Endocardial Versus Epicardial Differences of Intracellular Free Calcium Under Normal and Ischemic Conditions in Perfused Rat Hearts

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Transmural heterogeneity of myocardial metabolism and function are present in the left ventricle under normal and ischemic conditions. To determine if endocardial versus epicardial differences of $[Ca^{2+}]$, are also present, perfused rat heart studies using indo-1 fluorescence as an index of $[Ca^{2+}]$, were performed in the left ventricular epicardium and endocardium. Hearts were studied under control conditions and low-flow ischemia. Results demonstrated the following: 1) At a pacing rate of 1.5 Hz, endocardial levels of diastolic and systolic $[Ca^{2+}]$, (470±40 and 1,240±170 nM) were higher than epicardial levels (290±30 and 920±150 nM). 2) At a more physiological pacing rate of 5 Hz, endocardial levels of diastolic and systolic $[Ca^{2+}]$, (680±50 and 1,230±70 nM) were also higher than epicardial levels (390±20 and 950±60 nM). 3) During low-flow ischemia, endocardial levels of diastolic $[Ca^{2+}]$, rose to a greater degree (from 680±50 to 1,050±70 nM at 10% of control coronary flow) compared with epicardial levels (from 390±20 to 580±40 nM at 10% of control flow), suggesting that the endocardium is more susceptible to low-flow ischemia. 4) The amplitude of the $[Ca^{2+}]$, transient was the same at the endocardium (540±50 nM) and epicardium (560±50 nM) and did not change during low-flow ischemia, despite marked contractile dysfunction. These findings are consistent with the hypothesis that endocardial versus epicardial differences of $[Ca^{2+}]$, exist under normal and low-flow ischemic conditions and may, in part, account for the previous observations of transmural metabolic and functional gradients in the left ventricle of the whole heart. Furthermore, the contractile failure associated with low-flow ischemia is not due to a decrease of the $[Ca^{2+}]$, transient amplitude. (Circulation Research 1993;72:1082-1090)

Key Words • indo-1 • contractility • endocardium • epicardium

Pathological studies of patients with coronary artery disease have demonstrated that the endocardium has a greater degree of myocardial necrosis, fibrosis, and metabolic abnormalities than the epicardium.1,2 These findings suggest that the endocardium is more susceptible to ischemia than the epicardium. To determine the factors that predispose the endocardium to ischemic injury, investigators have examined the transmural distribution of numerous myocardial metabolic and functional parameters under normal physiological conditions and during low-flow and no-flow ischemia.1-23

Under normal physiological conditions, transmural gradients have been demonstrated for high-energy phosphate metabolites,3 glycolytic enzyme activities,4 substrate oxidative capacity,5 and oxygen tension and consumption,6,7 as well as coronary flow,6,8,9 intravascular pressure,10 sarcomere length,11,12 and myofiber strain.13 During low-flow and no-flow ischemia, transmural metabolic gradients have been shown for high-energy phosphates,14-18 intracellular pH,14,17 lactate concentration,15,19 and mitochondrial redox state.20 During ischemia, transmural heterogeneity has also been demonstrated for coronary flow,8,18,21 sarcomere length,11,12,22 and systolic contractile force.21,23 The mechanisms underlying this transmural heterogeneity of myocardial metabolic and mechanical function have not been determined. It has been suggested that transmural differences of wall stress, oxygen supply, and/or energy expenditure may account for these observed transmural gradients.3,6,10

The finding of transmural gradients of metabolic and mechanical function raises the possibility that transmural differences of intracellular calcium, an important regulator of myocyte metabolism and contractility, may also occur. Since elevated intracellular calcium levels have been implicated as a mediator of myocyte ischemic
injury, a transmural gradient of intracellular calcium may account for the greater susceptibility of the endocardium to ischemic injury. Although several studies have examined \([\text{Ca}^{2+}]\), in perfused hearts under normal physiological conditions and during low-flow and no-flow ischemia,\(^{24-27}\) no previous study has measured \([\text{Ca}^{2+}]\) at both the epicardium and endocardium.

Therefore, the goals of the present study were 1) to use a novel technique recently developed in our laboratory\(^{28}\) to estimate \([\text{Ca}^{2+}]\) at both the epicardium and endocardium of the left ventricle in a perfused rat heart model of low-flow ischemia, 2) to test the hypothesis that endocardial versus epicardial differences of \([\text{Ca}^{2+}]\) exist under normal physiological conditions, and 3) to test the hypothesis that endocardial versus epicardial differences of \([\text{Ca}^{2+}]\) develop during severe reductions of coronary flow. These hypotheses were tested in isolated perfused rat hearts using indo-1 calcium fluorescence to estimate \([\text{Ca}^{2+}]\) at the epicardium and endocardium during control and low-flow ischemic conditions.

