Ca^{2+} Transient Decline and Myocardial Relaxation Are Slowed during Low Flow Ischemia in Rat Hearts

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Abstract

The mechanisms that impair myocardial relaxation during ischemia are believed to involve abnormalities of calcium handling. However, there is little direct evidence to support this hypothesis. Therefore, we sought to determine whether the time constant of cytosolic calcium ($[Ca^{2+}]_c$) decline (τ_{Ca}) was increased during low flow ischemia, and if there was a relationship between the time constant of left ventricular pressure decline (τ_P) and τ_{Ca}. Isolated perfused hearts were studied using indo-1 fluorescence ratio as an index of $[Ca^{2+}]_c$. τ_P was used as an index of myocardial relaxation. The time constant of decline of the indo-1 ratio increased from 74±5 ms to 95±4, 144±10, and to 204±16 ms when coronary flow was reduced was reduced to 50, 20, and 10% of control, respectively. Indo-1 transients were calibrated to calculate τ_{Ca}. τ_{Ca} increased from 67±6 ms to 108±9 and 158±19 ms when coronary flow was reduced to 20 and 10% of control, respectively. There was a linear relationship between τ_{Ca} and τ_P (r = 0.82). These data support the hypothesis that during low flow ischemia, impaired myocardial relaxation may be caused by slowing of $[Ca^{2+}]_c$ decline. (*J. Clin. Invest.* 1994. 93:951–957.) Key words: myocardial relaxation • myocardial contraction • fluorescent dyes • indo-1 • coronary flow

Introduction

The ability of the left ventricle to pump blood depends on a complex interaction between systolic contraction and diastolic filling. The effects of myocardial ischemia on systolic contraction have been appreciated for many years. However, the importance of diastolic dysfunction in the pathophysiology of ischemic syndromes has only been recognized more recently (1).

A major manifestation of diastolic dysfunction during ischemia is impaired myocardial relaxation. The most reliable and least load-dependent index of myocardial relaxation is the time constant of left ventricular pressure decline (τ_P)[1] (2–4). Several studies have documented an increase of τ_P during ischemia in a variety of experimental preparations (5–9) and patients with coronary artery disease (10–12).

The mechanisms that impair myocardial relaxation during ischemia are believed to involve abnormalities in calcium handling. Calcium is removed from the cytosol into the sarcoplasmic reticulum and extracellular space against steep electrochemical and concentration gradients (13, 14). Therefore, it has been postulated that the metabolic consequences of ischemia may impair these processes and result in prolonged myocardial relaxation (13, 15, 16). However, there is little direct evidence that cytosolic calcium ($[Ca^{2+}]_c$) decline is abnormal during myocardial ischemia. Most previous studies have evaluated the effects of hypoxia on $[Ca^{2+}]_c$ decline in isolated cells or muscle strips and have found conflicting results. Silverman et al. (17) found no change in $[Ca^{2+}]_c$ decline (measured by indo-1 fluorescence) during hypoxia in isolated myocytes. In contrast, other investigators (18) found an increase in the time to 50% $[Ca^{2+}]_c$ decline (measured by aequorin) in isolated ferret papillary muscle exposed to hypoxia. However, these studies of hypoxia, performed in isolated cardiac cells and papillary muscles, may not be directly applicable to the clinical situation of myocardial ischemia in whole hearts.

Recently, investigators have monitored $[Ca^{2+}]_c$ transients during ischemia in whole hearts using indo-1 fluorescence (19) and aequorin bioluminescence (20). A broadening of the indo-1 fluorescence transient was observed during acute no flow ischemia (19). However, no attempt was made to quantify these changes or relate them to changes in myocardial relaxation. Furthermore, it may be difficult to assess the relationship between $[Ca^{2+}]_c$ transient decline and myocardial relaxation during no flow ischemia, since left ventricular pressure and metabolites are changing rapidly. In contrast, we previously demonstrated that graded reductions of coronary flow (low flow ischemia), produced stable levels of left ventricular pressure, phosphate metabolites, intracellular pH, and indo-1 fluorescence (21, 22). This suggests that low flow ischemia may be a useful intervention in which to study the relationship between $[Ca^{2+}]_c$ decline and myocardial relaxation.

