lies in CC. To see this write the coordinates of p from Eq. 2 as

$$L_m = \sum_{k=1}^{N} L(C_k)p_k$$
$$C_m = \sum_{k=1}^{N} C_k p_k. \tag{3}$$

If $N = 2$, then the fact that p is in CC follows from that set's definition as a convex set. Suppose that it is true for $N - 1$. We separate the first term from the sums in Eq. 3 to rewrite it as

$$L_m = p_1 L(C_1) + \left(\sum_{r=2}^{N} p_r\right) \sum_{k=2}^{N} \left[L(C_k)p_k \Big/ \left(\sum_{r=2}^{N} p_r\right)\right]$$
$$C_m = p_1 C_1 + \left(\sum_{r=2}^{N} p_r\right) \sum_{k=2}^{N} \left[C_k p_k \Big/ \left(\sum_{r=2}^{N} p_r\right)\right], \tag{4}$$

where the sum in parentheses has been multiplied and divided into each of the second terms. If we define

$$Q_1 \equiv \sum_{r=2}^{N} p_r \tag{5a}$$

$$Q_k \equiv \frac{p_k}{Q_1}; \qquad k = 2 \ldots N \tag{5b}$$

we can rewrite Eq. 4 as

$$L_m = p_1 L(C_1) + Q_1 \sum_{k=2}^{N} L(C_k) Q_k$$

$$C_m = p_1 C_1 + Q_1 \sum_{k=2}^{N} C_k Q_k. \tag{6}$$

But by construction the Q_k $(k = 2 \ldots N)$ form a set of $N - 1$ normalized probabilities, so that the sums in Eq. 6 form, by hypothesis, the coordinates of a point p' that lies in CC. Therefore, we can write in two-dimensional vector notation

$$\mathbf{p} = p_1(L[C_1], C_1) + Q_1 \mathbf{p}' \tag{7}$$

and, since $p_1 + Q_1 = 1$, we again find, since CC is convex and contains both $[L(C_1), C_1]$, which lies on S and p', it contains p. By induction on N, the arbitrary convex set CC containing S must contain every point p that is generated by a discrete, finite calcium distribution. But any point in CE generated by an arbitrary distribution can be expressed as a limit of points generated by discrete distributions, using the definition of the integrals in Eq. 2 as limits of finite sums. Since CC is a closed set, it contains all its limit points, and therefore any point in CE. By suitably generalizing the distributions in Eq. 2 to include singular (e.g., delta function) distributions, we can assume that CE includes its boundary points, i.e., is a closed set. Therefore, we have shown that any closed convex set containing S contains the closed convex set CE, so the latter must be the smallest such set. Thus Theorem one is proven.

We have now shown that the locus of points in the calcium-light plane that can occur for an arbitrary calcium distribution is the complex envelope of an S-shaped segment of the aequorin response curve L. This would appear to be rather abstract progress. However, we can explicitly construct the region CE, which is the area enclosed by an ideal rubber band stretched around the segment S. Fig. 2 shows this region schematically, (the curve L has been shown as more S-shaped than it is for clarity of illustration). The physical significance of this region is that it expresses the bounds of possible variability of true mean calcium in relation to measured aequorin light. In particular, if a horizontal line were drawn at the level of measured aequorin light, the possible values of true mean calcium lie on the intersection of this line with the region CR. Therefore, the intersections of this line with the boundaries of CR give the upper and lower bounds for the value of true mean calcium, given the measured light.

The region constructed, which we call CR (for rubber band), contains the segment S and is bounded by two segments of the curve S and the two chords CH1 and CH2 that begin at the points C_{min} and C_{max} on S and extend to the points A and B where they are tangent to the curve S. We take it as evident without formal proof that the region CR is convex. But every point of the boundary of CR is either on S or can be expressed as the convex sum of, at most, two points on S. Every point in the interior of CR lies on a chord connecting two points on the boundary, and so can be expressed as the convex sum of no more than four points on S. Therefore, all of CR is contained in the convex envelope CE. But CE is the smallest such convex set, so $CR = CE$, proving that the set constructed in Fig. 2 is in fact the set CE of all possible points in the plane which can result from any calcium distribution that lies within the range C_{min}–C_{max}.