Materials and Methods

Heart Perfusion and Measurement of Function

Male Sprague-Dawley rats (450–550 g) were heparinized (1,000 units i.p.) and anesthetized with ketamine 100 mg i.p. Hearts were excised and arrested in cold isosmotic saline (20 mM KCl). Isolated hearts were perfused retrograde via the aorta at a constant pressure of 71 mm Hg with a Krebs-Heinslieit perfusate containing (mM) NaCl 118, KCl 6.0, CaCl\(_2\) 2.5, NaHCO\(_3\) 25, MgSO\(_4\) 1.2, Na\(_2\)EDTA 0.5, glucose 4.0, and pyruvate 10.0, along with 20 units/i insulin. The perfusate was continuously bubbled with 95% O\(_2\)-5% CO\(_2\) and maintained at 37°C. A compliant latex balloon attached to a Trantec pressure transducer (American Edwards Laboratories, Irvine, Calif.) via rigid polyethylene tubing was inserted into the left ventricle to measure pressure. Left ventricular diastolic pressure was set at approximately 10 mm Hg by adjusting balloon volume. Developed pressure was recorded on a series 8000 chart recorder (Gould Electronics, Hayward, Calif.). Hearts were paced at 1.5 (after removal of the sinoatrial node) or 5 Hz with two platinum-tipped electrodes connected to a stimulus generator (model SD-5, Grass Instrument Co., Quincy, Mass.). Coronary flow was measured by collecting the heart effluent. Coronary flow reductions were produced using an in-line flowmeter that controlled coronary perfusion pressure (Gilmont Instruments Inc., Barrington, Ill.).

Fluorescence Measurements

Fluorescence instrumentation. Fluorescence studies were performed with a modified spectrofluorometer (model 48000S, SLM Instruments Inc., Urbana, Ill.). Excitation light from a 450-W xenon arc lamp (SLM Instruments) was filtered through a 350-nm interference filter and focused onto the in-going leg of a quartz bifurcated fiber bundle. For epicardial measurements, the common leg of this 0.69-mm-diameter fiber bundle, with a fixed rubber hood (1.09 mm o.d.) protruding 1.5 mm past the fiber bundle end, was girdled against the epicardial surface of the left ventricle, avoiding visible vessels. For endocardial measurements, this same fiber bundle was passed through the left atrium and mitral valve onto the left ventricular endocardial surface above the pressure balloon. A nylon girdle about the heart was used to hold the fiber bundle in place relative to the heart. Repeated repositioning of the fiber bundle on different parts of the heart surface (both endocardium and epicardium) resulted in reproducible fluorescence intensities and transient ratios. A shutter in front of the excitation light was opened for only seconds at a time during data acquisition to prevent bleaching of indo-1 fluorescence. The fluorescent signal was transferred via the outgoing leg of the bundle and separated by a dichroic mirror onto two photomultiplier tubes preceded by 385- and 456-nm interference filters. Signal acquisition rate was 200 acquisitions per second (40 acquisitions per cardiac cycle).

Indo-1 methods. After a 30-minute equilibration period, hearts were loaded for 30 minutes with the cell-permeable fluorescent calcium indicator indo-1 AM (Molecular Probes, Inc., Eugene, Ore.) by perfusion with Krebs-Heinslieit buffer containing 5 µM indo-1 AM (initially dissolved in 1.5 ml dimethylsulfoxide containing 0.165 g Pluronic F-127/ml) in the presence of 5% fetal calf serum. Residual indo-1 AM was washed out by perfusing with standard buffer for 45 minutes. Loading with indo-1 resulted in a 12±2-fold increase in fluorescence at the 385-nm emission wavelength compared with initial background fluorescence. Probenecid (0.1 mM) was added to all buffer solutions to slow the extrusion of indo-1 from myocytes. When this technique was used, high signal-to-noise fluorescence signals were present for more than 2 hours.

Calcium transient determination. \([\text{Ca}^{2+}]\) was monitored using the “corrected” ratio of indo-1 fluorescence intensities measured at emission wavelengths of 385 and 456 nm (F385 and F456, respectively). Several factors that could affect the indo-1 fluorescence ratio (and thus affect \([\text{Ca}^{2+}]\), determination) were taken into account. Motion artifact was minimized by securing the fiber bundle to the heart using a nylon girdle and by monitoring two emission wavelengths that are relatively close in the fluorescence spectrum, since this has been shown to further minimize motion artifact.\(^{29}\) Changes in tissue light absorption (predominantly due to myoglobin) were minimized by using emission wavelengths at which light absorbance was independent of the tissue oxygenation state.\(^{30,31}\) Changes of background fluorescence during coronary flow reductions (primarily due to NADH) were accounted for by subtracting mean background fluorescence intensities, previously determined in another set of experiments (see “NADH measurements” and “Experimental Design” below), from the appropriate control or low-flow indo-1 fluorescence signal at each emission wavelength. The corrected ratio was then calculated from the corrected F385 and F456 intensities and used to determine \([\text{Ca}^{2+}]\).

