ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/7419863

A Theoretical and Experimental Approach to the Use of Single Wavelength Calcium Indicators

ARTICLE *in* JOURNAL OF THEORETICAL BIOLOGY · MAY 2003

Impact Factor: 2.12 · DOI: 10.1006/jtbi.2003.3203 · Source: PubMed

CITATION
1

READS
5

2 AUTHORS, INCLUDING:

Luminita Pojoga
Harvard Medical School

41 PUBLICATIONS **630** CITATIONS

SEE PROFILE

All in-text references underlined in blue are linked to publications on ResearchGate, letting you access and read them immediately.

Available from: Luminita Pojoga
Retrieved on: 24 January 2016

J. theor. Biol. (2003) **221,** 543–557
doi:10.1006/jtbi.2003.3203, available online at http://www.idealibrary.com on IDEAL®

A Theoretical and Experimental Approach to the Use of Single Wavelength Calcium Indicators

L. H. POJOGA†‡ AND R. H. HILDERMAN*§

†*Department of Genetics and Biochemistry, Clemson University, Clemson, SC* 29634-0324, *U.S.A.* and §*Department of Genetics and Biochemistry and the South Carolina Experiment Station, Clemson University, Clemson, SC* 29634-0324, *U.S.A.*

(*Received on* 1 *August* 2002, *Accepted in revised form on* 29 *October* 2002)

Fluorescent Ca^{2+} indicators have been extremely valuable in understanding the role of intracellular Ca^{2+}. However, the presence of extracellular dye can confound interpretation of data due to indicator accumulation in the Ca^{2+}-rich medium, which induces an increase in the fluorescence signal. By using a mathematical approach, we show that overlooking extracellular dye usually leads to overestimating cytosolic Ca^{2+} ($[Ca^{2+}]$) levels. We propose an experimental design and provide mathematical formulations to make the appropriate correction. We applied our model to determine $[Ca^{2+}]$ in Fluo-3-loaded bovine aortic endothelial cells (BAECs). Our results indicate that for basal level Ca^{2+}, the uncorrected value overestimates by a factor of 2.7 the result obtained when extracellular dye was accounted for. We also showed that both bradykinin (BK) and ATP significantly increase $[Ca^{2+}]$ in BAECs. For the uncorrected values, BK and ATP induced 2.3- and 3.3-fold apparent increases in $[Ca^{2+}]$, respectively. When applying the correction, there was a 4.5- and 5.4-fold induction of $[Ca^{2+}]$ for BK and ATP, respectively. Our theoretical and experimental models provide explanations and, at least in part, solutions to the dye leakage problem, and should thus be a valuable tool in clarifying the proper usage of fluorescent dyes for Ca^{2+} measurements.

© 2003 Elsevier Science Ltd. All rights reserved.

1. Introduction

Intracellular calcium is a universal second messenger that can serve as a broad-based measure of receptor activity. Critical evaluation of the role of Ca^{2+} as an intracellular messenger requires quantitative measurement of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]$) and comparison with varied stimuli and cell responses. Since their discovery, fluorescent Ca^{2+} indicators have been routinely used when investigating intracellular Ca^{2+} metabolism in various cell systems. There are two types of indicators that are used to determine intracellular $[Ca^{2+}]$. Single-wavelength (SW) dyes are characterized by a unique pair of excitation–emission wavelengths, and their fluorescence intensity significantly changes between the Ca^{2+}-free and Ca^{2+}-bound forms. The second type of dyes are ratiometric ones, whose specific excitation (or emission) wavelength shifts after binding to Ca^{2+}. Not all the laboratories interested in $[Ca^{2+}]$ determination have access to a dual excitation or emission

‡On leave from the Institute of Cellular Biology and Pathology Nicolae Simionescu, 8 B.P. Hasdeu Street, Bucharest 79691, Romania.

*Corresponding author. Tel.: +1-864-656-3586; fax: +1-864-656-6879.

E-mail address: hilderr@clemson.edu (R. H. Hilderman).

0022-5193/03/$35.00

© 2003 Elsevier Science Ltd. All rights reserved.

device, but most have a single excitation–emission apparatus at hand; SW dyes will then be the indicators of choice. Among the recently developed SW [Ca^{2+}] indicators, Fluo-3 and Fluo-4 are most widely used, due to their Ca^{2+} binding affinities, their large increase in fluorescence intensity upon complexation with calcium, and to the fact that their excitation wavelengths lie within the visible range (Minta *et al.*, 1989; Thomas *et al.*, 2000). The relationship between [Ca^{2+}] and the fluorescent signal coming from the sample (F) is given, for SW dyes, by the well-known equation (Kao *et al.*, 1989):

$$[Ca^{2+}] = K_d \frac{F - F_{min}}{F_{max} - F}, \qquad (1)$$

where K_d is the dissociation constant for the dye–Ca^{2+} complex, F_{max} is the fluorescence intensity when all the dye is in the Ca^{2+} -bound form, and F_{min} is the fluorescent signal recorded when all the dye is Ca^{2+}–free. For SW dyes with a high K_d (such as Fluo-3 and Fluo-4), it is more difficult to saturate them with Ca^{2+} by ionophore permeabilization of the cells (Kao *et al.*, 1989, Merritt *et al.*, 1990). Therefore, F_{max} is usually obtained by lysing the cells with digitonin, thus releasing the dye in the Ca^{2+}-saturated environment; the usual calibration procedure follows by the subsequent addition of enough EGTA to determine F_{min}.

Incorrect determination of any of the parameters on the right-hand side of eqn (1) leads to erroneous values of calculated [Ca^{2+}], as discussed below. Equation (1) describes the behaviour of an ideal system, in which all fluorescence values correspond to the intracellular compartment only. Intensity changes arising from factors that are unrelated to changes in [Ca^{2+}] (assumed constant in resting cells), such as loss of indicator from the cytosol by leakage, can confound interpretation of F. It has been shown that fluorescent signals emitted by Ca^{2+} indicators from cell suspensions can vary with time and between cell types due to dye extrusion across the plasma membrane (Malgaroli *et al.*, 1987; Arkhammar *et al.*, 1989; Homolya *et al.*, 1993; Kao, 1994). The loss of indicator into the Ca^{2+}-containing media will lead to its saturation and thereby to an increase in fluorescence. When the measurements are done in a cuvette, accumulating extracellular dye will constitute a substantial part of the total fluorescence signal and present a serious problem in the determination of the cytoplasmic free Ca^{2+} concentrations, when using either SW or ratiometric dyes.

Another source of error when applying eqn (1) to calculate [Ca^{2+}] is known to be the use of a K_d value previously published for an arbitrary calibration solution. Since Ca^{2+}-sensitive dyes not only bind to Ca^{2+} but also to cellular constituents, it is reasonable to predict an increased K_d for the dye–Ca^{2+} complex in the intracellular environment (Merritt *et al.*, 1990; Uto *et al.*, 1991; Hove-Madsen & Bers, 1992; Harkins *et al.*, 1993; Bassani *et al.*, 1995; Gee *et al.*, 2000). In addition, K_d has been shown to be temperature-dependent (Merritt *et al.*, 1990; Kao, 1994; Thomas *et al.*, 2000), requiring that its value be adjusted with respect to the temperature at which the experiments are carried out. Samples are usually read at either room temperature or at 37°C, depending on the trade-off between physiological conditions (37°C) or more acceptable leakage rates (room temperature). As seen from eqn (1), the intrinsic effect of altering K_d is a direct multiplicative factor for [Ca^{2+}]. For example, doubling the K_d will double the calculated [Ca^{2+}] in both basal and agonist-stimulated cells without affecting the relative fold increase. Thus, choosing the correct K_d value is not essential when comparing relative fold induction values. However, for comparison of absolute values of [Ca^{2+}], similar K_d values must be used, otherwise discrepancies may confuse data interpretation in the context of other published results.

