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Spectral Evidence for Non-Calcium Interactions of Intracellular Indo-1

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Indo-1 is widely used to measure intracellular free calcium, $[Ca^{2+}]_i$, by comparing the fluorescence emission at 2 or more wavelengths with the emissions, which are assumed to be known, of Indo-1 when it is fully calcium-bound and when it is fully calcium-free. Accurate quantitation requires that these "reference" values be obtained on *intra*cellular dye, and the full spectra of this study show that the reason is a significant spectral shift of the calcium-free peak, but not the calcium-bound. A mathematical analysis shows that the new peak must be a new state of the Indo-1 molecule, since it cannot be simply due to residual calcium in the cell. When intracellular "reference" spectra were used in the data analysis, $[Ca^{2+}]_i$ could be calculated from whole spectra or from the ratio of observations at two wavelengths with good agreement. When extracellular "reference" spectra were used, the value calculated by the ratio method depended on the choice of wavelengths.

Fluorescent probes which bind calcium report changes in intracellular free calcium activity, $[Ca^{2+}]_i$, with rapid response times and minimal perturbation of the cell (1). Indo-1, which exhibits a spectral shift in its emission spectrum upon binding calcium (2), is a dye which has been widely used in numerous cell types including the B lymphocyte (3-6). In principle, the sample spectrum, at least at the wavelengths that are being monitored, must be compared with spectra of the bound and free forms of the dye. For the purpose of the analysis, the latter are assumed to be known. From this one infers the ratio of the concentrations of calcium-bound and calcium-free dye. Multiplying this ratio by the dissociation constant for calcium then generates a value for $[Ca^{2+}]$.

Often, measurements are made at just two wavelengths, and their ratio is used (2) in the calculation of $[Ca^{2+}]$. This determination is independent of dye concentration, but the limited amount of data do not allow any check of the assumptions that go into the calculation. In order to have more information, we have taken whole spectra of Indo-1, both inside and outside cells. The analysis then made use of all available spectral data from the dye. It was then checked by calculating a predicted spectrum and comparing it with the experimental. The predicted spectrum was generated by taking the assumed spectra for the bound and free dye, with height adjustments proportional to the respective concentrations, and then adding them together.

Comparisons of spectra showed that Indo-1 inside the lymphocyte was not in the same state as pentapotassium Indo-1 in cell-free buffer. Specifically, the difference was seen in the spectrum of the calcium-free form of the dye. The analysis showed that the shifted spectral peak was not due to residual intracellular calcium. Taking this shift into account significantly affected the calculation of $[Ca^{2+}]_i$ from spectral data.

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MATERIALS AND METHODS

Reagents: Indo-1, its acetoxymethylester Indo-am, and the detergent Pluronic F-127 (BASF) were obtained from Molecular Probes (Eugene OR). Ionomycin was obtained from Calbiochem. Unless otherwise noted, cells were suspended in Hanks Balanced Salt Solution (HBSS), buffered with 10 mM Hepes (pH 7.2) but without phenol red. Additions of Ca^{2+}, Mg^{2+}, and EGTA varied as specified.

Indo-1 Loading in Cells: BALB/c mouse spleen cells were isolated, red cells were lysed, and the sample was passed through Sephadex G-10 to remove adherent cells. Indo-am (20 μg) was dissolved in 5 μL of DMSO and 5 μL of a 12.5% (w/w in DMSO) solution of Pluronic F-127 was added (7-10), followed by 75 μL fetal bovine serum. This mixture was then added to the cell suspension (typically 4 ml at $20x10^6$ cells/ml in phosphate-saline with 10% serum) and incubated at room temperature for 7 minutes. After washing, cells were incubated 15 min. at 37°C (in similar medium) to ensure complete intracellular hydrolysis of Indo-am. Cells were then washed and stored on ice at 4.2×10^7/ml in HBSS, with 1.3 mM $CaCl_2$, 0.9 mM $MgCl_2$, 0.1% glucose (w/v) and fortified with 10% fetal bovine serum. An aliquot of cells which received no dye was stored under the same conditions for use in obtaining background signals in the fluorometer. Cell viability was better than 90%.

