II. Investigation of Factors Affecting Fluorometric Quantitation of Cytosolic [Ca^{2+}] in Perfused Hearts

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ABSTRACT The goal of these studies was to examine the effects of several factors that may artifactually influence quantitation of cytosolic [Ca^{2+}], $[Ca^{2+}]_c$, while using the fluorescent calcium indicator Indo-1. The following factors were investigated: 1) a possible fluorescence contribution from unhydrolized Indo-1/AM (by Mn^{2+} quenching), 2) Ca^{2+} buffering by Indo-1 (by varying [Indo-1]), 3) endothelial and mitochondrial Indo-1 loading (by bradykinin stimulation and calculations), and 4) effects of changing tissue fluorescence (predominantly NAD(P)H) on calculated $[Ca^{2+}]_c$ during hypoxia (by a new method which allowed simultaneous determination of $[Ca^{2+}]_c$ and changes in [NAD(P)H]). No significant contribution of Indo-1/AM was found. With increasing [Indo-1], calculated systolic $[Ca^{2+}]_c$ fell significantly. Indo-1 incorporation (<18%) into endothelial cells, caused a slight underestimation of systolic $[Ca^{2+}]_c$, while mitochondrial Indo-1 loading may cause overestimation of $[Ca^{2+}]_c$. With increased tissue fluorescence, during hypoxia, systolic $[Ca^{2+}]_c$ may be underestimated by ~27% (for Indo-1 loading factors three to five times original tissue fluorescence). These studies suggest conditions in which experimental artifacts could be minimized to allow reliable quantitation of $[Ca^{2+}]_c$ in intact perfused hearts using Indo-1 fluorometry. The major problem of obtaining reliable results depended on the ability to correct for changing NAD(P)H fluorescence while keeping [Indo-1] low.

GLOSSARY

Miscellaneous

λ	detection wavelength
g_λ	gain factor; depends on instrumental gain and tissue light absorbance at wavelength λ
K_d	Indo-1 dissociation constant for calcium
L_λ	loading factor (ratio between fluorescence intensities after Indo-1 loading and prior to loading)

Concentration, etc.

$[Ca^{2+}]_c$	cytosolic calcium concentration
$[Ca^{2+}]_m$	"noncytosolic" calcium concentration (e.g., concentration in mitochondria or endothelium)
$[Ca^{2+}]_{dia}$	cytosolic calcium concentration during diastole
$[Ca^{2+}]_{sys}$	cytosolic calcium concentration during systole
$n(\text{Indo})_c$	amount of cytosolic Indo-1 (e.g., units of moles)
$n(\text{Indo})_m$	amount of "noncytosolic" Indo-1 (e.g., mitochondrial or endothelial Indo-1; units of moles)
f_m	relative fraction of "noncytosolic" Indo-1

Intensities

Background intensities (Ca^{2+}-insensitive) and related terms

B_λ^N	background fluorescence intensity arising from NAD(P)H
B_λ^x	background fluorescence intensity arising from instrumentation, flavoproteins etc. ("non-NAD(P)H")
rB_λ^x	relative contribution of non-NAD(P)H fluorescence to total background fluorescence
$B_\lambda(\text{bl})$	background fluorescence intensity prior to Indo-1 loading ($= B_\lambda^N + B_\lambda^x$)
B_λ^{AM}	background fluorescence intensity arising from unhydrolized Indo-1/AM
$B_\lambda(\text{al})$	background fluorescence intensity after Indo-1 loading ($= B_\lambda(\text{bl}) + B_\lambda^{AM}$)
ΔN	fractional increase in [NAD(P)H] during hypoxia
ΔB_λ	fractional increase in background fluorescence intensity during hypoxia
κ	proportionality factor relating ΔB_{385} to ΔB_{456}

Indo-1 intensities (Ca^{2+}-sensitive) and related terms

I_λ^c	"standard" Indo-1 fluorescence intensity of cytosolic Indo-1 (per moles of Indo-1 and gain unit (Grynkiewicz et al., 1985))
I_λ^m	"standard" Indo-1 fluorescence intensity of noncytosolic Indo-1 (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 according to Eq. 2)
F'^{c}_λ	contribution to F_λ arising from cytosolic Indo-1 fluorescence
F'^{m}_λ	contribution to F_λ arising from noncytosolic Indo-1 fluorescence
F'^{min}_λ	detected Indo-1 fluorescence intensity, after background subtraction, in the absence of Ca^{2+}
F'^{max}_λ	detected Indo-1 fluorescence intensity, after background subtraction, with saturating [Ca^{2+}]
R	ratio of detected Indo-1 fluorescence intensities (after background subtraction)

Calibration constants

S_λ	calibration constant: Ratio between Indo-1 fluorescence intensities at a single wavelength (See Eq.6)

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bH	"gain-adjuster": slope of relationship F_{456} vs. F_{385} (used to correct for tissue light absorbance)
aH	intercept of relationship F_{456} vs. F_{385}
aH'	intercept of relationship F_{456} vs. F_{385} after background subtraction

INTRODUCTION

In a previous study, a new method to quantify cytosolic $[Ca^{2+}]$, $[Ca^{2+}]_c$ in whole perfused hearts using Indo-1 fluorometry was presented (Brandes et al., 1993). The calculated values in diastole (~187 nM) and systole (~464 nM) were obtained while assuming that several possible artifacts were insignificant: 1) unhydrolized Indo-1/AM was assumed to be negligible, 2) effects of Ca^{2+} buffering by Indo-1 was not investigated, but was minimized by using hearts with low Indo-1 loading, and 3) Indo-1 loading into an intracellular compartment such as mitochondria (Miyata et al., 1991) or endothelial cells (Lorell et al., 1990) ("noncytosolic" Indo-1) was assumed to be negligible. Furthermore, quantitation was not attempted during hypoxia during which tissue autofluorescence (background fluorescence) increase, mainly due to increased [NAD(P)H] (Chance et al., 1965). Such increased background fluorescence has previously been neglected during hypoxia or ischemia when calculating the Indo-1 fluorescence ratio (Lee et al., 1988), although it is expected to underestimate the ratio (and $[Ca^{2+}]_c$) for low Indo-1 loading.

Therefore, the goal of this study was twofold: first, to develop methods of quantifying several potential artifacts that may affect the calculated $[Ca^{2+}]_c$. Specifically, residual Indo-1/AM fluorescence, Ca^{2+} buffering and noncytosolic Indo-1 loading. Second, to develop methods to quantify $[Ca^{2+}]_c$ during hypoxia by accounting for the increase in background fluorescence during hypoxia. This increased background fluorescence was measured both in the absence of Indo-1, and in its presence by using a novel method which made it possible to measure changes in NAD(P)H fluorescence simultaneously with changes in Indo-1 fluorescence. This new method could be useful to study the simultaneous effects of hypoxia and ischemia on $[Ca^{2+}]_c$ and [NAD(P)H] (Sick et al., 1989; Fralix et al., 1991).

Experimental approach

The following factors, which were believed to affect quantitation of native $[Ca^{2+}]_c$, were investigated.

Background fluorescence

Background fluorescence, insensitive to calcium and manganese, may arise from NAD(P)H, other intracellular constituents, instrumental autofluorescence, and possibly unhydrolized Indo-1/AM. The sum of background fluorescence intensities (at each detection wavelength) were first measured prior to Indo-1 loading, and any additional background fluorescence from unhydrolized Indo-1/AM was assessed by subsequent quenching of Indo-1 (free acid) fluorescence by manganese. To obtain accurate Indo-1 fluorescence ratios, background fluorescence intensities were subtracted from the total measured fluorescence intensities prior to calculating the ratio (and $[Ca^{2+}]_c$). However, quantitation could be complicated by a change in background fluorescence during hypoxia due to increased [NAD(P)H] after Indo-1 loading (Koretsky et al., 1987; Sick et al., 1989) (see below).

