A Ca$^{2+}$-Insensitive Form of Fura-2 Associated with Polymorphonuclear Leukocytes

**ASSESSMENT AND ACCURATE Ca$^{2+}$ MEASUREMENT**

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The new, fluorescent Ca$^{2+}$ indicator, fura-2, promises to expand our understanding of the role of subcellular changes in Ca$^{2+}$ underlying cell function. During an investigation of the role of Ca$^{2+}$ in the polarization response of human polymorphonuclear leukocytes to formyl-methionyl-leucyl-phenylalanine, we found that fura-2 trapped by cells incubated with the acetoxy-methyl ester of fura-2, F2-AM, yielded measurements of Ca$^{2+}$ that were depressed at rest and during the response to formyl-methionyl-leucyl-phenylalanine. Fura-2, trapped by the cells, exhibited a spectrum in the presence of saturating Ca$^{2+}$ that differed from that of fura-2 free acid. We have shown that the cellular fluorescence can be spectrally decomposed into two components: one with Ca$^{2+}$ sensitivity identical to fully deesterified fura-2, and another which is Ca$^{2+}$-insensitive. The Ca$^{2+}$-insensitive component appears to be more fluorophore than F2-AM and is spectrally different from F2-AM. The insensitive form probably results from incomplete deesterification of F2-AM by the cells.

In order to accurately measure Ca$^{2+}$ in polymorphonuclear leukocytes, it is imperative to check for the presence of Ca$^{2+}$-insensitive fluorescence. The contribution of Ca$^{2+}$-insensitive fura-2 fluorescence can be assessed routinely from spectral data obtained by calibration of intracellular fura-2 with known [Ca$^{2+}$] using ionomycin. The end-of-experiment calibration step not only ensures accurate [Ca$^{2+}$] measurements in polymorphonuclear leukocytes and in other cell types that display Ca$^{2+}$-insensitive, contaminating fluorescence but also yields the spectral characteristics of the insensitive species.

Ca$^{2+}$ plays an important role in many biological systems exerting control over a wide range of subcellular processes. Subcellular measurements of [Ca$^{2+}$] are thus required in order to understand its role in cell function. The ability to monitor local changes in [Ca$^{2+}$] has been greatly aided by the new, fluorescent Ca$^{2+}$ indicator, fura-2 (1, 2). Fura-2 has been used in several cell types to examine local Ca$^{2+}$ changes in small regions of the cell in a nonperturbing manner. Ca$^{2+}$ changes following contractile activation of smooth muscle (3) and during mitosis of Pt K2 epithelial cells (4) are among processes successfully examined in detail using fura-2. On the other hand, there have been reports in the literature of methodological difficulty associated with the use of fura-2, suggesting that, in certain cell types, assumptions inherent in the use of fura-2 are not valid. For instance, some cells appear to be deficient in the esterase required to cleave F2-AM and, as a consequence, microinjection of these cell types with fura-2 free acid is necessary to obtain a reliable Ca$^{2+}$ signal (5-7). In other cell types, the fluorescent response of cell-associated fura-2 to Ca$^{2+}$ appears to be different from that of free acid, requiring calibration of cellular fluorescence with high viscosity gelatin solutions in order to mimic the intracellular environment (2). A recent report proposes that a modified form of F2-AM may become partially bound to isolated vesicles of skeletal muscle (8). Prior to this, we had reported in preliminary form on the presence of a Ca$^{2+}$-insensitive yet highly fluorescent form of fura-2 associated with polymorphonuclear leukocytes (PMNs) incubated with F2-AM (9, 10).

In this communication, we propose that the identity of the Ca$^{2+}$-insensitive fluorescence associated with PMNs represents intermediates of the deesterification of F2-AM. The presence of this fluorescence may also account for problems associated with the use of fura-2 in other cell types. Since the presence of the insensitive form of fura-2 precludes the use of the standard procedure for calculation of [Ca$^{2+}$] (1), which assumes only two intracellular forms of fura-2, we suggest a method which allows for accurate measurement of [Ca$^{2+}$] in all cells where intermediates of the intracellular deesterification of F2-AM, or F2-AM itself, are present. This method involves in situ calibration of cell-associated fura-2 by permeabilization of the cells to Ca$^{2+}$ using the Ca$^{2+}$ ionophore, ionomycin, with the assumption that the concentration of ionomycin is sufficient to equilibrate intracellular and extracellular [Ca$^{2+}$]. The calibration step not only checks for the presence of Ca$^{2+}$-insensitive fura-2 and leads to correct Ca$^{2+}$ measurement in spite of the insensitive dye, but it also provides a means for characterizing the insensitive moiety.