As seen from eqn (1), a third source of error when calculating [Ca^{2+}] lies in the use of incorrectly determined minimum and maximum fluorescence values (F_{min} and F_{max}) (Grynkiewicz *et al.*, 1985; Malgaroli *et al.*, 1987). These two fluorescence values should be proportional to the total amount of dye present in the cellular system (assuming that the indicator concentration is within the linearity range), amount which is much smaller than the dye concentrations routinely used to determine F_{min} and F_{max} *in vitro*. Therefore, if these values are determined in an *in vitro* (Malgaroli *et al.*, 1987; Homolya

et al., 1993) instead of an *in vivo* system, there will be inconsistencies between F and either F_{min} or F_{max}, which will lead to an incorrect estimate of $[Ca^{2+}]$. Even when using an *in vivo* calibration system, the yield of dye loading into the cells varies from day to day, suggesting that F_{min} and F_{max} should be determined for each sample. The error introduced by F_{min} and F_{max} is not of a multiplicative type, and it will differentially affect the calculated basal and agonist-stimulated levels of Ca^{2+}, thus inducing an erroneous estimate of the fold increase also.

While vast literature exists on the use of Ca^{2+}-sensitive fluorescent dyes, a disproportionately smaller literature has addressed the question of the accuracy of these determinations when extracellular dye is present in the system (Hesketh *et al.*, 1983; Rink & Pozzan, 1985; Di Virgilio *et al.*, 1988; McDonough & Button, 1989; Tsien & Pozzan, 1989; Gunter *et al.*, 1990). Most investigators incorrectly use eqn (1) without checking for indicator leakage, which does lead to an overestimation of the Ca^{2+} level inside the cells (see Theoretical Considerations below). For many purposes, a qualitative uncalibrated index of Ca^{2+} changes induced in various experimental conditions may suffice. However, for a more quantitative analysis, the extracellular dye — if present — needs to be accounted for.

In this communication, we discuss ways in which the presence of the Ca^{2+}-sensitive dye in the extracellular compartment can affect the correct determination of intracellular $[Ca^{2+}]$. We also propose mathematical formulations and an experimental design that can eliminate these extracellular dye interferences. As an example of how our theoretical findings can be applied to an experimental situation, we analysed the basal and ATP or bradykinin (BK)-induced levels of cytosolic Ca^{2+} in Fluo-3-loaded BAECs. From the same fluorescence data, three different sets of results were derived by calculating $[Ca^{2+}]$ with or without corrections for dye leakage in the extracellular compartment. As predicted by our mathematical approach, the uncorrected experimental results overestimate the values obtained when corrected for dye leakage. Although this paper was written with Fluo-3 in mind, the applicability of our findings extends to other comparable SW indicators, and (with appropriate adjustments for ratioing) even to ratiometric ones.

Theoretical Considerations

To calculate the intracellular level of free Ca^{2+} by using SW Ca^{2+} indicators, one relies on the previously determined eqn (1), which originates from describing the formation of the dye–Ca^{2+} complex:

$$[Ca^{2+}] = K_d \frac{n_2}{n_1}, \tag{2}$$

where n_1 and n_2 are the molar concentrations of dye in the Ca^{2+}-free and Ca^{2+}-bound forms, respectively (Rink & Pozzan, 1985). This equation is valid if all the dye molecules share the same K_d for binding to Ca^{2+} in a 1 : 1 complex. Since fluorescence intensity can be a linear function of the dye concentration (when the concentration range is sufficiently small), we define the "molar fluorescence coefficients", f_{min} and f_{max} (at a given light path), as the fluorescence of a 1 M dye solution in a Ca^{2+}-free and a Ca^{2+}-saturated environment, respectively. These proportionality coefficients are known to include factors such as the extinction coefficient, the path length, the quantum efficiency of the dye, the instrumental efficiency of photon collection, and the intensity of the incident light (Grynkiewicz *et al.*, 1985). Inside the cells, the Ca^{2+} concentration is limited and is exceeded by the concentration of dye, so the fluorescence originating from an ideal cell suspension can be written as follows:

$$F = n_1 f_{min} + n_2 f_{max}, \tag{3}$$

where the first and second terms describe the fluorescence coming from the Ca^{2+}-free and Ca^{2+}- bound indicators, respectively.

By using the same notations and in the assumption that $[Ca^{2+}]$ stays constant in resting cells, F_{max} and F_{min} can then be expressed as

$$F_{max} = (n_1 + n_2) f_{max}, \tag{4}$$

$$F_{min} = (n_1 + n_2) f_{min}. \tag{5}$$

By solving the system formed by eqns (2–5), one obtains the formula in eqn (1). However, eqn (1)

can only describe the behaviour of an ideal system, in which there is no dye present outside the cells, or the extracellular fluorescence has been subtracted from the experimental values. Changes in fluorescence intensity that are unrelated to changes in $[Ca^{2+}]$ (such as those induced by indicator leakage from the cells), can induce significant errors in data analysis and interpretation. The correct formula to use in such cases is the one given by eqn (2), which takes into account the ratio between the bound and unbound dye *inside* the cells. By using eqn (1) without taking into account the presence of extracellular indicator, one obtains an apparent Ca^{2+} level, $[Ca^{2+}]_{app}$, which will overestimate the result obtained by applying the formula in eqn (2) (see below).

The extracellular dye may be accounted for by subtracting from each component in eqn (1) the fluorescence coming from the extracellular compartment (by taking an aliquot of cell suspension, centrifuging it, and reading the supernatant in the exact same conditions as for the sample) (Gunter *et al.*, 1990; Lin *et al.*, 1999). However, this will only be an estimate of the external dye that is present in the cuvette, due to uncontrollable factors, such as pressure- or cell damage-induced extrusion of the dye during centrifugation (Di Virgilio *et al.*, 1988), possible non-homogeneities in the cell suspension, etc. Even so, this procedure may be applicable only to a system in which the dye extrusion rate can be neglected, because an active leakage phenomenon will greatly complicate this possibility. In an attempt to avoid this method, we will now discuss how to apply eqn (2) to two cases: (a) the extracellular dye concentration is sufficient to induce a significant additional fluorescence signal, but the leakage rate is either zero or can be neglected; and (b) both the leakage rate and the amount of external dye are significant.

CASE ONE: THE LEAKAGE RATE IS CONSIDERED NEGLIGIBLE BUT THE EXTRACELLULAR DYE CONCENTRATION IS SIGNIFICANT

This situation can occur in the case of dye release due to cell death or membrane damage, without an active dye extrusion mechanism from living cells. In this case, F, F_{max} and F_{min} can be written as follows:

$$F = n_1 f_{min} + n_2 f_{max} + n_3 f_{max},$$

$$F_{max} = (n_1 + n_2 + n_3) f_{max},$$

$$F_{min} = (n_1 + n_2 + n_3) f_{min},$$

where n_1 and n_2 are the concentrations of dye inside the cells in the Ca^{2+}-free and Ca^{2+}-bound forms, respectively, and n_3 is the extracellular dye concentration, which is initially all coupled to Ca^{2+}. By introducing these values in eqn (1), we obtain an apparent Ca^{2+} level:

$$[Ca^{2+}]_{app} = K_d \frac{(n_2 + n_3)(f_{max} - f_{min})}{n_1 (f_{max} - f_{min})}$$

$$= K_d \frac{n_2 + n_3}{n_1} = K_d \frac{n_2}{n_1} + K_d \frac{n_3}{n_1}$$

which is easily seen to be an overestimation of the real intracellular Ca^{2+} level [shown by eqn (2)] by the amount $K_d n_3 / n_1$. The problem becomes more complicated when trying to determine not only the resting, but also an agonist-induced level of Ca^{2+}. If the fluorescence intensity in the presence of an agonist has reached a peak value F_A, and by keeping in mind that inside the cells the dye molecules have redistributed between the bound and unbound fractions because of the agonist-induced Ca^{2+} increase, then F_A can be written:

$$F_A = (n_1 - n) f_{min} + (n_2 + n) f_{max} + n_3 f_{max},$$

where n is the intracellular dye concentration that was induced by the agonist to shift from a Ca^{2+}-free to a Ca^{2+}-bound form, and $(n_1 - n)$ and $(n_2 + n)$ are the new concentrations of dye inside the cells in the low and high fluorescence forms, respectively. The correct value of the induced Ca^{2+} level, $[Ca^{2+}]^A$, will then be estimated by a formula similar to the one in eqn (2):

$$[Ca^{2+}]^A = K_d \frac{n_2 + n}{n_1 - n}. \quad (6)$$

However, by applying eqn (1) and substituting F_A for F, we obtain the apparent concentration

of Ca^{2+}:

$$[Ca^{2+}]^{A}_{app} = K_d \frac{F_A - F_{min}}{F_{max} - F_A}$$
$$= K_d \frac{(n_2 + n + n_3)(f_{max} - f_{min})}{(n_1 - n)(f_{max} - f_{min})}$$
$$= K_d \frac{n_2 + n + n_3}{n_1 - n} = K_d \frac{n_2 + n}{n_1 - n} + K_d \frac{n_3}{n_1 - n}$$

which overestimates the real Ca^{2+} level from eqn (6) by $K_d n_3/(n_1 - n)$, a quantity even larger than the overestimation in the case of resting $[Ca^{2+}]$. Also, it can be easily seen that the ratio between the apparent induced and resting Ca^{2+} levels (the fold induction triggered by the agonist) underestimates the real fold increase.

