Effect of Ischemia on Calcium-Dependent Fluorescence Transients in Rabbit Hearts Containing Indo 1
Correlation with Monophasic Action Potentials and Contraction

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The effects of acute global ischemia on cytosolic calcium transients were studied in perfused rabbit hearts loaded with the fluorescent calcium indicator indo 1. Indo 1–loaded hearts were illuminated at 360 nm, and fluorescence was recorded simultaneously at 400 and 550 nm from the epicardial surface of the left ventricle. The $F_{400}/F_{550}$ ratio was calculated by an analog circuit, which allowed cancellation of optical motion artifact. Resulting calcium transients demonstrated a rapid upstroke and slow decay similar to those recorded in isolated ventricular myocytes. Global ischemia rapidly suppressed contraction, but it produced a concurrent increase in the systolic and diastolic levels of the calcium transients, together with an increase in the duration of the peak. The effects of ischemia were reversed by reperfusion, inhibited by verapamil, and mimicked by perfusion of nonischemic hearts with acidified (CO$_2$-rich) solution. In addition to elevation of the calcium transients, ischemia caused a pattern of intracellular calcium alternans that was discernible after 2–3 minutes. The pattern of alternans was stable at a given epicardial site, but it could be out of phase at different sites. Similar nonuniformities were observed in contraction strength and in the duration of monophasic action potentials recorded immediately adjacent to the fiber-optic probe. Abnormalities in intracellular calcium may be a causal factor in the loss of electrical and mechanical synchrony in the acutely ischemic heart. (Circulation 1988;78:1047-1059)

Cessation of blood flow causes rapid changes in the electrical and mechanical activity of the heart. The resting potential of ischemic cells declines promptly, which inactivates sodium channels and slows conduction. The duration of the action potential is modified to varying degrees so that recovery of excitability no longer occurs in the normal, orderly sequence. The combination of slowed conduction and nonuniform excitability leads to vulnerability of the ischemic heart to ventricular fibrillation. Changes in electrical activity are accompanied by profound impairment of mechanical performance. Ischemia causes a marked decrease in contraction strength, along with alternation of contraction strength from beat to beat. The mechanism of "mechanical alternans" during ischemia has not been established, but it presumably involves fluctuation in the level of cytosolic calcium ([Ca$^{2+}$]) that is transiently achieved during the action potential.

Changes in membrane potential during ischemia may also result from abnormalities in [Ca$^{2+}$]. "Calcium overload" mimicks the effects of ischemia by reducing the resting potential and by changing the configuration of the action potential in a characteristic way. These effects are mediated either by "calcium-activated" cation channels or by electrogeneic sodium-calcium exchange. Because both of these processes are practically instantaneous, variations in the [Ca$^{2+}$], transient from beat to beat would cause concomitant changes in the action potential plateau.

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To clarify the role of \([Ca^{2+}]\), in ischemia, we have devised a method of recording \([Ca^{2+}]-dependent fluorescence transients from rabbit hearts loaded with the fluorescent calcium indicator indo 1. This method involves arterial perfusion of the hearts with the cell-permeant form of the indicator, indo 1-AM, which has been used previously in isolated cardiac myocytes.12–14 The present experiments are designed to study changes in the calcium transients during ischemia as well as the mechanism by which the transients are altered. We find that ischemia causes rapid elevation of the calcium transients, which is simulated by elevation of CO\(_2\) and is prevented by pretreatment with verapamil. Ischemia also causes spatial and temporal variations in the amplitude of the transients, which correspond to inhomogeneities in contraction strength and action potential duration. These observations suggest that cytosolic calcium abnormalities contribute to the loss of synchronized excitation in the acutely ischemic heart.

