

Cytosolic and Mitochondrial $[Ca^{2+}]$ in Whole Hearts Using Indo-1 Acetoxymethyl Ester: Effects of High Extracellular Ca^{2+}

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ABSTRACT Assessment of free cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) using the acetoxymethyl ester (AM) form of indo-1 may be compromised by loading of indo-1 into noncytosolic compartments, primarily mitochondria. To determine the fraction of noncytosolic fluorescence in whole hearts loaded with indo-1 AM, Mn^{2+} was used to quench cytosolic fluorescence. Residual (i.e., noncytosolic) fluorescence was subtracted from the total fluorescence before calculating $[Ca^{2+}]_c$. Noncytosolic fluorescence was used to estimate mitochondrial $[Ca^{2+}]$. In hearts paced at 5 Hz ($N = 17$), noncytosolic fluorescence was 0.61 ± 0.06 and 0.56 ± 0.07 of total fluorescence at λ_{385} and λ_{456} , respectively. After taking into account noncytosolic fluorescence, systolic and diastolic $[Ca^{2+}]_c$ was 673 ± 72 and 132 ± 9 nM, respectively. noncytosolic $[Ca^{2+}]$ was 183 ± 36 nM and increased to 272 ± 12 when extracellular Ca^{2+} was increased from 2 to 6 mM. This increase in noncytosolic $[Ca^{2+}]$ was inhibited by ruthenium red, a blocker of Ca^{2+} uptake by mitochondria. We conclude that cytosolic and mitochondrial $[Ca^{2+}]$ can be determined in whole hearts loaded with indo-1 AM by using Mn^{2+} to quench cytosolic fluorescence.

INTRODUCTION

The central role of free cytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) in excitation-contraction coupling of cardiac myocytes has led to the development of several techniques to measure $[Ca^{2+}]_c$ in a variety of experimental preparations. In isolated whole hearts, $[Ca^{2+}]_c$ has been measured using the fluorescent indicators indo-1 (Lee et al., 1988; Wikman-Coffelt et al., 1991; Figueredo et al., 1992) and fura-2 (Ataka et al., 1992), the bioluminescent protein aequorin (Kihara et al., 1989), and ^{19}F -BAPTA NMR (Steenbergen et al., 1987; Kitakaze and Marban, 1989). Each of these techniques has advantages and limitations. An advantage of fluorescent indicators is that an excellent signal-to-noise ratio can be achieved with little Ca^{2+} buffering. Furthermore, these indicators can be loaded into cardiac myocytes using the cell-permeable acetoxymethyl (AM) form. However, when cardiac myocytes are loaded with the AM form of these indicators, a significant fraction of emitted fluorescence arises from subcellular organelles (Davis et al., 1987; Blatter and Wier, 1990; Spurgeon et al., 1990), thereby compromising the measurement of $[Ca^{2+}]_c$. Under these conditions, emitted fluorescence will be the sum of fluorescence from the cytosol and subcellular organelles. In this paper, all compartments other than the cytosol will be called the noncytosol.

Two major approaches have been developed to differentiate cytosolic from noncytosolic fluorescence in isolated cardiac myocytes and muscle strips. One approach is to directly load the cytosol using the free-acid form of these fluorescent indicators rather than the AM form. Because the free-acid form is not membrane permeable, direct injection using a microelectrode (Spurgeon et al., 1990; Backx and Ter Keurs, 1993) or reversible lysis of the cell membrane (Sollott et al., 1992) has been used to load the dye into the cytosol. However, both of these methods for loading the free-acid form of these indicators are technically demanding and cannot be used in whole hearts. A second approach for differentiating cytosolic from noncytosolic fluorescence is to load cardiac myocytes with the AM form of the indicator followed by selective quenching of cytosolic fluorescence by Mn^{2+} . By inference, the remainder of fluorescence is from noncytosolic compartments. Subtraction of noncytosolic fluorescence from total fluorescence yields cytosolic fluorescence (Spurgeon et al., 1990; Miyata et al., 1991, 1992). An advantage of this technique is that it can be used to assess mitochondrial $[Ca^{2+}]$, because previous investigators (Miyata et al., 1991) have demonstrated that noncytosolic fluorescence is derived primarily from mitochondria. However, this work was limited to isolated cardiac myocytes studied at room temperature.

Because several physiological and pathological processes (e.g., myocardial ischemia) are best studied using whole hearts that have intact coronary flow and myocardial structure, our laboratory has assessed $[Ca^{2+}]_c$ in whole hearts loaded with indo-1 AM. Previous studies have identified and minimized other sources of artifact and described a novel approach for the calibration of indo-1 fluorescence to $[Ca^{2+}]$ (Brandes et al., 1992, 1993a,b, 1994).

Because noncytosolic loading of indo-1 may affect the calculation of $[Ca^{2+}]_c$, the first goal of this study was to

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determine the fraction of fluorescence arising from the noncytosol in whole hearts loaded with indo-1 AM and to use these data to calculate $[Ca^{2+}]_c$. Quenching of cytosolic fluorescence by Mn^{2+} was used to determine the fraction of noncytosolic fluorescence.

Because noncytosolic fluorescence in cardiac myocytes arises predominantly from mitochondria (Miyata et al., 1991), a second goal was to explore the possibility that noncytosolic $[Ca^{2+}]$ ($[Ca^{2+}]_{nc}$) might be used to estimate mitochondrial free $[Ca^{2+}]$ in whole hearts. To this end, the effects of high extracellular $[Ca^{2+}]$ were determined, with and without ruthenium red, a blocker of Ca^{2+} uptake by mitochondria (Moore, 1971; Gupta et al., 1989). It was postulated that if noncytosolic fluorescence arises primarily from mitochondria, ruthenium red should prevent the increase in $[Ca^{2+}]_{nc}$ caused by high extracellular $[Ca^{2+}]$ without affecting the increase in $[Ca^{2+}]_c$.

MATERIALS AND METHODS

Isolated heart preparation

Male Sprague-Dawley rats weighing between 300 and 350 g were anesthetized with ketamine (100 mg/kg i.p.) and anticoagulated with heparin (1000 units/kg i.p.). Hearts were quickly excised and immediately arrested using a cold, high-potassium (KCl = 30 mmol/liter) saline solution. After cannulation of the aorta, perfusion according to Langendorff was started at a constant pressure of 72 mm Hg. The standard perfusate contained (mmol/liter): NaCl (118), KCl (6.0), $MgSO_4$ (1.2), $CaCl_2$ (2.0), $NaHCO_3$ (25), pyruvate (5.0), glucose (4.0), and insulin, 20 units/liter. The perfusate was continuously bubbled with a 95% O_2 /5% CO_2 gas mixture, which resulted in a pH of 7.35–7.45, and the temperature was maintained at 37°C. Isovolumic pressure was measured with a latex balloon placed inside the left ventricle through the mitral valve. The balloon was mounted on rigid tubing containing a high-fidelity micromanometer (Millar Instruments, Houston, TX). Pressure was recorded on a Gould series 8000 chart recorder (Gould Electronics, Hayward, CA) and digitized at 2-ms intervals with an SLM spectrofluorometer (see below). The LV balloon was filled to set the end-diastolic pressure (EDP) at 10 mm Hg. Hearts were paced at 5 Hz with a Grass Instruments SD-5 stimulator (Grass Instruments, Quincy, MA) by means of two platinum-tipped electrodes attached to the outflow tract of the right ventricle. Coronary flow was measured by collecting the heart effluent.