Ca^{2+}-buffering

Increased indicator concentration has been shown to decrease $[Ca^{2+}]_c$ because of increased Ca^{2+} buffering in isolated preparations such as myocytes and trabeculae (Baylor et al., 1988; Noble et al., 1991; Sollot et al., 1992; Backx et al., 1993). In this study, Ca^{2+} buffering was investigated in intact hearts by determining the relationship between developed pressure and Indo-1 loading, and between calculated $[Ca^{2+}]_c$ and Indo-1 loading.

Noncytosolic or nonmyocyte loading of Indo-1

Because cell permeable Indo-1/AM was used to load the hearts, it is possible that a fraction of Indo-1 was located in a noncytosolic intracellular compartment such as mitochondria (Miyata et al., 1991) or endothelial cells (Lorell et al., 1990). On the time scale of a heart beat, such noncytosolic Indo-1 would contribute a constant Indo-1 background signal to the total time-varying fluorescence intensity. Indo-1 loading into endothelial cells was evaluated using bradykinin (an endothelial cell stimulator), and the effects of a constant Indo-1 background signal on the determination of $[Ca^{2+}]_c$ was calculated.

Changes in background fluorescence

The magnitude of changes in background fluorescence was measured in two ways: First, the increase in background fluorescence during hypoxia was measured prior to Indo-1 loading. Second, a new method was developed to measure this increase in the presence of Indo-1. Effects of the measured change in background fluorescence on the fluorescence ratio was subsequently calculated as a function of Indo-1 loading.

MATERIALS AND METHODS

Heart perfusion and spectroscopic methods

Male Sprague-Dawley rats (450–550 g), $n = 33$, were excised and perfused by the isovolumic Langendorf method as described previously (Brandes et al., 1993). During normoxia, the standard Krebs-Heinseleit perfusate (Brandes et al., 1993) was equilibrated at 37°C with a 95% O_2/5% CO_2 gas mixture, and during "total" hypoxic insults, the hearts were switched to a second perfusion system containing standard perfusate equilibrated at 37°C with a 95% N_2/5% CO_2 gas mixture. Milder hypoxia was also produced by low flow ischemia, by reducing the flow to 10 and 20% of control flow (at 71 mm Hg) using an in-line valve. Flow rates were measured by collecting effluent perfusate over a fixed period of time. Unless stated, all hypoxic and low flow measurements were performed after a 3-min equilibration period. This time period was chosen based on stable readings of background fluorescence (partly caused by NAD(P)H) and developed pressure.

Indo-1 loading into hearts

Hearts were loaded with Indo-1 for 20–45 min as described previously (Brandes et al., 1993). The loading time was varied to obtain various levels of Indo-1 loading.

Saturating C perfusate

Saturating intracellular calcium conditions were achieved using perfusate containing 80 mM $CaCl_2$ (Brandes et al., 1993).

Indo-1 quenching M perfusate

To quench Indo-1 (but not Indo-1/AM), two different Indo-1 quenching perfusates were prepared using either: (*a*) a N_2-containing buffer, identical to the saturating Ca^{2+} perfusate, except that 80 mM $CaCl_2$ was replaced by 80 mM $MnCl_2$ or (*b*) standard (O_2-containing) perfusate, where $[Mn^{2+}]$ was gradually increased (over ~30–60 min) until no further reduction in fluorescence intensity was observed (final concentration was different for different hearts, but ≤10 mM). In one of the three hearts, using this latter protocol, 2 μM ionomycin was added to the Mn^{2+} perfusate at the final $[Mn^{2+}] = 1$ mM.

Bradykinin perfusate

To determine the possibility of Indo-1 loading into endothelial cells the fluorescence response to perfusion with standard perfusate containing 10 μM bradykinin was investigated.

Spectroscopic methods

A modified SLM 48000S spectrofluorometer (Urbana, IL) was used for all measurements, and heart fluorescence was measured using a bifurcated silica fiber optic bundle with its common leg positioned close to the heart (Brandes et al., 1993). The fluorescence emission was detected 385 and 456 nm, since tissue light transmission is independent of tissue oxygenation state at these two isosbestic wavelengths (Brandes et al., 1991; Fralix et al., 1990)

Statistical analysis

Values are reported as means ± standard error of the measurement (SEM). Statistical analysis were performed using Student's *t* test (paired where applicable), and differences were considered significant when $p < 0.05$.

THEORY AND METHODS

Measurement of background (Ca^{2+}-insensitive) fluorescence

To determine if it would suffice to correct Indo-1 fluorescence intensities, at wavelength λ, by subtraction of the background intensities obtained before loading, the background fluorescence was determined prior to Indo-1 loading: B_λ(bl), and after Indo-1 loading: B_λ(al). B_λ(bl) is composed of the sum of NAD(P)H fluorescence and other background fluorescence (e.g., instrumental autofluorescence, flavoproteins etc.). After Indo-1 loading, additional background fluorescence may arise from unhydrolized Indo-1/AM. B_λ(bl) was measured by a short (~1 s) time average (to minimize NAD(P)H bleaching), and B_λ(al) was measured similarly following quenching of cytosolic (and presumably any noncytosolic) Indo-1 fluorescence, using the quenching Mn^{2+} perfusate (see above). When the N_2-containing Mn^{2+} perfusate was used, an additional in increase background fluorescence was expected from increased [NAD(P)H]. The contribution of unhydrolized Indo-1/AM, B_λ^{AM}, to the background intensities after Indo-1 loading is then given by:

$$B_\lambda^{AM} = B_\lambda(\text{al}) - B_\lambda(\text{bl}) - \Delta B_\lambda \cdot B_\lambda(\text{bl}), \quad (1)$$

where ΔB_λ is the fractional increase in background fluorescence during application of the N_2-containing Mn^{2+} perfusate, and was determined in parallel experiments using hearts without Indo-1.

Factors that may influence calculated $[Ca^{2+}]_c$

Factors that may influence the calculated $[Ca^{2+}]_c$ include the intracellular value of the Indo-1 dissociation constant for Ca^{2+}, K_d, Indo-1 Ca^{2+} buffering, and a constant Indo-1 background signal from noncytosolic Indo-1 (in an intracellular compartment or endothelial cells). Increased [Indo-1] may cause a reduction in the cytosolic calcium concentration and thereby suppress mechanical function. Any fluorescence intensity arising from noncytosolic Indo-1 could spuriously affect calculated $[Ca^{2+}]_c$ unless independently measured and subtracted from the measured fluorescence intensities before calculating the fluorescence ratio. If the "noncytosol" contains a lower $[Ca^{2+}]$ than the cytosol (at any time in the heart cycle), $[Ca^{2+}]_c$ would be underestimated and vice versa.

Unless indicated, $[Ca^{2+}]_c$ was calculated using methods described previously (Brandes et al., 1993) (see also Appendix).

Effects of Indo-1 loading

To assess the cytosolic Indo-1 concentration, the loading factors, L_λ (defined as the ratio between fluorescence intensities after and before Indo-1 loading), were calculated. Before loading with Indo-1, the fluorescence intensity is equal to the sum of background intensities, B_λ(bl), and after Indo-1 loading, the total intensity, F_λ, is given by (Brandes et al., 1993):

$$F_\lambda = g_\lambda \cdot \{n(\text{Indo})_c \cdot I_\lambda^c + n(\text{Indo})_m \cdot I_\lambda^m\} + B_\lambda(\text{al}), \quad (2)$$

where g_λ is a gain factor that depends on instrumental gain and tissue light absorption, $n(\text{Indo})_c$ and $n(\text{Indo})_m$ are the amounts (e.g., moles) of cytosolic and noncytosolic Indo-1, respectively, I_λ^c and I_λ^m are the intensities (per gain-unit and mole of Indo-1 (Grynkiewicz et al., 1985)) of cytosolic and noncytosolic Indo-1, respectively, and B_λ(al) is the sum of various background fluorescence intensities after Indo-1 loading. The loading factor can be related to the cytosolic and noncytosolic Indo-1 concentrations, $[\text{Indo-1}]_c$ and $[\text{Indo-1}]_m$, respectively, using Eq. 2 and substituting B_λ(bl) according to Eq. 1 (with $\Delta B_\lambda = 0$):

$$\begin{aligned} L_\lambda &= F_\lambda / B_\lambda(\text{bl}) \\ &= 1 + g_\lambda \cdot \{[\text{Indo-1}]_c \cdot V_c \cdot I_\lambda^c + [\text{Indo-1}]_m \cdot V_m \cdot I_\lambda^m\} \\ &\quad + B_\lambda^{AM}/B_\lambda(\text{bl}), \end{aligned} \quad (3)$$

where V_c and V_m are the volumes of the cytosol and noncytosol, respectively. In the absence of noncytosolic Indo-1 and negligible unhydrolized Indo-1/AM, the loading factors (at each wavelength) would be linearly related to the cytosolic Indo-1 concentration (with intercept = 1) and to each other, i.e., $L_{385} = \alpha \cdot L_{456} + \beta$, where α and β are constants (and $L_{385} = L_{456} = 1$ when $[\text{Indo-1}]_c = [\text{Indo-1}]_m = 0$).