Fluorescence Spectroscopy: Fluorescence spectra were obtained in a Perkin-Elmer 650-10S scanning spectrofluorometer with cuvette at 35-37°C and an excitation wavelength of 330 nm, with 5 nm slits on both monochromators. Cells (4.2×10^6 cells in 1 ml) were maintained in suspension by frequent gentle shaking. The background from cell light scattering and autofluorescence, Raman scattering and buffer fluorescence was measured with unlabeled cells, and was usually less than 10% of the peak fluorescence of the corresponding labeled cells. For spectra with intracellular dye fully chelated, ionomycin was added (12.5 μM final concentration). Under the conditions in the cuvette (1% serum and 37°C), this was shown to be a saturating addition of ionophore. For all spectra, equivalent baselines were measured with the appropriate combinations of medium, or medium + cells which contained no dye, and these were subtracted from the measured spectra.

Spectral Analysis: The lab computer program (9) started with an experimental spectrum, $y_e(\lambda)$, where λ = wavelength, and two "reference" spectra, $y_b(\lambda)$ and $y_f(\lambda)$ which were assumed to represent bound and free dye, respectively. It then found the weighting factors W_b and W_f which made a linear combination of the form $W_b y_b(\lambda) + W_f y_f(\lambda)$ the best approximation to $y_e(\lambda)$, by the criterion of least squares.

RESULTS

Spectra of Intracellular and Extracellular Indo-1: The intracellular spectrum of calcium-bound Indo-1 was obtained by addition of ionomycin to cells suspended in medium containing 1 mM calcium ion. The extracellular Indo-1 spectrum was obtained from the pentapotassium salt of Indo-1 in "cytosol-like" buffer (2). These curves coincided extremely well, as can be seen in Fig. 1A. The agreement was not due to extensive leakage of Indo-1 from cells into the calcium-containing medium. A sample was centrifuged and resuspended and the supernatant contained only a minor fraction of the dye (depending on the time elapsed at 37° in the cuvette). The spectral shapes of the leaked dye and the dye in the resuspended cells (data not shown) were identical to Fig. 1A.

This spectral agreement showed that our system was free of two types of calcium-independent artifacts. The first was the presence of non-binding forms of the Indo-1 dye, such as unhydrolyzed acetoxymethyl ester (9-11) or photobleached dye (12). The second was the presence of background signals from light scattering, autofluorescence, etc. The latter were eliminated by subtracting appropriate baseline spectra of buffer, or medium with an equivalent number of cells which were not loaded with dye.

Calcium-free spectra of Indo-1, on the other hand, differed significantly between extracellular and intracellular dye. As can be seen in Fig. 1B, pentapotassium Indo-1 in EGTA-containing medium peaked at about 470 nm, whereas intracellular Indo-1 in calcium-depleted cells peaked at 450 nm. The peak at 450 nm was not an artifact of the EGTA treatment, since it was also visible in normal resting cells, as will be discussed below.

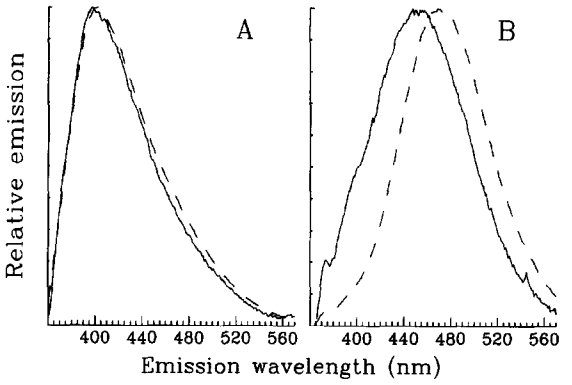

Figure 1
Emission spectra of intracellular Indo-1 (solid curves) or extracellular pentapotassium Indo-1 (dashed curves), normalized to the same height for comparison. Panel A: Calcium-bound Indo-1 in medium with 1.3 mM [Ca^{2+}], and ionomycin, in the case of the cell sample. Panel B: Essentially calcium-free Indo-1. Cells were suspended for 3-10 minutes in medium containing 20 mM EGTA, and free dye was in buffer containing approximately 1 mM EGTA and no added calcium or magnesium.

Spectral Analysis: A mathematical analysis was performed to test if Indo-1 in calcium-depleted cells was in the same chemical states (determined spectroscopically), as those of the dye in cell-free buffer. If it were, then the extracellular spectra for calcium-bound and -free Indo-1 would be appropriate representations of the dye inside the cell, and there would be some combination of these extracellular spectra which would provide a good fit to the intracellular curve. If this were the case, then the explanation of the shifted intracellular spectrum of Fig. 1B would be the presence of residual calcium in the cells.

