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I. Quantitation of Cytosolic [Ca^{2+}] in Whole Perfused Rat Hearts Using Indo-1 Fluorometry

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ABSTRACT Fluorometric determination of cytosolic calcium, $[Ca^{2+}]_c$, using Indo-1 in intact tissue, is limited by problems in obtaining calibration parameters for Indo-1 in vivo. Therefore, the goal of this study was to calibrate Indo-1 using in vitro constants, obtained from protein-containing reference solutions designed to produce similar Indo-1 spectral properties to those in vivo. Due to wavelength-dependent tissue light absorbance, the in vitro constants had to be absorbance-corrected using a novel method. The correction factor was calculated from the relationship between the Indo-1 fluorescence intensities at the two detection wavelengths. A mixture of proteins at ~28 mg/ml had a similar Indo-1 isosbestic wavelength (430 nm) to that found in vivo (427 nm), and a similar fluorescence ratio maximum with saturating Ca^{2+} to that found in vivo (after absorbance correction). Using calibration constants from this protein mixture, calculated $[Ca^{2+}]_c$ in a Langendorf perfused rat heart was 187 nM during diastole, and 464 nM in systole. This new calibration method circumvented the considerable experimental problems of previous methods which required measurements with the cytosol fully depleted and fully saturated with Ca^{2+}.

GLOSSARY

Summary of abbreviations

Miscellaneous

λ	detection wavelength
λ_{iso}	Indo-1 isosbestic wavelength
δ_λ	fluorescence amplitude of Ca^{2+} dependent Indo-1 transients
g_λ	gain factor; depends on instrumental gain and tissue light absorption at wavelength λ
K_d	Indo-1 dissociation constant for calcium

Concentration etc.

$[Ca^{2+}]_c$	cytosolic calcium concentration
$[Ca^{2+}]_{dia}$	cytosolic calcium concentration during diastole
$[Ca^{2+}]_{sys}$	cytosolic calcium concentration during systole
n(Indo)	amount of cytosolic Indo-1 (e.g. units of moles)

Intensities

B_λ	sum of various background fluorescence intensities
I_λ	"standard" Indo-1 intensity from cytosolic Indo-1 (per moles of Indo-1 and gain unit (Grynkiewicz et al., 1985))
I_λ^{max}	"standard" Indo-1 intensity with saturating $[Ca^{2+}]$ (per moles of Indo-1 and gain unit (Grynkiewicz et al., 1985))
I_λ^{min}	"standard" Indo-1 intensity in the absence of Ca^{2+} (per moles of Indo-1 and gain unit (Grynkiewicz et al., 1985))
F_λ	detected heart fluorescence intensity (from Indo-1 and background; related to I_λ by Eq. 2)
F'^{min}_λ	detected Indo-1 fluorescence intensity, after background subtraction: absence of Ca^{2+}
F'^{max}_λ	detected Indo-1 fluorescence intensity, after background subtraction: saturating $[Ca^{2+}]$

Fluorescence ratios

R	ratio of detected Indo-1 fluorescence intensities (after background subtraction)
R^{min}	ratio of detected Indo-1 intensities (after background subtraction): absence of Ca^{2+}
R^{max}	ratio of detected Indo-1 intensities (after background subtraction): saturating $[Ca^{2+}]$

Calibration constants

S_λ	calibration constant: ratio between Indo-1 fluorescence intensities at a single wavelength (Eq. 6)
S_R	reference ratio (calculated from calibration constants S_λ; see Eq. 7c)
bH	gain-adjuster: slope of relationship F_{456} vs. F_{385} (used to correct for inner filter effects)

INTRODUCTION

Reliable measurements of cytosolic Ca^{2+}, $[Ca^{2+}]_c$, are necessary to understand the control of cardiac contractility. Previous studies have used calcium indicators such as the bioluminescent indicator Aequorin (Kihara et al., 1989), the NMR indicator ^{5}F-BAPTA (Marban et al., 1990), and more recently the fluorescent indicators Fura-2 and Indo-1. The fluorescent indicators have the advantage of emitting bright light even at relatively low concentrations. This allows minimal loading of the indicator, thus minimizing interaction

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with myocyte function. Furthermore, both Fura-2 and Indo-1 shift their spectral characteristics upon calcium binding, and can therefore be used to calculate a fluorescence ratio between intensities at two wavelengths (Grynkiewicz et al., 1985). This has the advantage that the ratio is insensitive to factors which proportionally affect both intensities identically such as indicator concentration and motion artifacts (to a first approximation (Brandes et al., 1992)). The ratio can subsequently be used, in combination with appropriate calibration parameters, to calculate $[Ca^{2+}]_c$.

The relationship between the fluorescence ratio of cytosolic Indo-1, R, and $[Ca^{2+}]_c$ is ideally given by (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_c = K_d \cdot S_2 \cdot \{(R - R^{min})/(R^{max} - R)\},$$

where K_d is the indicator dissociation constant for Ca^{2+}, S_2 is the ratio between the fluorescence intensities, measured at the longer of the two detection wavelengths, in the absence of Ca^{2+} and in the presence of saturating $[Ca^{2+}]_c$, and R^{min} and R^{max} are the fluorescence ratios in the absence of Ca^{2+} and in the presence of saturating $[Ca^{2+}]_c$, respectively. Two different methods have previously been used to determine these four calibration parameters. The first in vivo method relies on obtaining in vivo measurements of R^{min} and R^{max} as well as titration of $[Ca^{2+}]_c$ to obtain K_d. It is, however, difficult to equilibrate $[Ca^{2+}]_c$ with low extracellular $[Ca^{2+}]$ using only ionophores (Poenie, 1990; Ikenouchi et al., 1991), especially in intact tissue. Therefore, a second in vitro method relies on obtaining representative values of K_d, S_2, R^{min}, and R^{max} from an in vitro reference solution where $[Ca^{2+}]$ can easily be controlled. There are, however, two major factors which could cause these parameters to differ in vivo and in vitro. First, the in vivo interaction between intracellular proteins and indicator may alter the fluorescence intensities differently at different wavelengths, causing S_2, R^{min}, and R^{max} to be different from the reference solution (Owen and Shuler, 1989; Ikenouchi et al., 1991; Poenie, 1990; Hove-Madsen and Bers, 1992; Konishi et al., 1988). Furthermore, proteins are also known to interact with the indicator-Ca^{2+} equilibrium, possibly altering K_d in vivo from that found in aqueous solutions in vitro (Hove-Madsen and Bers, 1992; Konishi et al., 1988). Second, the tissue light absorption, "inner filter," varies as a function of detection wavelength in the heart, causing different R^{min} and R^{max} in vivo compared to the values in vitro where the filter effect is absent (Koretsky et al., 1987).