Fluorescence measurements

Fluorescence instrumentation

Fluorescence measurements were performed as previously described in detail (Brandes et al., 1992; Figueredo et al., 1992) using a modified SLM 48000S spectrofluorometer (SLM Instruments, Rochester, NY). Briefly, light from a 450-W xenon arc lamp, filtered through a 350-nm band-pass filter, was used for excitation. A quartz bifurcated fiber bundle was used to transmit excitation and emitted light as previously described (Figueredo et al., 1992). To minimize photobleaching of indo-1, a shutter in front of the excitation light was opened for 3–5 s to acquire each scan. Emitted light was split into two beams with a dichroic mirror, filtered at 385 and 456 nm with bandpass (± 10 nm) filters, and monitored by photomultiplier tubes. These emission wavelengths were chosen because they were previously found to be isosbestic with regard to tissue light absorption during ischemia (Brandes et al., 1994; Camacho et al., 1994) and to minimize motion artifact (Brandes et al., 1992).

Indo-1 loading

After a 15-min equilibration period, heart autofluorescence (background) was measured and subtracted from all subsequent fluorescence measurements. Hearts were then loaded with indo-1 by retrograde perfusion for 35 min with standard buffer that contained indo-1 AM (6 μ M; dissolved in dimethylsulfoxide and Pluronic F-127, 8% weight/volume) (Molecular Probes, Eugene, OR) and fetal calf serum (6%). Probenecid (0.1 mmol/liter) was added to all buffer solutions to slow the extrusion of indo-1 from the myocytes (Arkhammar et al., 1990). Residual indo-1 AM was washed out by perfusing with standard buffer (i.e., without indo-1) for 25 min. In these experiments, diastolic (trough) fluorescence after loading of indo-1 was 13.2 ± 0.7 and 7.8 ± 0.3 times greater than autofluorescence at λ_{385} and λ_{456} , respectively. If fluorescence intensity at either emission wavelengths decreased to less than twice autofluorescence during any phase of the experimental protocol (see below), the experiment was discarded.

Experimental protocols

After a 15-min equilibration period, background fluorescence and hemodynamic parameters were recorded. Thereafter, hearts were divided into five experimental groups.

Mn^{2+} quenching of cytosolic fluorescence

To determine the fraction of total fluorescence arising from the noncytosol after loading with indo-1 AM, cytosolic fluorescence was quenched with Mn^{2+} . Because large increases of $[Ca^{2+}]$ during the cardiac cycle occur only in the cytosol, quenching of cytosolic fluorescence was determined by elimination of fluorescence transients. Residual fluorescence was inferred to arise from the noncytosol. Quenching of fluorescence transients was accomplished by adding $MnCl_2$ (17.5 μ M) to the perfusate. One-second fluorescence scans were recorded at 2-min intervals until fluorescence transients disappeared.

To determine the rate of fluorescence decline due to both washout of indo-1 and potential quenching of noncytosolic fluorescence, Mn^{2+} infusion was continued in a subset of hearts for 20 min after fluorescence transients had disappeared. The rate of fluorescence decline after fluorescence transients disappeared was calculated and compared with the rate of fluorescence decline in the absence of Mn^{2+} (see below). This provided an estimate of the rate of at which noncytosolic fluorescence may be quenched during Mn^{2+} infusion.

Fluorescence decline over time in the absence of Mn^{2+}

To correct for fluorescence decline during the Mn^{2+} quenching protocol due to washout of indo-1 (photobleaching was minimal), fluorescence was monitored in a group of indo-1-loaded hearts in the absence of Mn^{2+} . In this group, 1-s fluorescence scans were recorded at 2-min intervals for 12 min, then at 5-min intervals for a total of 60 min. The rate of fluorescence decline was then calculated and used to correct the data obtained in the Mn^{2+} -quenched hearts, as described in the Calculations section, below.

Autofluorescence during Mn^{2+} infusion

To determine whether the Mn^{2+} quenching protocol changed background fluorescence, hearts not loaded with indo-1 were studied during the protocol described above.

Effects of high extracellular Ca^{2+}

To determine the effects of high extracellular $[Ca^{2+}]$ on cytosolic and noncytosolic $[Ca^{2+}]$, indo-1-loaded hearts were studied during perfusion, with high $[Ca^{2+}]$ in the perfusate (6.0 mM). After hearts were loaded with indo-1 using the control perfusate, baseline fluorescence and hemodynamic

measurements were taken. Hearts were then perfused with high extracellular [Ca²⁺] for 15 min followed by Mn²⁺ quenching of cytosolic fluorescence, as described above.

When hearts were perfused with high [Ca²⁺] in the perfusate, a higher [Mn²⁺] in the perfusate (67.5 μ M) was also needed to quench fluorescence transients in 10 min. This is most likely because Ca²⁺ and Mn²⁺ compete for entry into cardiac myocytes via L-type Ca²⁺ channels (Payet et al., 1980). Thus, to achieve the same concentration of Mn²⁺ in the cytosol in 10 min, a threefold increase in [Mn²⁺] (67.5 μ M vs. 17.5 μ M) was needed when the [Ca²⁺] in the perfusate was increased by threefold (6.0 mM vs. 2.0 mM). Although it is unknown whether these [Mn²⁺] inhibited the Ca²⁺ current, excitation-contraction coupling was not significantly affected, because LV pressure did not change in hearts not loaded with indo-1 (see Results for the effects of Mn²⁺ in hearts loaded with indo-1).

Effects of high extracellular Ca²⁺ in the presence of ruthenium red

We postulated that if noncytosolic fluorescence primarily arises in mitochondria, ruthenium red, a blocker of mitochondrial Ca²⁺ uptake (Moore, 1971; Gupta et al., 1989), should prevent the increase in noncytosolic [Ca²⁺] caused by high extracellular [Ca²⁺]. Thus experiments were performed as described above using high [Ca²⁺] (6.0 mM) and ruthenium red (25 nM; Sigma Chemical Co.; not purified further) in the perfusate.

Potential effects of ruthenium red on fluorescence measurements

Cuvette experiments were also done to confirm that ruthenium red would not spuriously affect the indo-1 data because of effects on light transmission. In perfusate containing 25 nM ruthenium red, light transmission at both emission wavelengths (385 and 456 nm) was unchanged compared to controls (i.e., no ruthenium red). Therefore, at this concentration, ruthenium red does not affect light transmission. Because ruthenium red may become concentrated in cells, these experiments were repeated with a concentration of ruthenium red 100 times higher. At this concentration of ruthenium red (2500 nM), light transmission was 0.80 of control at both emission wavelengths. Because the ratio of F_{385}/F_{456} was used to calculate [Ca²⁺]_c, even this concentration of ruthenium red would not affect the calculation of [Ca²⁺]_c, because emitted fluorescence would be reduced by the same fraction at both wavelengths.