**MATERIALS AND METHODS**

Preparation of Human Polymorphonuclear Leukocytes—Whole blood was obtained by venipuncture of healthy donors and sedimented for 30 min over 6% dextran (Pharmacia P-L Biochemicals). The

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† The abbreviations used are: PMNs, polymorphonuclear leukocytes; fMLF, formyl-methionyl-leucyl-phenylalanine; HBSS, Hank's balanced salt solution; EGTA, N-ethylmethylendinitrilo]tetraacetic acid.
white cell-enriched supernatant was centrifuged at 1200 rpm for 15 min in a Sorvall RC2-B centrifuge at 4 °C. The pellet was resuspended in a small volume of Hanks' balanced salt solution (GIBCO); remaining red cells were removed by hypotonic lysis in 9.5 ml of distilled water for 30 s, followed by addition of 0.5 ml of 18% NaCl to restore normal osmolarity. After centrifugation at 1200 rpm for 10 min at 4 °C, the pellet was resuspended in Hanks' balanced salt solution containing 1% Knox gelatin (HBSS-gel), pH 7.0-7.2. The preparation contained between 10^6 and 10^7 PMNs/ml. The cells were kept at room temperature during the course of the experiment and remained viable for 6 h after collection.

Fura-2 Loading—Fura-2 and its acetoxy-methyl ester, F2-AM, were obtained from Molecular Probes, Eugene, OR. PMNs were loaded with fura-2 by addition of 1 μM F2-AM to the cell suspension (range, 2-6 x 10^6 cells/ml) for 1 h in a 37 °C incubator, 90% O₂, 10% CO₂. After incubation, the cells were centrifuged at 1200 rpm for 10 min to remove extracellular dye. Cells were postincubated at 37 °C for at least 30 min. The intracellular fura-2 concentration was calculated according to the method of Williams and Fay (17). The cell volume was taken as 5.2 x 10^-13 liters/cell. Cell density was determined with a hemocytometer and ranged from 2 x 10^6 to 6 x 10^6 cells/ml.

Spectrofluorimetric Studies—Spectrofluorimetric experiments were conducted on a Perkin-Elmer MPF-3 spectrofluorimeter in the laboratory of Dr. David Wolf, Worcester Foundation for Experimental Biology, Shrewsbury, MA, or on a Spex CM-1 spectrofluorimeter operated in ratio mode with rhodamine B quantum coatings. Slit widths were 4 nm in the Perkin-Elmer spectrofluorometer and 11 nm in the Spex CM-1. Cells were maintained at 37 °C by a circulating water bath and continually stirred by a small magnetic stirrer to ensure rapid and complete dispersal of agonist. Fluorescence was corrected for volume dilution after addition of agonist. Since autofluorescence of PMNs  to fMLP, we investigated the ability of fura-2, the resting level of [Ca++]

Changes in [Ca++] following addition of fMLP (10^-9 M) to PMNs. At time 60 s, chemoattractant was added to the medium bathing the cells (x) (9 x 10^6 cells in 3 ml, 37 °C). An equivalent volume of HBSS-gel was added to control cells ( ). [Ca++] was calculated from the ratio of fluorescent intensities measured at 340- and 380-nm excitation (510-nm emission) using the standard procedure described under “Materials and Methods.” PMNs stimulated with fMLP showed a 2-fold increase in [Ca++] Control cells did not display an increase in [Ca++] during the same period.