Effects of constant Indo-1 background fluorescence

An expression for the cytosolic Indo-1 fluorescence intensity, F'^{c}_{λ}, can be obtained after subtracting the constant noncytosolic Indo-1 background fluorescence, F'^{m}_{λ}, and other background fluorescence, $B_\lambda(\text{al})$, from F_λ according to (see Appendix):

$$F'^{c}_{\lambda} = \{F_\lambda - f_m \cdot F'^{m}_{\lambda} - B_\lambda(\text{al})\}/\{1 - f_m\}, \quad (4a)$$

where

$$f_m = n(\text{Indo})_m/\{n(\text{Indo})_m + n(\text{Indo})_c\}, \quad (4b)$$

$$F'^{m}_{\lambda} = F'^{\max}_{\lambda} \cdot ([Ca^{2+}]_m + S_\lambda \cdot K_d)/([Ca^{2+}]_m + K_d), \quad (4c)$$

$$F'^{c}_{\lambda} = F'^{\max}_{\lambda} \cdot ([Ca^{2+}]_c + S_\lambda \cdot K_d)/([Ca^{2+}]_c + K_d), \quad (4d)$$

$$F'^{\max}_{385} = (aH'/bH) \cdot (1 - S_{456})/(S_{456} - S_{385}), \quad (4e)$$

$$F'^{\max}_{456} = aH' \cdot (1 - S_{385})/(S_{456} - S_{385}), \quad (4f)$$

$$aH' = aH - B_{456}(\text{al}) + bH \cdot B_{385}(\text{al}). \quad (4g)$$

In Eq. 4, f_m is the fraction of noncytosolic Indo-1, $[Ca^{2+}]_m$ is the Ca^{2+} concentration in the noncytosol, aH and bH are the intercept and slope, respectively, obtained when the fluorescence intensity from the Indo-1 transients measured at 385 nm is plotted against the intensity at 456 nm (see Appendix and Brandes et al. (1993)) and S_λ is the ratio between the fluorescence intensities obtained in the absence of Ca^{2+} and with saturating $[Ca^{2+}]$ (obtained using a suitable reference solution (Brandes et al., 1993)). The measured parameters in Eq. 4 are: F_λ, aH, bH, and $B_\lambda(\text{al})$ (or $B_\lambda(\text{al})$; see Results), from which the following can be calculated: $F'^{\max}_{\lambda}$ (background-corrected, calculated intensity during saturating Ca^{2+} conditions (Brandes et al., 1993)) and aH' (background-corrected intercept). The unknown parameters in Eq. 4 are: f_m and $[Ca^{2+}]_m$, both which would affect the calculated F'^{c}_{λ} and consequently calculated $[Ca^{2+}]_c$ (by using Eq. 4d).

Effects of Indo-1 loading into endothelial cells

The fraction of Indo-1 in endothelial cells, f_m, can be obtained from Eq. 4a: Equation 4a is first rewritten with F_λ as the dependent variable. During stimulation with bradykinin, F'^{c}_{λ} is assumed to be constant (since $[Ca^{2+}]_c$ is constant), while F'^{m}_{λ} changes due to changing $[Ca^{2+}]_m$. Therefore, according to Eq. 4a, the difference between F_λ during stimulation, $F_\lambda(\text{stim})$, and control, $F_\lambda(\text{ctrl})$, is proportional to f_m or alternatively, by solving for f_m:

$$f_m = \{F_\lambda(\text{stim}) - F_\lambda(\text{ctrl})\}/\{F^{m}_{\lambda}(\text{stim}) - F^{m}_{\lambda}(\text{ctrl})\}. \quad (5)$$

$F^{m}_{\lambda}(\text{ctrl})$ and $F^{m}_{\lambda}(\text{stim})$ are the calculated (Eqs. 4c-g, with literature values for $[Ca^{2+}]_m$) Indo-1 fluorescence intensities arising from endothelial cells during control and stimulation, respectively. To suppress the fluorescence transients, caused by cytosolic $[Ca^{2+}]_c$ transients, $F_\lambda(\text{ctrl})$ and $F_\lambda(\text{stim})$ were determined after mathematical low-pass filtering of the measured signals. The cytosolic calcium concentration, $[Ca^{2+}]_c$, was then calculated using f_m (according to Eq. 5) and $[Ca^{2+}]_m$ (literature value during control conditions) according to Eq. 4.

Effects of Indo-1 loading into mitochondria

The effect of various degrees of Indo-1 loading into mitochondria, f_m, was evaluated by calculating $[Ca^{2+}]_c$ (according to Eq. 4), assuming a value for $[Ca^{2+}]_m$ of 420 nM, which was previously found in isolated myocytes paced at a similar frequency (4 Hz) (Miyata et al., 1991) as used in this study (5 Hz).

Effects of hypoxia on background fluorescence

During hypoxia (or ischemia), the background intensity is expected to increase because of increased [NAD(P)H]. Since this increase is not canceled by calculating the fluorescence ratio, the calculated $[Ca^{2+}]_c$ will be incorrect unless the background fluorescence during hypoxia is first subtracted from the individual intensities before calculating the fluorescence ratio, R, during hypoxia. The goal was therefore to determine the relative increase in the background fluorescence during hypoxia compared with normoxia.

Measurements of changes in background fluorescence (caused by hypoxia) in the absence of Indo-1

The effect of "total" hypoxia on the background fluorescence, prior to Indo-1 loading, was investigated by switching from standard perfusate to the hypoxic perfusate (see above). The effect of milder hypoxia was also measured by low flow ischemia at 10 and 20% of control flow. Because the detected background fluorescence is not exclusively caused by NAD(P)H, the fluorescence increase is not directly proportional to increased [NAD(P)H].

During hypoxia it is assumed that the NAD(P)H background fluorescence intensity, B^{N}_{λ}, increases by ΔN (%), and that other background fluorescence intensities, B^{x}_{λ}, remain constant. The relative increase in background fluorescence, ΔB_λ, during hypoxia is then given by:

$$\Delta B_\lambda = B_\lambda(N_2)/B_\lambda(O_2) - 1 = \Delta N \cdot (1 - rB^{x}_{\lambda}), \quad (6a)$$

where

$$rB^x_\lambda = B^x_\lambda/(B^N_\lambda + B^x_\lambda). \quad (6b)$$

In Eq. 6, $B_\lambda(O_2)$ (= $B^N_\lambda + B^x_\lambda$) and $B_\lambda(N_2)$ (= $B^N_\lambda + B^x_\lambda + \Delta N \cdot B^N_\lambda$) are the sums of background fluorescence intensities during normoxia and hypoxia respectively and rB^x_λ is the relative contribution of nonNAD(P)H fluorescence intensity to the total background fluorescence intensity. Because ΔN is independent of detection wavelength, ΔB_{385} is related to ΔB_{456} according to (using Eq. 6a at two detection wavelengths: 385 and 456 nm, and eliminating ΔN):

$$\Delta B_{385} = \Delta B_{456} \cdot \kappa, \quad (7a)$$

where

$$\kappa = (1 - rB^x_{385})/(1 - rB^x_{456}), \quad (7b)$$

In Eq. 7b, κ is a proportionality factor that relates the change in background intensity at 385 nm to that at 456 nm (independent of the degree of hypoxia). If non-NAD(P)H fluorescence is negligible or equal at 385 and 456 nm, then $\kappa = 1$. If non-NAD(P)H fluorescence is larger at 385 nm than at 456 nm, then $\kappa < 1$. If it is assumed that the fluorescence at 456 nm is caused exclusively by NAD(P)H, then $rB^x_{385} = 1 - \kappa$. To verify Eq. 7, ΔB_{385} and ΔB_{456} were measured during hypoxia and low flow ischemia at 10 and 20% of control flow.