NADH measurements. Changes of background fluorescence due to reductions in coronary flow (primarily due to NADH) were accounted for in two ways. First, the control background fluorescence intensities at F385 and F456 were measured in unloaded hearts at both the epicardium and endocardium at control flow (n=13) and during graded coronary flow reductions (50%, 20%, and 10% of control flow, n=5) in an identical fashion to the protocol used in indo-1–loaded hearts (see “Exper-
mM ionomycin (in the presence of 6% fetal calf serum) was added to the perfusate. To inhibit energy-dependent calcium transport processes, the perfusate also contained 1.0 mM iodoacetate and was bubbled with 100% N₂, thereby inhibiting glycolysis and oxidative phosphorylation. The resulting indo-1 fluorescence intensities at emission wavelengths F385 and F456, which peaked (F385감) or troughed (F456감) after 1–2 minutes, were recorded. In a parallel set of experiments using the same protocol in unloaded hearts, changes of the background fluorescence (F385감 and F456 감) were measured to correct F385 감 and F456 감 before calculation of R 감 (epicardium, n=3; endocardium, n=3), using the following equation:

$$R_{\text{감}} = \frac{(F385_{\text{감}} - F385_{\text{ 감}})}{(F456_{\text{ 감}} - F456_{\text{ 감}})}$$  \hspace{1cm} (1)$$

*Quantitative estimation of indo-1 fluorescence data.* To obtain a quantitative estimate of differences in epicardial and endocardial calcium levels, [Ca²⁺], was calculated from the following equation adapted from Gryniewicz et al:34

$$[\text{Ca}^{2+}] = K_d \cdot \frac{(R - R_{\text{ 감}})}{(R_{\text{ 감}} - R)} \cdot S456b/S456h$$  \hspace{1cm} (2)$$

where $K_d$ is the indo-1 dissociation constant for calcium in the myocyte, R is the observed background corrected indo-1 fluorescence ratio, R 감 is R at [Ca²⁺]=0, and S456/S456h is the ratio of calcium-free and calcium-saturated indo-1 free acid measured at F456 in a heart homogenate cuvette study. Based on the findings of Hove-Madsen and Bers in permeabilized myocytes, a value of 1,000 nM was used for $K_d$. $R_{\text{ 감}}$ was determined as described above. $R_{\text{ 감}}$ was calculated using the following equations adapted from Mohabir et al:25

$$F385_{\text{ 감}} = F385_{\text{ 감}} + (F385_{\text{ 감}} - F385_{\text{ 감}}) \cdot S385b/S385b$$  \hspace{1cm} (3)$$

$$F456_{\text{ 감}} = F456_{\text{ 감}} + (F456_{\text{ 감}} - F456_{\text{ 감}}) \cdot S456b/S456b$$  \hspace{1cm} (4)$$

$$R_{\text{ 감}} = \frac{(F385_{\text{ 감}} - F385_{\text{ 감}})}{(F456_{\text{ 감}} - F456_{\text{ 감}})}$$  \hspace{1cm} (5)$$

where F385 감 and F456 감 are the calculated indo-1 emission wavelength intensities at [Ca²⁺]=0, and S385 감, S385 감, and S456 감 are the ratios of calcium-free and calcium-saturated indo-1 free acid at each emission wavelength in heart homogenate cuvette experiments (described below).

To verify that no significant residual unhydrolyzed indo-1 AM remained after a 45-minute washout (which could spuriously affect quantitation) and that background fluorescence intensities (NADH) were unchanged after indo-1 loading, indo-1 fluorescence was quenched by subjecting hearts to a HEPES-buffered perfusate containing 80 mM MnCl₂ (in place of the CaCl₂), to quench the indo-1 fluorescence signal, 10 μM ionomycin, and 6% fetal calf serum. The resulting values of F385 (F385 감 감) and F456 (F456 감 감), which represent background fluorescence plus any residual unhydrolyzed indo-1 AM, reached a plateau after approximately 3 minutes and were recorded. S385 감 감 감 감 and S456 감 감 감 감 were determined in heart homogenate solutions prepared with four per-
fused rat hearts (approximately 6 g wet wt) and 15 ml HEPES (5 mM) perfusate containing 20 mM NaCl, 115 mM KCl, and 75 μM indo-1 free acid. Homogenate solutions were prepared using a Polytron blender (Brinkmann Instruments, Ontario, Canada) and ultra-centrifugation (100,000g for 1 hour). Before fluorescence measurements, either 20 mM EGTA (calcium-free indo-1) or 20 mM CaCl₂ (calcium-saturated indo-1) was added to this solution and placed in a cuvette (solutions corrected to pH 7.1). The isosbestic points for indo-1 in the heart homogenate and whole-heart fluorescence studies were similar (approximately 429 nm). S385f/S385b and S456f/S456b were then calculated from the indo-1 fluorescence intensities at emission wavelengths of 385 and 456 nm, respectively, for the calcium-free (S385f) and calcium-saturated (S385b and S456b) cuvette solutions.