To determine whether ruthenium red affected background fluorescence (i.e., autofluorescence), hearts not loaded with indo-1 were studied in the presence and absence of ruthenium red (25 nM). There was no change in background fluorescence at either emission wavelength (385 and 456) in the presence of ruthenium red.

Calculations

Fraction of noncytosolic fluorescence

Calculation of the noncytosolic contribution to total fluorescence was based on the concept that measurable [Ca²⁺] transients occur only in the cytosol (Spurgeon et al., 1990; Miyata et al., 1991). noncytosolic fluorescence was defined as the residual fluorescence, when transients were no longer evident, during Mn²⁺ infusion. Because it took ~10 min of Mn²⁺ infusion to quench fluorescence transients, data were corrected for the time-dependent decline of fluorescence that was independent of Mn²⁺ quenching. This time-dependent decline of fluorescence was describable by a monoexponential function (see Results). Therefore, the fraction of noncytosolic fluorescence at each emission wavelength (f_{λ}^N ; λ = 385 or 456 nm) was calculated by

$$f_{\lambda}^N \lambda = F_{\lambda}^N \lambda / F_{\lambda}^T \lambda * e^{-kt}, \quad (1)$$

where F_{λ}^N is the noncytosolic fluorescence, F_{λ}^T is the end-diastolic fluorescence just before Mn²⁺ infusion, k is the fractional rate of fluorescence decline in the absence of Mn²⁺, and t is the time to elimination of fluorescence transients (i.e., quenching of cytosolic fluorescence). In all equations presented in this paper, fluorescence (F) is measured fluorescence minus autofluorescence, which was determined in each heart before indo-1 loading.

Calculation of [Ca²⁺]_c

The ratio of cytosolic fluorescence at 385 nm (F_{385}^C) and 456 nm (F_{456}^C) was used to calculate [Ca²⁺]_c. This ratio (R^C) was calculated as follows:

$$F_{385}^C = F_{385}^T * (1 - f_{385}^N) \quad (2)$$

$$F_{456}^C = F_{456}^T - (1 - f_{456}^N) \quad (3)$$

$$R^C = F_{385}^C / F_{456}^C \quad (4)$$

[Ca²⁺]_c was calculated using the standard equation for fluorescent calcium indicators (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_c = K_d * \{(R^C - R_{min}) / (R_{max} - R^C)\} * (S_{456}), \quad (5)$$

where R_{min} and R_{max} are the fluorescence intensity ratios (F_{385}/F_{456}) at zero and saturating [Ca²⁺], respectively. K_d is the dissociation constant of indo-1 for calcium, and S_{456} is the ratio of fluorescence intensities during saturating and zero [Ca²⁺] at the 456-nm emission wavelength.

R_{max} and R_{min} were determined by an approach that has been described in detail (Brandes et al., 1993a) using the following equations:

$$R_{max} = S_R / bH \quad (6)$$

$$R_{min} = (R_{max})(S_{385}/S_{456}), \quad (7)$$

where S_{385} is the ratio of fluorescence intensities during saturating and zero [Ca²⁺] at the 385-nm emission wavelength; $S_R = (1 - S_{456}) / (1 - S_{385})$; and bH is the slope of the relationship between fluorescence intensities at 456 and 385 nm during a transient.

Because interaction with cell proteins alters indo-1 binding to calcium (K_d) and indo-1 fluorescence properties (S_{385} and S_{456}), these calibration parameters were determined using a protein solution prepared from heart homogenates. This solution matched indo-1 spectral parameters to those found in vivo (Baker et al., 1994). The K_d of indo-1 for calcium increased from 250 nM in a protein-free buffer solution to 594 nM in a solution containing 100 mg/ml of heart proteins. Because this is the estimated protein concentration in the cytosol (Hove-Madsen and Bers, 1992), a K_d of 594 nM was used to calculate [Ca²⁺]_c. At this protein concentration, S_{385} and S_{456} were 0.098 and 2.209, respectively.

Calculation of [Ca²⁺]_{nc}

The ratio F_{385}^N / F_{456}^N (R^N) was used to calculate [Ca²⁺]_{nc}, using the same equations and calibration parameters used to calculate [Ca²⁺]_c. An important assumption of this approach is that the calibration parameters are the same in the cytosolic and noncytosolic compartments. This assumption is based on previous studies suggesting that calibration parameters of fluorescent indicators are similar in the cytosol and mitochondria (Gunter et al., 1988; Miyata et al., 1991).

Statistical analysis

Results are presented as mean \pm SE. Exponential function was fit to data using commercially available software (SigmaPlot; Jandel Scientific, Corte Madera, CA). Comparisons between groups was performed using paired t -tests, with Bonferroni correction for multiple comparisons where appropriate. Comparisons of data at different time points were performed using

repeated measures analysis of variance (RMANOVA) with Tukey's post hoc test for multiple comparisons. Differences were considered significant when $p < 0.05$.

RESULTS

Mn²⁺ quenching of cytosolic fluorescence

Indo-1 fluorescence

To determine the fraction of total fluorescence arising from the noncytosol in whole hearts loaded with indo-1 AM, Mn²⁺ was used to quench fluorescence of indo-1 in the cytosol ($N = 17$). After loading of indo-1, transient increases of the fluorescence ratio were clearly evident during each cardiac cycle (Fig. 1). These cyclic changes of fluorescence, frequently referred to as "transients," report changes in $[Ca^{2+}]_c$ in the cytosol of myocytes during the cardiac cycle.

During Mn²⁺ infusion, F_{385} and F_{456} decreased significantly and fluorescence transients were no longer evident after 10 min of Mn²⁺ infusion (Fig. 1). However, there was significant residual fluorescence at both emission wavelengths after fluorescence transients were no longer evident. Fig. 2 shows the decrease in trough (i.e., diastolic) fluorescence at both emission wavelengths during Mn²⁺ infusion. The decrease in transient amplitude (i.e., peak minus

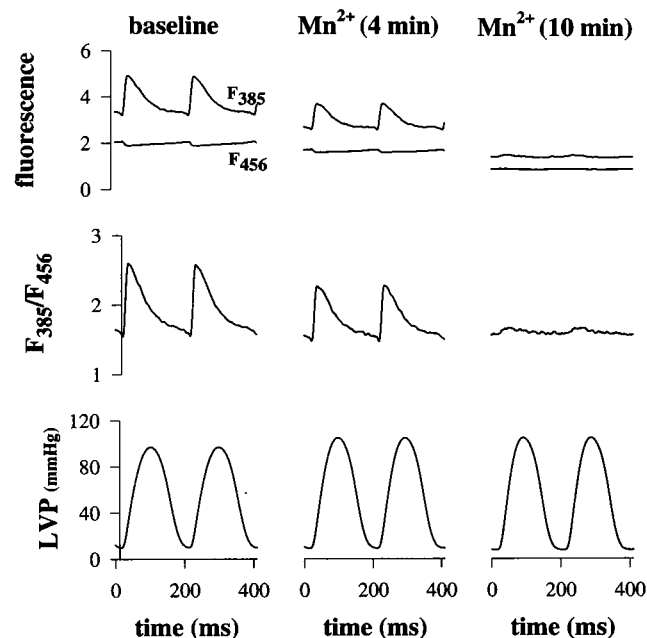


FIGURE 1 Indo-1 fluorescence and left ventricular pressure (LVP) during control, 4 min, and 10 min of Mn²⁺ infusion: single wavelength fluorescence (upper graphs), fluorescence ratio (middle graphs), and LVP (lower graphs). Note that indo-1 fluorescence transients were no longer evident at 10 min of Mn²⁺ infusion, but ~0.50 of control diastolic fluorescence remained at each wavelength. These data show that a significant fraction of indo-1 fluorescence in whole hearts loaded with indo-1 arises from noncytosolic compartments. LVP was slightly increased after Mn²⁺ quenching, most likely because of less buffering of $[Ca^{2+}]_c$ by indo-1.