Therefore, the purpose of this study was to test the hypothesis that during low flow ischemia, impaired myocardial relax-

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1. *Abbreviations used in this paper:* τ_P, time constant of left ventricular pressure decline; $[Ca^{2+}]_c$, cytosolic $[Ca^{2+}]$; τ_{Ca}, time constant of cytosolic calcium decline; τ_R, time constant of indo-1 fluorescence ratio decline; $t_{50\%}$, time from peak to 50% decline of a transient.

ation may be caused by slowing of the calcium transient decline. Specifically, we sought to determine whether the time constant of cytosolic calcium decline (τ_{Ca}) was increased during low flow ischemia and if there was a relationship between τ_P and τ_{Ca}. Isolated buffer-perfused hearts were studied using indo-1 fluorescence as an index of $[Ca^{2+}]_c$. Left ventricular pressure was simultaneously recorded to calculate τ_P as a measure of myocardial relaxation.

Methods

Isolated heart preparation. Male Sprague-Dawley rats (450–550 g) were anesthetized using 100 mg/kg i.p. ketamine and anticoagulated using 1,000 U/kg i.p. heparin. Hearts were excised and immediately arrested using cold saline containing KCl (20 mM). Hearts were then perfused according to Langendorff at a control perfusion pressure of 71 mmHg using Krebs-Henseleit buffer containing the following (mM): NaCl, 118; KCl, 6.0; $CaCl_2$, 2.5; $NaHCO_3$, 25; $MgSO_4$, 1.2; Na_2EDTA, 0.5; glucose, 4; pyruvate, 10; and insulin (20 U/liter). The perfusate was continuously bubbled using a 95% O_2/5% CO_2 gas mixture and maintained at 37°C. Hearts were paced at 300 bpm using a stimulus generator (model SD-5; Grass Instrument Co., Quincy, MA). Coronary flow was measured by collecting the heart effluent. Coronary flow reductions were produced using an in-line flow meter that controlled coronary perfusion pressure (Gilmont Instruments, Barrington, IL).

Left ventricular pressure was measured in two ways. In the first group of hearts (n = 3), left ventricular pressure was measured using a high fidelity micromanometer. A compliant latex balloon was attached to a 2-cm segment of rigid polyethylene tubing that was connected to a Y adapter. One end of the Y adapter was used to advance a 3 French, high fidelity micromanometer (Millar Instruments, Inc., Houston, TX) to the latex balloon. The other end of the Y adapter was used to fill the left ventricular balloon with bubble-free water in order to set the end diastolic pressure. The balloon was inserted through the left atrium into the left ventricle. Pressure was recorded on a chart recorder (series 8000; Gould Electronics, Hayward, CA) and also digitized at 3–5 ms intervals by the SLM spectrofluorometer. In this manner, high fidelity micromanometer pressure measurements were obtained in a system that contained water only in the left ventricular balloon and a 2-cm segment of rigid tubing. Because the micromanometer system is large and difficult to insert into the left ventricle, pressure was measured in a second group of hearts (n = 6) using a water-filled system. A 15-cm segment of rigid polyethylene tubing was connected to a pressure transducer (Trantec; American Edwards Laboratories, Irvine, CA) to form a short, fluid-filled system. As previously noted by other investigators (23), τ_P was found to be the same using both the solid-state and fluid-filled systems; left ventricular pressure and τ_P data were, therefore, grouped together. Left ventricular end-diastolic pressure was set to 10±1 mmHg during control.

Indo-1 fluorescence. Fluorescence measurements were performed, as previously described in detail (21, 22, 24), using a modified spectrofluorometer (model 48000S; SLM Instruments, Inc., Urbana, IL). Briefly, light from a 450-W xenon arc lamp filtered through a 360-nm interference filter was used for excitation. 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. Emitted fluorescent light was monitored by two photomultiplier tubes preceded by 385 and 456 nm interference filters that have bandwidths of ±5 nm. These wavelengths were chosen because they were found to be isosbestic in regards to tissue light absorption during ischemia (21, 25) and to minimize motion artifact (26).

Hearts were loaded with indo-1 via retrograde coronary perfusion using Krebs-Henseleit buffer containing fetal calf serum (5%) and indo-1 acetoxymethyl ester (5 μM), dissolved in dimethylsulfoxide and Pluronic F-127 (10% wt/vol). Residual indo-1 acetoxymethyl ester was washed out by perfusing with control Krebs-Henseleit buffer for 30 min. Loading with indo-1 increased emitted fluorescence 14±2 (385 nm) and 7±1 (456 nm) times over heart autofluorescence. Probenecid (0.1 mM) was added to all buffer solutions to slow the extrusion of indo-1 from the myocytes (27).