**Materials and Methods**

**Preparation of Isolated Hearts**

Albino male New Zealand rabbits weighing between 1.8 and 2.2 kg were killed by cervical fracture. The heart was rapidly excised and perfused with saline solution at a constant flow rate of 20–30 ml/min. The perfusate contained NaCl 115 mM, KCl 4.7 mM, CaCl\(_2\) 2.0 mM, MgCl\(_2\) 0.7 mM, NaHCO\(_3\) 28 mM, NaH\(_2\)PO\(_4\) 0.5 mM, glucose 20 mM, insulin 10 units/l, and fetal calf serum 0.1%; the perfusate was adjusted to pH 7.4, equilibrated with 95% O\(_2\)–5% CO\(_2\), and heated to maintain the heart at 30 ± 1°C. Left ventricular pressure was recorded with an isovolumic intracavitary latex balloon that contained a fiber-optic pressure transducer (Camino Laboratories, San Diego, California). Simultaneous recordings of local contractile activity were obtained with a piezoelectric epicardial strain gauge, the transducing pins of which were 4 mm apart. In some experiments, the ventricular action potential was recorded from the surface of the heart with a “contact” monophasic action potential electrode, which is known to respond linearly to changes in membrane potential.15,16 Most recordings were obtained during rapid pacing of the right ventricle (180 beats/min) by an epicardial plunge electrode.

**Fluorescence Recordings**

Recordings were obtained from the anterior surface of the left ventricle except where otherwise stated. Illumination from a 100 W mercury vapor lamp was filtered at 360 ± 5 nm and directed through a silica fiber-optic cable onto the surface of the heart. The fiber-optic cable terminated in a plastic receptacle, which was attached to the heart by a plastic sleeve and rubber girdle that were designed to minimize relative motion. The plastic sleeve was opaque, and it confined the illumination beam to a circular region of the ventricular surface 1 cm in diameter. Fluorescence emissions were collected by a ring of smaller fiber optics and were directed through a beam splitter into two photomultipliers fitted with optical band-pass filters centered at 400 and 550 nm. The output of the photomultipliers was passed into an electronic ratio circuit so that the fluorescence ratio \((F_{400}/F_{550})\) could be obtained. Fluorescence at individual wavelengths, the \(F_{400}\) and \(F_{550}\) ratio, and other physiological signals were displayed on a Gould-Brush strip chart recorder (Cleveland, Ohio). Indo 1-AM was solubilized in dimethyl sulfoxide containing pluronic F-127 (25% w/v) and infused into the heart at a final concentration of 2.5 \(\mu M\), in the presence of 5% fetal calf serum. Perfusion with indo 1-AM continued for 30 minutes, followed by a 30-minute washout. This procedure caused the fluorescence of the heart to increase by a factor of 5–12 compared with the fluorescence obtained before loading ( autofluorescence).

**Differential Centrifugation, Manganese Quenching, and Electron Microscopy**

To confirm that infused indo 1-AM is converted to the calcium-sensitive free acid, manganese quenching studies were performed. Low concentrations of manganese are known to quench completely the fluorescence of indo 1 free acid, while having no effect on the calcium-insensitive fluorescence of indo 1-AM.17 In three hearts loaded with indo 1, 20 mM MnCl\(_2\) was infused in the presence of the ionophore ionomycin (1.5 \(\mu M\)), which transports Mn\(^{2+}\) ions into the cell interior. Manganese promptly quenched more than two thirds of the fluorescence in all three hearts, which indicates that most of the fluorescence arose from indo 1 free acid. Postmanganese fluorescence was, however, significantly higher than the autofluorescence level (172 ± 5% of autofluorescence at 400 nm and 161 ± 5% of autofluorescence at 550 nm), which indicates that there was retention of incompletely hydrolyzed indo 1-AM. The presence of incompletely hydrolyzed indo 1-AM complicates conversion of the fluorescence ratios to \([Ca^{2+}]\).17