**Experimental Design**

After indo-1 loading and baseline measurements of coronary flow, developed pressure, and F385 and F456, hearts (n=8) were subjected to a 2.6-minute period of a coronary flow reduction (50%, 20%, or 10% of control flow), at which time measurements of coronary flow, developed pressure, and F385 and F456 were repeated. This 2.6-minute acquisition time was previously demonstrated to be the time at which a steady state was reached for approximately 30 seconds for developed pressure, high-energy phosphate metabolites, and F385 and F456, but of a short enough duration to prevent myocardial stunning or damage. A 15-minute recovery period followed each flow reduction, which allowed coronary flow and developed pressure to return to baseline levels. Baseline measurements were repeated before each of the flow reductions, which were performed in random order. This protocol was performed at both the epicardium (n=5) or at the endocardium (n=5) in a random order. At the end of each experiment, a set of control measurements was taken.

All studies were approved by the institutional animal experimentation committee and adhered to the guidelines set out by the National Institutes of Health guide for the care and use of laboratory animals.

**Statistical Analyses**

Values are reported as mean±SEM. Statistical analyses were performed using paired t tests, repeated-measures analysis of variance, or linear regression, when indicated. Results were considered significant at p<0.05. Results not found to be statistically significant were reported as p=NS.

**Results**

**Heart Function**

Loading with indo-1 resulted in a 33% decrease of baseline developed pressure from 92±3 to 61±4 mm Hg but had no effect on control diastolic pressure (10±1 mm Hg) or coronary flow (14.2±0.6 ml/g wet wt). There was no difference in control coronary flow or developed pressure between groups of hearts studied at the epicardium and endocardium. Reductions of coronary flow to 50%, 20%, and 10% of control flow produced expected and similar linear decreases of developed pressure in both unloaded and indo-1-loaded hearts. No differences between epicardial and endocardial experiments were observed in the fall of developed pressure after coronary flow reductions. Control measurements of coronary flow and diastolic pressure at the conclusion of experiments were unchanged when compared with baseline measurements before flow reductions. Developed pressure decreased approximately 8% over the course of each experiment.

**Background (NADH) Fluorescence Changes**

Control background fluorescence intensities were found to be similar and reproducible at the endocardium (F385, 0.57±0.02 intensity units; F456, 0.61±0.02 intensity units; n=13) and epicardium (F385, 0.57±0.03 intensity units; F456, 0.58±0.04 intensity units; n=13) (p=NS for endocardial versus epicardial). The percent increase of background fluorescence during coronary flow reductions to 50%, 20%, and 10% of control flow (primarily due to increased NADH) tended to be greater at the endocardium than at the epicardium but did not reach statistical significance (n=5). Background fluorescence at F456 increased 5±1% at the epicardium versus 8±1% at the endocardium at 50% of control flow; 17±2% versus 20±2%, respectively, at 20% of control flow; and 33±7% versus 40±5%, respectively, at 10% of control flow. It should be noted that subtraction of these increases in background fluorescence from the indo-1 signal during low-flow ischemia results in a reduction of the indo-1 fluorescence intensities of only 0.5–2.3% after background correction. This is the result of 12 times indo-1 loading compared with background fluorescence levels at 385 nm.

**Potential Nonmyocyte Contribution to Indo-1 Fluorescence**

To examine the potential contribution of alterations in endothelial cell calcium during ischemia to the indo-1 fluorescence signal in perfused rat hearts, indo-1-loaded hearts were perfused with bradykinin (10⁻⁵ M) to abruptly increase endothelial cytosolic calcium. Bradykinin is known to produce a vasodilatory response in rat endothelium that is mediated through an increase in endothelial cytosolic calcium, which in turn increases endothelium-derived relaxing factor activity.³⁶,³⁷ Perfusion with bradykinin resulted in a 16±4% increase of coronary flow (p<0.05) after 30 seconds because of its vasodilatory effect. Left ventricular pressure was unchanged. Bradykinin infusion caused an increase in fluorescence at F385 and a decrease at F456, which occurred and leveled off within 17–24 seconds (epicardium and endocardium were similar). The percent increase of the minimum corrected fluorescence ratio, an index of diastolic [Ca²⁺], was 9±4% at the epicardium and 7±2% at the endocardium. The percent increase of the minimum corrected fluorescence ratio, an index of systolic [Ca²⁺], was 6±2% at the epicardium and 7±3% at the endocardium.

To further confirm that the single endothelial cell layer on the endocardial surface does not disproportionately influence the indo-1 fluorescence signal from the endocardium, confocal fluorescence microscopy experiments were performed. Careful examination of the endocardium (and epicardium) of freshly indo-1-loaded hearts did not reveal a surface rim of increased fluorescence...
cience intensity above the baseline fluorescence of the remainder of the endocardium. This would suggest that differences in the indo-1 fluorescence ratio at the endocardium, relative to the epicardium, cannot be explained by changes in endothelial fluorescence.