The ratio of light intensities emitted at 385 and 456 nm was used as an index of $[Ca^{2+}]_c$. The following factors that can affect $[Ca^{2+}]_c$ determination were taken into account: (*a*) motion artifact (26); (*b*) tissue inner filter effect primarily caused by myoglobin (21, 25); (*c*) changes of background autofluorescence predominantly caused by NADH (25); and (*d*) potential contribution by nonmyocyte (bradykinin-sensitive) signal (21, 24).

In a subset of experiments, the fluorescence ratio was calibrated to absolute $[Ca^{2+}]_c$ using the method described previously (24). Briefly, the equation described by Grynkiewicz et al. (28) was used:

$$[Ca^{2+}]_c = K_d * [(R - R_{min})/(R_{max} - R)] * S456_f/S456_b,$$

where K_d is the indo-1 dissociation constant for calcium in myocytes (1,000 nM [29]), R_{min} is the ratio at 0 calcium, R_{max} is the ratio at saturating calcium, $S456_f/S456_b$ is the ratio of calcium-free and calcium-saturated indo-1 fluorescence measured at 456 nm in a heart homogenate, and R is the background-corrected indo-1 fluorescence ratio. The background terms were measured in hearts not loaded with indo-1 during control and low flow ischemia (21).

R_{max} was determined by subjecting a series of indo-1–loaded hearts (n = 8) to high calcium perfusate containing (mM): Hepes, 5; $CaCl_2$, 80; KCl, 6; $MgCl_2$, 1.2; and Na_2EDTA 0.5. To facilitate entry of perfusate calcium into the cell, ionomycin (10 μM, in the presence of 6% fetal calf serum) was added to the perfusate. To inhibit energy-dependent calcium transport, the perfusate also contained 1.0 mM iodoacetate and was bubbled with 100% N_2, thereby inhibiting glycolysis and oxidative phosphorylation. The resulting peak fluorescence intensities at emission wavelengths of 385 and 456 nm (*F385* and *F456*, respectively) were used to calculate R_{max} using the following equation:

$$R_{max} = (F385_{max} - F385_{bg})/(F456_{max} - F456_{bg}),$$

where $F385_{bg}$ and $F456_{bg}$ are the background fluorescence intensities during calcium saturating conditions determined using the same protocol in a group (n = 6) of hearts not loaded with indo-1.

R_{min} was determined in a manner analogous to that described by Mohabir et al. (30) using the ratio of calcium-free and calcium-saturated indo-1 fluorescence measured at 385 and 456 nm in a heart homogenate (24).

Experimental protocol. After a 30-min equilibration period, left ventricular pressure, coronary flow, and background fluorescence were measured (preloading values). After indo-1 loading and washout, control measurements were obtained. Coronary perfusion pressure was then decreased to produce the appropriate reduction of coronary flow. Previous studies have shown that a new steady-state of phosphate metabolites, intracellular pH, and indo-1 fluorescence was reached after 2.5 min of reduced coronary flow and was maintained for ≥ 3 min. Therefore, after 3 min of low flow fluorescence and left ventricular pressures were digitized during a 1-s period. The heart was exposed to excitation light for only brief periods of time to minimize bleaching of indo-1. A 12–15-min reperfusion period followed each coronary flow reduction. Control measurements were repeated after the reperfusion period. This reperfusion period allowed complete functional and metabolic recovery. Three levels of reduced coronary flow (50, 20, and 10% of control) were studied in a random order. In the subset of experiments used to calibrate indo-1 fluorescence, two levels of reduced coronary flow (20 and 10% of control) were studied.

Data analysis. Left ventricular pressure and indo-1 fluorescence data (digitized every 3–5 ms by the SLM spectrofluorometer) were stored in a personal computer. All curve fitting was performed using commercially available graphing software (SigmaPlot; Jandel Scientific, Corte Madera, CA).

τ_P was calculated assuming monoexponential kinetics. The decline portion of the pressure transient starting at minimum dP/dt until minimum left ventricular pressure was used to calculate τ_P. However, the

final 10% of this curve was deleted, since the terminal phase of pressure decline will be primarily determined by calcium-independent processes (31). The following equation was fit to the observed data:

$$P(t) = (P_0 - P_\infty) * \exp(-t/\tau_P) + P_\infty,$$

where P is left ventricular pressure, P_∞ is asymptotic pressure, t is time, and P_0 is left ventricular pressure at minimum dP/dt. P_∞ was allowed to vary, since this is believed to provide the most accurate description of left ventricular relaxation (2).