To define further the localization and fate of indo 1 in the intact heart, tissue homogenates were fractionated by differential centrifugation.18 One gram of myocardium was excised from the anterior left ventricular wall of an indo 1–loaded heart at the same site where \([Ca^{2+}]-dependent fluorescence transients had previously been recorded. The specimen was minced and suspended in 5 ml of a buffer containing 0.25 M sucrose, 115 mM KCl, 20 mM NaCl, 1.115 mM MgCl\(_2\), 1.115 mM ethyleneglycol-bis-(β-aminoethyl ether)-N,N',N'',N'''-tetraacetic acid (EGTA), and 10 mM N-(2-hydroxyethyl)piperazine-N''-2-ethanesulfonic acid (HEPES), which was adjusted to pH 7.05 at 4°C. The specimen was then homogenized with a Polytron instrument (Brinkman Instruments, Westbury, New York) operated twice at full speed for 30 seconds. The crude homogenate was spun at 40g in a Damon/IEC HN-SII centrifuge (Needham Heights, Massachusetts) for 10 minutes to
remove intact cells. Ultracentrifugations were then performed with a TY 65 rotor in a Beckman L5-65 ultracentrifuge (Irvine, California). The supernatant from the crude homogenate was spun at 1,000g for 20 minutes to remove large organelles and subcellular debris (nuclear fraction). The supernatant was then spun at 10,000g for 20 minutes to isolate mitochondria (mitochondrial fraction). A third ultracentrifugation was then performed at 105,000g for 120 minutes to obtain the microsomal fraction, with the final supernatant being the cytosol fraction. The three ultracentrifugation pellets were resuspended in 5-ml aliquots of buffer, and 1 ml of each fraction was placed in a quartz cuvette for measurement of fluorescence. Fluorescence was recorded at an excitation wavelength of 360 nm and an emission wavelength of 400 nm. For each cuvette, calcium-specific fluorescence was determined as the difference between $F_{\text{max}}$ and $F_{\text{Mn}^2+}$, where $F_{\text{max}}$ was obtained by addition of $25 \mu l$ 100 mM CaCl$_2$ to the cuvette and $F_{\text{Mn}^2+}$ was obtained by addition of $50 \mu l$ 100 mM MnCl$_2$. The total calcium-specific fluorescence of the homogenate was then calculated as the sum of calcium-specific fluorescence for each of the three ultracentrifugation pellets plus the calcium-specific fluorescence of the final supernatant. The percentage of total calcium-specific fluorescence in each fraction was then determined with the following results: cytosol fraction = 72.2%, mitochondrial fraction = 3.6%, microsomal fraction = 3.4%, and nuclear fraction = 20.8%. These results indicate that most of the calcium-specific fluorescence, which arises from indo 1 free acid, was located in the soluble cytosolic fraction. Very little indo 1 free acid was contained in the mitochondria. Although it is possible that some of the mitochondria were disrupted during homogenization of the tissue, a conclusion similar to ours has been reached by Steinberg and others, who used a nondisruptive technique (rhodamine dual fluorescence microscopy) to study the subcellular fate of indo 1-AM.

The distribution of incompletely hydrolyzed indo 1-AM in the tissue homogenate could be deduced from the amount of fluorescence not quenched by manganese ($F_{\text{Mn}^2+}/F_{\text{max}}$; expressed in a percentage). For the cytosolic fraction, only 2.1% of the $F_{\text{max}}$ fluorescence was resistant to quenching, which implies that practically all of the cytosolic indo 1 is composed of the free acid. In contrast, a greater portion of the fluorescence was resistant to quenching in the other fractions (36.6%, 31.5%, and 17.7% for the mitochondrial, microsomal, and nuclear fractions, respectively). These observations suggest that retained indo 1-AM is largely associated with organelles.

To further confirm the distribution of indo 1 described above, a sample of the four fractions was submitted for electron microscopy. The microscopist was blinded as to the identity of the fractions. Electron microscopy showed that mitochondria were abundantly present in the mitochondrial fraction and were not present in other fractions. The microsomal fraction contained clear membrane-bound vesicles that were presumably of sarcoplasmic reticular origin, whereas the cytosol fraction contained no electron dense material.