Measurements of changes in background fluorescence (caused by hypoxia) in the presence of Indo-1

A new method was developed to measure relative changes in background fluorescence, ΔB_λ, simultaneously with Indo-1 fluorescence. After Indo-1 loading, during normoxic conditions, the intercept of the relationship between the intensity at 385 vs. 456 nm, $aH = aH(O_2)$, was given by Eq. A4c (see Appendix). During hypoxia, the background fluorescence increases (by $\Delta B_\lambda \cdot B_\lambda(bl)$ at both wavelengths), and therefore result in another intercept, $aH(N_2)$, given by:

$$aH(N_2) = g_{456} \cdot \{n(Indo)_c + n(Indo)_m\} \cdot a + B_{456}(al) + \Delta B_{456} \cdot B_{456}(bl) - bH \cdot \{B_{385}(al) + \Delta B_{385} \cdot B_{385}(bl)\}, \quad (8)$$

where a is a constant (see Appendix, Eq. A4). The difference between $aH(N_2)$ and $aH(O_2)$ is therefore given as (using Eqs. A4c and 8):

$$aH(N_2) - aH(O_2) = \Delta B_{456} \cdot B_{456}(bl) - bH \cdot \Delta B_{385} \cdot B_{385}(bl). \quad (9a)$$

By substituting ΔB_{385} (according to Eq. 7a), and solving for ΔB_{456} in Eq. 9a, the following is obtained:

$$\Delta B_{456} = \frac{aH(N_2) - aH(O_2)}{B_{456}(bl) - bH \cdot \kappa \cdot B_{385}(bl)}. \quad (9b)$$

In the presence of Indo-1, $aH(N_2)$ and $aH(O_2)$ are the intercepts during hypoxia and control (normoxia), respectively, κ was determined from an hypoxic insult prior to Indo-1 loading, and ΔB_{385} was calculated using Eq. 7a.

Effect of Indo-1 loading on apparent changes in the Indo-1 fluorescence ratio during hypoxia

In the absence of a change in $[Ca^{2+}]_c$, an increase in [NAD(P)H], and therefore ΔB_λ, would cause a change of the Indo-1 ratio. The magnitude of this change depends on ΔB_λ and Indo-1 loading, L_λ. The Indo-1 background-corrected fluorescence ratio (Brandes et al., 1993) during normoxia, $R(O_2)$, and hypoxia, $R(N_2)$, are given by (see Eq. 2):

$$R(O_2) = \frac{g_{385}}{g_{456}} \cdot \frac{n(Indo)_c \cdot I^c_{385} + n(Indo)_m \cdot I^m_{385}}{n(Indo)_c \cdot I^c_{456} + n(Indo)_m \cdot I^m_{456}}, \quad (10)$$

$$R(N_2) = \frac{g_{385} \cdot [n(Indo)_c \cdot I^c_{385} + n(Indo)_m \cdot I^m_{385}] + \Delta B_{385} \cdot B_{385}(bl)}{g_{456} \cdot [n(Indo)_c \cdot I^c_{456} + n(Indo)_m \cdot I^m_{456}] + \Delta B_{456} \cdot B_{456}(bl)}. \quad (11)$$

Substituting the concentration-dependent terms in Eqs. 10 and 11 with the loading factors (Eq. 3, assuming that $B^{AM}_\lambda = 0$; see Results), and then dividing Eqs. 10 and 11 gives:

$$\frac{R(N_2)}{R(O_2)} = \frac{(L_{385} - 1 + \Delta B_{385}) \cdot (L_{456} - 1)}{(L_{456} - 1 + \Delta B_{456}) \cdot (L_{385} - 1)}. \quad (12)$$

Because $L_{385} > L_{456} > 1$ and $\Delta B_{456} > \Delta B_{385} > 0$, increased [NAD(P)H] causes a reduction of the relative Indo-1 ratio, $R(N_2)/R(O_2)$, during hypoxia. The magnitude of this reduction was calculated using values of ΔB_λ which were determined prior to or after Indo-1 loading, and measured values of L_λ during diastole and systole, L_λ(dia) and L_λ(sys), respectively, for 24 hearts.

RESULTS AND DISCUSSION

Measurement of background (Ca^{2+}-insensitive) fluorescence

Unhydrolized Indo-1/AM fluorescence intensity was assessed for in two ways. First, a Mn^{2+} quenching solution was applied to the heart after saturating Ca^{2+} conditions (normally used to determine calibration parameters (Brandes et al., 1993)). Approximately 10 min after application of the Mn^{2+} solution, both F_{385} and F_{456} decreased in intensity and approached that of the original background intensities before loading. Quenched intensities were 26 ± 9% and 2 ± 12% larger than the pre Indo-1 loading intensities at 385 and 456 nm, respectively ($n = 5$). This slight increase might have been caused by increased [NAD(P)H] during the application of the hypoxic Mn^{2+} solution. The quenching solution (but without Mn^{2+}) was therefore also applied to hearts ($n = 4$) in the absence of Indo-1 loading which caused the background intensities to increase by 26 ± 4% and 48 ± 5% at 385 and 456 nm, respectively. Therefore, after Indo-1 quenching, the fluorescence intensities, F_{385} and F_{456}, were

slightly smaller (0.3 ± 9% and 31 ± 13%, respectively) than the pre-Indo-1 loading intensities. The slight reduction in the quenched intensity may have been caused by a reduction in absolute [NAD(P)H] from initial conditions. These results demonstrate that there was not a significant amount of fluorescence intensity from unhydrolized Indo-1/AM (compared to the fully hydrolyzed Indo-1 intensities).

In the presence of high extracellular [Ca^{2+}], the Indo-1 fluorescence intensities have been found to decrease with time (Brandes et al., 1993), suggesting Indo-1 leakage due to membrane permeabilization. Therefore, it is possible that also some unhydrolized Indo-1 had leaked out of the cytosol before quenching. The second approach was therefore to avoid saturating Ca^{2+} conditions, and to directly titrate additions of Mn^{2+} to the standard perfusate until no further reduction in fluorescence intensity was observed. In this case, the final quenched intensities were 34 ± 18% and 30 ± 17% (n = 3) larger than the pre-Indo-1 loading intensities at 385 and 456 nm, respectively. In one of these hearts, ionomycin was added toward the end of the experiment, and the quenched intensities were then only −1 and 8% larger than the pre-Indo-1 loading intensities at 385 and 456 nm, respectively. These results confirmed that levels of unhydrolized Indo-1/AM were negligible. Therefore, it would be sufficient to correct the Indo-1 fluorescence intensities using the background fluorescence intensities measured prior to Indo-1 loading (provided that the background intensities were constant throughout the experiment, see below).

These findings are in contrast to previous studies in whole rabbit hearts which suggested that some Indo-1/AM was not hydrolyzed (Lee et al., 1988), and also from measurements on endothelial cells where unhydrolized Indo-1/AM significantly contributed to the fluorescence signals (Luckhoff, 1986).