The time constant of indo-1 ratio transient decline (τ_R) was calculated in a manner analogous to τ_P. τ_R was calculated from the decline portion of the fluorescence transient starting at minimum dR/dt and continuing to the end of the transient minus the final 10%. To determine whether the nonlinearity of the indo-1 response to calcium would affect the results, τ_{Ca} was calculated in a subset of experiments using fluorescence transients calibrated to yield $[Ca^{2+}]_c$. For comparison with previous studies (18), the time to 50% $[Ca^{2+}]_c$ decline ($t_{50\%}$) was also measured.

Statistical analysis. Values are reported as means±SEM. Comparison between interventions was performed using repeated measures analysis of variance with Tukey's post hoc test for multiple comparison. Differences were considered significant at $P < 0.05$. Linear regression was used to determine whether there was a relationship between τ_P and τ_{Ca}.

Results

Hemodynamic changes produced by indo-1 and low-flow ischemia. Control coronary perfusion pressure was 71 mmHg and mean coronary flow was 21±0.4 ml/min (n = 13). Loading and washout of indo-1 did not affect coronary flow. Coronary flow returned to control during the recovery period after each episode of reduced coronary flow.

Peak systolic and end-diastolic pressures in the left ventricle were 101±5 and 9±1 mmHg, respectively, during control. After loading and washout of indo-1, peak systolic pressure was 71±5 mmHg; end diastolic pressure was 9±1 mmHg. Therefore, the only hemodynamic effect of loading with indo-1 was to decrease peak systolic pressure by 30±4%. To determine how much of this decrease was caused by the passage of time during indo-1 loading and washout (1 h), a group of unloaded hearts (n = 7) was perfused for this period of time. Perfusion for 1 h reduced peak systolic pressure from 108±4 to 100±5 mmHg (a 6±5% decrease). End diastolic pressure was unchanged. Therefore, it is likely that most of the decrease in peak systolic pressure after loading with indo-1 was caused by calcium buffering by indo-1.

Peak systolic pressure was reduced to 46±4, 28±5, and 18±2 mmHg when coronary flow was reduced to 50, 20, and 10% of control, respectively (see Table I). Peak systolic pressure returned to control during the recovery period after each episode of reduced coronary flow. End diastolic pressure did not change during these brief periods of low flow, as has been observed by other investigators (7, 9).

$[Ca^{2+}]_c$ transient decline during low flow ischemia. The first question addressed by this study was whether the decline of the $[Ca^{2+}]_c$ transient was slowed during low flow ischemia. Indo-1 fluorescence ratio transients were used as an index of $[Ca^{2+}]_c$. Fig. 1 illustrates the progressive nature of changes in fluorescence ratio decline as coronary flow was reduced from 100 to 10% of control. In this figure, the transient amplitudes are scaled to the same height to illustrate the changes in fluorescence ratio decline. Fig. 2, *A* and *B*, show representative fluorescence ratio transients (*dashed lines*) during control and during reduced coronary flow (20% of control). To quantify the changes in fluorescence ratio decline produced by low flow ischemia, τ_R was determined. In Fig. 2, the solid lines are the monoexponential fit used to determine τ_R. In this example, τ_R increased from 62 ms during control to 116 ms during 20% of control. The group data show that mean τ_R increased from 74±5 ms during control to 95±4, 144±11, and 204±16 ms when coronary flow was reduced to 50, 20, and 10% of control,

Table I. Left Ventricular Pressure, Indices of Pressure and Calcium Decline, and Free Cytosolic Calcium

Coronary flow (% control)	100	50±1*	20±1*	10±0.3*
LVSP (mmHg)	71±5	43±4*	27±3*	18±2*
LVEDP (mmHg)	9±1	8±2	8±1	8±1
τ_R (ms)	74±5	95±4	144±11*	204±16*
τ_{Ca} (ms)	67±6		108±9*	158±19*
$t_{50\%}$ (ms)	54±2		72±2*	81±2*
τ_P (ms)	35±3	39±4	104±11*	
Systolic $[Ca^{2+}]_c$ (nM)	897±84		973±87	1,074±122*
Diastolic $[Ca^{2+}]_c$ (nM)	383±22		468±26	589±45*

Data from experiments in isolated rat hearts (n = 13) subjected to 3 min of low flow ischemia. Peak systolic pressure (LVSP) and end diastolic pressure (LVEDP) were determined using an isovolumic balloon. Indo-1 fluorescence ratio was used as an index of $[Ca^{2+}]_c$. The time constant of indo-1 ratio transient decline (τ_R) was determined assuming monoexponential kinetics. The time constant of $[Ca^{2+}]_c$ decline (τ_{Ca}) was determined in a subset of experiments (n = 8) using calibrated indo-1 ratio transients during severe coronary flow reductions (20 and 10% of control). $t_{50\%}$ was also determined, and τ_P was determined during coronary flow reductions to 50 and 20% of control. However, during the most severe flow reduction (10% of control), pressure decline could not be fit to a monoexponential. Data are mean±SE. (* $P < 0.05$ when compared to control).