RESULTS AND DISCUSSION

As a first step in using the new Ca++-sensitive dye fura-2 to determine subcellular changes in [Ca++] underlying the polarization of PMNs to fMLP, we investigated the ability of fura-2 to detect changes in [Ca++] in a population of PMNs in suspension. Cells were incubated with F2-AM as described under “Materials and Methods,” washed, and postincubated at room temperature for 30 min. The postincubation step was included with the assumption, later shown not to be valid, that it would facilitate complete cleavage of all acetoxy-methyl groups. PMNs loaded with fura-2 were then exposed to 10^-9 M fMLP, a concentration of chemoattractant known to yield maximal polarization (11). The time course of the [Ca++] change, calculated using the standard procedure for ratiometric [Ca++] measurements (1), is shown for a typical experiment in Fig. 1. The magnitude and the time course of the polarizing effect of fMLP (not shown) was similar to that reported by other investigators (13). In contrast, although increases in [Ca++] in response to fMLP were apparent from the change in cellular fluorescence of fura-2, the resting level of [Ca++], as well as the magnitude and duration of the Ca++ transient, were both depressed relative to values reported using the Ca++ indicator quin2 (14, 15). [Ca++] rose from 35 to 75 nM within 10 s of addition of fMLP and returned to prestimulus levels within 20 s; control cells maintained a basal [Ca++] of 50 nM. These differences were unexpected as the cellular Ca++ buffering directly added by fura-2 (125 μM) should be much less than that contributed by the millimolar intracellular concentrations of quin2 generally used (14, 15) and, as a consequence, less attenuation of Ca++ transients would be expected.

There are two possible explanations for the diminished Ca++ transient observed in Fig. 1. The first is that an assumption inherent in the use of fura-2 fluorescence to calculate [Ca++] is erroneous. Alternatively, the Ca++ transient might appear to be attenuated if the response to fMLP of individual cells within the suspension was asynchronous. Fluorescence measurements, using digital imaging microscopy, of single cells loaded with fura-2 suggested that the latter possibility is unlikely as the mean [Ca++] calculated in individual, nonpolarized cells (44.8 ± 2.5 nM, n = 54) and in cells polarized in response to a linear gradient of fMLP (85.0 ± 10.8 nM, n = 21) is similar to bulk measurements of nonpolarized and polarized cells in suspension.

It is necessary, therefore, to consider the possibility that, under these circumstances, in PMNs loaded with fura-2, at least one assumption underlying the traditional, ratiometric measurement of [Ca++] using dual excitation wavelengths (340 and 380 nm) is in error (see “Materials and Methods”). The central premise in the ratiometric method is that the fluorescence of the cell represents the weighted average of only two fluorescent species within the cell, Ca++-bound fura-2 (F2-AM) and Ca--insensitive fura-2.
Ca\textsuperscript{2+}-insensitive Form of Fura-2 in PMNs

Ca\textsuperscript{2+} and unbound fura-2 (F2), which possess properties identical to fura-2 free acid in solution (Fig. 2A). There were several observations made during the course of experimentation which raised questions regarding this assumption. We found that experimental values of the ratio of fluorescence at 340 and 380 nm (R) were occasionally less than R\textsubscript{min}, the predicted value for fura-2 in Ca\textsuperscript{2+}-free solution, and led to calculation of apparent negative [Ca\textsuperscript{2+}] values. Moreover, after addition of 5 \mu M ionomycin, cellular values of R increased but not to R\textsubscript{max}, the predicted value of fura-2 in Ca\textsuperscript{2+}-saturating solution. Since ionomycin is a potent and selective Ca\textsuperscript{2+} ionophore, the cellular Ca\textsuperscript{2+} gradient should be dissipated, allowing for the saturation of intracellular fura-2 with Ca\textsuperscript{2+}; however, R after ionomycin addition to cells in suspension was always considerably lower than R\textsubscript{max} (R = 5.06±45, n = 6; R\textsubscript{max} = 11.18±82, n = 6). Total cell lysis by Triton X-100 in the presence of 1.8 mM Ca\textsuperscript{2+} resulted in R values which still remained lower than R\textsubscript{max} (R = 5.11±0.98, n = 4; R\textsubscript{max} = 12.36±2.36, n = 4); however, values of R after Triton X-100 lysis were not significantly different from values obtained after ionomycin treatment, suggesting that ionomycin had effectively equilibrated intracellular and extracellular Ca\textsuperscript{2+}, thereby fully saturating fura-2 with Ca\textsuperscript{2+}. Furthermore, the similarity between R values obtained after ionomycin permeabilization and Triton X-100 lysis suggests that the intracellular environment has little effect on the behavior of cell-associated fura-2.