The goal of this study was to calibrate $[Ca^{2+}]_c$ from in vivo fluorescence measurements of Indo-1 in perfused rat hearts. Because of difficulties of equilibrating $[Ca^{2+}]_c$ with extracellular Ca^{2+} in an intact heart, we used the second in vitro calibration approach in which reference solutions were created by adding various proteins to aqueous buffered solutions. The suitability of the references were verified by comparing their spectral characteristics with those in vivo. The expected in vivo values of R^{max} and R^{min} were then calculated by combining the in vitro calibration constants with a correction constant which was used to correct for heart light absorption. A novel method was developed to determine this correction constant using the relationship between Indo-1 fluorescence intensities detected at two emission wavelengths.

The Indo-1 dissociation constant, K_d, was not determined. Using single myocytes and Indo-1, K_d ~250 nM (similar to that in solution) has been reported (Spurgeon et al., 1990; Ikenouchi et al., 1991). In contrast, using solutions with soluble myocyte proteins, K_d ~1000 nM (Hove-Madsen and Bers, 1992) was reported. Despite this uncertainty in the indicator K_d, an important result of calibration is to "linearize" the $[Ca^{2+}]_c$ versus ratio relationship, and this is accomplished regardless of the exact value of K_d since $[Ca^{2+}]_c$ is directly proportional to K_d. Because most investigators have used $K_d = 250$ nM (Wikman-Coffelt et al., 1991; Sollot et al., 1992; Mohabir et al., 1991; Ikenouchi et al., 1991; Cleeman and Morad, 1991) it was also used here in order to facilitate comparison of calculated $[Ca^{2+}]_c$.

MATERIALS AND METHODS

Preparation of reference solutions for determination of calibration constants

In order to calibrate Indo-1 fluorescence transients (see below), calibration constants were determined from the following reference solutions.

Preparation and use of albumin and protein reference

Albumin (500 mg/ml) and a protein mixture (500 mg/ml) containing aldolase (46%), creatine kinase (33%), and glyceraldehyde dehydrogenase (21%) (Konishi et al., 1988) were separately dialyzed against 10 mM PIPES buffer for ~16 h. Dialyzed albumin or protein mixture was used to prepare stock solutions (110 mM KCl, 10 mM PIPES, pH 7.04). Equal amounts of either albumin or the protein mixture stock solutions were added to three different cuvettes with buffer (10 mM pipes, 110 mM KCl, pH 7.04). Two of these cuvettes contained Indo-1 (~16 μM) and 11 mM $CaCl_2$ or 6.8 mM EGTA, respectively. Emission spectra (see below) were acquired from all three cuvettes and corrected spectra were obtained after subtraction of the spectrum from the Indo-1 free ("blank") cuvette.

Preparation and use of homogenate reference

Hearts (approximately 1.5 g wet weight) were first perfused with standard perfusate (see below) for ~20 min to remove blood. Four hearts were immersed in 15 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 5 mM) buffer with 20 mM NaCl and 115 mM KCl and homogenized using a Polytron blender (Brinkman Instruments, Ontario, Canada). The soluble protein fraction was collected following centrifugation (ending with ultracentrifugation at 100,000 g for 1 h). All liquid was retained throughout, and the final concentration of soluble protein therefore corresponded to that obtained from 0.4 g of tissue/ml added solution. Indo-1 was added to the homogenate and the solution was divided into two parts containing 75 μM Indo-1 and 20 mM $CaCl_2$ or 20 mM EGTA, respectively (solutions were adjusted to pH 7.1). Emission spectra were obtained for both solutions as well as for a "blank," containing only homogenate.

Heart perfusion methods

Male Sprague-Dawley rats (450–550 g), $n = 24$, were anticoagulated using heparin (1 ml intraperitoneally) and anesthetized using ketamine (1 ml intraperitoneally). Hearts were then excised and immediately arrested in cold

isosmotic saline containing 20 mM KCl. Isolated hearts were perfused retrograde via the aorta at a control pressure of 71 mm Hg by the isovolumic Langendorf method. A perfusion apparatus was constructed using water-jacketed glassware (Radnotti Glass, Monrovia, CA). The standard Krebs-Heinseleit perfusate contained (in millimolar): NaCl (118), KCl (6.0), $CaCl_2$ (2.5), $MgSO_4$ (1.2), Na_2EDTA (0.5), dextrose (4.0), $NaHCO_3$ (25.0), pyruvate (10.0), and insulin (20 units/liter), and was equilibrated at 37°C with a 95% O_2/5% CO_2 gas mixture. A compliant latex balloon, attached to a Trantec pressure transducer (American Edwards Laboratories, Irvine, CA) via rigid polyethylene tubing, was inserted into the left ventricle to measure pressure. Left ventricular diastolic pressure was set to approximately 10 mm Hg by adjusting the balloon volume. Hearts were paced at 300 beats/min using two platinum-tipped electrodes connected to a Grass Instruments (Quincy, OR) SD-5 stimulus generator. Developed pressure was recorded on a Gould (Cleveland, OH) series 8000 chart recorder and also digitized and stored in an IBM personal computer (via the SLM 48000S digitizer, see below).

Indo-1 loading into hearts

Hearts were perfused for 15 min using the standard perfusate, before loading for 20–30 min with 150 ml of recirculating standard perfusate containing 5 μM Indo-1/AM (cell permeable ester form of Indo-1; Molecular Probes, Eugene, OR), initially dissolved in 1.5 ml of dimethylsulfoxide containing 8% (w/v) Pluronic F-127, 5% calf serum, and 0.1 mM probenecid. Residual Indo-1/AM was washed out by perfusing the heart with standard perfusate for at least another 30 min before any measurements were made.

Background correction of Indo-1 fluorescence

The background fluorescence intensities, at each wavelength, were determined prior to Indo-1 loading and subsequently subtracted from the fluorescence intensities after loading to obtain corrected Indo-1 intensities and ratios (Brandes et al., 1993).

Determination of in vivo R^{max}

In vivo R^{max} was determined during saturating intracellular calcium conditions, using HEPES (5 mM) buffered (pH 7.4) perfusate containing 80 mM $CaCl_2$, 6.0 mM KCl, 1.2 mM $MgCl_2$, 0.5 mM Na_2EDTA, 1.0 mM iodoacetate, 6% fetal calf serum, and 10 μM ionomycin (added together with the calf serum). To minimize energy-dependent calcium transport processes, the perfusate contained iodoacetate (1 mM) to inhibit glycolysis, and was also equilibrated with 100% N_2 to minimize oxidative phosphorylation.