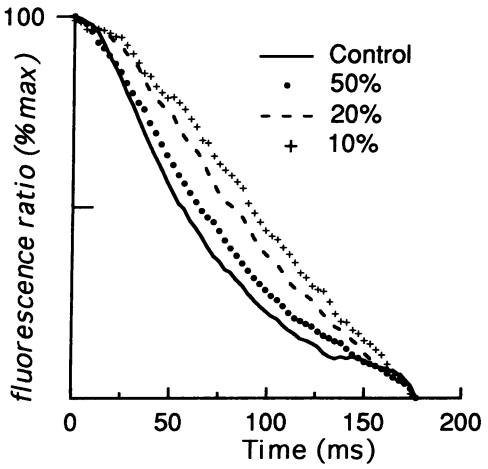

Figure 1. Representative fluorescence data showing the decline portion of the ratio transients. The transient amplitudes were scaled to the same height so that changes in transient decline during reduced coronary flow may be appreciated. A slowing of the transient decline was evident as coronary flow was reduced from 100 to 10% of control. In this example, the time constants of fluorescence ratio decline (τ_R) increased from 62 ms during control to 95, 116, and 186 ms during 50, 20, and 10% of control, respectively.

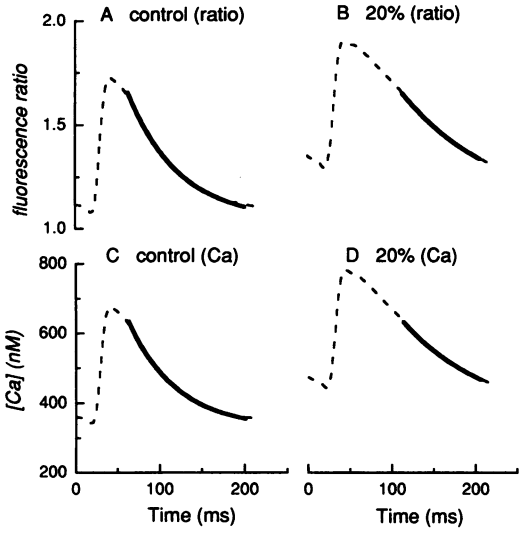

Figure 2. A and *B* show representative indo-1 ratio transients during control and 20% of control coronary flow. All tracings begin at the time of the pacer stimulus. The dotted lines are the fluorescence ratio reported in arbitrary intensity units. The solid lines are the exponential fit of the decline used to determine τ_R (see Methods). In this example, τ_R increased from 62 ms during control to 116 ms during 20% of control. *C* and *D* show the same data calibrated to absolute $[Ca^{2+}]_c$. The dotted lines are the data, and the solid lines are the exponential fit of the data used to determine τ_{Ca}. In this example, τ_{Ca} increased from 57 ms during control to 100 ms during 20% of control.

respectively (see Table I). To confirm that the increase of τ_R during low flow ischemia was reversible with reperfusion, τ_R was determined before each period of low flow (see "Experimental protocol"). Mean τ_R was not significantly different during the three control periods (75±6, 73±5, and 78±6 ms; P = NS).

Because the response of the indo-1 fluorescence ratio to calcium is not linear, the changes in τ_R might differ from changes in τ_{Ca}. $[Ca^{2+}]_c$ was, therefore, calculated in a subset of experiments (n = 8) and τ_{Ca} was determined using the calibrated transients. Fig. 2, *C* and *D*, show representative $[Ca^{2+}]_c$ transients (*dashed lines*) determined from the fluorescence ratio transients shown in Fig. 2, *A* and *B*. The solid lines are the monoexponential fit of these data. In this example, τ_{Ca} increased from 57 ms during control to 100 ms when coronary flow was reduced to 20% of control. To demonstrate that it was reasonable to fit the $[Ca^{2+}]_c$ transient decline to a monoexponential model, Fig. 3 shows the fit portion of the $[Ca^{2+}]_c$ decline plotted on a semilogarithmic scale. Since the asymptote is not 0, the asymptote value was subtracted from each data point (32). Fig. 3 shows that these plots are linear with correlation coefficients > 0.99. This demonstrates that it is reasonable to fit the $[Ca^{2+}]_c$ transient decline to a monoexponential model as has been previously demonstrated for the left ventricular pressure decline (2). The group data show that mean τ_{Ca} increased from 67±6 ms during control to 108±9 and 158±19 ms when coronary flow was reduced to 20 and 10% of control, respectively (Table I).