The differences between experimental and predicted R values for the Ca\textsuperscript{2+}-saturating condition indicate that PMNs incubated with F2-AM contain a fluorescent species other than fura-2 acid (F2 or F2-Ca\textsuperscript{2+}). The species may represent additional Ca\textsuperscript{2+}-sensitive forms with spectral characteristics that differ from those of free acid or, alternatively, F2-AM or a form of F2-AM that has been converted into a moiety that is also Ca\textsuperscript{2+}-insensitive. These possibilities were investigated by determining the spectral characteristics of the Ca\textsuperscript{2+}-sensitive species associated with PMNs. This process involved subtraction of the excitation spectrum of ionomycin-treated cells (Fig. 2B) obtained in the absence of Ca\textsuperscript{2+} from that in the presence of saturating Ca\textsuperscript{2+}. This difference spectrum for ionomycin-treated cells is compared to that of fura-2 free acid in solution (Fig. 2C). The two curves are nearly identical, suggesting that the only Ca\textsuperscript{2+}-sensitive species associated with PMNs is free acid. Although, the Ca\textsuperscript{2+}-sensitive spectra are extremely similar, the excitation spectra of cell-associated fura-2 and free acid in high and low Ca\textsuperscript{2+} environments are clearly different (see Fig. 2, A and B). In the presence of saturating Ca\textsuperscript{2+}, there is higher fluorescence at 380 nm associated with the ionomycin-treated cells than with free acid. The increased fluorescence intensity at 380 nm is consonant with a value of R (after ionomycin treatment) that is diminished in comparison with R\textsubscript{max}. Since the Ca\textsuperscript{2+}-difference spectra indicate that the Ca\textsuperscript{2+}-sensitive component associated with the PMNs appears to be spectrally identical to fura-2 free acid, the observed difference between the excitation spectra of ionomycin-treated cells and free acid must be due to an additional fluorescent component that is Ca\textsuperscript{2+}-insensitive.

The spectral characteristics of the Ca\textsuperscript{2+}-insensitive form of fura-2 were dissected from the spectra of ionomycin-treated cells obtained in the presence of Ca\textsuperscript{2+}-saturating and Ca\textsuperscript{2+}-free solutions (see Fig. 2B). Ionomycin treatment of cells in the presence of 1.8 mM Ca\textsuperscript{2+} results in the dissociation of F2-Ca\textsuperscript{2+} to F2; F2-INS is the Ca\textsuperscript{2+}-sensitive form of fura-2: F2-Ca\textsuperscript{2+} and the fluorescent yet Ca\textsuperscript{2+}-insensitive species, F2-INS. The addition of 3.6 mM EGTA (pH 8.5) results in the dissociation of F2-Ca\textsuperscript{2+} to F2; F2-INS is the Ca\textsuperscript{2+}-sensitive form of fura-2 free acid, the observed difference between the excitation spectra of ionomycin-treated cells and free acid must be due to an additional fluorescent component that is Ca\textsuperscript{2+}-insensitive.

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The value of \( z \) is determined from the fluorescences of a known amount of free acid in solution, in \( \text{Ca}^{2+} \)-free and \( \text{Ca}^{2+} \)-saturating buffers.