Factors which may influence calculated $[Ca^{2+}]_c$

Two factors which may influence the calculated $[Ca^{2+}]_c$ were investigated: 1) buffering of Ca^{2+} by Indo-1 and 2) effects of a constant background signal from Indo-1 localized in endothelial cells or mitochondria.

Effects of Indo-1 calcium buffering on $[Ca^{2+}]_c$ and developed pressure

Fig. 1 *A* shows the calculated (Brandes et al., 1993) $[Ca^{2+}]_c$ during diastole and systole, $[Ca^{2+}]_{dia}$, and $[Ca^{2+}]_{sys}$, respectively, as a function of L_{456}(dia). Although the $[Ca^{2+}]_c$ showed a large variability, the calculated $[Ca^{2+}]_{sys}$ increased significantly at low Indo-1 loading. These results suggest that $[Ca^{2+}]_{sys}$ depended on Indo-1 loading, which may have been caused by increased calcium buffering with increased [Indo-1]. The actual concentration of Indo-1 was not measured here, but may be estimated from the loading factors. In a previous single cell study (Peeters et al., 1987) an intracellular [Indo-1] = 50 μM corresponded to a loading factor of 6–7 times (at 456 nm). If it is assumed that the background

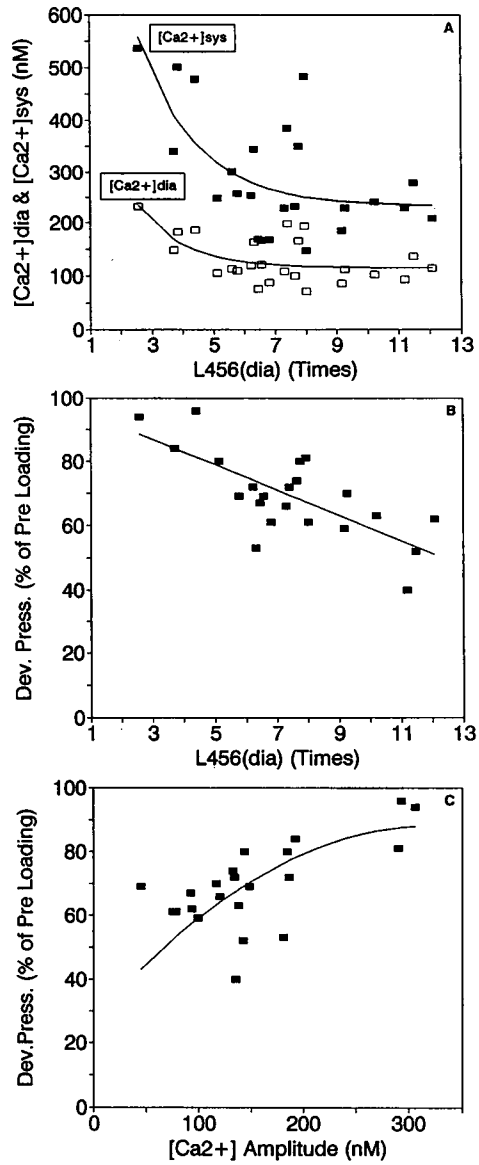

FIGURE 1 (*A*) Calculated $[Ca^{2+}]_c$ during systole (■) and diastole (□) as a function of Indo-1 loading factors (L_{456}(dia)). The data points were fit (R^2 = 0.43 for $[Ca^{2+}]_{sys}$) to exponentially decaying functions with a constant term. (*B*) Developed pressure (percent of developed pressure prior to Indo-1 loading) as a function of L_{456}(dia). The relationship was fitted to a straight line: Dev. Press. (%) = 99 − 4 · L_{456}(dia). (*C*) Developed pressure (%) as a function of Ca^{2+} amplitude = $[Ca^{2+}]_{sys}$ − $[Ca^{2+}]_{dia}$. The data points were fitted by a second degree polynomial.

fluorescence intensity in the single cell study was similar to that observed here, then the range of L_{456}(dia) = 2.6–12 in the present study would correspond to $[Indo\text{-}1]_c$ = 19–91 μM. (This would be an underestimation of $[Indo\text{-}1]_c$ if Indo-1 was also partly compartmentalized or loaded into endothelial cells). With low loadings, L_{456}(dia) = 2.6–4.4 times, corresponding to [Indo-1] ~19–33 μM, $[Ca^{2+}]_{dia}$ = 187 nM and $[Ca^{2+}]_{sys}$ = 464 nM, which is similar to the $[Ca^{2+}]_c$ obtained in rat myocytes with comparable [Indo-1] (Sollot et al., 1992; Spurgeon et al., 1990).

Fig. 1 *B* shows that there was a reduction of developed pressure which was related to the Indo-1 loading factor,

L_{456}(dia). This relationship was fitted by linear regression, resulting in a slope = −4.0 ± 0.8% (per times loading) and intercept 99 ± 9%. This suggests that Indo-1, at high concentrations, buffers enough Ca^{2+} to affect contractility.

Fig. 1 *C* confirms that the developed pressure was related to the calculated Ca^{2+}-transient amplitude ($[Ca^{2+}]_{sys}$ − $[Ca^{2+}]_{dia}$). Therefore, during high Indo-1 loading, the reduction of calculated $[Ca^{2+}]_c$ was most likely caused by a real reduction in $[Ca^{2+}]_c$, and less likely caused by an apparently reduced $[Ca^{2+}]_{sys}$ due to preferential loading of Indo-1 into a low $[Ca^{2+}]$-containing compartment (e.g., mitochondria or endothelial cells; see below).

The effects of [Indo-1] on $[Ca^{2+}]_c$ and function in this study were similar to the effects of [Fura-2] on the Ca^{2+} transients in trabeculae, which demonstrated that $[Ca^{2+}]_{sys}$ was ~40% larger at [Fura-2] < 35 μM compared with [Fura-2] ~70 μM, and a 22% reduction in force was obtained at a loading of 3–5.5 times (Backx et al., 1993).

Closely related to the effects of calcium buffering, are the consequences of Ca^{2+} binding kinetics. In myocytes, the peak of the calcium transient (measured with Fura-2) is significantly increased when the in vivo derived estimates for rate constants of calcium binding to Fura-2 are used to obtain kinetically corrected transients (Sipido et al., 1991; Baylor et al., 1988; Konishi et al., 1991). Therefore, it is possible that kinetic corrections of the transients in this study would likewise increase the estimates of $[Ca^{2+}]_{sys}$.

Effects of constant Indo-1 background fluorescence

Fig. 2 shows that the diastolic loading factors, L_{385}(dia) and L_{456}(dia), appeared to be linearly related with slope = 1.56 ± 0.09. The loading factors were therefore 56% larger at 385 nm than at 456 nm. This relationship showed a somewhat larger variability with higher dye loading.

Effects of Indo-1 loading into endothelial cells—Addition of bradykinin resulted in time-dependent changes in the fluorescence intensities which peaked after 17–24 s. At the peak, the fluorescence ratio (F'_{385}/F'_{456}) increased by 9 ± 2%, and coronary flow increased by 16 ± 4% (n = 9). After ~3 min of bradykinin infusion, the intensities had returned to control levels. These changes were much smaller than those previously observed in rabbit hearts (Lorell et al., 1990). In the heart with the largest changes in fluorescence intensities, the intensity increased by 13% at 385 nm, decreased by 4.3% at 456 nm, and increased by 18% in the fluorescence ratio. Assuming an endothelial $[Ca^{2+}]_m$ ~132 nM during control conditions, and $[Ca^{2+}]_m$ ~800 nM during bradykinin stimulation (as has been observed previously (Luckhoff, 1986)), the observed fluorescence changes of + 13% at 385 nm and −4.5% at 456 nm, corresponded to (using Eq. 5) an endothelial Indo-1 loading, f_m ~18%.