Spectroscopic measurements

A modified SLM 48000S spectrofluorometer (Urbana, IL) was used for all measurements. The excitation light, from a 450-watt xenon arc lamp, was filtered at 350 nm using an interference filter (bandwidth 10 nm; Corion, Holliston, MA) and a visible blocking filter (Hoya U-340, Fremont, CA), and was focused onto the in-going leg of a bifurcated silica fiber optic bundle (Dolan-Jenner, Woburn, MA). The common leg of the bundle was positioned so that its tip was 2–3 mm away from the surface of the perfused heart. The heart was partially immobilized by hanging it from both the aortic cannula and the catheter used to inflate the left ventricular balloon (see above). To eliminate swinging motions of the heart with regard to the tip of the fiber optic bundle, a ferrule (Oriel, Stratford, CT) was attached to the tip of the bundle and the heart was held against it with nylon mesh and suture. To protect the heart against mechanical damage, a rubber O-ring was placed between the ferrule and the heart. Using this arrangement, a 2–3-mm circular region on the heart left ventricular surface was irradiated, and motion artifacts mainly arose from the motion of this surface as the heart was beating. To avoid bleaching of Indo-1, a shutter in front of the arclamp was only opened for seconds during each measurement. The light emitted from the heart was collected by the fiber optic tip of the common leg and transferred through the outgoing leg of the bundle. The light beam was then filtered by a UV-blocking filter (Schott KV-380, Duruyea, PA), split into two beams by a dichroic mirror (long-pass 420 nm; Reynard Enterprises, Laguna Niguel), and each beam was further filtered by tilted interference filters set to transmit at 385 and 456 nm (nominally 390 and 460, respectively, with a 10-nm bandwidth; Corion, Holliston, MA). The emission wavelengths were selected at 385 and 456 nm since tissue light transmission is independent of tissue oxygenation state at these two isosbestic wavelengths (Brandes et al., submitted for publication; Fralix et al., 1990; Brandes et al., 1991). In some experiments, the beamsplitter was replaced by the built-in monochromator set to 4-nm bandwidth, and in other experiments, a 50%/50% mirror (Reynard Enterprises, Laguna Niguel) was used in combination with the dichroic mirror and the monochromator to obtain a third detection channel. The filtered light beams were detected by photo multiplier tubes (PMTs), digitized, and stored in an IBM personal computer for data processing.

Statistical analysis

Values are reported as means ± S.E. of the measurement (SEM). Statistical analysis were performed using Student's t test, and differences were considered significant when $P < 0.05$.

THEORY AND METHODS OF $[CA^{2+}]_C$ QUANTITATION

(See Glossary for abbreviations.)

In order to calculate intracellular cytosolic calcium, $[Ca^{2+}]_c$, the established "ratio method" (Grynkiewicz et al., 1985) was used. According to this method, $[Ca^{2+}]_c$, was determined using the fluorescence ratio, R, calculated from the ratio between cytosolic Indo-1 fluorescence intensities, measured at two different emission wavelengths: 385 and 456 nm:

$$[Ca^{2+}]_c = K_d \cdot S_{456} \cdot \{(R - R^{min})/(R^{max} - R)\}, \quad (1)$$

where all the parameters were defined above. Because of problems of completely depleting (or saturating) the cytosol of Ca^{2+}, especially in an intact perfused heart, S_{456} was directly measured in a suitable reference solution, and R^{min} and R^{max} were calculated from calibration constants (determined in the reference solution) in combination with the inner filter correction constant: the gain-adjuster, bH (determined in the heart).

Calculation of the calibration parameters R^{min} and R^{max}

The fluorescence intensity, at wavelength λ, of Indo-1 in solution, I_λ, is ideally given by Grynkiewicz et al. (1985):

$$I_\lambda = \{I_\lambda^{max} \cdot [Ca^{2+}]_c + I_\lambda^{min} \cdot K_d)/([Ca^{2+}]_c + K_d\}, \quad (2a)$$

where I_λ^{min} and I_λ^{max} are the intensities of Indo-1 (per gain-unit and mole Indo-1) in the absence of Ca^{2+} and with saturating $[Ca^{2+}]$, respectively. In an Indo-1 loaded heart, at any particular time, the total measured fluorescence intensities at wavelength λ, F_λ, are related to I_λ, but also depend on tissue

light absorption, the actual amount of Indo-1, and background fluorescence according to:

$$F_\lambda = g_\lambda \cdot n(\text{Indo}) \cdot I_\lambda + B_\lambda, \quad (2b)$$

where g_λ is a gain factor that depends on instrumental gain and tissue light absorption (tissue filtering) at wavelength λ. This tissue filter depends on light scattering and light absorbance caused by myoglobin, Indo-1, and other absorbing molecules. n(Indo) is the amount (e.g., moles) of cytosolic Indo-1, and B_λ is the sum of various background fluorescence intensities after Indo-1 loading. In Eq. 2b, it was assumed that all Indo-1 fluorescence intensity arise from the cytosol of myocardial cells (i.e., n(Indo) in endothelial cells or in a cytosolic subcompartment was negligible (Brandes et al., 1993)).

In the absence of Ca^{2+}, and with saturating $[Ca^{2+}]$, the Indo-1 ratios, R^{min} and R^{max}, respectively, are given by:

$$R^{min} = F'^{min}_{385}/F'^{min}_{456} = \{g_{385}/g_{456}\} \cdot \{I^{min}_{385}/I^{min}_{456}\} \quad (3a)$$

$$R^{max} = F'^{max}_{385}/F'^{max}_{456} = \{g_{385}/g_{456}\} \cdot \{I^{max}_{385}/I^{max}_{456}\}, \quad (3b)$$

where F'^{min}_{385} *and* F'^{max}_{385} are the fluorescence intensities after background subtraction of B_λ (Eq. 2b) in the absence of Ca^{2+} (Eq. 2a with $I_\lambda = I^{min}_\lambda$), and with saturating $[Ca^{2+}]$ (Eq. 2a with $I_\lambda = I^{max}_\lambda$), respectively. According to Eq. 3, in order to calculate R^{min} and R^{max} in the heart, the ratio-gain, $\{g_{385}/g_{456}\}$, must independently be determined in the heart and gain-independent calibration constants (related to I^{min}_λ and I^{max}_λ) may be determined from the reference solution.

Ratio-gain and gain-adjuster (from heart)

The ratio-gain can be obtained from the relationship between the originally measured intensities, F_{385} and F_{456}, based on the following: consider the relationship between $[Ca^{2+}]_c$ and the Indo-1 intensities given by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_c = K_d \cdot \{(I_\lambda - I^{min}_\lambda)/(I^{max}_\lambda - I_\lambda)\}. \quad (4a)$$

Because $[Ca^{2+}]_c$ is independent of detection wavelength (e.g., λ = 385 or 456 nm), the following relationship between I_{385} and I_{456} is obtained (using Eq. 4a):

$$\{(I_{385} - I^{min}_{385})/(I^{max}_{385} - I_{385})\} = \{(I_{456} - I^{min}_{456})/(I^{max}_{456} - I_{456})\}. \quad (4b)$$

If Eq. 2b is rewritten with I_λ as the dependent variable, and I_{385} and I_{456} are substituted into Eq. 4b, it follows that F_{456} is linearly related to F_{385} with slope bH and intercept aH (aH will not be used in this study, but see Brandes et al. (1993)) according to:

$$F_{456} = bH \cdot F_{385} + aH, \quad (5a)$$

where

$$bH = \{g_{456}/g_{385}\} \cdot (I^{max}_{456} - I^{min}_{456})/(I^{max}_{385} - I^{min}_{385}) \quad (5b)$$

and

$$aH = g_{454} \cdot n(\text{Indo-1}) \cdot a + B_{456} - bH \cdot B_{385} \quad (5c)$$

with

$$a = (I^{min}_{456} \cdot I^{max}_{385} - I^{max}_{456} \cdot I^{min}_{385})/(I^{max}_{385} - I^{min}_{385}) \quad (5d)$$

The slope, bH, defined as the gain-adjuster, is directly proportional to the ratio-gain and can therefore be used to correct for the effect of tissue filtering in the heart. The gain-adjuster could differ in different hearts (and as a function of time), and must therefore be determined for each heart and data set separately. (Note that Eq. 5 would also be valid if a fraction of Indo-1 were loaded into endothelial cells or mitochondria (Brandes et al., 1993).)