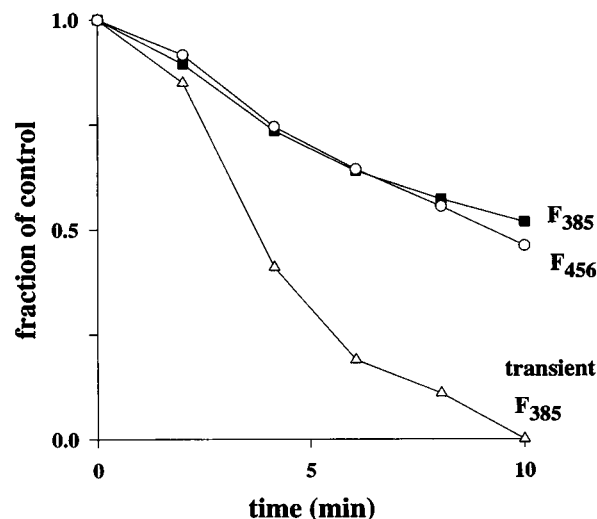


FIGURE 2 Data from a representative Mn²⁺ quenching experiment. Fluorescence data are presented as a fraction of control during Mn²⁺ infusion. Indo-1 fluorescence transients (peak-trough) were abolished at 10 min of Mn²⁺ perfusion, but residual fluorescence was 0.52 and 0.46 of control fluorescence λ_{385} and λ_{456} , respectively. This residual fluorescence at the time when fluorescence transients were abolished arises from noncytosolic compartments. Because Mn²⁺ quenching took 10 min, a correction for washout of indo-1 was determined as illustrated in Fig. 3.

trough) at λ_{385} is also shown. In this example, the transient amplitude was zero at 10 min of Mn²⁺ infusion. However, 0.52 and 0.46 of diastolic fluorescence remained at λ_{385} and λ_{456} , respectively.

In the entire group, mean time to quenching of the fluorescence transients was 10 ± 0.4 min. When fluorescence transients were quenched, residual fluorescence was 0.53 ± 0.02 and 0.49 ± 0.02 at λ_{385} and λ_{456} , respectively. These data show that a significant fraction of total fluorescence in whole hearts loaded with indo-1 AM arises from the noncytosol. However, these data do not take into account indo-1 washout during the time of Mn²⁺ infusion (see the following section).

Hemodynamics

Table 1 lists the hemodynamic parameters of hearts in this group. Indo-1 loading had no effect on coronary flow or LV end-diastolic pressure. In contrast, LV peak (i.e., systolic) pressure decreased from 136 ± 3 mm Hg before indo-1 loading to 105 ± 2 mm Hg after the indo-1 loading and

TABLE 1 Hemodynamics of hearts loaded with indo-1 AM in which cytosolic fluorescence was quenched by Mn²⁺ infusion

Parameter	Control (before indo-1)	Baseline (after indo-1)	10 min Mn ²⁺
Coronary flow (ml/min)	18 ± 0.2	19 ± 0.3	19 ± 0.3
End-diastolic pressure (mm Hg)	10 ± 0.2	11 ± 0.4	11 ± 0.4
Peak systolic pressure (mm Hg)	136 ± 3	$105 \pm 2^*$	$116 \pm 2^*$

$N = 17$; * $p < 0.05$ vs. control.

washout period. This 23% decrease in systolic pressure is similar to that observed in previous studies (Figueredo et al., 1992; Camacho et al., 1994) and is likely caused by buffering of Ca²⁺ by indo-1 (Camacho et al., 1994).

Fig. 1 shows that, during Mn²⁺ infusion, LV systolic pressure increased slightly, even though fluorescence transients were quenched (Fig. 1). This finding suggests that Mn²⁺ infusion did not adversely affect excitation-contraction coupling. The group data show that mean LV systolic pressure increased from 105 ± 2 to 116 ± 2 during Mn²⁺ infusion (Table 1). This 11% increase in LV systolic pressure was likely due to a decrease in Ca²⁺ buffering by indo-1, because this increase did not occur during Mn²⁺ infusion in hearts not loaded with indo-1. The group data also show that Mn²⁺ infusion had no effect on coronary flow or end-diastolic pressure (Table 1). Thus, Mn²⁺ did not affect coronary vascular resistance or diastolic properties of the heart.

Correction for fluorescence decline due to washout of indo-1

To account for the decrease in fluorescence during the 10 min of Mn²⁺ infusion, which was due to washout of indo-1, fluorescence was monitored in a group of hearts loaded with indo-1 but not exposed to Mn²⁺ ($N = 4$). A monotonic decrease in fluorescence was observed over time, as illustrated in Fig. 3. This decrease in fluorescence was most likely caused by washout of indo-1, because photobleaching was minimized (see Materials and Methods). The rate of this fluorescence decrease was the same at both emission wavelengths and could be fit by a monoexponential function with a rate constant (k) of $0.014 \pm 0.002 \text{ min}^{-1}$. This rate constant was used to correct the Mn²⁺ quenching data as described in Eq. 1. After correcting for this decrease in fluorescence caused by washout of indo-1, mean f_{385}^N was 0.61 ± 0.02 and f_{456}^N was 0.56 ± 0.02 .

Potential Mn²⁺ quenching of noncytosolic fluorescence

A possibility that must be considered is that Mn²⁺ infusion quenches the cytosol and noncytosol simultaneously at the same rate. If this were the case, the rate of fluorescence decline before and after transients were quenched would be the same. Therefore, in a group of hearts loaded with indo-1, Mn²⁺ infusion was continued for 20 min after transients had disappeared ($N = 4$). Fig. 3 shows a representative example illustrating that fluorescence decline was appreciably slower after transients were quenched, suggesting that the cytosol and noncytosol were not quenched simultaneously at the same rate. These data could be described by a biexponential function with two rate constants describing the rates of fluorescence decline before and after quenching of the transients. The mean rate constant of fluorescence decline for the group was significantly higher before than after tran-