**In Vitro pH Sensitivity of Indo 1**

For indo 1 to be accepted as an indicator of $[Ca^{2+}]_i$ during ischemia, it was necessary to determine whether the fluorescence or calcium sensitivity of the indicator is altered by moderate acidification. These determinations were made by illuminating vials containing indo 1 free acid at varying pH with the same fiber-optic apparatus that was used for intact hearts. Three titration curves (pH vs. the $F_{400}/F_{550}$ ratio) were constructed, one for an indo 1 solution that was saturated with calcium, one for an indo 1 solution in the calcium-free form, and one for a solution in which indo 1 was partially saturated so that the fluorescence ratio was similar to that obtained in the intact heart. All three solutions contained

![Graph](image-url)
(mM) KCl 115, NaCl 20, MgCl₂ 1.15, and HEPES 10, and they were adjusted to an initial pH of 7.4. The calcium-free solution contained EGTA 1.115 mM and indo 1 1 µM. The calcium-saturated solution contained CaCl₂ 5 mM and indo 1 1 µM. The partially saturated solution contained indo 1 21 µM and CaCl₂ 10 µM. Each solution was acidified by stepwise addition of small aliquots (10–50 µl) of 1N HCl. Resulting pH changes were measured with an Instrumentation Laboratory (Lexington, Massachusetts) Model 282 Co-oximeter. Measurements of the F₄₁₀/F₅₅₀ ratio in the unloaded hearts were obtained at four to eight different pH values for each solution, which ranged between 5.8 and 7.4.

**Results**

[Ca²⁺]_i-Dependent Fluorescence Transients

Under Baseline Conditions

Hearts loaded with indo 1 exhibit [Ca²⁺]_i-dependent fluorescence transients (Figure 1), which are not present before loading. Calcium transients rise more steeply than the contraction (Figure 2) and have the same shape as those observed in cardiac myocytes containing indo 1. Transients are clearest in the F₄₁₀/F₅₅₀ ratio (Figure 1, middle trace), which reflects reciprocal changes at the two emission wavelengths. Transients are also clear in the individual fluorescence signals, where the systolic increase in [Ca²⁺], produces a phasic increase in fluorescence at 400 nm (top trace) and a phasic decrease in fluorescence at 550 nm (bottom trace). The calcium transients represent, a 5% and 7% variation in the total fluorescence at 400 and 550 nm, respectively. Corresponding autofluorescence levels are 9% and 12%.

Onset of the fluorescence transients coincides with, or slightly precedes, the onset of ventricular contraction (Figure 2, right panel). The contractions of indo 1-loaded hearts are not reduced in amplitude or increased in duration compared with unloaded hearts, which indicates that there is no significant buffering of [Ca²⁺], by the indicator. Fluorescence transients are uniform from one heart to the next and can be recorded from any portion of the left ventricular surface or from the right ventricle. Figure 3 shows calcium transients recorded from three nonoverlapping regions of the left ventricle of a single heart. All 15 transients are similar in time course and have similar ratio values.

[Ca²⁺]_i-Dependent Fluorescence Transients During Ischemia

A primary reason for recording calcium transients in intact hearts is to determine the effects of

**FIGURE 2.** Simultaneous recordings of calcium transients (top right) and ventricular pressure (bottom right) in a heart loaded with indo 1. Isovolumic left ventricular pressure is measured with an intracavitary latex balloon containing a fiber-optic pressure transducer. Peak systolic pressure is 100 mm Hg. The left-hand recording shows intraventricular pressure in the same heart before loading with indo 1. Loading of the heart with indo 1 does not reduce systolic pressure and does not slow the time course of relaxation compared with the control recording (bottom left). These observations indicate that there is no significant buffering of [Ca²⁺], by the indicator. F₄₁₀/F₅₅₀ ratio was not recorded in this heart before loading with indo 1, but results of such a recording are shown in Figure 4B. Possible effects of dimethyl sulfoxide and Pluronin F-127 alone were tested in one heart, and no change in ventricular pressure was found.