**Standardization and Quantitation of Indo-1 Fluorescence**

$R_{\text{max}}$ was determined at both the epicardium and endocardium to rule out the possibility that differences in the tissue filter effect existed that could confound comparison of indo-1 fluorescence intensities between the epicardium and endocardium. $R_{\text{max}}$ was similar at the epicardium and the endocardium (6.2±0.2 versus 6.8±0.3, $p=\text{NS}$). Background fluorescence changes due to NADH under conditions of complete metabolic inhibition and saturating calcium were also similar; the percent increase in background fluorescence was 55±5% at the epicardium and 50±7% at the endocardium ($p=\text{NS}$). Of note, this corresponds to a further subtraction of background contribution from the indo-1 signal at 385 nm of only 4.2–4.5%. These findings suggest that differences in the tissue filter effect that could confound comparison of indo-1 fluorescence intensities at the endocardium and epicardium do not exist.

To provide a quantitative means for converting indo-1 fluorescence data to [Ca$^{2+}$], the following parameters were also determined: S385/S385, and S456/S456, were 0.07 and 2.12, respectively, and the calculated $R_{\text{min}}$ values were 0.20±0.04 at the endocardium and 0.20±0.03 at the epicardium. Because $R_{\text{max}}$ and $R_{\text{min}}$ were the same at the endocardium and epicardium, results were pooled ($R_{\text{max}}$, 6.5±0.2; $R_{\text{min}}$, 0.20±0.03).

After quenching of the indo-1 fluorescence, there was no difference observed between F385$_{\text{Mg}^{2+}}$ and F456$_{\text{Mg}^{2+}}$ values, which represent background fluorescence and any residual unhydrolyzed indo-1 AM, and control background fluorescence intensities (before indo-1 loading). Nor was there a difference between the epicardial and endocardial F385$_{\text{Mg}^{2+}}$ and F456$_{\text{Mg}^{2+}}$ values. This suggests that there was no significant residual unhydrolyzed indo-1 AM in the myocyte using the present loading protocol at the endocardium or epicardium.

**[Ca$^{2+}$], Under Normal Physiological Conditions**

Representative corrected fluorescence ratios at the epicardium and endocardium are shown in Figure 1. The maximum and minimum corrected fluorescence ratios, indexes of systolic and diastolic [Ca$^{2+}$], were higher at the endocardium than the epicardium under control conditions. To demonstrate the magnitude of this difference in more physiologic terms, indo-1 fluorescence intensities, an index of [Ca$^{2+}$] levels, were converted to nanomolar values. Figure 2 shows the calculated [Ca$^{2+}$], at the epicardium and endocardium under control conditions and during graded reductions of coronary flow in hearts paced at a physiological rate of 5 Hz. At control flow, values for diastolic and systolic [Ca$^{2+}$] were higher at the endocardium (680±50 and 1,230±70 nM, respectively) compared with the epicardium (390±20 and 950±60 nM, respectively) ($p<0.05$ for epicardial versus endocardial diastolic and systolic concentrations). As shown in Figure 3, despite the difference in diastolic [Ca$^{2+}$], the size of the [Ca$^{2+}$] transient was similar at the endocardium (540±40 nM) and epicardium (560±50 nM) under control conditions.

When hearts were paced at 1.5 Hz under control conditions (epicardium, $n=5$; endocardium, $n=4$), values for diastolic and systolic [Ca$^{2+}$] were also higher at the endocardium (470±40 and 1,240±170 nM, respec-

**Figure 1.** Representative tracings of the epicardial (panel A) and endocardial (panel B) indo-1 calcium fluorescence ratio (R) under control conditions (solid line) and during a coronary flow reduction to 10% of control flow (dashed line) in a perfused rat heart. R, an index of intracellular free calcium (predominantly cytosolic free calcium, [Ca$^{2+}$]), is the ratio of the single-emission wavelength indo-1 calcium fluorescence intensities at 385 and 456 nm after excitation at 350 nm. After corrections for changes of background fluorescence (primarily NADH), R is converted into [Ca$^{2+}$].

**Figure 2.** Graphs showing diastolic (closed squares) and systolic (open squares) [Ca$^{2+}$], (predominantly cytosolic free calcium) under control conditions and during coronary flow reductions to 50%, 20%, and 10% of control flow in the epicardium (panel A) and the endocardium (panel B). Pooled data ($n=8$) are presented as mean±SEM. Values for endocardial [Ca$^{2+}$], are shown to be higher than values for epicardial [Ca$^{2+}$], under control and low-flow conditions. At coronary flow reductions to 20% and 10% of control flow, diastolic [Ca$^{2+}$] increased relative to control levels at both the epicardium and the endocardium (*p<0.05).
tively) compared with the epicardium (290±30 and 920±150 nM, respectively) (p<0.05 for epicardial versus endocardial diastolic [Ca2+]).

\[ \text{[Ca}^{2+}] \text{, During Low-Flow Ischemia} \]

Figure 1 demonstrates the greater rise of the minimum corrected fluorescence ratio, an index of diastolic [Ca2+]i, at the endocardium during a coronary flow reduction to 10% of control flow compared with the increase at the epicardium. As shown in Figure 4, a coronary flow reduction to 10% of control flow resulted in a rise of the [Ca2+]i transient, determined from an elevation of the corrected fluorescence ratio, which occurred because of the expected increase of the indo-1 fluorescence at 385 nm and decrease at 456 nm, after increased [Ca2+]i.