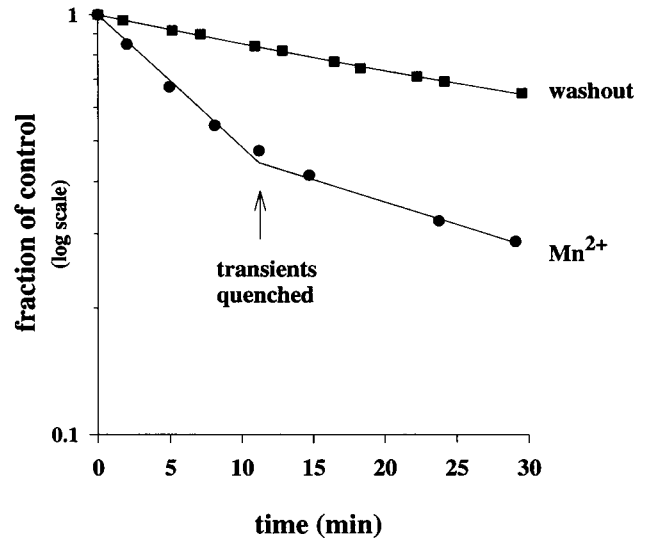


FIGURE 3 Data from an indo-1 washout experiment and a Mn²⁺ quenching experiment. Fluorescence at λ_{385} is presented as a fraction of control. To determine the rate of fluorescence decline due to washout of indo-1, fluorescence was monitored in hearts loaded with indo-1 in the absence of Mn²⁺ (*top tracing*). The decline of fluorescence could be fit by a monoexponential function (*solid line*) to determine the rate constant fluorescence decline (0.014 min^{-1} in this example). These data were then used to calculate the fraction of noncytosolic fluorescence as described in Eq. 1. To determine the combined effects of indo-1 washout and potential quenching of noncytosolic fluorescence by Mn²⁺, fluorescence was monitored in hearts loaded with indo-1 for 30 min of Mn²⁺ infusion (*bottom tracing*). Fluorescence transients were quenched at 10 min of Mn²⁺ infusion. The rate of fluorescence decline was slower after transients were quenched, suggesting that the cytosol and noncytosol were not quenched simultaneously at the same rate. These data could be fit by a biexponential function (*solid line*) to determine the rate constants of fluorescence decline before and after quenching of cytosolic fluorescence (0.073 and 0.025 min^{-1} , respectively, in this example).

sients were quenched (0.070 ± 0.006 and $0.031 \pm 0.006 \text{ min}^{-1}$, respectively; $p < 0.05$). These data suggest that Mn²⁺ preferentially gained access to the cytosol during the time that fluorescence transients were being quenched.

On the other hand, if there were no quenching of noncytosolic fluorescence during Mn²⁺ infusion, the rate of fluorescence decline after transients were quenched would be the same as in the absence of Mn²⁺ (i.e., due to washout of indo-1 as noted above). However, the rate constant of fluorescence decline after transients were quenched was greater than the rate constant in the absence of Mn²⁺ (0.031 ± 0.006 and 0.014 ± 0.002 , respectively; $p < 0.05$). Therefore, it is likely that after fluorescence transients were quenched, the decrease in fluorescence was due to a combination of indo-1 washout and some quenching of noncytosolic fluorescence. This implies that some quenching of noncytosolic fluorescence also occurred during the 10 min required to quench the fluorescence transients. Therefore, the rate of fluorescence decline after fluorescence transients were quenched was also used to calculate f_{385}^N and f_{456}^N (Eq. 1). The mean values of f_{385}^N and f_{456}^N calculated in this manner were 0.66 ± 0.02 and 0.60 ± 0.02 , respectively.

Autofluorescence during Mn^{2+} infusion

To determine whether the Mn^{2+} quenching protocol changed background fluorescence, hearts not loaded with indo-1 were studied during the Mn^{2+} quenching ($N = 5$). There was no change in background fluorescence at any point during the Mn^{2+} quenching protocol, so no correction for changes in autofluorescence was required.

Effect of accounting for noncytosolic fluorescence on the calculation of $[Ca^{2+}]_c$

Fig. 4 A illustrates the effect of accounting for noncytosolic fluorescence on fluorescence transients. The bold line is a transient of total fluorescence ratio (i.e., F_{385}^T/F_{456}^T), which was derived from indo-1 in both the cytosol and noncytosol. The light line is a transient of cytosolic fluorescence (F_{385}^C/F_{456}^C), which was calculated by subtracting noncytosolic fluorescence from total fluorescence at each wavelength before calculating the ratio (see Eqs. 2–4). The main effect of subtracting noncytosolic fluorescence was that systolic F_{385}^C/F_{456}^C was higher than systolic F_{385}^T/F_{456}^T .

Table 2 shows the effect of accounting for noncytosolic fluorescence on F_{385}/F_{456} in the group data. Mean systolic F_{385}^T/F_{456}^T was 2.704 ± 0.058 , and systolic F_{385}^C/F_{456}^C was 4.182 ± 0.209 ($p < 0.05$). This difference suggests that if F_{385}^T/F_{456}^T were used to calculate $[Ca^{2+}]_c$, systolic $[Ca^{2+}]_c$ would be significantly underestimated.

Fig. 4 B illustrates the effect of accounting for noncytosolic fluorescence on calculated $[Ca^{2+}]_c$, using the same data as shown in Fig. 4 A. Calibration of the total fluorescence transient yielded a systolic (peak) $[Ca^{2+}]_c$ of 300 nM and a diastolic (minimum) $[Ca^{2+}]_c$ of 130 nM. Calibration of the cytosolic fluorescence transient yielded a systolic $[Ca^{2+}]_c$ of 660 nM and a diastolic $[Ca^{2+}]_c$ of 100 nM. Therefore, if total fluorescence had been used to calculate $[Ca^{2+}]_c$, systolic $[Ca^{2+}]_c$ would have been underestimated

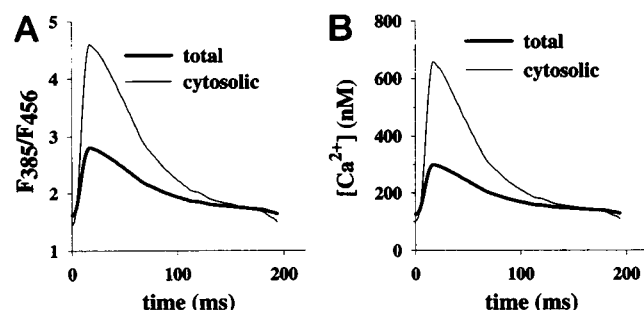


FIGURE 4 (A) Representative fluorescence ratio transient before and after correction for noncytosolic fluorescence (i.e., F_{385}^T/F_{456}^T and F_{385}^C/F_{456}^C , respectively). The main effect of subtracting noncytosolic fluorescence was that systolic (peak) F_{385}^C/F_{456}^C was much higher than systolic F_{385}^T/F_{456}^T . In contrast, diastolic (minimum) F_{385}^C/F_{456}^C was slightly higher than diastolic F_{385}^T/F_{456}^T . (B) $[Ca^{2+}]_c$ transients calculated from the fluorescence transients shown in A. These data illustrate that not accounting for noncytosolic fluorescence would result in underestimation of systolic $[Ca^{2+}]_c$ and overestimation of diastolic $[Ca^{2+}]_c$.