Calibration constants (from reference solution)

Gain-independent calibration constants can be found from the reference solution by calculating the fluorescence ratios, S_λ, between the detected Indo-1 intensities in the absence of Ca^{2+}, I^{min}_λ, and with saturating $[Ca^{2+}]$, I^{max}_λ, according to:

$$S_\lambda = I^{min}_\lambda / I^{max}_\lambda. \quad (6)$$

Because S_λ is calculated as a ratio between I^{min}_λ and I^{max}_λ at the same wavelength, the gain-factors cancel, and S_λ is therefore independent of wavelength-dependent filtering effects. It will be assumed that S_{385} and S_{456} are intrinsic properties of Indo-1 and are therefore constants, identical to those in any heart (provided that the reference solution properly mimics the intracellular interactions with Indo-1).

An expression for R^{max} as a function of bH and S_λ can be obtained by multiplying R^{max} with bH (Eqs. 3b and 5b) and simplifying by using the expression for S_λ (Eq. 6) according to:

$$R_{max} \cdot bH = \frac{(I^{max}_{385}/I^{max}_{456}) \cdot (I^{max}_{456} - I^{min}_{456})}{I^{max}_{385} - I^{min}_{385}} = \frac{1 - S_{456}}{1 - S_{385}}, \quad (7a)$$

which is equivalent to

$$R^{max} = S_R/bH \quad (7b)$$

where

$$S_R = (1 - S_{456})/(1 - S_{385}). \quad (7c)$$

In Eq. 7, S_R is defined as the "reference ratio." An expression for R^{min} as a function of R^{max} and S_λ can be obtained by dividing Eq. 3a with 3b and simplifying by using Eq. 6 according to:

$$R^{min} = R^{max} \cdot \{I^{min}_{385}/I^{min}_{456}\}/\{I^{max}_{385}/I^{max}_{456}\} = R^{max} \cdot S_{385}/S_{456}. \quad (8)$$

Eqs. 7 and 8 demonstrate that the calibration parameters, R^{min} and R^{max}, needed to calculate $[Ca^{2+}]_c$ from the Indo-1 fluorescence ratio, R, can be calculated by combining the gain-adjuster, bH, obtained from the heart with S_{385} and S_{456} obtained from an appropriate reference solution. Conversely,

the reference ratio, S_R, could be obtained from the heart during saturating $[Ca^{2+}]_c$ in order to verify the calibration constants S_{385} and S_{456} (see below).

Correction for motion artifacts

Because the Indo-1 intensity transients measured in the heart by F_{385} and F_{456} are slightly altered by a motion artifact, a new method was developed to calculate the motion function which modulated the measured intensities (see Appendix). Motion corrected intensities were then calculated by division with the modulation function, and the gain-adjuster was calculated as described above.

Verification of calibration constants S_{385} and S_{456} obtained from reference solution.

The calibration constants S_{385} and S_{456} were determined in the three different types of reference solutions described above: an aqueous buffered solution with albumin, a protein mixture at various concentrations, and a heart homogenate (containing soluble heart proteins). Because S_{385} and S_{456} differed in different solutions (see Results), they were selected from a reference solution where the spectral characteristics of Indo-1 were believed to match those of the heart. There are two criteria which could be used to compare the spectral characteristics of Indo-1 in vitro and in vivo: 1) the Indo-1 isosbestic wavelength in the reference solution should match that observed in vivo, and 2) the calculated value of S_R, using the measured values of S_λ (from the reference solution), should match that obtained in the heart during saturating $[Ca^{2+}]$ (Eqs. 7c and 7b, respectively). If the reference solution has a similar isosbestic wavelength and calculated S_R compared to the heart, then the Indo-1 spectral characteristics in vivo are considered to be appropriately mimicked by the reference solution. It is, however, possible that S_{385} and S_{456} could nevertheless differ in vitro, and in vivo since various combinations of S_{385} and S_{456} could produce identically calculated values of S_R. Thus it is likely, but not unequivocally proven, that S_{385} and S_{456} determined from the reference solution would be identical to those in the heart.

Comparison of Indo-1 in-vivo and in-vitro isosbestic wavelengths

Because of the wavelength-dependent tissue light absorption, it is impossible to directly compare the complete spectral characteristics of Indo-1 in the heart with those in the reference solutions. However, the Indo-1 isosbestic wavelength, λ_{iso}, is independent of tissue light absorption and background fluorescence (the Indo-1 intensities would be independent of $[Ca^{2+}]_c$ only at λ_{iso}, and light absorbance or background fluorescence would only change the detected intensity at λ_{iso}).

Variation of $[Ca^{2+}]_c$ was obtained naturally by using a beating heart, and the Indo-1 isosbestic wavelength was determined by simultaneously detecting the fluorescence intensities at 385 nm (F_{385}), x nm (F_x) and 456 nm (F_{456}), where x was varied between 410 and 440 nm. The motional modulation function was then calculated from F_{385} and F_{456}, as described in Appendix, and used to motion-correct F_x. The diastolic and systolic values of F_x, F_x(dia), and F_x(sys), respectively, were used to calculate the amplitude $\delta_x = F_x(sys) - F_x(dia)$. A polynomial was used to fit the amplitude versus wavelength relationship, and the Indo-1 isosbestic wavelength in the heart was obtained at the wavelength where the fitted $\delta_x = 0$.

In solution, the isosbestic wavelength was determined at the wavelength where $I_\lambda^{min} = I_\lambda^{max}$ using Ca^{2+}-depleted and Ca^{2+}-saturated solutions.

Comparison of S_R in reference solution and in the heart

In the reference solution, S_R was calculated from S_{385} and S_{456} according to Eq. 7c. To verify that an appropriate reference solution was chosen, S_R was also independently determined directly in the heart by switching the standard perfusate to the saturating Ca^{2+} perfusate (see above). The maximum fluorescence intensities, F_λ^{max}, was then used to calculate R^{max} (after background subtraction; Eq. 3b) and subsequently S_R (Eq. 7b).

RESULTS AND DISCUSSION

Measurements of calibration constants S_{385} and S_{456} and λ_{iso} obtained from reference solution.