To further confirm that the $[Ca^{2+}]_c$ transient decline was prolonged during low flow ischemia, $t_{50\%}$ was determined. Mean $t_{50\%}$ increased from 54±2 during control to 72±2, and to 81±2 when coronary flow was reduced to 20 and 10% of control, respectively (Table I). In summary, a progressive increase of τ_R, τ_{Ca}, and $t_{50\%}$ was observed during graded coronary flow reductions.

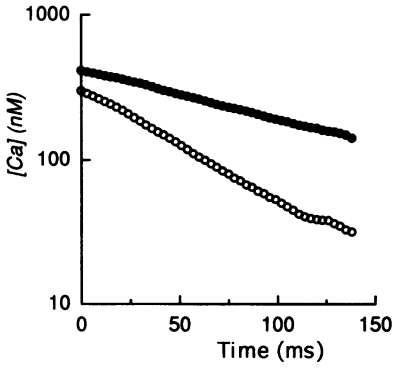

Figure 3. The portion of the $[Ca^{2+}]_c$ transients shown in Fig. 2 that were fit to a monoexponential are shown on a semilogarithmic scale. The hollow and filled circles are individual data points during control and 20% of control coronary flow, respectively. Since the minimum $[Ca^{2+}]_c$ value (asymptote) was not 0, the asymptote value was subtracted from each data point. These plots are linear with correlation coefficients of 0.99. This demonstrates that it was reasonable to fit these data to monoexponential functions.

Relationship between $[Ca^{2+}]_c$ and pressure decline during ischemia. The second question addressed by this study was whether there was a relationship between τ_P and τ_{Ca} during low flow ischemia. Fig. 4 shows representative left ventricular pressure transients that correspond to the fluorescence and $[Ca^{2+}]_c$ transients shown in Fig. 2. The dashed lines are the left ventricular pressure measurements; the solid lines are the monoexponential fit for this data set. In this example, τ_P increased from 30 ms during control to 100 ms when coronary flow was reduced to 20% of control. Mean τ_P increased from 35±3 ms during control to 39±4 and 104±11 ms when coronary flow was reduced to 50 and 20% of control, respectively. Left ventricular pressure could not be fit to a monoexponential function when coronary flow was reduced to 10%, most likely because of the markedly reduced developed pressure (see Table I). However, the pressure data obtained when coronary flow was 20% of control demonstrates that myocardial relaxation was impaired during low flow ischemia. To confirm that the increase of τ_P during low flow ischemia was reversible with reperfusion, τ_P was determined prior to each period of low flow (see "Experimental protocol"). Mean τ_P was not significantly different during the three control periods (34±3, 36±3, and 34±3 ms; P = NS). Time to 50% pressure decline was not calculated, since

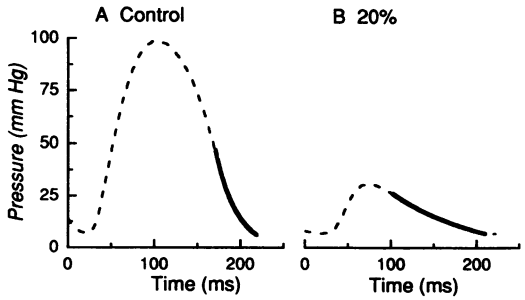

Figure 4. Left ventricular pressure tracings, obtained simultaneously with the indo-1 ratio transients in Fig. 2, are shown. The dotted lines are the left ventricular pressure values and the solid lines are the exponential fits. In this example, τ_P increased from 30 ms during control to 100 ms during 20% of control.

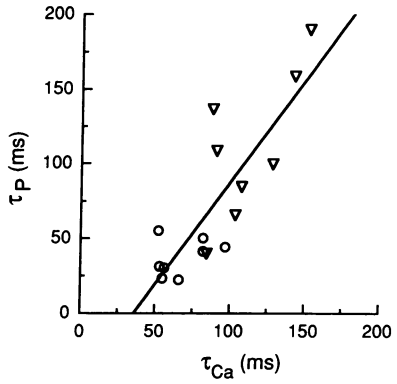

Figure 5. Individual τ_P are plotted as a function of τ_{Ca}. Data were obtained during 100 and 20% of control flow (circles and inverted triangles, respectively). There was a linear relationship between τ_P and τ_{Ca} ($r = 0.82$).

this index is unreliable when developed pressure changes significantly (2).