To obtain the values of n_1, n_2, n_3 and n that would help one get a better estimate of intracellular Ca^{2+} levels, the experimental design needs to be changed. One possibility is to add EGTA to the cell suspension at the end of the experiment, before lysing the cells with digitonin, and then to add enough Ca^{2+} to the lysate to give a reliable excess of total Ca^{2+} over EGTA. This calibration alternative, described elsewhere (Tsien & Pozzan, 1989), allows correct determination of the $[Ca^{2+}]$ level immediately prior to chelating the extracellular Ca^{2+}. It has been shown that, if F_1 and F_2 are the fluorescence levels before and immediately after adding EGTA, then the intracellular Ca^{2+} level corresponding to F_1 is given by the following expression (Tsien & Pozzan, 1989), very much like eqn (1):

$$[Ca^{2+}] = K_d \frac{F_2 - F_{min}}{F_{max} - F_1}. \qquad (7)$$

However, as the authors mention in their comments, one has to assume that the intracellular Ca^{2+} concentration has not changed between the moments when F_1 and F_2 are measured. This assumption does not hold in the case when cytosolic Ca^{2+} levels are determined sequentially in resting and agonist-induced conditions; the stimulated Ca^{2+} usually drops rapidly to either the baseline or an intermediate plateau level. In such cases, the formula above will incorrectly estimate at least one of the $[Ca^{2+}]$ levels. This can be easily verified for an extreme, although not most commonly encountered situation : the fluorescence intensity raises to a short-lasting peak due to agonist stimulation, followed by a drop to the basal level. Let us denote by F_1 and F_A the fluorescence intensities coming from the cell suspension in resting and stimulated conditions, respectively, and by F_2 the one obtained when adding EGTA, after the signal has dropped to the initial level. The equations for this case are:

$$F_1 = n_1 f_{min} + n_2 f_{max} + n_3 f_{max},$$

$$F_A = (n_1 - n) f_{min} + (n_2 + n) f_{max} + n_3 f_{max},$$

$$F_2 = n_1 f_{min} + n_2 f_{max} + n_3 f_{min},$$

$$F_{max} = (n_1 + n_2 + n_3) f_{max},$$

$$F_{min} = (n_1 + n_2 + n_3) f_{min}.$$

By introducing these values in eqn (7), one obtains for the basal and induced levels of Ca^{2+}, respectively:

$$[Ca^{2+}]_{app} = K_d \frac{F_2 - F_{min}}{F_{max} - F_1} = K_d \frac{n_2}{n_1},$$

$$[Ca^{2+}]^{A}_{app} = K_d \frac{F_2 - F_{min}}{F_{max} - F_A} = K_d \frac{n_2}{n_1 - n}$$
$$= K_d \frac{n_2 + n}{n_1 - n} - K_d \frac{n}{n_1 - n}.$$

While the resting value of Ca^{2+} concentration $[Ca^{2+}]_{app}$ coincides with the true $[Ca^{2+}]$ shown by eqn (2), the apparent agonist-induced $[Ca^{2+}]^{A}_{app}$ is an underestimation of the real $[Ca^{2+}]^{A}$ by the quantity $K_d n/(n_1 - n)$ [compare to eqn (6)]. On the other extreme, if the agonist does not induce a peak, but a sustained response (a stable plateau), similar equations can be written, and one can verify that applying eqn (7) will now overestimate the basal level of Ca^{2+}, while giving a correct value for $[Ca^{2+}]^{A}$. Anywhere in between these extremes, both resting and stimulated Ca^{2+} levels will be incorrectly estimated by using eqn (7).

An alternative experimental design employs the quenching properties of Mn^{2+}, which greatly reduces the dye fluorescence (to a level intermediate between the minimum and maximum signals (Minta *et al.*, 1989), characterized by the molar fluorescence, f_{Mn}) and also eliminates the problems related to the pH-changes associated with the use of EGTA. It has been shown that f_{max}, f_{min} and f_{Mn} (as well as the corresponding fluorescence values F_{max}, F_{min} and F_{Mn} — the last one being the fluorescent signal when all the dye present in the system is Mn^{2+}-bound) are linear over a suitable range of concentrations (Minta *et al.*, 1989; Kao *et al.*, 1989). This can be written as follows:

$$f_{Mn} = k_2 f_{min}, \tag{8}$$

$$f_{max} = k_1 f_{Mn}, \tag{9}$$

$$f_{max} = k_1 k_2 f_{min}, \tag{10}$$

where k_1 and k_2 can be determined from the coefficients f_{max}, f_{min} and f_{Mn} as shown by eqns (8–10). The fluorescence coefficients, f_{max}, f_{min} and f_{Mn}, can be determined *in vitro*, by calculating the slopes of the linear dependencies between fluorescence intensity and low indicator concentration, in either a Ca^{2+}-saturated, a Ca^{2+}-free or a Mn^{2+}-saturated environment. In the original paper, Minta *et al.* (1989) have found that $k_1 = 5$ and $k_2 = 8$ (and thus $k_1 k_2 = 40$) for the initial lot of dye they used, but other authors have shown that newer lots of Fluo-3 have much higher values for k_1 and k_2, and consequently for $k_1 k_2$ (Harkins *et al.*, 1993; Gee *et al.*, 2000). We have found that $k_1 = 7$ and $k_2 = 20$ (and thus $k_1 k_2 = 140$), and these discrepancies from the initially reported values can greatly influence the Ca^{2+} level measurement. It has been shown that, when using manganese, the relationship between $[Ca^{2+}]$ and the fluorescent signal F is (Kao, 1994):

$$[Ca^{2+}] = K_d \frac{F - F_{Mn}/k_2}{k_1 F_{Mn} - F} \tag{11}$$

which is equivalent to eqn (1). By quenching the extracellular fluorescence at the end of the experiment, either before or after lysing the cells, errors will occur in the calculated values for either the basal or the stimulated level of $[Ca^{2+}]$ or for both, as shown above in the case of EGTA. In order to be able to correctly estimate both these intracellular Ca^{2+} concentrations, we found that, following stimulation, one needs to wait for the fluorescence to reach a stable plateau level (F_a), before adding either EGTA or Mn^{2+}. To avoid the pH-related problems that may occur after adding EGTA, we chose to use the quenching properties of Mn^{2+} in our experiments. If n' is the change from the initial dye concentrations inside the cells, $(n_1 - n')$ and $(n_2 + n')$ represent the intracellular dye concentrations (in the Ca^{2+}-free and Ca^{2+}-bound forms, respectively), all corresponding to the F_a level, then the set of equations characteristic to this experimental design can be written as follows:

$$F = n_1 f_{min} + n_2 f_{max} + n_3 f_{max}, \tag{12}$$

$$F_A = (n_1 - n) f_{min} + (n_2 + n) f_{max} + n_3 f_{max}, \tag{13}$$

$$F_a = (n_1 - n') f_{min} + (n_2 + n') f_{max} + n_3 f_{max}, \tag{14}$$

$$F_1 = (n_1 - n') f_{min} + (n_2 + n') f_{max} + n_3 f_{Mn}, \tag{15}$$

$$F_{Mn} = (n_1 + n_2 + n_3) f_{Mn}, \tag{16}$$

where F and F_A are the fluorescence signals in resting and stimulated conditions, respectively, F_a — the stable plateau level reached after stimulation, F_1 — the fluorescence after the addition of enough Mn^{2+} to quench all the extracellular fluorescence, and F_{Mn} the signal obtained after lysis, when all the dye molecules are released in the Mn^{2+}-saturated medium. The analytical solution of this system (n_1, n_2, n_3, n and n') is too cumbersome to be presented here, but it can be easily obtained by using software that allows equation processing (e.g. Maple V, see Materials and Methods). The mathematical expressions for n_1, n_2 and n, obtained by solving this system, can be further introduced in eqns (2) and (6) to obtain the true intracellular basal and agonist-induced Ca^{2+} levels.