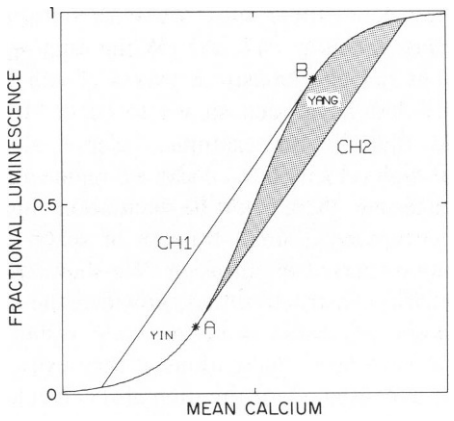

FIGURE 2 Schematic of the construction of the region CE (see text), which is the complex envelope of the segment of the aequorin response curve lying between the minimum and maximum values of calcium. The region CE represents all possible combinations of mean calcium and measured aequorin light that could be obtained from any distribution of calcium inhomogeneities lying within the given bounds. The region CE is bounded by the chords CH1 and CH2 running from the minimum and maximum calcium to the points of tangency A and B with the curve. The aequorin curve (shown here with an exaggerated S-shape for clarity) divides the region CE into two lobes, Yin, in which aequorin overestimates calcium and Yang, in which it underestimates. If the range of calcium fluctuations lies entirely on one side of the inflection point, only one lobe will be present.

We have now accomplished the first two steps in our analysis, leading to a geometrical construction of the set CE of possible combinations of mean calcium and mean light arising from any degree of calcium inhomogeneity, provided only that local instantaneous calcium remains between C_{min} and C_{max}. C_{min} will usually be close to zero in practice. If C_{max} lies below the inflection point of the curve, it is intuitively obvious that aequorin will always overestimate calcium. If C_{min} and C_{max} lie on opposite sides of the inflection point then, as shown in the figure, the region CE consists of two lobes, Yin lying above the curve L and Yang (shaded) lying below the curve. For any distribution of calcium inhomogeneities that generates a point in the Yang region, aequorin will underestimate mean calcium.

For this to occur, there must be excursions of local, instantaneous calcium >47 μM. While this is a large number, it is not enormously in excess of concentration fluctuations that have been shown to occur. It is quite imaginable that if, for example, release of calcium occurred at high velocity into a localized region around the terminal cisternae, there could be fluctuations much larger, albeit occupying a small fraction of space and time before being dispersed by diffusion. We show, next, from the geometrical construction that, provided the measured mean calcium lies below a certain safe value, we can assume that such large fluctuations, if they exist, are rare enough not to cause underestimation of mean calcium due to aequorin saturation.

Examining the region CE closely shows that the lowest value of C_m, the true mean calcium, for which a point of CE can actually lie below (as opposed to on) the curve L, is found at the point of tangency A. Since the curve L is monotonically increasing, this point also gives the lowest value of L_m and, therefore, of C_{ap}, for which a point can lie below the curve (i.e., for which aequorin can underestimate mean calcium). Therefore, we have found the following theorem.

THEOREM 2

If the true or apparent mean calcium lies below the value C_{tan}, at which the chord from the point $[C_{max}, L(C_{max})]$ is tangent to the curve L, the apparent mean calcium is always an overestimate of the true mean calcium, regardless of the details of the calcium distribution.

The value of C_{tan} depends on the assumed maximum calcium in the distribution (C_{max}). If C_{max} is below the inflection point, the Yang lobe is absent, and the apparent mean calcium is always an overestimate regardless of its value (it must, of course, be less than C_{max}).

To make use of this result we must determine C_{tan} analytically. The condition that the chord CH2 be tangent at point A can be expressed as

$$\frac{dL(C_{tan})}{dC} = \frac{L(C_{max}) - L(C_{tan})}{C_{max} - C_{tan}}. \quad (8)$$

Inserting the function $L(C)$ from Eq. 1, performing the differentiation and rearranging algebraically converts Eq. 8 to

$$3kK(1 + kC_{tan})^2(C_{max} - C_{tan}) = L(C_{max})(1 + K + kC_{tan})^4 - (1 + kC_{tan})^3(1 + K + kC_{tan}), \quad (9)$$

which is a quartic equation in C_{tan}, whose smallest positive real root is the desired value expressing the safe level of mean calcium as a function of C_{max}. Eq. 9 can actually be solved analytically to give $C_{tan}(C_{max})$, but the resulting expression involves over a page of square and cube roots nested four deep. It is more convenient to solve it numerically by an iterative method. The result is shown in Fig. 3. The solid curve gives C_{tan} as a function of C_{max} for $C_{max} >$ C_{inf}, the inflection point. For $C_{max} < C_{inf}$, the curve continues as a straight line of equality, expressing the fact that the mean calcium must be less than C_{max} (or else C_{max} was chosen incorrectly). For any assumed value of C_{max}, the region under the curve gives values of apparent mean calcium, such that the aequorin estimate can be assumed to be an upper limit for the true mean calcium. The curve is slowly asymptotic to zero for large C_{max}, showing that, if sufficiently large values of calcium are permitted with suitably low probability, it is possible to construct highly skewed distributions of calcium with arbitrarily small mean calcium for which aequorin will underestimate the mean calcium. The curve is plotted only out to C_{max} = 1,000 μM, certainly a conservative estimate of the highest intracellular calcium likely to be present in a physiologic state; at this point C_{tan} = 7.25 μM, so that if the measured apparent mean calcium is less than this value, it is safe to assume that it is an overestimate of the true mean calcium, even if fluctuations are present that rise considerably above the aequorin inflection point. By choosing plausible limits on C_{max} in various situations, Fig. 3 may be used to determine the safe range of measured calcium over which the aequorin measurement gives an upper bound for true mean calcium.