Fig. 3 (*heavy solid line*) shows that without correcting for endothelial cell loading (i.e., assuming f_m = 0), $[Ca^{2+}]_{dia}$ ~129 nM and $[Ca^{2+}]_{sys}$ ~300 nM (in a heart with loading factor, L_{456}(dia) ~6 times). Fig. 3 (*solid line*) demonstrates that by correcting for the endothelial cell loading, using f_m = 18%, calculated $[Ca^{2+}]_{sys}$ increased only by 60 nM (and $[Ca^{2+}]_{dia}$ did not change). This 20% correction of $[Ca^{2+}]_{sys}$ was the largest observed in nine hearts, and therefore suggests that possible endothelial Indo-1 loading can be neglected when calculating $[Ca^{2+}]_{sys}$.

In a previous study of endothelial cells loaded with Indo-1/AM, it was found that ~50% of Indo-1/AM was not hydrolyzed after loading (Luckhoff, 1986). However, as was found in this study, all Indo-1/AM was hydrolyzed, suggesting that insignificant amount of Indo-1/AM and therefore Indo-1 were located in endothelial cells. This finding confirms that endothelial Indo-1 loading can be neglected when calculating $[Ca^{2+}]_c$ in perfused rat hearts.

Effects of Indo-1 loading into mitochondria—Fig. 3 (*dotted line*) shows the calculated $[Ca^{2+}]_c$ assuming that the mitochondria contained 18% Indo-1 and $[Ca^{2+}]_m$ = 420 nM (Miyata et al., 1991) (and no endothelial cell loading). In this case, $[Ca^{2+}]_{dia}$ ~95 nM and $[Ca^{2+}]_{sys}$ ~280 nM. At higher mitochondrial Indo-1 loadings, for example 40%, $[Ca^{2+}]_{dia}$ ~41 nM and $[Ca^{2+}]_{sys}$ ~241 nM (not shown). The assumption of a mitochondrial Indo-1 loading would therefore result

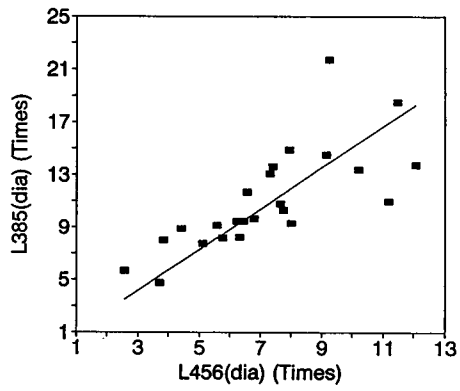

FIGURE 2 Relationship between loading factors measured at 456 and 385 nm during diastole. The relationship was fitted to a straight line, forced to pass through L_{385}(dia) = L_{456}(dia) = 1.

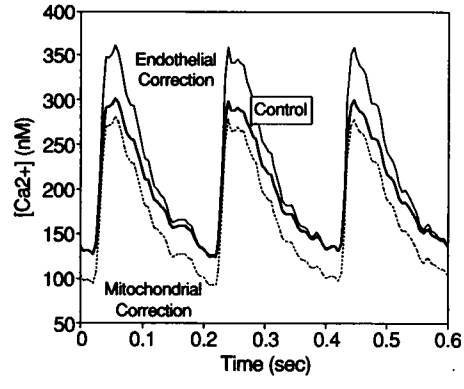

FIGURE 3 Effect of constant Indo-1 background on calculated $[Ca^{2+}]_c$. Assuming no background (i.e., f_m = 0) (*heavy solid line*). Assuming endothelial cell Indo-1 loading with f_m = 18% (as found from bradykinin stimulation) and $[Ca^{2+}]_m$ = 132 nM (*light solid line*). Assuming mitochondrial Indo-1 loading with f_m = 18% and $[Ca^{2+}]_m$ = 420 nM (*dotted line*).

in a lower calculated $[Ca^{2+}]_c$ than if it assumed that the mitochondria do not contain any Indo-1. Furthermore, since the hearts were paced at 5 Hz here (rather than 4 Hz (Miyata et al., 1991)), it is possible that $[Ca^{2+}]_m$ would be even larger than 420 nM, and this would further reduce the corrected $[Ca^{2+}]_c$. Therefore, provided that $[Ca^{2+}]_m \geq 420$ nM, it is unlikely that mitochondrial loading of Indo-1 is a major factor responsible for the apparently lower $[Ca^{2+}]_{sys}$ with high Indo-1 loading. These results are consistent with those of Lee et al. (1988), who did not find a significant amount of Indo-1 in the mitochondria using whole perfused rabbit hearts.

Effects of hypoxia on background fluorescence, Indo-1 ratio and calculated $[Ca^{2+}]_c$

Measurement of changes in background fluorescence (caused by hypoxia) in the absence of Indo-1

To evaluate the changes in background fluorescence during hypoxia, measurements were first performed in the absence of Indo-1 loading. Fig. 4 *A* shows that the intensity increased by 60% at 456 nm, and by 13% at 385 nm. In six hearts (nine hypoxic insults), the fluorescence increased by 59 ± 4% at 456 nm and by 21 ± 3% at 385 nm, corresponding to κ = 0.36 ± 0.05. If it is assumed that the increased intensity during hypoxia at 456 nm is solely caused by a 59% rise in [NAD(P)H], then the non-NAD(P)H background fluorescence at 385 nm was 64 ± 5% of the total detected intensity during normoxia. In contrast, if there were a significant contribution of non-NAD(P)H fluorescence at 456 nm as well as at 385 nm, then the rise in [NAD(P)H] was less than 59%, and non-NAD(P)H fluorescence consequently accounted for more than 64% at 385 nm. Therefore, measurements of changes in NAD(P)H might be underestimated, depending on detection wavelength, unless non-NAD(P)H background fluorescence is accounted for.

To ascertain whether κ, and therefore non-NAD(P)H fluorescence, could be determined using any level of hypoxia, the fluorescence increase was also measured during reduced coronary flows at 20 and 10% of control flow. At 20 and 10% of control flow, the intensity increased by 31 ± 7% and 47 ± 6%, respectively, when detected at 456 nm. Fig. 4 *B* shows that, when the flow was reduced, the intensities increased in proportion at 385 and 456 nm so that $\Delta B_{385} = \kappa \cdot \Delta B_{456}$, where κ = 0.39 ± 0.03. Thus, the non-NAD(P)H background fluorescence found using various levels of hypoxia was similar to that found during total hypoxia (κ = 0.36 ± 0.05), and κ is therefore independent of the degree of hypoxia.

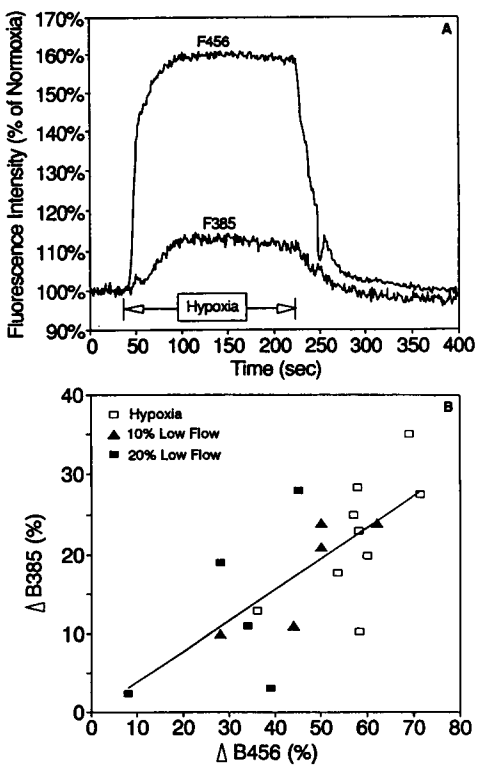

FIGURE 4 Increased background fluorescence measured at 385 and 456 nm during hypoxia in the absence of Indo-1. (*A*) Example of time-dependent changes during total hypoxia (N_2-containing perfusate). (*B*) Percent increase in background fluorescence during: total hypoxia (□, n = 9), 10% of control flow (▲, n = 5) and 20% of control flow (■, n = 5). The changes at 385 and 456 nm were linearly related by the fitted line: $\Delta B_{385} = 0.39 \cdot \Delta B_{456}$ (intercept forced to zero).