The simplest Indo-1 reference solution for determining S_λ is an aqueous buffered solution with Indo-1 in the absence of Ca^{2+} and in the presence of saturating $[Ca^{2+}]$. Fig. 1 *A* shows that the resulting spectra peak at ~400 nm with saturating $[Ca^{2+}]$ (maxCa; *heavy solid line*) and at ~470 nm in the absence of Ca^{2+} (0Ca; *heavy dashed line*), as has been shown previously (Grynkiewicz et al., 1985). However, because the cytosol contains a large number of proteins, Indo-1 spectra might be different intracellularly versus in aqueous solution (Owen and Shuler, 1989; Hove-Madsen and Bers, 1992). Fig. 1 *A* shows that the major effect of added albumin was a blue shift and an increase in intensity of the spectrum obtained in the absence of Ca^{2+} (*dashed line*), while the intensity during saturating $[Ca^{2+}]$ (*solid line*) only increased slightly in intensity. The Indo-1 isosbestic wavelength, λ_{iso}, consequently moved from 450 to 427 nm (at 0.3 mg/ml albumin). Fig. 1 *B* shows the isosbestic wavelength as a function of albumin concentration, [Alb] (*solid line*) and protein mixture concentration [Prot] (*dashed line*). The λ_{iso} decreased with increasing [Alb] or [Prot] until it reached a minimum of λ_{iso} ~415 nm and λ_{iso} ~430 nm for [Alb] ~10 mg/ml and [Prot]~ 100 mg/ml, respectively. The relatively small effect of [Prot] on λ_{iso} and other dye properties have also been observed previously for Indo-1 (Owen and Shuler, 1989; Hove-Madsen and Bers, 1992) and Fura-2 (Konishi et al., 1988).

The spectral changes shown in Fig. 1 *A* also demonstrated that S_{385}, S_{456}, and S_R were dependent on [Alb] and [Prot]. Fig. 1 *C* shows the fitted relationship (using second order

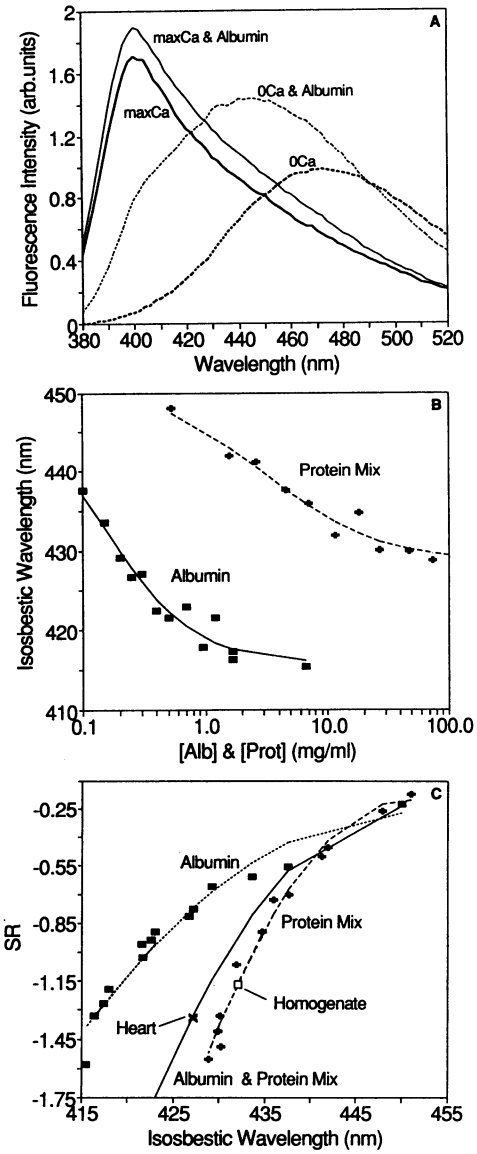

FIGURE 1 Spectral properties of reference solutions used to estimate S_{385} and S_{456}. (*A*) Emission spectra of Indo-1 in: aqueous solution with saturating $[Ca^{2+}]$ (*heavy solid line*) and in the absence of Ca^{2+} (*heavy dotted line*). Aqueous solution with 0.3 mg/ml albumin and saturating $[Ca^{2+}]$ (*light solid line*), and in the absence of Ca^{2+} (*light dotted line*). The spectra show a left shift of the Indo-1 isosbestic wavelength for calcium when albumin was added. (*B*) Effect of albumin concentration, [Alb] (■, *solid line*) and protein mixture concentration [Prot] (+, *dashed line*) on the Indo-1 isosbestic wavelength. The data were fitted to logistic functions. (*C*) Relationship between λ_{iso} and S_R for different concentrations of albumin (■, *dotted line*) and protein mixture (+, *dashed line*), homogenate (□) and the heart (*X*). Calculated linear combination of the albumin and protein mixture fitted polynomials (*solid line*).

polynomials) between the isosbestic wavelength and S_R for different [Alb] (*dotted line*) and [Prot] (*dashed line*) as well as the homogenate (*open square*; at fixed protein concentration). The relationship between λ_{iso} and S_R were different for the albumin and protein mixture, but identical to the protein mixture and the homogenate. Furthermore, each point (given by S_R and λ_{iso}) on these curves corresponded to a unique set of values for S_{385} and S_{456} (which were similar for the protein mixture and the homogenate; see Table 1 for examples). Knowledge of S_R and λ_{iso} therefore unequivocally determines the values of S_{385} and S_{456}.

Measurement of gain-adjuster in Indo-1-loaded hearts

Fig. 2 *A* (*dashed lines*) shows representative heart Indo-1 fluorescence intensity transients, F_{385} and F_{456}, in the presence of a motion artifact. As $[Ca^{2+}]_c$ increased, the fluorescence intensity increased at 385 nm and decreased at 456 nm. The maximal change was an increase of 42% at 385 nm (43 ± 2%; n = 24) and a decrease by 15% (13 ± 0.6% ; n = 24) at 456 nm. Fig. 2 *B* (thin loop-like curves) shows the same data with F_{456} plotted against F_{385}. Because the measured intensities were modulated by a motion artifact, their relationship deviated from the predicted straight line (see Eq. 5a), and therefore resulted in the loop-like structure. With each transient in Fig. 2 *A*, the tracing in Fig. 2 *B* completes one loop with the minimum and maximum of the transient corresponding to the upper left and lower right corners, respectively.

Correction of motion artifacts

The motion artifact influenced the detected intensity at each individual wavelength. To eliminate it, the motional modulation function was calculated, and motion corrected intensities were obtained by dividing the original intensity transients with the modulation function (see Appendix). Fig. 2 *A* (*bottom heavy solid line*) shows that the calculated motional modulation function from the transients in Fig. 2, *A* and *B*, had an amplitude of ~3%. Using this modulation function, the motion corrected transients in Fig. 2 *A* (*solid lines*) and Fig. 2 *B* (*heavy solid line*) were obtained. Fig. 2 *B* shows that the motion corrected relationship between F_{385} and F_{456} transformed the loop-like structure of the relationship to a straight line, with slope bH = −0.15.