Fig. 5 shows individual τ_P plotted as a function of τ_{Ca}. There was a close relationship between τ_P, an index of myocardial relaxation, and τ_{Ca}, an index of $[Ca^{2+}]_c$ transient decline. The good correlation between τ_P and τ_{Ca} suggests that during low flow ischemia, impaired myocardial relaxation may be caused by slowing of the $[Ca^{2+}]_c$ transient decline.

Discussion

The major finding of this study was that during graded low flow ischemia, there was a progressive increase of τ_{Ca}. This demonstrates that the $[Ca^{2+}]_c$ transient decline was slowed during ischemia. Furthermore, there was a close relationship between τ_P and τ_{Ca}. Taken together, these data support the hypothesis that impaired myocardial relaxation during low flow ischemia may be caused by slowing of the $[Ca^{2+}]_c$ transient decline.

Impaired $[Ca^{2+}]_c$ transient decline during ischemia. Although the importance of myocardial relaxation to cardiac function is well established (1, 4), the cellular mechanisms that regulate relaxation remain incompletely understood. It has long been postulated that abnormal calcium cycling is an important factor in causing impaired myocardial relaxation during ischemia. However, there has been little experimental data to support this hypothesis and conflicting results have been reported. Allen and Orchard studied the effect of hypoxia and metabolic inhibition (18) on aequorin light transients (an index of $[Ca^{2+}]_c$) in ferret papillary muscle. They noted a modest increase in the $t_{50\%}$ of the aequorin light transient during hypoxia and acidosis. In contrast, other investigators (17) found no change in $t_{50\%}$ of the indo-1 fluorescence transient during hypoxia in isolated cardiac myocytes from rats. Therefore, it is not certain whether the $[Ca^{2+}]_c$ transient is prolonged during myocardial hypoxia. Furthermore, the applicability of these data to the clinical situation of myocardial ischemia is uncertain, since these experiments were performed in isolated cells and papillary muscles that were exposed to hypoxia.

The current study was the first to systematically assess the effect of myocardial ischemia on the $[Ca^{2+}]_c$ transient decline in whole hearts. A progressive increase of τ_R, τ_{Ca}, and $t_{50\%}$ was observed during graded reductions of coronary flow. These data demonstrate that the $[Ca^{2+}]_c$ transient decline was slowed during low flow ischemia. This observation is in agreement with a recent study (19), where prolongation of the indo-1 fluorescence transient was noted during acute no flow ischemia. However, these investigators did not quantify the changes in the indo-1 fluorescence transient or relate them to changes in left ventricular pressure relaxation.

Although the current experiments demonstrate that the $[Ca^{2+}]_c$ transient decline was slowed during low flow ischemia, the mechanisms that may be responsible for this abnormality were not addressed. The $[Ca^{2+}]_c$ transient is the net result of several cellular processes. The major factors that affect the decline portion of the $[Ca^{2+}]_c$ transient are transport of calcium out of the cytosol and binding by calcium buffers in the cytosol. The major routes of calcium removal from the cytosol are the sarcoplasmic reticulum Ca^{2+}-ATPase pump and the sarcolemmal Na^+-Ca^{2+} exchanger (13). Experimental evidence suggests that the activity of these processes may be impaired during ischemia and contribute to slowing of the $[Ca^{2+}]_c$ transient decline (15, 33, 34). In addition, ischemia may also affect the various calcium buffers in the cell and thereby prolong the $[Ca^{2+}]_c$ transient decline. Therefore, further studies are required to determine the specific factors that prolong the $[Ca^{2+}]_c$ transient decline during ischemia.