CASE TWO: BOTH THE LEAKAGE RATE AND THE EXTRACELLULAR DYE CONCENTRATION ARE SIGNIFICANT

It has been shown that the dye loss rate for mammalian cells is strongly temperature-dependent (Malgaroli *et al.*, 1987; Schilling *et al.*, 1988; Tsien & Pozzan, 1989; Gunter *et al.*, 1990; Kao, 1994; Lin *et al.*, 1999); therefore, Ca^{2+} measurements are often performed at low temperatures to stem this loss. However, even at reasonably low temperatures, some cell types show a significant dye leakage rate with the subsequent accumulation of important amounts of indicator in the extracellular space, where it will be in the Ca^{2+}-bound form (Rink & Pozzan, 1985; Malgaroli *et al.*, 1987; Di Virgilio *et al.*, 1988; McDonough & Button, 1989). Therefore, the rate of indicator leakage needs to be taken into account when calculating the Ca^{2+} concentrations (Di Virgilio *et al.*, 1988; McDonough & Button, 1989).

Under the reasonable assumption that Ca^{2+} concentration inside the cells is not affected by leakage and stays constant for unstimulated cells, the only dye molecules that may be extruded from the cells have to be Ca^{2+}-free. The dye leakage phenomenon is accompanied by a series of rapidly occurring events in both the cytosol and the Ca^{2+}-rich extracellular medium. The diagram presented in Fig. 1 proposes a succession of events illustrated for the case of nine dye molecules, of which three are Ca^{2+}-bound, with the assumption that intracellular [Ca^{2+}] does not change. As shown above, if three dye particles leak out, they necessarily come from the Ca^{2+}-free pool of dye molecules [Fig. 1(A) and (B)]; as a consequence, the internal equilibrium between Ca^{2+}-bound and Ca^{2+}-free fractions is temporarily disturbed, their ratio shifting in this case from 1 : 2 to 1 : 1. Since the extracellular medium is high in Ca^{2+}, the dye molecules that leak out of the cell rapidly become Ca^{2+}-bound [Fig. 1(C)]. Meanwhile, the intracellular dye redistributes between the Ca^{2+}- free and Ca^{2+}-bound fractions,

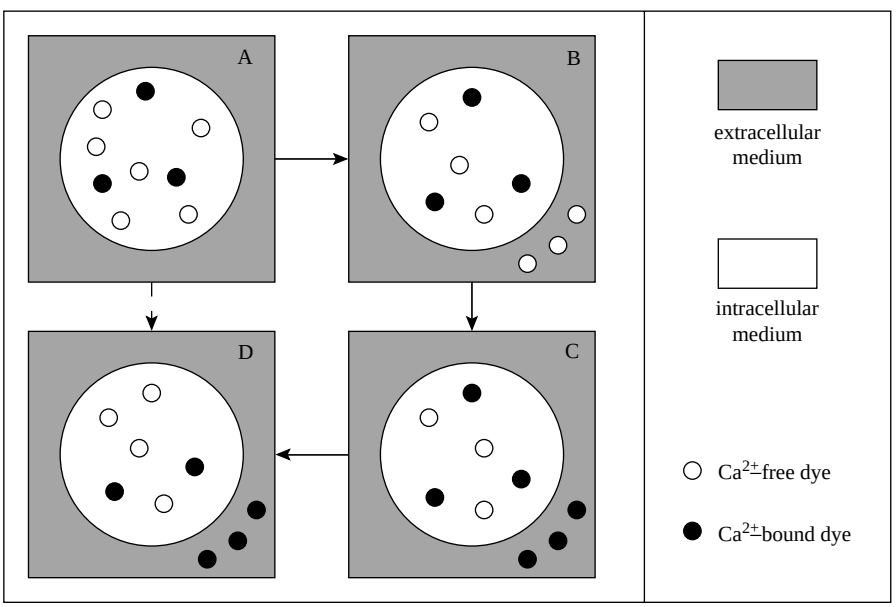

FIG. 1. Proposed sequence of events during dye leakage from the intracellular medium into the Ca^{2+}-rich extracellular environment: (A) in the example shown, nine dye molecules are distributed inside the cytosol in a 1 : 2 ratio (Ca^{2+}-bound : Ca^{2+}-free), corresponding to the intracellular [Ca^{2+}] level; (B) three Ca^{2+}-free molecules are extruded from the cytosol; the internal equilibrium between the Ca^{2+}-bound and Ca^{2+}-free fractions is temporarily disturbed (1 : 1 ratio); (C) the dye molecules that leaked out of the cell, bind Ca^{2+} from the extracellular medium; (D) the intracellular dye molecules redistribute between the bound and unbound fractions, in order to restore the initial equilibrium with Ca^{2+}: one Ca^{2+}-bound molecule becomes Ca^{2+}-free, thus shifting the system back to the initial 1 : 2 ratio. The overall result of this series of events [compare (A) to (D)] looks as if and is mathematically equivalent to one Ca^{2+}-bound and two Ca^{2+}-free dye molecules leaked out of the cell.

so that their ratio, directly linked to Ca^{2+} level through eqn (2), stays constant; in our case a Ca^{2+}-bound molecule becomes Ca^{2+}-free and the ratio shifts back to the initial value of 1 : 2 [Fig. 1(C) and (D)]. A step-by-step theoretical approach of this problem is very cumbersome due to the complexity of the sequence of events. However, the overall result of this process [going directly from Fig. 1(A) to Fig. 1(D)] looks as if one Ca^{2+}-bound and two Ca^{2+}-free molecules have leaked out of the cell. This greatly simplifies the mathematical treatment, inasmuch as it allows one to describe the leakage phenomenon as an extrusion of both Ca^{2+}-free and Ca^{2+}-bound dye with the corresponding apparent rates dn/dt and dn'/dt. The real rate of indicator loss from the cells is given by the sum of these two apparent rates. Since the Ca^{2+}-bound fraction will not change its fluorescence after being extruded in the Ca^{2+}-saturated buffer, one can determine experimentally only the apparent rate of leakage of the free dye (dn/dt), which will undergo a shift in its fluorescence from f_{min} (inside the cells) to f_{max} (in the extracellular medium) (see Materials and Methods).

As discussed earlier, we found that the correct determination of $[Ca^{2+}]$ involves the addition of Mn^{2+} after the fluorescence went through an agonist-induced transient peak and reached a stable plateau level. The correct system of equations that takes into account the non-negligible leakage rates (under the assumption that these are constant over time, as if leakage was a linear phenomenon), and allows one to find the true $[Ca^{2+}]$ and $[Ca^{2+}]^A$ from eqns (2) and (6), can be written as follows:

$$F = n_1 f_{min} + n_2 f_{max} + n_3 f_{max}, \tag{17}$$

$$\begin{aligned} F_A = &\left[n_1 - n - \frac{dn}{dt}(t_2 - t_1)\right] f_{min} \\ &+ \left[n_2 + n - \frac{dn'}{dt}(t_2 - t_1)\right] f_{max} \\ &+ \left[n_3 + \left(\frac{dn}{dt} + \frac{dn'}{dt}\right)(t_2 - t_1)\right] f_{max}, \end{aligned} \tag{18}$$

$$\begin{aligned} F_a = &\left[n_1 - n' - \frac{dn}{dt}(t_3 - t_1)\right] f_{min} \\ &+ \left[n_2 + n' - \frac{dn'}{dt}(t_3 - t_1)\right] f_{max} \\ &+ \left[n_3 + \left(\frac{dn}{dt} + \frac{dn'}{dt}\right)(t_3 - t_1)\right] f_{max}, \end{aligned} \tag{19}$$