Because the motion artifact was relatively small, 7 and 20% of the detected transients at 385 and 456 nm, respectively, the corrected transients in Fig. 2 *A* are almost superimposable on the uncorrected ones. This is especially noticeable during the initial edge (rising at 385 nm, falling at 456 nm) of the fluorescence transient which occurs before any pressure development (Mohabir et al., 1991). The initial edges of the transients in Fig. 2 *A* corresponds to the parallel traces between the upper left and lower right corners in Fig. 2 *B*, and a constant part (unity except for random noise) of the motional modulation function (Fig. 2 *A*, *bottom trace*). These traces are parallel with the thick line in Fig. 2 *B* which describes the motion corrected relationship, and the slope of the traces could therefore be used directly as an approximation of bH.

An additional benefit of plotting the two fluorescence intensities against each other is that it provides a novel technique to quantify the motion artifact contribution to the

TABLE 1 Calculated $[Ca^{2+}]_c$ as a function of calibration constants S_{385} and S_{456}

Solution	λ_{iso}	S_{385}	S_{456}	S_R	$[Ca^{2+}]_{dia}$	$[Ca^{2+}]_{sys}$
	nM				nM	nM
Aqueous	450	0.07	1.22	−0.22	<0	<0
Albumin (0.3 mg/ml)	427	0.22	1.60	−0.49	245 ± 40	1564 ± 335
Albumin (1.7 mg/ml)	417	0.28	1.96	−1.34	83 ± 13	297 ± 34
Homogenate	432	0.072	2.09	−1.18	201 ± 20	552 ± 58
Protein mix* (28 mg/ml)	430	0.051	2.27	−1.34	187 ± 17	464 ± 43
Whole heart	427	?	?	−1.34		

* Used as final reference solution.

Ca^{2+}-dependent Indo-1 transients. This method could therefore be used to minimize any mechanical factors responsible for the motion artifact, especially since calculation of the fluorescence ratio does not completely eliminate it (Brandes et al., 1992).

Verification of calibration constants S_{385} and S_{456} obtained from reference solution

The solution-derived values of S_{385} and S_{456} were verified by two independent methods as described below. The first method relied on matching λ_{iso} of the reference solutions (e.g., at a particular protein concentration) with that of the heart. The second method relied on matching the calculated S_R, using the solution derived values of S_{385} and S_{456} with the in vivo determined S_R.

Comparison of Indo-1 in vivo and in vitro isosbestic wavelengths

Fig. 3 shows the amplitudes of the motion corrected fluorescence transients measured as a function of detection wavelength during control conditions in a particular heart. The Indo-1 in vivo isosbestic wavelength was determined at that wavelength were the transient amplitude $\delta_x = 0$, which occurred at $\lambda_{iso} = 427$ nm ($n = 2$).

Since the motion correction required simultaneous measurements at three wavelengths, a simpler method of analysis was also evaluated; the derivative of the uncorrected transients was calculated to suppress any motion artifact relative to the (derivative of the) Ca^{2+}-dependent part of the Indo-1 transients. (Because the Ca^{2+}-dependent, very steep initial edge of the transient occurs before any motion artifact, the derivative emphasizes it relative to a less steep edge caused by any motion artifact). The isosbestic wavelength was then found at the wavelength where the derivative of the transient changed sign. This analysis method also resulted in $\lambda_{iso} = 427$ nm (not shown).

The in vivo isosbestic wavelength of 427 nm could be matched exactly in vitro by an albumin solution with [Alb]

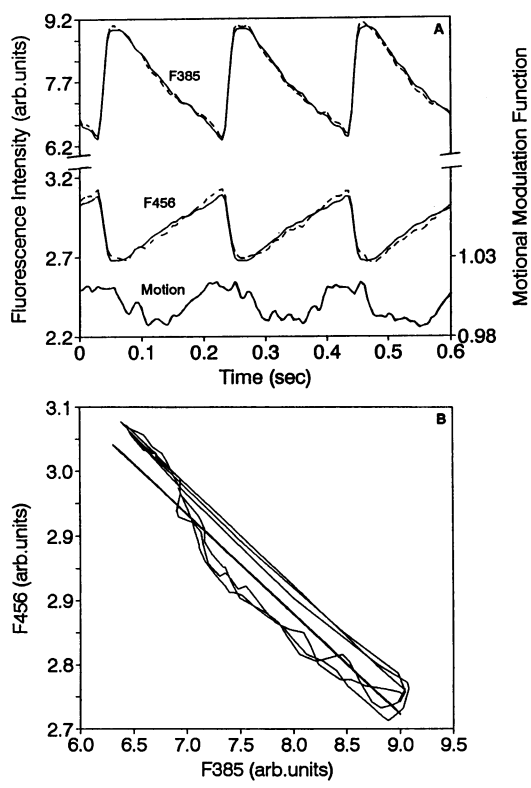

FIGURE 2 Indo-1 fluorescence transients measured at 385 and 456 nm in the presence of a motion artifact. (*A*) Intensities before motion correction (*dashed line*), intensities after motion correction (*light solid line*), and motional modulation function (*heavy solid line*). (*B*) Intensity measured at 456 nm as a function of intensity at 385 nm. The loop-like trace corresponds to uncorrected transients (*dashed line* in *A*) and the straight line (*heavy solid line*) to motion corrected transients (*light solid lines* in *A*).

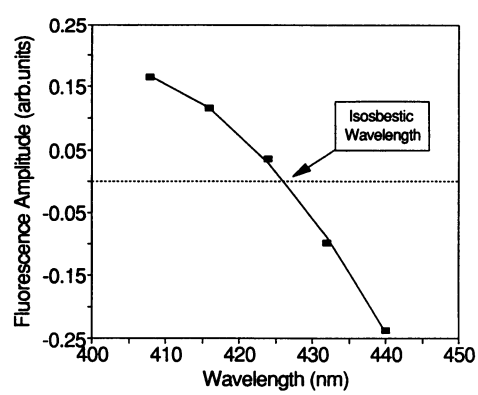

FIGURE 3 Example of in vivo determination of Indo-1 isosbestic wavelength, λ_{iso}. Indo-1 transient data were motion corrected to obtain the amplitudes (■) as a function of detection wavelength. The data were fitted to a quadratic polynomial (*solid line*), and λ_{iso} was found at the wavelength where the fitted polynomial was zero.

= 0.3 mg/ml but only approximately by the protein mixture, $\lambda_{iso} \geq 430$ nm ([Prot] $\leq$ 100 mg/ml) or homogenate, λ_{iso} = 432 nm (see Fig. 1 *B* and Table 1).

In contrast to the more conventional steady state measurements using high and low $[Ca^{2+}]$ to determine λ_{iso}, the transient method ensures that only cytosolic Indo-1 was monitored since noncytosolic $[Ca^{2+}]$ is not expected to vary during the heart cycle in a compartment such as mitochondria (Miyata et al., 1991) or in endothelial cells.