Figure 2 shows calculated [Ca2+]i at the epicardium and endocardium during graded reductions of coronary flow. Values of diastolic and systolic [Ca2+]i, were unchanged during a mild coronary flow reduction to 50% of control flow at both the endocardium and epicardium (p=NS at both the epicardium and endocardium). In contrast, diastolic and systolic [Ca2+]i increased after more severe reductions of flow to 20% and 10% of control flow. Figure 2 shows that at the epicardium, diastolic [Ca2+]i increased from 390±20 to 500±20 nM at 20% of control flow (p<0.05) and to 580±40 nM at 10% of control flow (p<0.05). Endocardial diastolic [Ca2+]i increased from an initial control value of 680±50 to 880±70 nM at 20% of control flow (p<0.05) and to 1,050±70 nM at 10% of control flow (p<0.05). Thus, at coronary flow reductions to 20% and 10% of control flow, the relative increase of diastolic [Ca2+]i was greater at the endocardium than at the epicardium (p<0.05). At 10% of control flow, endocardial diastolic [Ca2+]i, rose 370±40 nM while epicardial diastolic [Ca2+]i, increased 190±30 nM (p<0.05). However, despite changes in diastolic [Ca2+]i, the [Ca2+]i transient amplitude did not significantly change at any coronary flow reduction (Figure 3). At a coronary flow reduction to 10% of control flow, the [Ca2+]i transient was 490±70 nM at the epicardium and 510±60 nM at the endocardium (p=NS versus control values).

\[ \text{Discussion} \]

This study uses a novel technique developed in this laboratory28 for comparing [Ca2+]i at the epicardium and endocardium of the left ventricle in a perfused heart model during control and low-flow ischemic conditions. The major findings of this study were as follows: 1) Levels of endocardial diastolic and systolic [Ca2+]i were higher than epicardial levels during control conditions. 2) During low-flow ischemia, diastolic and systolic [Ca2+]i increased more at the endocardium than at the epicardium. 3) The amplitude of the [Ca2+]i transient was the same at the endocardium and epicardium under normal physiological conditions. 4) Despite contractile failure, the amplitude of the [Ca2+]i transient did not change with low-flow ischemia. These findings are consistent with the hypothesis that endocardial versus epicardial differences of [Ca2+]i exist under normal physiological conditions and during low-flow ischemia, paralleling previous observations of transmural metabolic and functional gradients in the left ventricular wall of the whole heart. Furthermore, the contractile failure...
associated with low-flow ischemia is not due to a decrease of the [Ca\textsuperscript{2+}] transient.

**Endocardial Versus Epicardial Differences Under Control Conditions**

Transmural gradients of coronary flow, metabolism, and mechanical function in the left ventricle have been extensively documented in in vivo and perfused heart studies under normal physiological conditions.\textsuperscript{3-7,9,12-23} However, the possibility of a transmural gradient of intracellular calcium, an important regulator of cell metabolism and function, has not been previously examined. In the present study, both epicardial and endocardial [Ca\textsuperscript{2+}] were measured in a whole heart. Results demonstrated that levels of diastolic and systolic [Ca\textsuperscript{2+}] were elevated in the endocardium compared with the epicardium under normal physiological conditions. Two possibilities that may account for higher [Ca\textsuperscript{2+}] at the endocardium are 1) qualitative differences in intracellular calcium homeostasis between the endocardium and epicardium, i.e., a different set point for intracellular calcium handling, or 2) a decreased ability of the endocardial myocyte to reuptake cytosolic calcium on a beat-to-beat basis because of relative ischemia at the endocardium under normal physiological conditions. This second possibility seems less likely given the findings of the present study and those of Kanaide et al.,\textsuperscript{20} which demonstrated that NADH levels were similar at the epicardium and endocardium at control coronary flows. This would suggest that, under control conditions, the endocardium is not flow or O\textsubscript{2} limited.

An interesting possibility is that the observed endocardial versus epicardial differences of [Ca\textsuperscript{2+}] may, in part, provide an explanation for the previously observed transmural heterogeneity of metabolic and mechanical function.\textsuperscript{3-7,9,12-23} The relative elevation of endocardial [Ca\textsuperscript{2+}X] could account for previously demonstrated transmural metabolic gradients for rates of substrate oxidation\textsuperscript{4} and glycolytic enzyme activities.\textsuperscript{4} Elevated endocardial [Ca\textsuperscript{2+}] may also provide an explanation for the previous findings of shorter mean sarcomere and myofiber lengths at the endocardium under normal physiological conditions.\textsuperscript{11-13} An alternative explanation is that muscle length (i.e., shorter mean sarcomere and myofiber lengths at the endocardium\textsuperscript{11-13}) influences diastolic [Ca\textsuperscript{2+}], as suggested by recent observations of Allen et al.,\textsuperscript{38} who used aequorin to measure calcium in ferret papillary muscle at varying lengths.