The spectral characteristics of the \( \text{Ca}^{2+} \)-insensitive species are plotted in Fig. 3A, along with the excitation spectrum of \( \text{F}2\text{-AM} \), the starting material used to load the PMNs. It is clear that the \( \text{Ca}^{2+} \)-insensitive fluorescence trapped by the cells is not \( \text{F}2\text{-AM} \) but rather a form of fura-2 which is more fluorescent than \( \text{F}2\text{-AM} \). Furthermore, the \( \text{Ca}^{2+} \)-insensitive species associated with the cells exhibits a broad excitation spectrum from 300 to 430 nm, clearly different from \( \text{F}2\text{-AM} \) whose spectral characteristics exhibit a peak at 380 nm with relatively little contribution below 340 nm. We considered the possibility that the intracellular environment changes the spectral characteristics of \( \text{F}2\text{-AM} \), leading to the spectrum displayed by the unknown component in Fig. 3A, but found that environmental effects were not the cause of the observed spectrum. Specifically, addition of \( \text{F}2\text{-AM} \) to unloaded cells treated with ionomycin resulted in spectral characteristics displayed by the unknown component in Fig. 3A, but found that environmental effects were not the cause of the observed spectrum. Specifically, addition of \( \text{F}2\text{-AM} \) to unloaded cells treated with ionomycin resulted in spectral characteristics displayed by the unknown component in Fig. 3A, but found that environmental effects were not the cause of the observed spectrum. Specifically, addition of \( \text{F}2\text{-AM} \) to unloaded cells treated with ionomycin resulted in spectral characteristics displayed by the unknown component in Fig. 3A, but found that environmental effects were not the cause of the observed spectrum. Specifically, addition of \( \text{F}2\text{-AM} \) to unloaded cells treated with ionomycin resulted in spectral characteristics displayed by the unknown component in Fig. 3A, but found that environmental effects were not the cause of the observed spectrum. Specifically, addition of \( \text{F}2\text{-AM} \) to unloaded cells treated with ionomycin resulted in spectral characteristics displayed by the unknown component in Fig. 3A, but found that environmental effects were not the cause of the observed spectrum.

In Fig. 3B, the spectral characteristics of the \( \text{Ca}^{2+} \)-insensitive species of cells from another experiment, loaded identically to those exhibited in Fig. 3A, are shown. The \( \text{Ca}^{2+} \)-difference spectra after ionomycin treatment (not shown) indicate that the \( \text{Ca}^{2+} \)-sensitive species is identical to fura-2 free acid. The \( \text{Ca}^{2+} \)-insensitive species in this experiment is different from that illustrated in Fig. 3A as well as from \( \text{F}2\text{-AM} \). In this experiment, the unknown species associated with the cells exhibits at least two peaks of fluorescence: one at 380 nm and the other at 430 nm. The difference between the spectral characteristics of the unknown species illustrated in Fig. 3, A and B suggests that, although in both cases the unknown species is not \( \text{F}2\text{-AM} \), the spectral characteristics of the \( \text{Ca}^{2+} \)-insensitive moiety varies among PMNs from different donors in spite of the fact that the incubation conditions are identical in all experiments.

We believe that the \( \text{Ca}^{2+} \)-insensitive species associated with PMNs is incompletely deesterified \( \text{F}2\text{-AM} \). We base this hypothesis on the results of the \textit{in vitro} hydrolysis of \( \text{F}2\text{-AM} \) by bovine esterase. The intermediates of this hydrolysis show progressive increases in fluorescence intensity which precede the acquisition of \( \text{Ca}^{2+} \) sensitivity. \( \text{Ca}^{2+} \)-difference spectra, obtained at various times during the hydrolysis, indicate that the \( \text{Ca}^{2+} \)-sensitive species is always identical to fura-2 and that the absolute concentration of this species appears to increase progressively with time. In addition, spectra of the \( \text{Ca}^{2+} \)-insensitive intermediates, at the same time points, exhibit maxima at 400 nm, generally similar to the \textit{in vitro} situation shown in Fig. 3B.

In our experiments, there appear to be at least two causes for incomplete deesterification of \( \text{F}2\text{-AM} \). In many cases, the problem is specific to cell type. In quiescent BALB/c 3T3 cells, no significant deesterification takes place, whereas, in PMNs, deesterification does not always continue to completion. We suggest that quiescent BALB/c 3T3 cells do not possess the esterase specific for the intracellular deesterification of \( \text{F}2\text{-AM} \) and that, in PMNs, \( \text{F}2\text{-AM} \) may not remain in contact with intracellular esterase for a sufficient period of time to ensure complete deesterification. In this situation, the cell-associated fura-2 moieties, containing from one to four acetoxy-methyl esters on the \( \text{Ca}^{2+} \)-binding portion of the molecule, would not be expected to be \( \text{Ca}^{2+} \)-sensitive. Incomplete deesterification was also occasionally apparent in normal muscle cells, a cell type in which deesterification generally proceeds to completion. The source of \( \text{Ca}^{2+} \)-insensitive fluorescence in smooth muscle cells was found to depend on the lot of \( \text{F}2\text{-AM} \) used, and the problem could be circumvented by switching to a new lot of dye, suggesting that at times the inability of cells to fully deesterify "\( \text{F}2\text{-AM} \)" may have been due to impurities in the dye.