Measurement of changes in background fluorescence (caused by hypoxia) in the presence of Indo-1

The changes in background fluorescence was also measured in the presence of Indo-1 using a novel technique, and low Indo-1 loading (to minimize any Ca-buffering). Fig. 5 *A* shows a representative example (L_{456}(dia) = 4.4 times) of the effect of hypoxia on the motion-corrected linear relationship between F_{385} and F_{456} (Brandes et al., 1993). During hypoxia, the fluorescence intensities increased at 385 and 456 nm (due to increased [NAD(P)H]), causing the line, which describes the relationship, to move up toward the upper right corner. Consequently, *aH* increased from 4.04 during normoxia (*heavy solid line*) to 4.32 during hypoxia (*solid line*). Before loading with Indo-1, κ = 0.39 (as determined by an hypoxic insult prior to loading), and therefore ΔB_{456} = 38% and ΔB_{385} = 14%. However, Fig. 5 *A* also demonstrates that during a subsequent normoxic measurement (12 min after the first; *dashed line*), the line moved towards the lower left corner. This can be explained by Indo-1 leakage between the two measurements.[1] It was therefore necessary to correct the intensities during hypoxia by estimating the leak rate (from interpolation of the normoxia intensities prior to and after hypoxia), resulting in ΔB_{456} = 50% and ΔB_{385} = 19%. Prior to Indo-1 loading this heart showed a slightly larger response to hypoxia: ΔB_{456} = 71% and ΔB_{385} = 28%. Three hearts (four hypoxic insults) were analyzed in this manner, and it was found that ΔB_{456} = 30 ± 6% and ΔB_{385}

[1] Parallel experiments during normoxia demonstrated that Indo-1 leaked out with an exponential time constant of ~1.2 h (not shown)

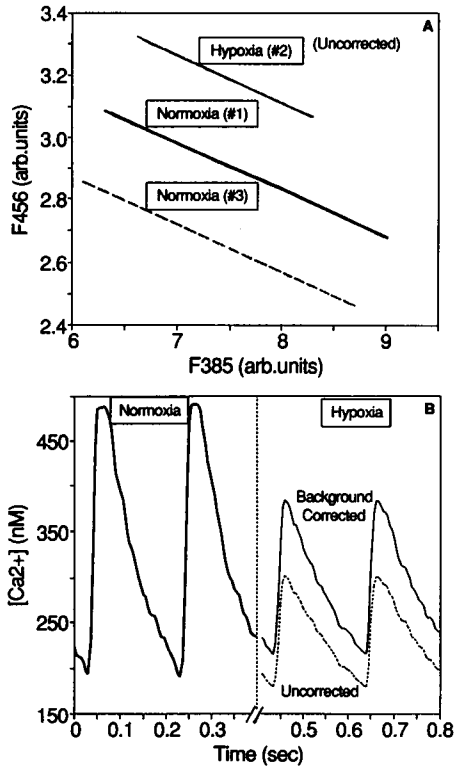

FIGURE 5 Effects of total hypoxia on Indo-1 intensities and $[Ca^{2+}]_c$. (*A*) Relationship between (motion-corrected (Brandes et al., 1993)) F_{385} and F_{456} during normoxia (*1, heavy solid line*), hypoxia (*2, light solid line*), and subsequently hypoxia again (*3, dashed line*). The right upward shift of this relationship corresponds to an increase in background fluorescence (at 456 nm) of ~50% (see text). (*B*) Effect of hypoxia on $[Ca^{2+}]_c$: normoxia (*heavy solid line*), hypoxia, uncorrected for the increase in background fluorescence (*dotted line*) and hypoxia, where the intensities were corrected for the increased background before calculating the Indo-1 ratio (see text) (*light solid line*).

= 13 ± 2% prior to leak correction, and ΔB_{456} = 48 ± 6% and ΔB_{385} = 19 ± 2% after the correction.

This method of simultaneous observation of changes in NAD(P)H and $[Ca^{2+}]_c$ provides a new tool to study questions relating [NAD(P)H] and $[Ca^{2+}]_c$ such as activation of NAD(P)H dehydrogenases by $[Ca^{2+}]_c$ (Fralix et al., 1991).

Calcium transients in the presence of changing background fluorescence

Fig. 5 *B* shows the calculated $[Ca^{2+}]_c$ (Brandes et al., 1993) (assuming $f_m = 0$) obtained from the intensities presented in Fig. 5 *A*. The heavy solid line shows $[Ca^{2+}]_c$ during normoxia, and the dashed line shows $[Ca^{2+}]_c$ during hypoxia (without correcting for the increased background fluorescence). Both $[Ca^{2+}]_{dia}$ and $[Ca^{2+}]_{sys}$ were smaller during hypoxia than during normoxia. However, when the fluorescence intensities were corrected for the measured increase in background fluorescence during hypoxia (using the values found in the presence of Indo-1: 50% at 456 nm and 19% at 385 nm), then the solid line demonstrates that $[Ca^{2+}]_{dia}$ actually increased slightly.

To illustrate the importance of NAD(P)H corrections with regard to quantitation of $[Ca^{2+}]_c$ during hypoxia, three different hearts were subjected to hypoxic insults (total of four insults). To minimize calcium buffering by Indo-1, loading was kept low: L_{456}(dia) = 4.1 ± 0.9 times. During hypoxia, the uncorrected fluorescence ratio decreased by 4 ± 8% during diastole, and by 24 ± 13% during systole. Consequently, in the absence of any correction, $[Ca^{2+}]_{dia}$, $[Ca^{2+}]_{sys}$ and the amplitude, $\delta[Ca^{2+}] = [Ca^{2+}]_{sys} - [Ca^{2+}]_{dia}$, appeared to change with −6.0 ± 12%, −36 ± 12%, and −56 ± 12%, respectively, during hypoxia. By correcting the intensities using the ΔB_λ values found in the presence of Indo-1, $[Ca^{2+}]_{dia}$, $[Ca^{2+}]_{sys}$, and $\delta[Ca^{2+}]$ changed with + 12 ± 9%, −21 ± 10%, and −41 ± 11%, respectively. These results demonstrate that incorrect values of $[Ca^{2+}]_c$ would be obtained unless the fluorescence ratio is corrected for increased background fluorescence during hypoxia.

Effect of Indo-1 loading on apparent changes in Indo-1 fluorescence ratio during hypoxia

According to Eq. 12, increased Indo-1 loading is expected to reduce the underestimation of the Indo-1 ratio during hypoxia. Fig. 6 shows the reduction in the fluorescence ratio during hypoxia (if the fluorescence intensities were not corrected) using the loading values shown in Fig. 2, and the relative increases in background fluorescence obtained in the presence of Indo-1: ΔB_{456} = 50% and ΔB_{385} = 19%. As expected, the underestimation was small for high Indo-1 loading, but became severe when the loading was less than ~5 times. The average loading was L_{456}(dia) = 7.2 ± 2.5 times (L_{385}(sys) = 15.8 ± 6 times), and the ratio was, on the average, underestimated by 7 ± 0.8% in diastole and by 8 ± 0.9% in systole. At a lower loading of 2.6–5.1 times, the ratio was underestimated by 12% in diastole, and by 16% in systole, corresponding to ~27% underestimation of $[Ca^{2+}]_{sys}$ (at a control $[Ca^{2+}]_{sys}$ = 480 nM) (Brandes et al., 1993). As discussed above, in order to prevent buffering of Ca^{2+} by Indo-1, the loading should be kept as low as possible, making it necessary to correct the fluorescence ratio for increased background fluorescence, especially for desirable loading factors of <5 times. In the absence of a correction, $[Ca^{2+}]_c$ might otherwise incorrectly be underestimated during hypoxia.