**Endocardial Versus Epicardial Differences During Low-Flow Ischemia**

Transmural gradients of coronary flow, metabolism, and mechanical function in the left ventricle have been extensively documented during low-flow and no-flow ischemia.\textsuperscript{8-10,12,14-22} Greater reductions of coronary flow\textsuperscript{8,18,21} and systolic contractile force\textsuperscript{21} and more marked alterations of energy metabolism\textsuperscript{14-20} occur at the endocardium than at the epicardium during low-flow and no-flow ischemia. Taken together, these previous studies strongly suggest that the endocardium is more prone to ischemia after an alteration of flow or substrate supply. Since intracellular calcium cycling is a highly energy-dependent process, transmural differences of intracellular calcium handling during low-flow ischemia would be expected. In the present study, diastolic and systolic [Ca\textsuperscript{2+}] increased more during severe reductions of coronary flow (10% and 20% of control flow) in the endocardium than in the epicardium. Given the greater vulnerability of the endocardium to ischemia, a decreased ability of the endocardial myocyte sarcoplasmic reticulum to reuptake cytosolic calcium (due to inhibition of Ca\textsuperscript{2+}-ATPase activity) could account for the elevated endocardial [Ca\textsuperscript{2+}] during low-flow conditions. Furthermore, the finding that diastolic [Ca\textsuperscript{2+}] was more elevated in the endocardium than in the epicardium during low-flow ischemia suggests that [Ca\textsuperscript{2+}] may either mediate or be the result of greater shortening of mean sarcomere lengths in the endocardium during acute ischemia.\textsuperscript{11-13,38}

In contrast, during a mild coronary flow reduction to 50% of control flow, there was no change in the diastolic [Ca\textsuperscript{2+}], at either the endocardium or the epicardium. This finding is in agreement with previous observations from this laboratory.\textsuperscript{28} This suggests that, during milder reductions of coronary flow, contractile depression is not due to alterations of intracellular calcium handling transmurally but is probably due to an alteration of the [Ca\textsuperscript{2+}]-pressure relation, possibly mediated through a decrease of vascular pressure or increased intracellular acidosis or inorganic phosphate.\textsuperscript{28} Furthermore, these results indicate that, at milder reductions of coronary flow (i.e., less than a 50% reduction of flow), the endocardium is not sufficiently ischemic to affect intracellular calcium handling. Thus, during mild coronary flow reductions, differences in [Ca\textsuperscript{2+}] between the endocardium and epicardium are not due to ischemic conditions in the endocardium but possibly a different set point for calcium homeostasis in the endocardial myocyte compared with the epicardial myocyte.

**[Ca\textsuperscript{2+}] Transient Amplitude**

The present study also demonstrated that the amplitude of the [Ca\textsuperscript{2+}] transient did not change at any reduction of coronary flow at either the endocardium or epicardium. Previous studies examining the effect of complete or partial reductions of coronary flow on [Ca\textsuperscript{2+}] in the perfused heart\textsuperscript{24-27} have reported conflicting results. Consistent with the present finding that contractile dysfunction during reductions of coronary flow is not due to a decrease of the [Ca\textsuperscript{2+}] transient amplitude, Kihara et al.,\textsuperscript{24} using aequorin, and Mohabir et al.,\textsuperscript{25} using indo-1, demonstrated that, after 90–180 seconds of no-flow ischemia, the epicardial [Ca\textsuperscript{2+}] transient did not decrease but remained unchanged or increased as contractility fell. This is in contrast to studies by Wikman-Coffelt et al.,\textsuperscript{26} using indo-1, and Kitakaze and Marban,\textsuperscript{27} using 5F-BAPTA, that demonstrated decreases in the [Ca\textsuperscript{2+}] transient amplitude during mild reductions of perfusion pressure. Differences between the present study and these studies are most likely due to differences in experimental design and methodology. Kitakaze and Marban used a perfusate calcium concentration of 8 mM to partially compensate for the calcium-buffering effect of 5F-BAPTA.\textsuperscript{27} Furthermore, 5F-BAPTA resulted in developed pressures of approximately 20–40 mm Hg and produced end-diastolic pressures of 40–60 mm Hg (authors’ unpublished data). A reduction of perfusion pressure by 25% produced a nonphysiological depression of devel-
oped pressure of greater than 50%. This suggests that hearts loaded with 5F-BAPTA in the presence of high perfusate calcium may have an altered relation between perfusion pressure and contractility. Wikman-Coffelt et al,26 who used indo-1, monitored emission wavelengths that are not isosbestic with regard to alterations of tissue absorbance (due to changes of the myoglobin oxygenation state). A change in the tissue filter effect after a decrease in perfusion pressure could cause a spurious change in the [Ca$^{2+}$], transient amplitude. Furthermore, Wikman-Coffelt et al used superphysiological coronary perfusion pressures and a lower bath calcium (1 mM). This suggests that the conditions under which their measurements were made were less physiological than those of the present study. The results of the present study support the previous finding that, during graded reductions of coronary flow in a perfused heart model that more closely approximates normal physiological conditions, the fall of contractility is not due to a decrease of the [Ca$^{2+}$], transient.28 This is true at both the endocardium and epicardium.