$$\begin{aligned} F_1 = &\left[n_1 - n' - \frac{dn}{dt}(t_4 - t_1)\right] f_{min} \\ &+ \left[n_2 + n' - \frac{dn'}{dt}(t_4 - t_1)\right] f_{max} \\ &+ \left[n_3 + \left(\frac{dn}{dt} + \frac{dn'}{dt}\right)(t_4 - t_1)\right] f_{\mathrm{Mn}}, \end{aligned} \tag{20}$$

$$F_{\mathrm{Mn}} = (n_1 + n_2 + n_3) f_{\mathrm{Mn}}, \tag{21}$$

where t_1, t_2, t_3 and t_4 are the time moments at which F, F_A, F_a and F_1 are being measured. Equations (17) and (21) are identical to eqns (12) and (16), but the other three [eqns (18–20)] take into account both the leakage rates and the time elapsed from the start of the experiment. Under the reasonable assumption that the Ca^{2+} level inside the cells is the same at moments t_3 and t_4, then eqn (2) implies that the ratio between bound and unbound dyes inside the cytosol will stay constant. Based on the above equations, this ratio can be written as follows:

$$\begin{aligned} \frac{Ca^{2+}\text{-bound}}{Ca^{2+}\text{-free}} &= \frac{n_2 + n' - (dn'/dt)(t_3 - t_1)}{n_1 - n' - (dn/dt)(t_3 - t_1)} \\ &= \frac{n_2 + n' - (dn'/dt)(t_4 - t_1)}{n_1 - n' - (dn/dt)(t_4 - t_1)} \end{aligned}$$

which is equivalent to:

$$\frac{dn'}{dt} = \left(\frac{n_2 + n'}{n_1 - n'}\right)\frac{dn}{dt}. \tag{22}$$

By introducing this result in the system formed by eqns (17–21), one obtains, after simplification,

$$F = n_1 f_{min} + n_2 f_{max} + n_3 f_{max}, \tag{23}$$

$$F_A = \left[n_1 - n - \frac{dn}{dt}(t_2 - t_1)\right] f_{min} + \left[n_2 + n + n_3 + \frac{dn}{dt}(t_2 - t_1)\right] f_{max}, \quad (24)$$

$$F_a = \left[n_1 - n' - \frac{dn}{dt}(t_3 - t_1)\right] f_{min} + \left[n_2 + n' + n_3 + \frac{dn}{dt}(t_3 - t_1)\right] f_{max}, \quad (25)$$

$$F_1 = \left[n_1 - n' - \frac{dn}{dt}(t_4 - t_1)\right] f_{min} + \left[n_2 + n' - \frac{dn}{dt}\left(\frac{n_2 + n'}{n_1 - n'}\right)(t_4 - t_1)\right] f_{max} + \left[n_3 + \frac{dn}{dt}\left(\frac{n_1 + n_2}{n_1 - n'}\right)(t_4 - t_1)\right] f_{Mn}, \quad (26)$$

$$F_{Mn} = (n_1 + n_2 + n_3) f_{Mn}. \quad (27)$$

This system can be easily solved by using software that allows equation processing and its solution (n_1, n_2, n_3, n and n') will not be presented here. The mathematical expressions for n_1, n_2 and n can be further introduced in eqns (2) and (6) to obtain the corrected intracellular Ca^{2+} basal and agonist-induced levels.

Materials and Methods

MATERIALS

BAECs were supplied by Dr. Robert Auerbach of the University of Wisconsin. Minimal essential medium (MEM) was purchased from GIBCO (Grand Island, NY, U.S.A.). ATP, BK, probenecid, penicillin and streptomycin were purchased from Sigma (St. Louis, MO, U.S.A.). Heat-inactivated fetal bovine calf serum (FBS) was purchased from HyClone (Logan, UT, U.S.A.). Fluo-3/AM and Pluronic F-127 were purchased from Molecular Probes Inc. (Eugene, OR, U.S.A.). All other reagents were of analytical grade or better.

CELL CULTURE

Passage 10 BAECs were cultured in 100 cm^2 Petri dishes as previously described (Campbell *et al.*, 1999) until they reached the early growth phase (2.5–5.0 $\times$ 10^4 cells cm^{-2}).

FLUO-3/AM LOADING INTO BAECS

We have found that 1.5 mM probenecid effectively inhibits dye leakage from the cytosol (unpubl. obs.). Therefore, all buffers and media used throughout the loading procedure and the fluorescence measurements contained 1.5 mM probenecid to diminish dye extrusion from the cytosol. BAECs were washed with Krebs–Henseleit buffer (10 mM HEPES, 120 mM NaCl, 4.6 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM NaH_2PO_4, 0.7 mM Na_2HPO_4, 10 mM glucose; pH 7.2). To load the cells with Fluo-3, we incubated the plates with 400 nM Fluo-3/AM in cold MEM, supplemented with 10 $\mu g\,ml^{-1}$ BSA and 0.02% Pluronic F-127. Loading was performed for 1 h in the following sequence: 15 min at room temperature, 15 min at 37°C, followed by 30 min at room temperature. Incubations at room temperature were performed with continuous shaking. Plates were subsequently washed with MEM for 15 min at room temperature and then allowed to recover at 37°C for another 15 min in MEM supplemented with 10% FBS. Cells were isolated from plates by trypsinization, resuspended in MEM with 10% FBS, and then washed three times by centrifugation in Krebs–Henseleit buffer. After resuspension in Krebs–Henseleit buffer, cell density was determined by using a hemacytometer. Cell suspensions with viability values less than 85%, as determined by trypan blue exclusion, were discarded. Cell density was adjusted to 1.5 $\times$ 10^6 cells ml^{-1} with Krebs–Henseleit buffer, and a 2 ml aliquot of cell suspension was transferred to a cuvette for fluorescence measurements.

FLUORESCENCE MEASUREMENTS AND $[Ca^{2+}]$ DETERMINATION

Measurements were performed by using a SLM 8000C spectrofluorometer (SLM AMINCO, Urbana, IL, U.S.A.); the cuvette holder was connected to a magnetic stirrer, so that cell suspensions were under continuous stirring conditions during the experiments. The wavelengths used for excitation and emission were 504 and 526 nm, respectively (bandpass 4 nm).

Time was carefully monitored (starting with $t = 0$ corresponding to 60 min after the beginning of trypsinization) so that each fluorescence measurement corresponds to a precise time or time frame. Prior to each experiment (at $t = 0$) the sample was read for 500 s and the observable leakage rate (dn/dt) determined by the least-squares fit method, this method being more reliable than cell suspension centrifugation (Di Virgilio *et al.*, 1988). All measurements were performed at room temperature and in the presence of 1.5 mM probenecid; in these conditions, the leakage was linear for at least 1 h and the leakage rate (dn/dt) did not significantly differ from the rate recorded in the time frame in which the experiments were performed (data not shown). The average leakage rate obtained in these conditions from 13 experiments was as little as 0.22 ± 0.04 nM min^{-1}. At $t = 10$ min, the baseline fluorescence in resting cells (F) was first read for 60 s prior to the addition of 1μM ATP or BK. Following stimulation, the fluorescent signal was recorded throughout the transient peak (F_A) and the stable plateau (F_a). The plateau level F_a was recorded for 60 s, followed by the addition of 700μM $MnCl_2$; the corresponding drop in fluorescence to a stable level was recorded for an additional 60 s (F_1). We have shown that this Mn^{2+} concentration quenches the extracellular Fluo-3 only and does not enter the cells, as its effect can be completely reversed by the addition of diethylenetriaminepentaacetic acid (DTPA) (data not shown). At the end of the experiment, cells were lysed by adding 20μM digitonin, and the signal recorded for at least 60 s (F_{Mn}). Previous attempts to determine F_{Mn} in Fluo-3-loaded BAECs, by using the ionophore 4-Br A23187 instead of digitonin, were unsuccessful (unpubl. obs.).