The appropriate use of indo-1 fluorescence as an index of $[Ca^{2+}]_c$ in whole hearts requires the recognition of potential sources of artifact. First, light-based measurements are susceptible to motion artifact. A major advantage of using indo-1 fluorescence is that the effects of motion can be effectively suppressed by taking the ratio of two emission wavelengths. We have previously demonstrated that motion artifact was reduced to < 8% in our system (26). Second, indo-1–$[Ca^{2+}]$ dissociation kinetics need to be sufficiently rapid to allow accurate analysis of the rate of $[Ca^{2+}]_c$ decline based on indo-1 ratio decline. Stop-flow kinetic analysis has shown that the time constant of indo-1–$[Ca^{2+}]$ dissociation is 8 ms in vitro (35). This is approximately eight times faster than τ_R and τ_{Ca} during control. Furthermore, Spurgeon et al. (36) measured $[Ca^{2+}]_c$ in single cardiac myocytes and found that the decline of $[Ca^{2+}]_c$ was not affected by kinetic corrections for the time constant of indo-1–$[Ca^{2+}]$ dissociation. Therefore, we conclude that the dissociation kinetics of indo-1 are sufficiently rapid to permit accurate assessment of the $[Ca^{2+}]_c$ transient decline. Third, the indo-1 fluorescence response to calcium is nonlinear. This nonlinearity becomes especially important at high $[Ca^{2+}]$. Therefore, in a subset of experiments, the fluorescence data was calibrated using a previously reported method (24) to yield absolute $[Ca^{2+}]$. The calibrated transients were then used to determine τ_{Ca}. Both τ_R and τ_{Ca} progressively increased during ischemia although the magnitude of the changes were different. Fourth, a portion of indo-1 fluorescence may arise from bradykinin-sensitive (presumably endothelial) cells (37) or subcellular compartments such as mitochondria (38). We previously demonstrated that rat hearts do not significantly load indo-1 into bradykinin-sensitive cells (21, 24). Furthermore, since the concentration of free calcium in these compartments does not significantly vary on a beat-to-beat basis, it is unlikely that the results of these experiments would be qualitatively affected.

Impaired myocardial relaxation during ischemia. During graded low flow ischemia, the increase of τ_{Ca} was associated with a parallel increase of τ_P. τ_P is a well-established index of myocardial relaxation that has been used extensively in experimental and clinical situations (2, 4). τ_P is the index of myocardial relaxation that is least affected by peak left ventricular pressure, end systolic fiber length, heart rate, and changes of end diastolic volume or pressure (2, 3). Our finding that τ_P increased during ischemia is in agreement with previous obser-

vations in humans (10–12), large animal models (3), and isolated perfused hearts (7–9). However, previous studies in isolated perfused hearts studied only mild reductions of coronary flow to ~ 50% (8) and 33% (7) of control. The current study extends these previous observations and demonstrates a progressive increase of τ_P during more severe low flow ischemia.

Relationship between $[Ca^{2+}]_c$ transient decline and myocardial relaxation. The linear relationship between τ_P and τ_{Ca} is consistent with the hypothesis that impaired myocardial relaxation may be caused by slowing of the $[Ca^{2+}]_c$ transient decline. This suggest that during low flow ischemia, calcium handling may be an important factor in regulating myocardial relaxation. However, the regulation of contraction and relaxation by calcium-dependent processes is a complex process. This is evident by the time delay between corresponding portions of the $[Ca^{2+}]_c$ and pressure transients (e.g., peak and nadir). Furthermore, calcium-independent factors may also be important in regulating myocardial relaxation. Previous work (31, 39, 40) suggests that in the absence of myocardial ischemia, cross-bridge dissociation kinetics may be important in determining the relaxation properties of myocardium. Therefore, it is likely that the control of myocardial relaxation involves both calcium-dependent and calcium-independent mechanisms. The current study emphasizes the importance of calcium-dependent processes in causing impaired relaxation during ischemia. However, the effect of ischemia on calcium-independent mechanisms needs to be studied further.

Relationship between diastolic and systolic dysfunction during ischemia. The current study has focused on the mechanisms of diastolic dysfunction during ischemia. However, ischemia causes abnormalities of both systolic and diastolic function (i.e., force of contraction and relaxation/filling, respectively). This raises the question of whether the mechanisms that impair systolic and diastolic function may be interrelated. Several lines of experimental evidence suggest that hypoxia and ischemia impair systolic function primarily by reducing myofilament responsiveness to calcium (19–22, 41). For example, Marban and Kusuoka (41) noted a decrease of maximal Ca^{2+}-activated pressure (determined by tetanization) during hypoxia. Recently, several investigators (19–22) have demonstrated a decrease of developed pressure without a significant decrease of the $[Ca^{2+}]_c$ transient. Taken together, these data strongly suggest that myofilament responsiveness to calcium is reduced during ischemia and may, in part, be responsible for reduced systolic contraction. However, reduced myofilament responsiveness to calcium would be expected to decrease τ_P, and is therefore unlikely to cause diastolic dysfunction during ischemia. This further emphasizes the potential role of impaired calcium handling, assessed by an increase of τ_{Ca}, in causing diastolic dysfunction during ischemia.

Summary and conclusion. During graded reductions of coronary flow, τ_{Ca} was progressively increased, which demonstrates that $[Ca^{2+}]_c$ decline was slowed. Furthermore, there was a close relationship between τ_P and τ_{Ca}. These findings support the hypothesis that slowing of the $[Ca^{2+}]_c$ transient decline may be an important factor in causing impaired myocardial relaxation during low flow ischemia.

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