Limitations

Many factors can spuriously affect endocardial and epicardial measurements of [Ca$^{2+}$], using indo-1 fluorescence. These include 1) possible endocardial versus epicardial differences in the tissue filter (myoglobin) effect, 2) motion artifacts, 3) an endothelial cell contribution to the indo-1 signal, and 4) differences in control and ischemic levels of NADH background fluorescence.28-31 To determine whether endocardial versus epicardial differences in tissue filtering exist, experiments were performed to determine $R_{\text{max}}$ at both the endocardium and epicardium. The finding that $R_{\text{max}}$ was similar at the endocardium and epicardium suggests that differences in tissue filtering are not responsible for the observed endocardial versus epicardial fluorescence differences. Motion artifact and changes of background autofluorescence (primarily NADH) were accounted for as previously described (see References 28-30 and "Materials and Methods"). The contribution of the indo-1 fluorescence signal from changes in endothelial cell calcium has been shown to be significant in perfused rabbit hearts by infusion with bradykinin, an endothelial cell calcium agonist.32 As previously shown in another laboratory,26 the present study demonstrated that bradykinin effect is small in perfused rat hearts and suggests that endothelial contribution is a small component of the observed increases of the indo-1 fluorescence transient during low-flow ischemia. Furthermore, confocal fluorescence microscopy experiments of the endocardial (and epicardial) rim of freshly indo-1-loaded hearts demonstrated no preferential indo-1 uptake versus the endocardium or the epicardium.

Two other artifacts that must be considered when interpreting indo-1 calcium fluorescence data are intracellular residual unhydrolyzed indo-1 (calcium insensitive) and compartmentalized dye. In this study, we demonstrated that, after quenching of the indo-1 fluorescence with MnCl$_2$, the residual fluorescence was the same as the initial background autofluorescence. This would suggest that there was no significant residual unhydrolyzed indo-1 AM remaining in the myocyte using the described loading protocol. With regard to the possibility of dye compartmentation, Miyata et al reported significant mitochondrial trapping of indo-1 in single myocytes. However, other investigators have found that approximately 5–10% of indo-1 is compartmentalized in their myocyte preparations.40-42 In a perfused whole-heart preparation (with loading conditions similar to those of the present study), Lee et al43 found that very little of the Mn$^{2+}$-quenchable indo-1 fluorescence was present in the mitochondria. It may be that the amount of compartmentation is due to different loading conditions (some cell preparations use much higher concentrations of indo-1) and/or differences in the myocyte’s esterase efficiency between models. Further studies will be required to determine the importance of dye compartmentation when interpreting indo-1 calcium fluorescence data.

To demonstrate the magnitude of endocardial versus epicardial differences of indo-1 calcium fluorescence data in more quantitative terms, relative indo-1 fluorescence intensities were converted to absolute concentrations. There are a number of difficulties in quantitating [Ca$^{2+}$], in whole hearts with fluorescent calcium indicators.28,32,34,43 The major difficulty lies in achieving accurate intracellular measurements of indo-1 calcium fluorescence intensities under conditions of saturating and zero calcium (to determine $R_{\text{ax}}$ and $R_{\text{min}}$) and in determining the $K_d$ of indo-1 for calcium within the myocyte. Several investigators have previously used cuvette values of $K_d$ to calibrate indo-1 fluorescence data.25,26 However, using data from cuvette studies may not reflect the possible effects of the intracellular milieu on $K_d$. A recent study by Hove-Madsen and Bers,35 using permeabilized myocytes, demonstrated that a more appropriate $K_d$ for the dissociation of calcium from indo-1 in the myocyte was approximately 1,000 nM. In the present study, the resulting epicardial diastolic [Ca$^{2+}$], was found to be slightly higher than previously observed diastolic [Ca$^{2+}$], from several perfused heart,24-26,44 papillary muscle,45 and isolated myocyte46,46 studies. This is likely due to the higher (and more physiological) pacing rate of 5 Hz used in the present study, which does not allow complete diastolic relaxation of [Ca$^{2+}$], before reexcitation. Pacing at a slower rate of 1.5 Hz resulted in lower epicardial [Ca$^{2+}$], which compares well with previous measurements,24-26,40,44-46

Conclusions

Although the present method of quantitation provides only an estimate of absolute [Ca$^{2+}$], the present study demonstrates that relative differences in control and low-flow ischemic [Ca$^{2+}$], exist between the epicardium and endocardium. Thus, these findings are consistent with the hypothesis that endocardial versus epicardial differences of [Ca$^{2+}$], exist under control conditions. Furthermore, additional differences of [Ca$^{2+}$], develop during low-flow ischemia between the endocardium and epicardium. These endocardial versus epicardial differences of calcium homeostasis parallel, and may partly account for, previous observations of transmural metabolic and functional gradients in the left ventricle of the whole heart.

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