Typical fluorescence recordings during such experiments are shown in Fig. 2 (A) and (B), for BK and ATP, respectively. F, F_a, F_1 and F_{Mn} were determined by averaging the signal over the corresponding time frames (see Fig. 2). Autofluorescence was determined by measuring the signal coming from unloaded cells, suspended in the same buffer at the same density, and was subtracted from each experimental value obtained from loaded cells. Since every addition to the cell suspension slightly diluted it, data were multiplied by the corresponding dilution factors before being employed in calculations. To determine the uncorrected values of $[Ca^{2+}]$, we have employed eqn (11) and the experimental fluorescence values. The corrected Ca^{2+} concentrations at rest and agonist-induced were determined by using eqns (2) and (6) after mathematically calculating the concentrations n_1, n_2 and n. When the correction was made for the extracellular dye only, n_1, n_2 and n were calculated by solving the system (12–16). For the Ca^{2+} concentrations corrected for both the extracellular dye and the leakage rate, we have determined n_1, n_2 and n from (23–27). All equations were solved by using the Maple V (version 5.1) software. To determine Ca^{2+} concentrations from eqns (2), (6) and (11), we used a K_d value of 400 nM which has been previously determined for vertebrate ionic strength (Kao *et al.*, 1989). The values for f_{max}, k_1 and k_2 were previously determined to be $f_{max} = 539.01$ fluorescence units nM^{-1}, $k_1 = 7$, and $k_2 = 20$, respectively (unpubl. obs.).

STATISTICAL ANALYSIS

Results from at least five experiments are reported as mean value ±SEM. Student's two-tailed t-test was used to determine the statistical significance of a difference between means. A p-value smaller than 0.05 was considered to denote significance.

Results and Discussion

In the previous sections, we have analysed the inherent problems that occur as a consequence of SW dye leakage from the cytosol during $[Ca^{2+}]$ measurements, which usually lead to an overestimation of intracellular $[Ca^{2+}]$ levels. We have also developed a mathematical approach to treat these problems; however, the utility of our model would be limited if the amount of the predicted overestimation were not significant. To check its applicability, we tested our model in an experimental situation (endothelial cells in resting and ATP- or BK-stimulated conditions) and we compared $[Ca^{2+}]$ levels calculated with or without the mentioned corrections.

It has been shown that the loss of indicator into the extracellular environment occurs by

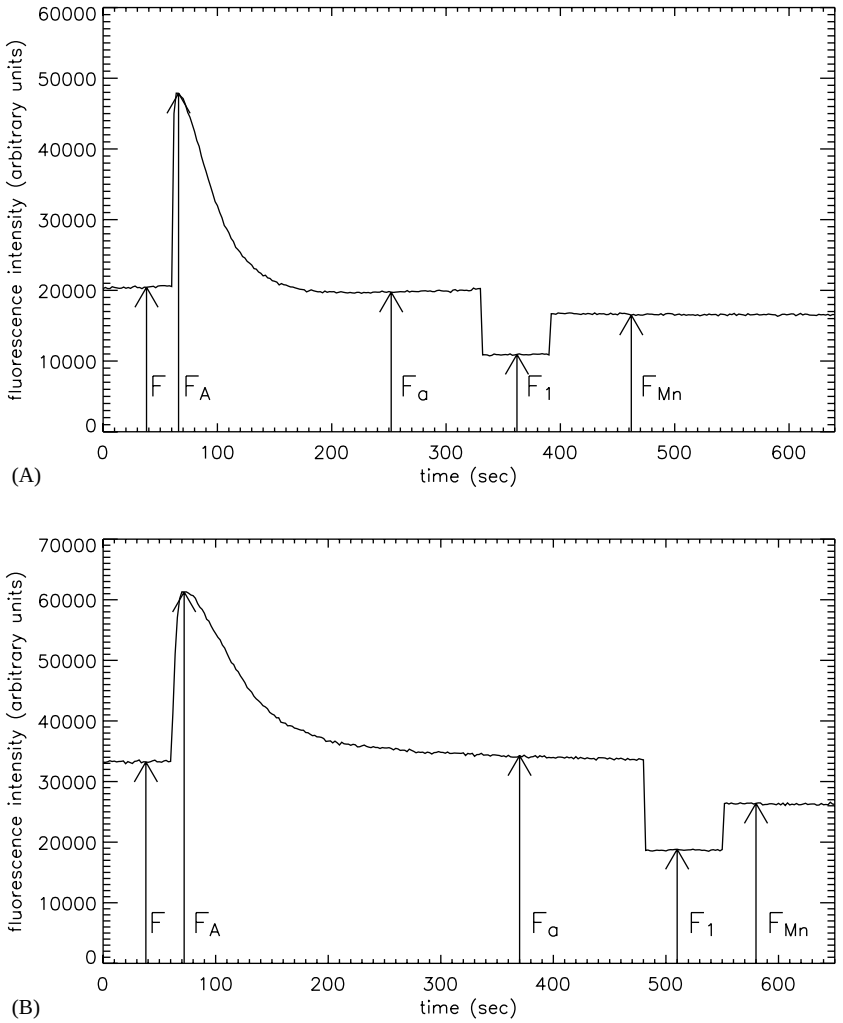

FIG. 2. Representative fluorescence traces of Fluo-3-loaded BAECs stimulated with 1μM ATP or BK during typical experiments. Fluorescence was recorded at 504 nm excitation and 526 nm emission. Loaded cells were suspended in Krebs–Henseleit buffer (1.5×10^6 cells ml^{-1}) and after reading the baseline (F), the cells were challenged with the agonist (peak F_A, plateau F_a). Mn^{2+} was then added to the cuvette to quench extracellular dye fluorescence (F_1), and cells were subsequently lysed with digitonin (F_{Mn}). The sequence of events, indicating the concentrations and times of the additions was as follows: (A) 1μM ATP at $t = 60$ s, 700μM $MnCl_2$, at $t = 330$ s, and 20μM digitonin at $t = 390$ s from the beginning of the experiment; (B) 1μM BK at $t = 60$ s, 700μM $MnCl_2$, at $t = 480$ s, and 20μM digitonin at $t = 550$ s from the beginning of the experiment.

an extrusion mechanism for organic anions (Di Virgilio *et al.*, 1988) and can be blocked effectively by inhibitors of uric acid transport, such as probenecid and sulphinpyrazone (Di Virgilio *et al.*, 1988; Arkhammar *et al.*, 1989; Lin *et al.*, 1999). However, the leakage rate usually cannot be reduced to zero, but can be substantially decreased and maintained constant. We have found that 1.5 mM probenecid used throughout the experiment effectively decreases the Fluo-3 leakage rate from BAECs, while greatly improving cell loading (unpubl. obs.).

In order to avoid intracellular Ca^{2+} buffering by excess dye (Rin & Pozzan, 1985; Tsien & Pozzan, 1989), the concentration of Fluo-3/AM in the loading solution was lowered from 2.0 μM to 400 nM. Minimizing the amount of dye loaded into the cells also has the beneficial effect of reducing the chemical by-products, formaldehyde, acetate and protons (Tsien, 1981; Rink & Pozzan, 1985), that could otherwise be harmful

to the cells. Assuming an endothelial cell volume of 0.5–1.8 μl (10^6 cells)$^{-1}$ (Rodgers *et al.*, 1984; Colden-Stanfield *et al.*, 1987; Schneider *et al.*, 1997), we estimated that the BAECs were effectively loaded with an intracellular Fluo-3 concentration between 65 and 400 μM. This dye concentration greatly exceeds the amounts of indicator needed to measure Ca^{2+} concentrations compatible with a living cell system, even in the case of a high K_d indicator, such as Fluo-3. Further evidence that there is sufficient dye within the cells to bind such Ca^{2+} concentrations can be also seen from Fig. 2. Lysing the cells with digitonin induces a significant increase in fluorescence (compare F_1 to F_{Mn}), which suggests that, before being released in the extracellular space, most of the intracellular dye was in a Ca^{2+}-free, low fluorescence form (f_{min}).

Our results indicate that 1 μM of either BK or ATP induces a rapid and transient increase in the fluorescence intensity [see Fig. 2 (A) and (B)], which corresponds to an increased Ca^{2+} level inside the BAECs. These results are in good agreement with previous reports of these agonists being responsible for inducing intracellular Ca^{2+} mobilization in endothelial cells (Schilling *et al.*, 1988; Adams *et al.*, 1989; Mo *et al.*, 1991; Busse & Fleming, 1996; Duchêne & Takeda, 1997; Pieper & Dondlinger, 1997; Stachon *et al.*, 1998; Viana *et al.*, 1998). To determine the extent of this mobilization, as well as the initial level of [Ca^{2+}], data originating from the same set of experiments were processed in three different ways, as described in the Materials and Methods Section. The comparison of uncorrected and corrected results for dye leakage and/or extracellular dye is presented in Table 1.