However, the best approximation to the intracellular curve that was possible with a combination of the extracellular calcium-bound and -free spectra was the very poor fit in the dotted line of Fig. 2. Since the best fit was unacceptable, there was no combination of the calcium-bound and -free extracellular spectra that satisfactorily matched the spectrum of the intracellular dye. The best fit was so poor because the extracellular spectra peaked too far apart relative to their width. This had the result that composites showed distinct peaks, instead of merging in a way that would have allowed them to mimic the single peak of the observed intracellular spectrum.

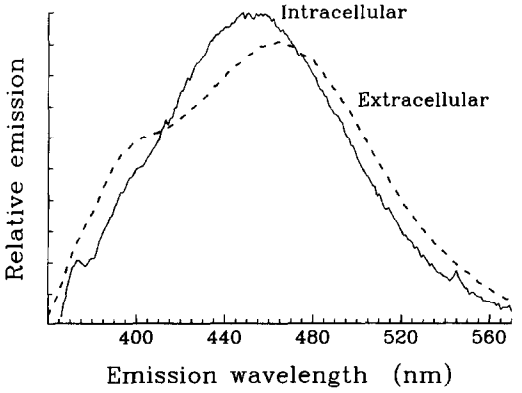

Figure 2
Intracellular Indo-1 in cells depleted of Ca, by incubation at 37°C in medium containing 20 mM EGTA. The dotted line shows the nearest computer fit (9) that can be found in an attempt to approximate the spectrum with a combination of the extracellular calcium-bound and -free Indo-1 spectra.

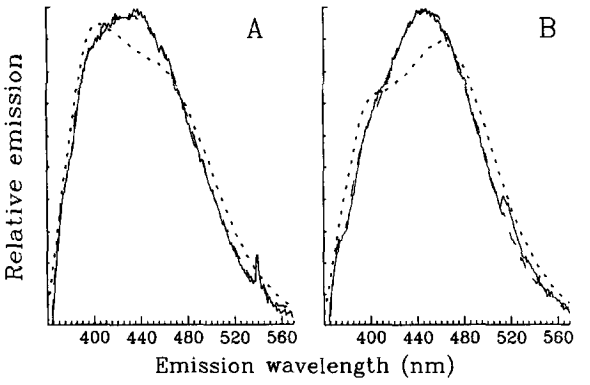

Figure 3

Spectra of intracellular Indo-1 in resting lymphocytes. The best fit to the observed spectrum using intracellular "reference" spectra for calcium-bound and -unbound dye is shown as a dashed curve, which fits the solid data curve too well to be visible over most of the spectrum. The best fit using extracellular "reference" spectra is shown as a dotted curve which deviates clearly from the experimental curve. Panel A shows lymphocytes at 37°C in medium containing 1 mM free calcium. In panel B the medium contained no added calcium and 1 mM EGTA. No corrections have been made for the leakage of a small portion of the dye from the cells.

Spectra of Indo-1 in resting cells. The same analysis was applied to lymphocytes resting in medium containing 1 mM calcium or 1 mM EGTA, as shown in Fig. 3. Neither spectrum could be accounted for by a combination of calcium-bound and -free extracellular Indo-1 spectra. The best approximations based on extracellular spectra (dotted curves) exhibited distinct peaks, just as were seen in Fig. 2.

On the other hand, if one took the intracellular curve of Fig. 1B as the "reference" spectrum for calcium-free Indo-1 in resting lymphocytes, the computer could produce composites of intracellular spectra which were excellent fits to the resting-cell curves (dashed curves). This indicates that the spectral shift seen in Fig. 1B is not just an artifact of the EGTA treatment of the cells but correctly represents the state of intracellular Indo-1 that is not bound to calcium in normal resting cells.