**FIGURE 3.** Recordings of calcium transients from three different portions of the surface of the left ventricle of a rabbit heart containing indo 1. Each recording is from a 1-cm circular region whose boundary is defined by the edge of the plastic sleeve that contains the fiber-optic cables. The recordings have been obtained by manually positioning the fiber-optic assembly on the anterior surface (top trace), the apical surface (middle trace), and the lateral surface (bottom trace) of the left ventricle. The recordings are nonsimultaneous but are aligned in the figure to facilitate comparison among the calcium transients. All fifteen transients have approximately the same time course and the same fluorescence ratio values. Different results are obtained when the heart has been ischemic for several minutes (see text).
ischemia, which can only occur in perfused tissue. Figure 4A shows the effect of a 60-second episode of global ischemia, during which the heart is paced at 180 beats/min. Ischemia produces a prompt increase in the peak amplitude of the calcium transients, along with an upward shift of the baseline, so that the $F_{400}/F_{550}$ ratio at end diastole approaches the normal systolic value. In most cases, the systolic fluorescence ratio increases more rapidly than the diastolic ratio so that the net amplitude of the calcium transients becomes larger (Figures 5 and 6). Net amplitude reaches a maximum value between 45 and 60 seconds, after which both ratios increase more slowly. The shape of the calcium transients also changes during ischemia so that the peak becomes broader and the terminal decay more abrupt (Figure 7). All of these effects can be discerned in recordings obtained at single wavelengths (Figure 5) as well as in the $F_{400}/F_{550}$ ratio. The effects of ischemia are reversed by reperfusion for 60–90 seconds (Figure 4A), are reproducible during a series of three to six ischemic trials, and are consistent in a series of hearts for which the mean change in $F_{400}/F_{550}$ ratio is shown in Figure 6.

Elevation of the calcium transients during ischemia is accompanied by an equally prompt decrease in contraction strength (Figure 4A), which is presumably due to a decrease in the calcium sensitivity of the myofilaments.20,21 The contractile response recovers during reperfusion, and there is a temporary impairment of relaxation, which may be due to persisting elevation of $[Ca^{2+}]_{i}$. 

**Effects of Ischemia Are Not Due to Changes in Autofluorescence**

The effects of ischemia on the calcium transients are not due to changes in epicardial temperature, changes in pH (see below), or changes in autofluorescence. There are three reasons why autofluorescence changes cannot explain the effects of ischemia on the $F_{400}/F_{550}$ ratio:

1) In contrast to the behavior of indo 1 fluorescence, changes in autofluorescence during ischemia are parallel at 400 and 550 nm; both signals show a moderate increase. These changes cancel in the $F_{400}/F_{550}$ ratio so that the ratio remains constant (Figure 4B). When recordings from the same heart are compared before and after loading with indo 1,
Figure 5. Recordings of effects of ischemia on calcium transients at individual wavelengths. Fluorescence transients at 400 nm (top trace) and 550 nm (bottom trace) are shown together with the F_{400}/F_{550} ratio (middle trace). Right panels obtained at 50 seconds of ischemia, and left panels obtained immediately before ischemia. Ischemia increases the net amplitude of the calcium transient in all three recordings. End-diastolic fluorescence increases at 400 nm and decreases at 550 nm, which leads to a comparable increase in the end-diastolic fluorescence ratio. As in Figure 4A, the effects are reversed by reperfusion (not shown). Recordings are obtained from the same heart as Figure 1, but the heart is now paced at 180 beats/min. Dotted lines in right panels show initial end-diastolic signal levels.

the effect of ischemia at 550 nm is converted from a fluorescence increase to a fluorescence decrease (Figure 5, bottom traces). This fluorescence decrease is indicative of a rise in [Ca^{2+}].

2) Changes in autofluorescence could not, in principle, explain the increased net amplitude of the fluorescence transients. Changes in autofluorescence should have no effect on the amplitude of fluorescence transients at single wavelengths. Furthermore, an increase in autofluorescence at both wavelengths should diminish the transients observed in the ratio.

3) Changes in indo 1 fluorescence during ischemia are larger than the changes in autofluorescence. This point is particularly important for the 400 nm signal, where the effect of a [Ca^{2+}] increase can be mimicked by an increase in autofluorescence. For the experiment in Figure 5, the increase in 400 nm end-diastolic fluorescence during 90 seconds of ischemia is 2.7 times greater than the increase in autofluorescence during an ischemic trial that preceded infusion of indo 1-AM. Although the increase in autofluorescence causes some overestimation of indo 1 fluorescence at 400 nm (Figure 5, top), this effect is balanced by partial masking of the [Ca^{2+}]-dependent fluorescence decrease at 550 nm. Net effects have been determined by deconvolution of the F_{400}/F_{550} ratio, and these calculations show that autofluorescence changes have no significant effect on the ratio and do not contribute to the increase in the ratio during ischemia.