For the uncorrected data, the basal [Ca^{2+}] level was 89.9 ± 5.86 nM, and the addition of 1 μM BK caused a 2.3-fold increase in [Ca^{2+}] (235 ± 16.4 nM), significantly above the resting level ($p < 0.001$). Similarly, the addition of 1 μM ATP induced intracellular Ca^{2+} mobilization up to 250 ± 15.0 nM, corresponding to a 3.3-fold increase ($p < 0.001$ vs. basal level).

Correcting for the extracytosolic dye only, leads to the following values (nM): 32.7 ± 1.10 for basal, 144 ± 7.32 for BK- and 196 ± 11.0 for ATP-induced Ca^{2+} levels. These values, significantly different from the uncorrected results (see Table 1), reflect a 4.7- and 5.6-fold induction for BK and ATP, respectively.

If corrections were applied for both the extracellular Fluo-3 and its leakage from the BAECs, the resting level of [Ca^{2+}] was found to be of 35.1 ± 1.24 nM and stimulation with 1 μM agonist triggered an increase in cytosolic Ca^{2+} up to 146 ± 7.51 and 198 ± 11.6 nM for BK and ATP, respectively. The corresponding fold increase values were 4.4 for BK and of 5.3 for ATP. One can see that both the Ca^{2+} level and fold induction show a statistically significant difference from the uncorrected values, which confirms our previous theoretical finding that neglecting the dye outside the cells overestimates the intracellular Ca^{2+} level. However, the values obtained by applying either correction are very similar to each other ($p > 0.3$). These results can be explained by the effectiveness of probenecid on decreasing the Fluo-3 extrusion through the plasma membrane, confirming that the leakage rate has been brought to a negligible level. In obtaining these results, an important role could also have been played by the short time frame during which the experiments were performed. Since the leakage rate, dn/dt, is always multiplied by the time elapsed from the start of the experiment, the decreased rate is potentiated by these short intervals (usually approximately 10 min for the whole experiment). A larger time combined with the same extrusion rate may yield significant differences between the two correction methods.

The fact that previous reports have indicated an intracellular basal level of Ca^{2+} higher (approximately 100 nM), (Hallam & Pearson, 1986; Pieper & Dondlinger, 1997; Stachon *et al.*, 1998; Sato *et al.*, 1999; Suh *et al.*, 1999) than the one we found for endothelial cells is not surprising if one keeps in mind the mathematical consequences of overlooking the effect of extracellular dye. This possibility is sustained by the fact that, in the absence of extracellular Ca^{2+}, several investigators have found a decreased cytosolic Ca^{2+} level for resting and/or agonist-induced conditions, as compared to the levels obtained when Ca^{2+} was present during the experiment (Schilling *et al.*, 1988; Oliver & Chase, 1992; Bassani *et al.*, 1995; Duchêne &

TABLE 1
Intracellular Ca^{2+} concentrations determined in BAECs in basal and agonist-stimulated conditions.

	Uncorrected	Corrected for extracellular dye only	Corrected for extracellular dye and leakage rate
Basal [Ca^{2+}] level (nM)	89.9 ± 5.86	32.7 ± 1.10*	35.1 ± 1.24*
ATP (1 μM)			
Stimulated [Ca^{2+}] level (nM)	250 ± 15.0†	196 ± 11.0†‡	198 ± 11.6†‡
Fold induction	3.34 ± 0.09	5.58 ± 0.24*	5.28 ± 0.19*
BK (1 μM)			
Stimulated [Ca^{2+}] level (nM)	235 ± 16.4†	144 ± 7.32*†	146 ± 7.51*†
Fold induction	2.30 ± 0.04	4.70 ± 0.23*	4.43 ± 0.19*

The cells were challenged with 1 μM BK or ATP, as shown in Fig. 2. Experimental data were processed either without any correction or by applying corrections for Fluo-3 leakage rate and/or extracellular Fluo-3 as described in Materials and Methods. When applying the correction for both extracellular dye and leakage rate, individual dn/dt values were obtained for each sample (range: 0.07–0.54 nM min^{-1}) and further used in calculations.

*$p < 0.001$ vs. uncorrected values.

†$p < 0.001$ vs. basal level.

‡$p < 0.05$ vs. uncorrected values.

Takeda, 1997). These differences have sometimes been interpreted as an active contribution of extracellular Ca^{2+} to the cytoplasmic Ca^{2+} concentrations; however, they may as well reflect the discussed overestimation due to the leakage of indicator in the Ca^{2+}-saturated (as compared to a Ca^{2+}-free) environment. On the other hand, other authors have found, in various cell types, [Ca^{2+}] levels similar to ours (Colden-Stanfield *et al.*, 1987; Baylor & Hollingworth, 1988; McDonough & Button, 1989; Gunter *et al.*, 1990; Mo *et al.*, 1991; Buckley *et al.*, 1995; Grøndahl & Langmoen, 1998; Viana *et al.*, 1998) when applying corrections or using a system in which extracellular dye was negligible. These results are significantly smaller when compared to previously reported, uncorrected values. Another possible explanation for this consists in the fact that we and some of the above-cited authors used an *in vitro* K_d value, which probably underestimates the real value in the cytosolic environment (Colden-Stanfield *et al.*, 1987; McDonough & Button, 1989; Mo *et al.*, 1991). However, this influence could explain these results only in part, since it would reflect only on the absolute cytoplasmic Ca^{2+} level and not on the fold induction value, as discussed in the Introduction.

In this paper, we have developed a theoretical and experimental approach for intracellular [Ca^{2+}] determination, which may help solve some of the problems implied by the extrusion of fluorescent indicators from the cytosol. Both our theoretical and experimental results point to the fact that the extracellular dye can greatly interfere with the results, generally by overestimating the cytosolic [Ca^{2+}] in both basal and agonist-induced conditions, and must be corrected for. The correction we have designed has an advantage over previously published correction methods in that it can be applied to suspensions of any cell types that have been loaded with any SW indicator. Most assumptions we made in order to develop this model are consistent with the theory upon which relies the very use of these dyes for Ca^{2+} measurements (low dye concentrations, and a constant [Ca^{2+}] inside resting cells). However, the assumption that the dye leakage rate is constant over time may not hold for all systems; in such cases, the use of probenecid may help linearize the leakage phenomenon. Our findings, together with the fact that probenecid can effectively inhibit the dye leakage rate in BAECs, can provide a valuable tool for [Ca^{2+}] determinations in future experiments.

This research was supported in part by National Science Fundation MCB-9816681, Greenville Hospital System and the South Carolina Experiment

Station Grant SC01630. The authors thank Drs. Gary Powell and James Zimmerman of Clemson University for critically evaluating the manuscript. The authors would also like to thank Dr. Cristian Ianculescu of Clemson University for his technical help in solving the equation systems.

REFERENCES

Adams, D. J., Barakeh, J., Laskey, R. & Van Breemen, C. (1989). Ion channels and regulation of intracellular calcium in vascular endothelial cells. *FASEB J.* **3,** 2389–2400.

Arkhammar, P., Nilsson, T. & Berggren, P.-O. (1989). Glucose-stimulated efflux of fura-2 in pancreatic β-cells is prevented by probenecid. *Biochem. Biophys. Res. Commun.* **159,** 223–228.

Bassani, J. W. M., Bassani, R. A. & Bers D. M. (1995). Calibration of Indo-1 and resting intracellular $[Ca^{2+}]_i$ in intact rabbit cardiac myocytes. *Biophys. J.* **68,** 1453–1460.

Baylor, S. M. & Hollingworth, S. (1988). Fura-2 calcium transients in frog skeletal muscle fibres. *J. Physiol.* **403,** 151–192.

Buckley, B. J., Mirza, Z. & Whorton, A. R. (1995). Regulation of Ca^{2+}-dependent nitric oxide synthase in bovine aortic endothelial cells. *Am. J. Physiol.* **269,** C757–C765.