TABLE 2 Effect of accounting for noncytosolic fluorescence on F_{385}/F_{456} and $[Ca^{2+}]_c$

	Total (cytosolic and noncytosolic)	Cytosolic
F_{385}/F_{456} (intensity units)		
Systole	2.70 ± 0.06	$4.18 \pm 0.21^*$
Diastole	1.72 ± 0.03	$1.53 \pm 0.05^*$
$[Ca^{2+}]_c$ (nM)		
Systole	322 ± 15	$673 \pm 72^*$
Diastole	159 ± 8	$132 \pm 9^*$

$N = 17$; $*p < 0.05$ vs. total.

by 50% in this example. In contrast, diastolic $[Ca^{2+}]_c$ would have been overestimated by 30%.

Table 2 shows the effect of accounting for noncytosolic fluorescence on $[Ca^{2+}]_c$ transients in the group data. Mean systolic $[Ca^{2+}]_c$, calculated from total fluorescence, was only 322 ± 15 nM. However, mean systolic $[Ca^{2+}]_c$, calculated from cytosolic fluorescence, was 672 ± 72 nM, which is in good agreement with determinations of systolic $[Ca^{2+}]_c$ made with indo-1 free acid (Sollott et al., 1992; Backx and Ter Keurs, 1993). Likewise, mean diastolic $[Ca^{2+}]_c$, calculated from total fluorescence, was 159 ± 8 nM, which decreased to 132 ± 9 nM when cytosolic fluorescence was used. Therefore, not accounting for noncytosolic fluorescence would result in a 52% underestimation of systolic $[Ca^{2+}]_c$ and a 20% overestimation of diastolic $[Ca^{2+}]_c$. These data emphasize the importance of accounting for noncytosolic fluorescence when calculating $[Ca^{2+}]_c$ in whole hearts loaded with indo-1 AM.

$[Ca^{2+}]_{nc}$ in whole hearts

The ratio of F_{385}^N/F_{456}^N provides an index $[Ca^{2+}]_{nc}$ that is independent of indo-1 concentration. The mean F_{385}^N/F_{456}^N was 1.880 ± 0.040 , which is significantly greater than diastolic F_{385}^C/F_{456}^C , which was 1.526 ± 0.045 ($p < 0.05$). Assuming that the K_d of Ca^{2+} for indo-1 and the spectral parameters of indo-1 are similar in the cytosol and noncytosol, as suggested by previous investigators (Miyata et al., 1991), the finding that F_{385}^N/F_{456}^N is greater than diastolic F_{385}^C/F_{456}^C suggests that $[Ca^{2+}]_{nc}$ is higher than diastolic $[Ca^{2+}]_c$.

Calibration of F_{385}^N/F_{456}^N yielded a mean $[Ca^{2+}]_{nc}$ of 183 ± 36 nM, which was statistically greater than the mean diastolic $[Ca^{2+}]_c$ value of 132 ± 9 nM ($p < 0.05$). If noncytosolic fluorescence arises primarily from the mitochondria (see below), this finding suggests that mitochondrial $[Ca^{2+}]$ was slightly higher than diastolic $[Ca^{2+}]_c$ in these whole hearts paced at 5 Hz.

Effects of extracellular Ca^{2+}

To determine whether noncytosolic fluorescence arises primarily from the mitochondria, experiments were performed to determine whether high extracellular Ca^{2+} would in-

crease noncytosolic [Ca²⁺]_i and, if so, whether a blocker of mitochondrial Ca²⁺ uptake could prevent this increase. After 15 min of perfusion with high extracellular Ca²⁺ (i.e., perfusate [Ca²⁺]_o of 6.0 mM), cytosolic fluorescence was quenched with Mn²⁺. Calibration of F_{385}^N/F_{456}^N resulted in a mean [Ca²⁺]_{nc} of 272 ± 12 in these hearts ($N = 5$), which was significantly greater than the mean [Ca²⁺]_{nc} of 183 ± 36 in the control group perfused with a [Ca²⁺]_o of 2.0 mM ($p < 0.05$). This 49% increase in [Ca²⁺]_{nc} during high extracellular Ca²⁺ demonstrates that the noncytosolic compartment is sensitive to changes in extracellular [Ca²⁺].

To determine the effects of high extracellular Ca²⁺ on [Ca²⁺]_c, cytosolic fluorescence transients were calibrated after subtracting noncytosolic fluorescence. Mean systolic [Ca²⁺]_c was 1110 ± 107 nM, and mean diastolic [Ca²⁺]_c was 294 ± 14 nM, which were both significantly greater than the values observed in the control group (see Table 2) ($p < 0.05$). This increase in [Ca²⁺]_c was associated with a 39% increase in mean developed pressure from 93 ± 7 during control to 129 ± 8 mm Hg during high extracellular Ca²⁺. These data show that systolic [Ca²⁺]_c, diastolic [Ca²⁺]_c, and developed pressure are sensitive to changes in extracellular [Ca²⁺].

We postulated that if noncytosolic fluorescence primarily reports mitochondrial [Ca²⁺], ruthenium red, a blocker of Ca²⁺ uptake by mitochondria (Moore, 1971; Gupta et al., 1989), should inhibit the increase in [Ca²⁺]_{nc} during high extracellular [Ca²⁺]_o without affecting the increase in [Ca²⁺]_c. Ruthenium red had no significant effect on [Ca²⁺]_{nc} or [Ca²⁺]_c or developed pressure during perfusion with control [Ca²⁺]_o in the perfusate (2.0 mM). After 15 min of perfusion with high [Ca²⁺]_o in the perfusate (6.0 mM) and ruthenium red, mean [Ca²⁺]_{nc} was 215 ± 15 nM ($N = 5$). This was significantly lower than the mean [Ca²⁺]_{nc} of 272 ± 12 determined with high [Ca²⁺]_o in the perfusate and no ruthenium red ($p < 0.05$).

In contrast, ruthenium red did not inhibit the increase in systolic and diastolic [Ca²⁺]_c with high extracellular [Ca²⁺]_o. After 15 min of high [Ca²⁺]_o and ruthenium red in the perfusate, systolic and diastolic [Ca²⁺]_c were 1059 ± 28 and 268 ± 13 nM, respectively. These values were not significantly different from those observed in the absence of ruthenium red (1110 ± 107 and 294 ± 14 nM, respectively). Furthermore, ruthenium red did not inhibit the increase in developed pressure to 138 ± 2 mm Hg.

These data demonstrate that ruthenium red prevented the increase in [Ca²⁺]_{nc} caused by high extracellular [Ca²⁺]_o without affecting the increase in [Ca²⁺]_c. Because ruthenium red blocks the uptake of Ca²⁺ by mitochondria (Moore, 1971; Gupta et al., 1989), these data strongly suggest that [Ca²⁺]_{nc} provides a measure of mitochondrial [Ca²⁺].

DISCUSSION

The first major finding of this study was that a large fraction of total fluorescence in whole hearts loaded with indo-1 AM

arises from the noncytosol (0.61 ± 0.06 at λ_{385} and 0.56 ± 0.07 at λ_{456}). Thus, if noncytosolic fluorescence had not been taken into account when calculating [Ca²⁺]_c, systolic [Ca²⁺]_c would have been underestimated by 52%. More accurate determinations of [Ca²⁺]_c can be made in whole hearts loaded with indo-1 AM by accounting for noncytosolic fluorescence using Mn²⁺ quenching.