Not only is the shifted intracellular curve of Fig. 1B *necessary* to explain experimental curves in resting lymphocytes, but the close agreement of calculated composites and experimental curves indicates that the two intracellular spectra of Fig. 1 are *sufficient*. Thus, there is no

Table 1

		Apparent[a] $[Ca^{2+}]_i$		
Cells	Reference data[b]	Curve-fitting[c]	400-490 Ratio[d]	400-440 Ratio[e]
Fig. 3A	Intracellular	112 nM	113 nM	98 nM
	Extracellular	220 nM	226 nM	114 nM
Fig. 3B	Intracellular	28 nM	31 nM	26 nM
	Extracellular	103 nM	109 nM	55 nM

[a] Calcium concentration obtained assuming a dissociation constant of 250 nM (2).
[b] Source for fluorescence data for fully-bound and fully-free dye (shown in Fig. 1).
[c] Calculation by curve fitting program (9).
[d] Calculation (2) by ratio of readings at only 2 wavelengths: 400 nm and 490 nm.
[e] Calculation (2) by ratio of readings at only 2 wavelengths: 400 nm and 440 nm.

evidence that Indo-1 in any state other than in the two seen in the solid curves of Fig. 1, contributes to the cell fluorescence. In particular, there is no evidence for any of the dye being in the same chemical state as occurs extracellularly (the dotted curve of Fig. 1B).

Calculation of $[Ca^{2+}]$. Table 1 compares the values of $[Ca^{2+}]_i$ which were calculated from the fluorescence of two selected samples (Figs. 3A and 3B). Either the intracellular or the extracellular spectra of Fig. 1 were used as the "reference" data which were assumed to describe the fully bound and free dye. For either choice the analysis of the percentage of dye bound to calcium was carried out by 3 methods: the curve fitting program (9), or the 2-wavelength ratio method (2) with two different choices for the wavelength pair. When the intracellular "reference" data were used, the calculated values for $[Ca^{2+}]_i$ were essentially unaffected by the method of calculation. When extracellular "reference" data were used, the calculated values varied by nearly a factor of 2. Furthermore, calculations with the ratio method were dependent on the wavelengths chosen.

DISCUSSION

The use of intracellular Indo-1 (or Fura-2) to measure intracellular calcium activity presupposes knowledge of the appropriate "reference" spectra of the dye in its fully calcium-bound and calcium-free states. With this knowledge, the ratio of bound to free dye can be inferred from experimental measurements at two wavelengths (2), or over the entire spectrum (9). A composite spectrum using the correct calcium-bound and -free "reference" spectra in the same proportion should then agree in detail with the experimental spectrum.

However, in agreement with reports from other laboratories (2, 13-16), we find that calcium-bound and -free spectra which are measured in a cell-free environment can disagree with the corresponding data from cells. As a specific example, Rabinovitch *et al*. (13) reported that calcium-depleted cells showed significantly more fluorescence in the 400-440 nm range than did cell-free dye in EGTA buffer. These authors (13) naturally attributed this to residual calcium in the cells, but our mathematical analysis of the spectra indicates otherwise. The curve cannot be represented by any combination of calcium-bound and -free extracellular Indo-1 spectra.

We conclude that the importance of using intracellular "reference" spectral data for calcium-bound and -free dye arises because the dye is in a spectrally distinct state inside the lymphocyte, and not because of background fluorescence, unhydrolyzed dye, or other artifacts. It is unclear, as yet, what component of the intracellular milieu could be interacting with Indo-1 so as to produce the observed spectral shift without interfering in any detectable way with chelation of calcium. Further studies, which are still in progress, have given a preliminary indication that the apparent dissociation constant for calcium is essentially unchanged by the environment in the cell. This would eliminate the possibility that a competing divalent cation such as Zn^{2+} could have bound most of the calcium-free dye.

Popov *et al*. (14) showed variations of Indo-1 spectra with polarity of solvent and suggested that cytosolic polarity might account for observed spectral shifts. Solvent polarity appeared to affect their spectra in both the calcium-bound and -free forms of the dye, however. This is in contradistinction to the intracellular effect reported here which was seen in the calcium-free state only.

Two corrections may be mentioned which could be applied to the analysis above but would not be expected to alter the main conclusions of this paper. These are the facts that (1) a small

percentage of the dye in the samples shown in Fig. 3 would have been outside the cells due to slow leakage while the cells were stored on ice, and (2) the intracellular-dye curve in Fig. 1B, which was taken to be the free-dye standard in the analysis, still contains a slight shoulder at 400 nm which might be due to residual intracellular free calcium. The leakage would have artifactually raised the observed calcium concentration in Fig. 3A (cells in 1 mM calcium ion) and lowered it in the case of Fig. 3B. If the curve which was used as the free-dye curve actually contained a trace contribution from calcium-bound dye, this would have artifactually lowered the calculated calcium ion concentration for all samples analyzed with the intracellular standard.

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