Busse, R. & Fleming, I. (1996). Molecular responses of endothelial tissue to kinins. *Diabetes* **45** (Suppl. 1)**,** S8–S13.

Campbell, S. A., Kemerling, A. J. & Hilderman, R. H. (1999). P^1, P^4-diadenosine 5'-tetraphosphate binding on bovine aortic endothelial cells. *Arch. Biochem. Biophys.* **364,** 280–285.

Colden-Stanfield, M., Schilling, W. P., Ritchie, A. K., Eskin, S. G., Navarro, L. T. & Kunze, D. L. (1987). Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. *Circ. Res.* **61,** 632–640.

Di Virgilio, F., Steinberg, T. H., Swanson, J. A. & Silverstein, S. C. (1988). Fura-2 secretion and sequestration in macrophages. *J. Immunol.* **140,** 915–920.

Duchêne, A. D. & Takeda, K. (1997). P2y- and P2u-mediated increases in internal calcium in single bovine aortic endothelial cells in primary culture. *Endothelium* **5,** 277–286.

Gee, K. R., Brown, K. A., Chen, W.N. Bishop-Stewart, J., Gray, D. & Johnson, I. (2000). Chemical and physiological characterization of fluo-4 Ca^{2+}-indicator dyes. *Cell Calcium* **27,** 97–106.

Grøndahl, T. O. & Langmoen, I. A. (1998). Confocal laser scanning microscopy used to monitor intracellular Ca^{2+} changes in hippocampal CA neurons during energy deprivation. *Brain Res.* **785,** 58–65.

Grynkiewicz, G., Poenie, M. & Tsien, R. Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260,** 3440–3450.

Gunter, T. E., Zuscik, M. J., Puzas, J. E., Gunter, K. K. & Rosier, R. N. (1990). Cytosolic free calcium concentrations in avian growth plate chondrocytes. *Cell Calcium* **10,** 171–180.

Hallam, T. J. & Pearson, J. D. (1986). Exogenous ATP raises cytoplasmic free calcium in fura-2 loaded piglet aortic endothelial cells. *FEBS Lett.* **207,** 95–99.

Harkins, A. B., Kurebashi, N. & Baylor, S. M. (1993). Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with Fluo-3. *Biophys. J.* **65,** 865–881.

Hesketh, T. R., Smith, G. A., Moore, J. P., Taylor, M. V. & Metcalfe, J. C. (1983). Free cytoplasmic calcium concentration and the mitogenic stimulation of lymphocytes. *J. Biol. Chem.* **258,** 4876–4882.

Homolya, L., Holló, Z., Germann, U. A., Pastan, I., Gottesman, M. M. & Sarkadi, B. (1993). Fluorescent cellular indicators are extruded by the multidrug resistance protein. *J. Biol. Chem.* **268,** 21493–21496.

Hove-Madsen, L. & Bers, D. M. (1992). Indo-1 binding to protein in permeabilized ventricular myocytes alters its spectral and Ca binding properties. *Biophys. J.* **63,** 89–97.

Kao, J. P. Y., Harootunian, A. T. & Tsien, R. Y. (1989). Photochemically generated cytosolic calcium pulses and their detection by Fluo-3. *J. Biol. Chem.* **264,** 8179–8184.

Kao, J. P. Y. (1994). Practical aspects of measuring $[Ca^{2+}]$ with fluorescent indicators. In: *Methods in Cell Biology*, A Practical Guide to the Study of Calcium in Living Cells (Nuccitelli, R., ed.), Vol. 40, pp. 155–181. San Diego: Academic Press.

Lin, K., Sade, W. & Quillan, J. M. (1999). Rapid measurements of intracellular calcium using a fluorescence plate reader. *BioTechniques* **26,** 318–326.

Malgaroli, A., Milani, D., Meldolesi, J. & Pozzan, T. (1987). Fura-2 measurement of cytosolic free Ca^{2+} in monolayers and suspensions of various types of animal cells. *J. Cell. Biol.* **105,** 2145–2155.

McDonough, P. M. & Button, D. C. (1989). Measurement of cytoplasmic calcium concentration in cell suspensions: correction for extracellular Fura-2 through use of Mn^{2+} and probenecid. *Cell Calcium* **10,** 171–180.

Merritt, J. E., McCarthy, S. A., Davies, M. P. A. & Moores, K. E. (1990). Use of Fluo-3 to measure cytosolic Ca^{2+} in platelets and neutrophils. *Biochem. J.* **269,** 513–519.

Minta, A., Kao, J. P. Y. & Tsien, R. Y. (1989). Fluorescent indicators for cytosolic calcium based on rhodamine and fluorescein chromophores. *J. Biol. Chem.* **264,** 8171–8178.

Mo, M., Eskin, S. G. & Schilling, W. P. (1991). Flow-induced changes in Ca^{2+} signalling of vascular endothelial cells : effect of shear stress and ATP. *Am. J. Physiol.* **260,** H1698–H1707.

Oliver, J. A. & Chase Jr, H. S. (1992). Changes in luminal flow rate modulate basal and bradykinin-stimulated cell $[Ca^{2+}]$ in aortic endothelium. *J. Cell. Physiol.* **151,** 37–40.

Pieper, G. M. & Dondlinger, L. (1997). Glucose elevations alter bradykinin-stimulated intracellular calcium accumulation in cultured endothelial cells. *Cardiovasc. Res.* **34,** 169–178.

Rink, T. J. & Pozzan, T. (1985). Using Quin2 in cell suspensions. *Cell Calcium* **6,** 133–144.

Rodgers, R. J., O'Shea, J. D. & Bruce, N. W. (1984). Morphometric analysis of the cellular composition of the ovine corpus luteum. *J. Anat.* **138,** 757–769.

Sato, N., Az-Ma, T., Fujii, K. & Yuge, O. (1999). Protamine induces elevation of cytosolic free Ca^{2+} in

cultured porcine aortic endothelial cells. *J. Pharm. Pharmacol.* **51,** 949–952.

Schilling, W. P., Ritchie, A. K., Navarro, L. T. & Eskin, S. G. (1988). Bradykinin-stimulated calcium influx in cultured bovine aortic endothelial cells. *Am. J. Physiol.* **255,** H219–H227.

Schneider, S. W., Yano, Y., Sumpio, B. E., Jena, B. P., Geibel, J. P., Gekle, M. & Oberleithner, H. (1997). Rapid aldosterone-induced cell volume increase of endothelial cells measured by the atomic force microscope. *Cell. Biol. Int.* **21,** 759–768.

Stachon, A., Stegemann, H., Hohage, H., Rahn, K. H. & Schlatter, E. (1998). Effects of diadenosine polyphosphates on the intracellular Ca^{2+} concentration in endothelial cells. *Cell. Physiol. Biochem.* **8,** 175–184.

Suh, S. H., Vennekens, R., Manolopoulos, V. G., Freichel, M., Schweig, U., Prenen, J., Flockerzi, V., Droogmans, G. & Nilius, B. (1999). Characterisation of explanted endothelial cells from mouse aorta: electrophysiology and Ca^{2+} signalling. *Pflugers Arch.* **438,** 612–620.

Thomas, D., Tovey, S. C., Collins, T. J., Bootman, M. D., Berridge, M. J. & Lipp, P. (2000). A comparison of fluorescent Ca^{2+} indicator properties and their use in measuring elementary and global Ca^{2+} signals. *Cell Calcium* **28,** 213–223.

Tsien, R. Y. (1981). A non-disruptive technique for loading calcium buffer and indicators into cells. *Nature* **290,** 527–528.

Tsien, R. & Pozzan, T. (1989). Measurement of cytosolic free Ca^{2+} with quin2. *Methods Enzymol.* **172,** 230–262.

Uto, A., Arai, H. & Ogawa, Y. (1991). Reassessment of Fura-2 and the ratio method for determination of intracellular Ca^{2+} concentrations. *Cell Calcium* **12,** 29–37.

Viana, F., De Smedt, H., Droogmans, G. & Nilius, B. (1998). Calcium signalling through nucleotide receptor P2Y2 in cultured human vascular endothelium. *Cell Calcium* **24,** 117–127.