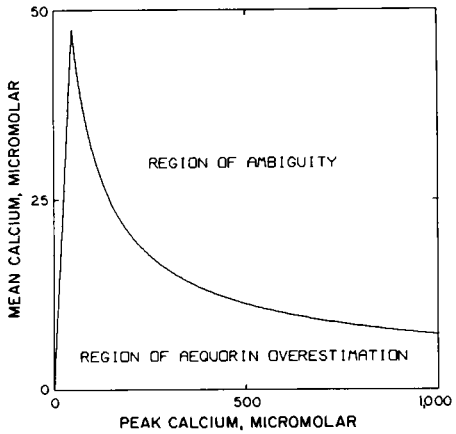

FIGURE 3 The maximum safe mean calcium, as a function of peak calcium fluctuation, by numerical solution of Eq. 9. For a given peak calcium concentration, measured mean concentrations below the curve will always be overestimates, while in the region above the curve, aequorin may either over- or underestimate mean calcium, depending on the details of the calcium distribution. For very high peak calcium concentrations, the curve is slowly asymptotic to zero, showing that, in principle, arbitrarily small measured calcium concentrations may be underestimates if the distribution of calcium fluctuations is sufficiently skewed.

DISCUSSION

The nonlinearities of aequorin are usually assumed to lead to overestimation of mean calcium concentration when inhomogeneity in space or time is present. It is intuitively obvious that if sufficiently large fluctuations of calcium are present to cause saturation of aequorin, the opposite might occur. The conditions for the underestimation of calcium

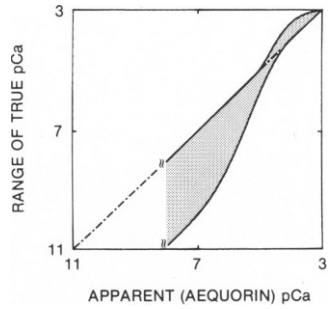

FIGURE 4 An aequorin calibration diagram produced by constructing the region *CE* (Fig. 2) analytically, and mapping it into the apparent-*p*Ca/true *p*Ca plane using the aequorin response function. The diagram was constructed assuming calcium fluctuations of up to 1 mM are possible. The shaded area shows the possible range of true *p*Ca, as a function of the apparent *p*Ca calculated from aequorin light. The dashed line is the line of identity (calibration in the absence of inhomogeneity). For mean calcium <7.25 μM (*p*Ca 5.14) aequorin always overestimates; above 95.2 μM (*p*Ca 4.02) it always underestimates.

concentration to happen appear to be rather extreme but, as we have argued above, rapid processes, such as local calcium release from the sarcoplasmic reticulum, might cause just such extremely skewed distributions of calcium. What is less intuitively obvious is that if the distribution of calcium is sufficiently skewed, the mean value need not be particularly extreme when underestimation occurs. If we admit that, during rapid calcium release from the sarcoplasmic reticulum, calcium in a local aequorin-containing space might reach equilibrium with the intravesicular concentration, then mean calcium at which underestimation sets in reaches down to systolic levels. It would be circular reasoning to assume that no such large fluctuations are occurring because aequorin (which overestimates calcium) does not show them. Conversely, the analysis above shows that we are quite safe in assuming that aequorin readings below 1 μM do represent an overestimate, given any imaginable fluctuation that could occur in an intact cell.

As mentioned above, the region *CR* itself gives the limits on true mean calcium in relation to measured light, regardless of whether the measurement is in the safe region. By using Eq. 9 to construct the region explicitly, we can produce an aequorin calibration diagram for any postulated limit C_{max} on the degree of inhomogeneity. Such a diagram, plotted on a log-log (*p*Ca) scale, is shown in Fig. 4, for the extreme case C_{max} = 1 mM. Similar diagrams can be constructed for any assumed limit C_{max}; the one shown represents the upper limits of aequorin variability likely to occur in actual practice.

It is somewhat surprising that an analytical expression for the safe limit on aequorin measurements can be obtained using only the upper limit C_{max} for fluctuations of calcium, without reference to the details of the calcium distribution. The form of Eq. 9 is certainly not intuitively obvious. Accordingly, the analysis presented here may prove useful in designing experiments to decide whether large inhomogeneities of intracellular calcium in fact occur.

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