The second major finding of this study was that [Ca²⁺]_{nc} may provide an estimate of mitochondrial [Ca²⁺] in whole hearts. High extracellular [Ca²⁺]_o caused a 49% increase in [Ca²⁺]_{nc} that was inhibited by ruthenium red, a blocker of Ca²⁺ uptake by mitochondria (Moore, 1971; Gupta et al., 1989). In contrast, ruthenium red did not prevent the increase in [Ca²⁺]_c or developed pressure that occurred during perfusion with high extracellular [Ca²⁺]_o. These data suggest that [Ca²⁺]_{nc} provides a useful method for estimating mitochondrial [Ca²⁺] in whole hearts.

Mn²⁺ quenching to differentiate cytosolic from noncytosolic fluorescence

A limitation of using indo-1 AM to assess [Ca²⁺]_c is that a significant fraction of the dye may load into noncytosolic compartments. To circumvent this limitation, other investigators (Spurgeon et al., 1990; Miyata et al., 1991, 1992) described a method for determining the fraction of noncytosolic fluorescence by using Mn²⁺ quenching. These studies were performed in isolated cardiac myocytes at room temperature (23°C). The current study extends this approach to whole hearts studied at physiological temperature (37°C).

Mn²⁺ infusion can be used to quench cytosolic fluorescence because Mn²⁺ crosses the sarcolemma and enters the cytosol, most likely via L-type Ca²⁺ channels (Payet et al., 1980; Hunter et al., 1981). Uptake of Mn²⁺ has been demonstrated in isolated rat hearts using radioactive manganese at a concentration of Mn²⁺ (5 μ M) similar to that used in the current study (Hunter et al., 1981). This uptake was blocked by verapamil and stimulated by isoproterenol, suggesting that the uptake of Mn²⁺ was via L-type Ca²⁺ channels. More recently, similar observations were made in isolated cardiac myocytes (Haworth et al., 1989). Once Mn²⁺ gains access to the cytosol, it binds to indo-1 with a 20-fold greater affinity than that of Ca²⁺ (Miyata et al., 1991). Binding of Mn²⁺ to indo-1 quenches indo-1 fluorescence (Grynkiewicz et al., 1985). Therefore, loss of fluorescence transients during Mn²⁺ infusion was taken as evidence that cytosolic fluorescence was quenched. The residual fluorescence was inferred to be from indo-1 in noncytosolic compartments.

The major difference in the Mn²⁺ quenching protocol used in the current experiments and previous studies in cardiac myocytes (Miyata et al., 1991) was the concentration of Mn²⁺ used to quench cytosolic fluorescence. In the current experiments, we infused 17.5 μ M Mn²⁺ via the coronary vasculature; in contrast, Miyata et al. (1991) su-

perfused 100 μM Mn^{2+} over isolated cardiac myocytes. Despite the lower concentration of Mn^{2+} used in our experiments, the time to complete quenching of fluorescence transients was shorter (10 ± 0.4 vs. 28 ± 9 min). These differences suggest that in our experiments, Mn^{2+} influx into the cytosol was facilitated by the higher temperature (37°C vs. 23°C) and pacing frequency (5 Hz vs. 0.2 Hz).

Fraction of noncytosolic fluorescence (f^{N}) estimated by Mn^{2+} quenching

Despite major differences in experimental preparation, the fraction of noncytosolic fluorescence (f^{N}) in the current study of whole hearts is similar to that previously reported in isolated cardiac myocytes (Miyata et al., 1991). Before correcting for time-dependent fluorescence decline during Mn^{2+} quenching, we found that noncytosolic fluorescence was 0.53 ± 0.02 and 0.49 ± 0.02 of total fluorescence at λ_{385} and λ_{456} , respectively. These values were not significantly different when hearts were loaded with indo-1 at room temperature ($N = 5$, data not shown). This is in good agreement with the results of Miyata et al. (1991), who found that residual fluorescence was 0.47 ± 0.03 and 0.42 ± 0.03 at λ_{410} and λ_{490} , respectively, in cardiac myocytes studied at room temperature.

However, these values for noncytosolic fluorescence do not take into account the decrease in fluorescence caused by indo-1 washout during Mn^{2+} infusion. Therefore, we determined the rate of fluorescence decline in the absence of Mn^{2+} and used this rate to correct the measurement of noncytosolic fluorescence (Eq. 1). However, this correction was small because the rate of decline in the absence of Mn^{2+} was much slower than the rate of fluorescence decline during Mn^{2+} quenching of cytosolic transients. After this correction, noncytosolic fluorescence was calculated to be 0.61 ± 0.06 at λ_{385} and 0.56 ± 0.07 at λ_{456} in this study.

The decline in fluorescence during the 10 min required to quench cytosolic transients may also be due to Mn^{2+} quenching of indo-1 in the noncytosol (e.g., mitochondria) and/or more rapid washout of indo-1 in the presence Mn^{2+} (e.g., Mn^{2+} might block the effects of probenecid that retard washout of indo-1). Therefore, correcting for indo-1 washout in the absence of Mn^{2+} would result in underestimation of the fraction of noncytosolic fluorescence. Experiments were thus performed to determine the rate of fluorescence decline during continued Mn^{2+} infusion after transients were quenched. Using this rate to correct the measurement of noncytosolic fluorescence should account for both washout of indo-1 and Mn^{2+} quenching of the noncytosol. However, this resulted in a relatively small increase in the calculated fraction of noncytosolic fluorescence to 0.66 at λ_{385} and 0.60 at λ_{456} , which translates into an 8% increase in diastolic $[\text{Ca}^{2+}]_{\text{c}}$.

Furthermore, this is a worst-case scenario. Because of the high affinity of Mn^{2+} for indo-1 ($K_{\text{d}} \approx 10$ nM) (Miyata et al., 1991), it is likely that free $[\text{Mn}^{2+}]$ in the cytosol was low

during the time of Mn^{2+} infusion when transients were being quenched. Thus mitochondrial uptake of Mn^{2+} was likely to be minimal during this time. Once the transients were quenched, all of the indo-1 in the cytosol was bound to Mn^{2+} , and continued Mn^{2+} infusion would increase free $[\text{Mn}^{2+}]$. This would result in an increase in the rate of Mn^{2+} uptake by mitochondria. Therefore, it is possible that the rate of fluorescence decline during continued Mn^{2+} infusion (i.e., after transients were quenched) overestimated the rate of noncytosolic quenching during the time cytosolic transients were being quenched. For this reason, the rate of fluorescence decline in the absence of Mn^{2+} was used to calculate the fraction of noncytosolic fluorescence. As noted above, even in a worst-case scenario, this would result in only an 8% underestimation of diastolic $[\text{Ca}^{2+}]_{\text{c}}$.

A limitation of the current study is that the concentrations of Mn^{2+} and indo-1 in the cytosol and mitochondria could not be directly measured. Therefore, quenching of fluorescence transients, which report beat-to-beat changes in cytosolic Ca^{2+} , was used to indicate whether cytosolic indo-1 had been quenched. A possibility to be considered is that fluorescence transients were abolished, despite incomplete quenching of cytosolic indo-1. However, previous investigators have shown that when the sarcolemma of cardiac myocytes was permeabilized after fluorescence transients were abolished by Mn^{2+} , the residual fluorescence did not decrease further (Miyata et al., 1991). This suggests that after transients are abolished by Mn^{2+} , there are no significant amounts of unquenched indo-1 in the cytosol; thus, loss of fluorescence transients appears to be a reasonable indicator of quenching of indo-1 in the cytosol.