In any situation in which there is a form of fura-2 associated with cells in addition to \( \text{F}2\text{-AM} \) and \( \text{F}2\text{-AM} \), direct application of the standard procedure for \( \text{Ca}^{2+} \) measurement, which includes the parameters, \( R_{\text{max}} \), \( R_{\text{min}} \), and \( \beta \) for the free acid, leads to erroneous results. We have developed a method to account for \( \text{Ca}^{2+} \)-insensitive fluorescence and to accurately measure \( [\text{Ca}^{2+}] \) within PMNs. This method is applicable to any cell type which exhibits intracellular, \( \text{Ca}^{2+} \)-insensitive fluorescence. The method requires treatment of cells with ionomycin, in the presence of \( \text{Ca}^{2+} \)-free and \( \text{Ca}^{2+} \)-saturating buffers, to generate \( R_{\text{max}} \) and \( R_{\text{min}} \) which reflect the cell-associated, minimum and maximum values of \( R \), and \( \beta' \). If \( R_{\text{max}} \), \( R_{\text{min}} \), and \( \beta' \) are then used in the standard equation to measure cellular \( [\text{Ca}^{2+}] \), an assumption in the method is that the concentration of ionomycin used is sufficient to equilibrate intracellular and extracellular \( [\text{Ca}^{2+}] \). Equilibration is attained when the value of \( R \) is unchanged by further ionomycin addition, working near the \( K_c \) for fura-2.

We verified the use of this modified method by determining the spectral characteristics of the \( \text{Ca}^{2+} \)-insensitive fluorescence associated with PMNs, as described earlier, and subtracting them from the total cell fluorescence (measured prior to treatment with ionomycin) at selected wavelengths (i.e. 340
and 380 nm. The resultant Ca\(^{2+}\)-sensitive fluoroences at these wavelengths can then be used according to the standard procedure of [Ca\(^{2+}\)] calculation \(1\), as described under “Materials and Methods.” The results of either method are identical. An advantage to the second method is that it assesses the forms of fura-2 fluorescence associated with the cell and allows for use of the appropriate procedure for calculating [Ca\(^{2+}\)].

Using \(R'_{\text{min}}, R'_{\text{max}}\), and \(\beta'\) in place of \(R_{\text{min}}, R_{\text{max}},\) and \(\beta\), we have recalculated [Ca\(^{2+}\)] measurements from the experiment described earlier (Fig. 1). The results are plotted in Fig. 4 and now indicate significant changes in [Ca\(^{2+}\)] upon stimulation with fMLP. Within 12 s of fMLP stimulation, [Ca\(^{2+}\)] increased severalfold and then fell to a level higher than the resting level for the remainder of the experiment. The time course of Ca\(^{2+}\) change is representative of more than 20 experiments using 10\(^{-9}\) M fMLP; the results are independent of the amount of Ca\(^{2+}\)-insensitive fura-2 within PMNs.

The most striking aspect of the work described here is the pitfall of making assumptions about the intracellular behavior of fura-2, based solely on theoretical grounds. In order to use fura-2 in any cell type, and with each new lot of F2-AM, the simplest way to test for cell-associated Ca\(^{2+}\)-insensitive fluorescence is by an in situ calibration of cell-associated dye with ionemycin, in the presence of Ca\(^{2+}\)-saturating and Ca\(^{2+}\)-free buffers. If these \(R\) values do not match those of free acid in Ca\(^{2+}\)-saturating and Ca\(^{2+}\)-free buffer, then the assumption of only two intracellular forms of the dye, F2-Ca\(^{2+}\) and F2, is not valid and the use of the standard procedure for [Ca\(^{2+}\)] measurement, using \(R_{\text{max}}\) and \(R_{\text{min}}\), is not possible. Having identified the nature of the Ca\(^{2+}\) insensitivity in PMNs loaded with fura-2, and with methods for valid [Ca\(^{2+}\)] measurement, we are now able to use fura-2 as a Ca\(^{2+}\) indicator in PMNs. The results of experiments on changes in [Ca\(^{2+}\)] after fMLP stimulation will be described in a future paper.

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