Comparison of S_R in reference solution and in the heart

Fig. 4 shows a representative experiment using saturating $[Ca^{2+}]$ in the heart to obtain S_R. After application of the Ca^{2+} saturating solution, F_{385} increased and reached a maximum after ~2 min. The intensity then slowly decayed during the following 5 min. In contrast, F_{456} immediately decreased and then increased slightly to reach a maximum at the same time (2 min) as F_{385}. Thereafter F_{456} decreased in intensity in parallel with F_{385}. It is possible that the measured peak intensities were affected by a common factor (e.g., Indo-1 leakage) that changed the detected intensities in parallel. To eliminate any such factors, which may influence F_{385} and F_{456} proportionally, the ratio R was calculated (using background corrected intensities), and R^{max} was approximated by the peak of R with saturating $[Ca^{2+}]$. Fig. 4 (*heavy solid line*) demonstrates that the ratio is more stable than the individual intensities. By using the measured R^{max} in combination with the measured *bH* in this example, it was found that the peak of S_R was −1.12. In the five hearts investigated, peak S_R = −1.34 ± 0.07 which is significantly lower than S_R = − 0.49 obtained from the albumin solution (0.3 mg/ml; λ_{iso} = 427 nm) but identical to S_R = −1.34 in the protein solution (28 mg/ml; λ_{iso} = 430 nm).

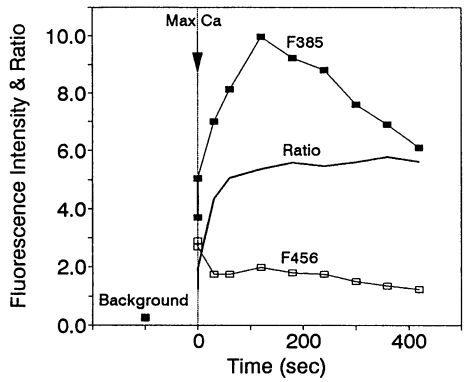

FIGURE 4 Example of Indo-1 in vivo response to saturating $[Ca^{2+}]$. Fluorescence intensities (measured at 385 nm (■) and at 456 nm (□)) were first recorded prior to Indo-1 loading (time < 0). Heart was loaded with Indo-1, and transients were recorded (time = −0). The saturating Ca^{2+} perfusate was then applied (at time = +0), which resulted in the increased intensity at 385 nm and decreased intensity at 456 nm. The ratio, R, was calculated between the background subtracted intensities measured at 385 and 456 nm (*solid line*). The data points have been connected for clarity.

Choice of S_{385} and S_{456}

Independent of [Alb] or [Prot], an appropriate reference solution could not be prepared to exactly account for both the measured λ_{iso} and S_R in the heart (Fig. 1 *C*, *cross symbol*). However, although the protein λ_{iso} (at 28 mg/ml) differed slightly from that in the heart, S_R was identical in the protein solution and in the heart. The protein S_λ values, using the 28 mg/ml concentration solution (S_{385} = 0.05 and S_{456} = 2.27), were therefore determined to be a reasonable approximation of the intracellular S_λ values.

It is possible that the in vivo Indo-1 spectral characteristics could be better mimicked by an appropriate mixture between albumin and the proteins. This is shown mathematically in Fig. 1 *C*, where the solid line was calculated from a linear combination of the protein fitted polynomial (*dashed line*; 58%) and the albumin fitted polynomial (*dotted line*; 42%). The calculated mixture (*solid line*) passed through the in vivo value (*cross symbol*).

Calculation of $[Ca^{2+}]_c$

To calculate $[Ca^{2+}]_c$ from the fluorescence ratio, R^{max} and R^{min} were first calculated by determining *bH* in each heart, and using the values of S_{385} and S_{456} determined in a reference solution. To demonstrate the dependency of calculated $[Ca^{2+}]_c$ on S_{385} and S_{456}, the diastolic and systolic values of $[Ca^{2+}]_c$, $[Ca^{2+}]_{dia}$ and $[Ca^{2+}]_{sys}$ were calculated (n = 4) using S_λ values determined from the various reference solutions (Table 1). Because it was shown previously (Brandes et al., 1993) that the contribution from unhydrolized Indo-1/AM was negligible, the fluorescence intensities measured at 385 and 456 nm were corrected by subtracting the background fluorescence measured prior to loading with Indo-1 (Brandes et al., 1993). However, any background intensity from compartmentalized or endothelial Indo-1 was not quantified, and was therefore neglected (set to zero) as a first approximation (Brandes et al., 1993). In order to avoid any buffering of intracellular calcium by Indo-1, the dye-loading was kept low (fluorescence intensity at 456 nm was less than five times background fluorescence prior to Indo-1 loading). Both $[Ca^{2+}]_{dia}$ and $[Ca^{2+}]_{sys}$ as well as the variation between different hearts (as indicated by their relative SEM) depended on the exact values of S_{385} and S_{456} (Table 1). By using S_λ obtained from the protein mixture (S_{385} = 0.05 and S_{456} = 2.27), the values were: $[Ca^{2+}]_{dia}$ = 187 ± 17 nM and $[Ca^{2+}]_{sys}$ = 464 ± 43 nM.

Table 2 shows several literature values for $[Ca^{2+}]_{dia}$ and $[Ca^{2+}]_{sys}$ in isolated myocytes as well as in intact tissue. Sollot et al. (1992) measured $[Ca^{2+}]_c$ in rat myocytes by loading with Indo-1/AM or Indo-1. They found $[Ca^{2+}]_{dia}$ = 136 nM, and $[Ca^{2+}]_{sys}$ = 354 and 482 nM when using Indo-1/AM and Indo-1, respectively. Their systolic value, using Indo-1, was similar to that obtained here, ($[Ca^{2+}]_{sys}$ = 482 vs. 464 nM) although their diastolic value was smaller than that found here ($[Ca^{2+}]_{dia}$ = 136 vs. 187 nM), and might be explained by the slower pacing rate used in their study (0.5 vs. 5 Hz in this study).

TABLE 2 Comparison of calculated $[Ca^{2+}]_{dia}$ and $[Ca^{2+}]_{sys}$ in different preparations, using in vivo or in vitro calibration of dual-wavelength fluorescent dyes

Preparation	Indicator	Calibration method	$[Ca^{2+}]_{dia}$	$[Ca^{2+}]_{sys}$	Reference
			nM*		
Whole rat hearts	Indo-1/AM	In vitro/in vivo	187	464	This study
Rat myocytes	Indo-1/AM	In vivo	136	354	(Sollot et al., 1992)
Rat myocytes	Indo-1	In vivo	136	482	(Sollot et al., 1992)
Rat myocytes	Indo-1	In vitro	120	512	(Cleeman and Morad, 1991)
Whole rat hearts	Indo-1/AM	In vitro/in vivo	330	638	(Wikman-Coffelt et al., 1991)
Whole rabbit hearts	Indo-1/AM	In vitro/in vivo	313	626	(Mohabir et al., 1991)
Whole rat trabeculae	Fura-2/AM	In vivo	122	427	(Backx and ter Keurs, 1993)
Whole rat trabeculae	Fura-2	In vivo	122	693	(Backx and ter Keurs, 1993)
Chick embryo myocytes	Indo-1/AM	In vivo	269	750	(Ikenouchi et al., 1991)

* Using K_d = 250 nM for Indo-1 and 224 nM for Fura-2 (Grynkiewicz et al., 1985).