Another possibility to be considered is that indo-1 in nonmyocytes may contribute to total fluorescence in whole hearts loaded with indo-1 AM. Other investigators have demonstrated that a significant portion of total fluorescence in rabbit hearts loaded with indo-1 AM arises from bradykinin-sensitive compartments, most likely endothelial cells (Lorell et al., 1990). However, this is not the case in rat hearts loaded with indo-1 AM (Wikman-Coffelt et al., 1991; Figueredo et al., 1993). Furthermore, confocal microscopy of tissue sections from rat hearts loaded with indo-1 AM showed no evidence of significant endothelial cell loading (Figueredo et al., 1993). Therefore, it is unlikely that a significant fraction of total fluorescence arises from nonmyocytes in rat hearts loaded with indo-1 AM.

Fraction of noncytosolic fluorescence (f^{N}) estimated by other methods

Other investigators, using other experimental approaches in different species, have estimated the fraction of noncytosolic fluorescence after loading the AM form of indo-1. Using techniques to assess intracellular diffusion and binding of indo-1, Blatter and Wier (1990) estimated the fraction of noncytosolic fluorescence to be 0.35 in isolated guinea pig myocytes. Using differential centrifugation of whole heart

homogenates, another group (Lee et al., 1988) estimated the fraction of noncytosolic fluorescence to be 0.28 in rabbits. The lower values reported in these studies may be due to methodological differences in the determination of noncytosolic fluorescence. However, there may also be species differences in esterase activity and/or cell volume occupied by organelles likely to load indo-1 (e.g., mitochondria).

Effect of noncytosolic fluorescence on the calculation of [Ca²⁺]_c

A major finding of this study was that using total fluorescence (which includes noncytosolic fluorescence) to calculate [Ca²⁺]_c results in a 52% underestimation of systolic [Ca²⁺]_c and a 20% overestimation of diastolic [Ca²⁺]_c. This finding is in agreement with Backx and ter Keurs (1993), who reported that mean systolic [Ca²⁺]_c was 38% lower in a group of muscle strips loaded with fura-2 AM compared to a group microinjected with the free-acid form. Furthermore, mean systolic and diastolic [Ca²⁺]_c (673 ± 72 and 132 ± 9, respectively) obtained in the current study are in good agreement with literature values obtained with a variety of indicators (Perreault et al., 1992; Sollott et al., 1992; Backx and Ter Keurs, 1993).

[Ca²⁺]_{nc} as an index of mitochondrial [Ca²⁺]

In addition to affecting the calculation of [Ca²⁺]_c, noncytosolic fluorescence may also provide an estimate of mitochondrial [Ca²⁺]. Miyata et al. (1991) provided strong evidence that in isolated cardiac myocytes, noncytosolic fluorescence arises predominantly from mitochondria and can be used to assess mitochondrial [Ca²⁺]. These investigators found that when [Ca²⁺]_c was increased by decreasing the bathing Na²⁺, there was an increase in the noncytosolic fluorescence ratio (F_{410}^N/F_{490}^N), suggesting an increase in mitochondrial [Ca²⁺]. When this experiment was repeated in the presence of ruthenium red, an inhibitor of Ca²⁺ uptake by mitochondria, there was no increase in F_{410}^N/F_{490}^N . These findings suggest that noncytosolic fluorescence can be used to calculate mitochondrial [Ca²⁺] in isolated cardiac myocytes.

Because indo-1 fluorescence in whole hearts loaded with indo-1 AM arises predominantly from cardiac myocytes, it may be possible to estimate mitochondrial [Ca²⁺] in whole hearts using noncytosolic fluorescence (F_{385}^N/F_{456}^N). Assuming this to be the case, our findings suggest that mitochondrial [Ca²⁺], estimated from [Ca²⁺]_{nc}, was 183 ± 36 nM in whole hearts paced at 5 Hz and 37°C. Given the differences in the experimental preparations and temperature, this value is in reasonable agreement with that of Miyata et al. (1991), who reported that mitochondrial [Ca²⁺] was ~200 nM in myocytes paced at 3 Hz and 23°C. Our results are also in excellent agreement with those of Allen et al. (1992), who reported a mean [Ca²⁺] of 172 ± 23 nM in isolated mitochondria that were loaded during whole heart perfusion with

fura-2 AM. In addition, our data suggest that mitochondrial [Ca²⁺] was slightly higher than diastolic [Ca²⁺]_c (183 ± 36 vs. 132 ± 9 nM; $p < 0.05$) under our experimental conditions.

It is important to emphasize that these estimates of mitochondrial [Ca²⁺] assume that the calibration parameters of indo-1 are similar in the cytosol and mitochondria. Although it is not technically feasible to test this assumption in whole hearts, other investigators have found that calibration parameters of fluorescent indicators, including indo-1, are similar in the cytosol and mitochondria (Gunter et al., 1988; Miyata et al., 1991).

To provide experimental support for the idea that [Ca²⁺]_{nc} may be used as an index of mitochondrial [Ca²⁺], the effects of high extracellular Ca²⁺ on [Ca²⁺]_{nc} and [Ca²⁺]_c were determined in the presence and absence of ruthenium red, a blocker of Ca²⁺ uptake by mitochondria (Moore, 1971; Gupta et al., 1989). During high extracellular Ca²⁺, [Ca²⁺]_{nc} was 49% higher compared to control extracellular Ca²⁺. Furthermore, the expected increase in [Ca²⁺]_c and left ventricular developed pressure was observed. In the presence of ruthenium red, the increase in [Ca²⁺]_{nc} during high extracellular [Ca²⁺] was inhibited, even though the expected increase in [Ca²⁺]_c and developed pressure was still observed. These data provide strong evidence that [Ca²⁺]_{nc} provides an index of mitochondrial [Ca²⁺] in whole hearts.

The possibility that ruthenium red may have affected [Ca²⁺]_c, by inhibiting the release of Ca²⁺ from the sarcoplasmic reticulum, should also be considered. This is unlikely for two reasons. First, ruthenium red did not affect developed pressure. Second, ruthenium red did not affect the indo-1 transient, and we previously demonstrated that the indo-1 transient is sensitive to small changes in [Ca²⁺]_c (Figueredo et al., 1992).

CONCLUSIONS

This study shows that a large fraction of total fluorescence in whole hearts loaded with indo-1 AM arises from the noncytosolic compartments. These compartments do not have measurable fluorescence transients during the cardiac cycle. Therefore, if noncytosolic fluorescence is not taken into account when calculating [Ca²⁺]_c, systolic (peak) [Ca²⁺]_c during a transient will be underestimated by 52%. However, [Ca²⁺]_c can be calculated in whole hearts loaded with indo-1 AM by determining the fraction of noncytosolic fluorescence at each emission wavelength using Mn²⁺ quenching. Furthermore, because noncytosolic fluorescence arises mainly from mitochondria, [Ca²⁺]_{nc} provides a measure of mitochondrial [Ca²⁺] in whole hearts.

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