SUMMARY

It was found that increasing loading of Indo-1 into the heart caused reduced calculated and real $[Ca^{2+}]_{sys}$ and consequently reduced developed pressure. Calculated $[Ca^{2+}]_c$ did not change appreciably by considering possible loading of Indo-1 into endothelial cells, and endothelial cell loading could therefore be neglected in the calculations. If a mitochondrial $[Ca^{2+}]_m$ larger than ~400 nM was assumed (Miyata et al., 1991), the calculated $[Ca^{2+}]_{sys}$ would be overestimated and, therefore, smaller than expected, suggesting

that mitochondrial Indo-1 loading may also be neglected. With low Indo-1 loading factors (~3–5 times) $[Ca^{2+}]_{sys}$ may be underestimated by ~27% during hypoxia because of increased [NAD(P)H]. Therefore, high loading with Indo-1 has the advantage of minimizing the possible error introduced by changes in [NAD(P)H] (e.g., caused by hypoxia or ischemia). The disadvantage of high loading is intracellular calcium buffering; conversely, low Indo-1 loading causes less calcium buffering but may result in incorrect (underestimated) $[Ca^{2+}]_c$ due to increased [NAD(P)H]. This underestimation could possibly lead to incorrect conclusions regarding calcium homeostasis during hypoxia, unless the fluorescence measurements were corrected using the techniques described in this study.

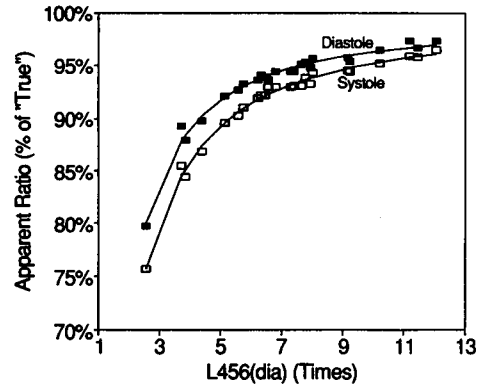

FIGURE 6 Calculated "apparent" Indo-1 ratio during hypoxia (% of normoxic ratio) in diastole (■) and systole (□) caused by a change in background fluorescence (ΔB_{385} = 50% and ΔB_{456} = 19%) as a function of Indo-1 loading factors. The data were fitted to logistic functions.

APPENDIX

Calculation and calibration of cytosolic Indo-1 fluorescence

An expression involving fluorescence intensities from cytosolic and noncytosolic Indo-1, I_λ^c, and I_λ^m, respectively, was given by Eq. 2. These intensities are related to $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ according to (Grynkiewicz et al., 1985):

$$I_\lambda^c = \{I_\lambda^{max} \cdot [Ca^{2+}]_c + S_\lambda \cdot I_\lambda^{max} \cdot K_d\}/\{[Ca^{2+}]_c + K_d\} \quad \text{(A1a)}$$

$$I_\lambda^m = \{I_\lambda^{max} \cdot [Ca^{2+}]_m + S_\lambda \cdot I_\lambda^{max} \cdot K_d\}/\{[Ca^{2+}]_m + K_d\}, \quad \text{(A1b)}$$

where

$$S_\lambda \cdot I_\lambda^{max} = I_\lambda^{min}. \quad \text{(A1c)}$$

I_λ^{max} is the intensity of Indo-1 (per gain unit and mole of Indo-1) with saturating $[Ca^{2+}]$, assumed to be identical in the cytosol and noncytosol (Spurgeon et al., 1990), and S_λ is an intrinsic Indo-1 constant (determined in a reference solution; S_{385} = 0.051 and S_{456} = 2.27 (Brandes et al., 1993)), that relates the intensity of Indo-1, in the absence of Ca^{2+}, I_λ^{min}, to that with saturating $[Ca^{2+}]$, I_λ^{max}. The total (background subtracted) intensities during saturating conditions, F'^{max}_λ, are given by (e.g., see Eq. 2 with $I_\lambda^c = I_\lambda^m = I_\lambda^{max}$):

$$F'^{max}_\lambda = g_\lambda \cdot I_\lambda^{max} \cdot \{n(\text{Indo})_c + n(\text{Indo})_m\}. \quad \text{(A2)}$$

Substituting I_λ^{max} from Eq. A2 into Eqs. A1a–b, and thereafter substituting I_λ^c and I_λ^m into Eq. 2 gives:

$$F_\lambda = \{1 - f_m\} \cdot F'^c_\lambda + f_m \cdot F'^m_\lambda + B_\lambda(\text{al}), \quad \text{(A3a)}$$

where

$$F'^m_\lambda = F'^{max}_\lambda \cdot ([Ca^{2+}]_m + S_\lambda \cdot K_d)/([Ca^{2+}]_m + K_d) \quad \text{(A3b)}$$

$$F'^c_\lambda = F'^{max}_\lambda \cdot ([Ca^{2+}]_c + S_\lambda \cdot K_d)/([Ca^{2+}]_c + K_d) \quad \text{(A3c)}$$

$$f_m = n(\text{Indo})_m/\{n(\text{Indo})_m + n(\text{Indo})_c\}. \quad \text{(A3d)}$$

In Eq. A3, f_m is the fraction of Indo-1 in the noncytosol ($1 - f_m$ is the fraction in the cytosol), and F'^c_λ and F'^m_λ represent the total (background subtracted) fluorescence intensity of cytosolic and noncytosolic Indo-1, respectively (corresponding to I_λ^c and I_λ^m in Eq. A1a–b). If f_m, $[Ca^{2+}]_m$, and F'^{max}_λ as well as the calibration constants S_λ and K_d were known, the "true" $[Ca^{2+}]_c$ could be calculated. (Calculation can be done at either emission wavelength, and should provide identical results.) The remaining obstacle is to determine F'^{max}_λ.

As described previously, the maximum Indo-1 ratio, R^{max}, could be calculated without application of saturating $[Ca^{2+}]$ (Brandes et al., 1993). A similar approach can be used to calculate F'^{max}_λ according to the following; If the fluorescence intensities measured at 385 and 456 nm were plotted against each other, a linear relationship with slope *bH* and intercept *aH* would be obtained (Brandes et al., 1993):

$$F_{456} = bH \cdot F_{385} + aH, \quad \text{(A4a)}$$

where

$$bH = (g_{456}/g_{385}) \cdot b \quad \text{(A4b)}$$

$$aH = g_{456} \cdot \{n(\text{Indo})_c + n(\text{Indo})_m\} \cdot a + B_{456}(\text{al}) - bH \cdot B_{385}(\text{al}) \quad \text{(A4c)}$$

and

$$b = (I_{456}^{max} - I_{456}^{min})/(I_{385}^{max} - I_{385}^{min}) \quad \text{(A4d)}$$

$$a = (I_{456}^{min} \cdot I_{385}^{max} - I_{456}^{max} \cdot I_{385}^{min})/(I_{385}^{max} - I_{385}^{min}) \quad \text{(A4e)}$$

The background corrected Indo-1 intercept *aH'* is defined as:

$$aH' = aH - B_{456}(\text{al}) + bH \cdot B_{385}(\text{al}). \quad \text{(A5)}$$

By combining Eqs. A1c, A4b, A4d–e, and A5, the following relationships are obtained:

$$(aH'/bH) \cdot \frac{1 - S_{456}}{S_{456} - S_{385}} = I_{385}^{max} \cdot g_{385} \cdot \{n(\text{Indo})_c + n(Indo)_m\} \quad \text{(A6a)}$$

$$aH' \cdot \frac{1 - S_{385}}{S_{456} - S_{385}} = I_{456}^{max} \cdot g_{454} \cdot \{n(\text{Indo})_c + n(\text{Indo})_m\}. \quad \text{(A6b)}$$

The right-hand side of Eqs. A6a and A6b are identical to F'^{max}_{385} and F'^{max}_{456}, respectively, given by Eq. A2. (Eq. A6a–b can easily be verified by direct substitution of *aH'*, *bH*, and S_λ given above). Thus, by calculating *aH'* and *bH* and using the calibration constants S_{385} and S_{456}, F'^{max}_{385} and F'^{max}_{456} can be calculated.

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