Wikman-Coffelt et al. (1991) attempted to compensate for the tissue light absorbance in intact hearts by measuring R^{min} and R^{max} in an aqueous reference solution (without protein), placed inside a balloon in the left ventricle, and transmitting excitation (and emission light) through the heart wall. However, this approach does not appropriately compensate for light absorbance, since the transmitted light and epicardial fluorescence intensities are affected by different volume elements (fluorescence is detected from within the first millimeter of the epicardium (Stewart, 1985)).

Recently, Mohabir et al. (1991), using whole rabbit hearts, avoided the problems of tissue light absorbance by calibrating intensities at a single detection wavelength (400 nm). The calibration parameter during saturating Ca^{2+} conditions, F_{400}^{max}, were obtained using a similar protocol as described here, but S_{400} (= 0.18) was derived from an aqueous reference solution (Grynkiewicz et al., 1985). Using high dye loading, 5–12 times (Lee et al., 1988), they found $[Ca^{2+}]_{dia}$ = 313 nM and $[Ca^{2+}]_{sys}$ = 626 nM (Mohabir et al., 1991), both which were higher than the values obtained here. However, if they would have used a larger S_{400} (as was found here in the presence of proteins), their calculated F_{400}^{min} would increase, resulting in significantly lower $[Ca^{2+}]_c$. Although the problem of light filtering is eliminated by using a single rather than dual wavelengths, single wavelength calibration complicates $[Ca^{2+}]_c$ quantitation since it can only be determined at the time of calibration (due to Indo-1 leakage). Furthermore, inaccurate estimates of F_{400}^{max} may be obtained due to Indo-1 intensity variations during saturating Ca^{2+} conditions (see Fig. 4).

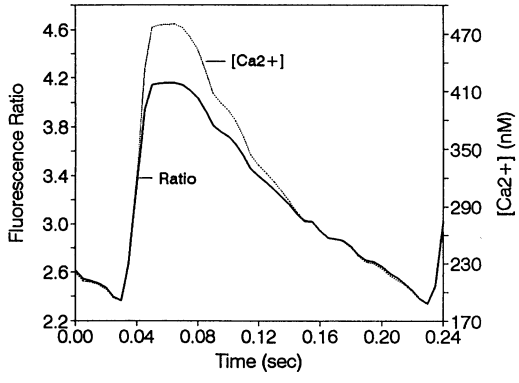

FIGURE 5 Example of calibrating the Indo-1 ratio (*solid line*) to obtain $[Ca^{2+}]_c$ (*dashed line*) in a single heart beat. Calibration demonstrated the nonlinear response of Indo-1 to increasing $[Ca^{2+}]_c$.

Fig. 5 illustrates the effects of calibrating the Indo-1 ratio to obtain $[Ca^{2+}]_c$ as a function of time in a single heart beat. As $[Ca^{2+}]_c$ increased, the ratio was progressively less sensitive to changes in $[Ca^{2+}]_c$ due to the nonlinear dye response (Grynkiewicz et al., 1985). For example, a 20% reduction of the maximum of the ratio (in systole) would correspond to a 33% reduction of $[Ca^{2+}]_{sys}$.

Because the Indo-1 dissociation constant, K_d, was not determined here, the absolute value of $[Ca^{2+}]_c$ might need to be corrected by a constant factor (see Eq. 1). The K_d of Indo-1 in the cytosol is currently uncertain. Some investigators reported no differences between the K_d of Indo-1 in vitro and in vivo (Spurgeon et al., 1990; Ikenouchi et al., 1991). In contrast, a recent report suggested that the K_d of Indo-1 in the cytosol is four times larger than in vitro (Hove-Madsen and Bers, 1992).

Fractional changes in $[Ca^{2+}]_c$ can nevertheless be determined if the ratio is calibrated using the methods described here, although K_d and therefore absolute $[Ca^{2+}]_c$ is unknown. In contrast, without calibration, fractional changes of the ratio may be misinterpreted if absolute $[Ca^{2+}]_c$ would change. An example would be a similar increase in the fluorescence ratio during diastole and systole, possibly being interpreted as a constant amplitude of the calcium transient, while in fact the calcium amplitude may have increased. The methods presented here circumvent this problem and provides estimates of $[Ca^{2+}]_c$ which could be improved with accurate knowledge of the intracellular K_d.

APPENDIX

Correction for motion artifacts

In order to obtain the linear relationship between the two measured fluorescence intensities, as described by Eq. 5a, any motion artifact must be eliminated. This can partly be accomplished by mechanically restraining the heart motion relative to the fiber optic probe (Brandes et al., 1992).

However, this might not always be adequate, and a mathematical method was therefore used to calculate the motion-corrected F_{385} and F_{456}.

Any motion artifact is expected to modulate the fluorescence intensity at each wavelength similarly. In this case, Eq. 5a can be rewritten as:

$$aH^*(t) = F_{456} \cdot \epsilon(t) - bH \cdot F_{385} \cdot \epsilon(t), \qquad \text{(A1)}$$

where

$$aH^*(t) = aH \cdot \epsilon(t). \qquad \text{(A2)}$$

$\epsilon_\lambda(t)$ is the motional modulation function which cause the modulation of the detected intensities, $F_{456} \cdot \epsilon(t)$ and $F_{385} \cdot \epsilon(t)$. In Eq. A1 it was assumed that instrumental autofluorescence is negligible, and to a first approximation, $\epsilon(t) \sim \epsilon_{456}(t) \sim \epsilon_{385}(t)$ (Brandes et al., 1992).

Any time-dependent variation of the detected intercept, $aH^*(t)$, is therefore caused by motion, provided that bH is known. For an incorrect bH, the variation in $aH^*(t)$ would contain an additional contribution from Ca^{2+}-dependent Indo-1 fluorescence fluctuations. A least squares regression analysis was therefore performed to calculate bH; bH was varied until the motion artifact (Brandes et al., 1992) of $aH^*(t)$ was as close to zero as possible, i.e.:

$$\frac{\text{Max}[aH^*(t)] - \text{Min}[aH^*(t)]}{\text{Max}[aH^*(t)] + \text{Min}[aH^*(t)]} \sim 0. \qquad \text{(A3)}$$

The motional modulation function, $\epsilon(t)$, is then given by Brandes et al. (1992):

$$\epsilon(t) = \frac{2 \cdot aH^*(t)}{\text{Max}[aH^*(t)] + \text{Min}[aH^*(t)]}, \qquad \text{(A4)}$$

and the motion corrected intensities, F_λ, are obtained after dividing the detected intensities with $\epsilon(t)$.

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