Alternans Behavior of Calcium Transients

Consecutive calcium transients remain uniform throughout the 1st minute of ischemia. However, in the 2nd or 3rd minute, a characteristic nonuniformity develops in which every other transient is diminished in amplitude and arises from a higher end-diastolic level (Figure 7A). This pattern of alternation is stable at any given site on the ventricular surface. However, when the fiber-optic probe is moved to a different site during a continuous recording, the pattern of alternation may reverse so that if odd numbered transients were larger at the first site, they are smaller at the second site. This observation indicates that the alternans behavior of calcium transients within a 1-cm circular region is independent of the behavior in other regions.

Nonuniformity of the calcium transients is associated with similar variations in contraction strength. Contractions in Figure 7B, for example, exhibit alternations in amplitude during ischemia that are similar to the alternations of the calcium transient described above. Alternations are seen in both the intraventricular pressure recording (top trace) and in the contractions recorded by a strain gauge transducer, which has an inter-pin distance of 4 mm (bottom trace). However, as shown by the arrows, the pattern of alternation is out of phase in the two recordings so that weak contractions recorded by

Figure 6. Plot of relative change in the calcium transient produced by ischemia and reperfusion in 11 hearts. Fluorescence ratios from each heart have been converted to a percentage of the end-diastolic value at the onset of ischemia. Ischemia causes a progressive increase in peak systolic and end-diastolic fluorescence ratio, which is reversed by reperfusion. Net amplitude of the calcium transient (peak systolic ratio minus end-diastolic ratio) increases during ischemia and tends to be largest at 45–60 seconds. Bars indicate SEM.
the strain gauge correspond to strong contractions recorded from the entire ventricle. This result implies that variations in contraction strength occur independently in localized regions of the heart. This interpretation has been confirmed by simultaneous recordings with two strain gauge transducers. Ischemia causes alternations in the strength of contraction recorded by each transducer, but these alternations do not develop simultaneously, and they are often out of phase.

Correlation of Calcium Transients With Monophasic Action Potentials During Ischemia

Changes in the calcium transient during ischemia are accompanied by changes in membrane potential. Ischemia causes reduction of the resting potential, which occurs within the 1st minute of rapidly paced occlusions and is reversed by reperfusion. Reversible depolarization during ischemia can be monitored with "floating" microelectrodes, or with monophasic action potential electrodes. Recordings of the latter type are illustrated in the top trace of Figure 8. Reduction of the resting potential is accompanied by an upward shift in the calcium transient recorded from an adjacent site. The increase in the diastolic fluorescence ratio in Figure 8 would be consistent with the possibility that increased [Ca2+]i causes changes in membrane permeability that reduce the resting potential of the ischemic cells.

Ischemia also causes initial broadening of the action potential (Figure 8, top right), which has been demonstrated previously by microelectrode impalement in the dog heart, and by suction monophasic action potential recordings in the pig heart. Broadening of the action potential is accompanied by elevation and broadening of the calcium transient (Figure 8, bottom right), which is reversed by reperfusion. Elevation of the calcium transient in Figure 8 could be a consequence of action potential broadening or could cause this broadening by inducing calcium-activated inward currents across the membrane of the myocardial cell. The latter interpretation is suggested by the marked shortening of the action potential that occurs when the calcium transients of rabbit myocardium are deliberately abolished by the drug ryanodine, which inhibits calcium release from the sarcoplasmic reticulum.

**Figure 7.** Recordings of alternation of contraction strength and calcium transients after 3 minutes of ischemia. Panel A shows alternation of the F400/F550 fluorescence ratio. Even numbered beats have smaller calcium transients that arise from a higher end-diastolic level and are followed by more complete decay. Despite the alternans pattern, all of the calcium transients have higher peak systolic and end-diastolic values than those recorded before ischemia (not shown). Panel B shows alternation of contractions recorded by an intracavity balloon (top trace) and by an epicardial strain gauge transducer (bottom trace). Alternans pattern is similar in the two recordings, but it is out of phase so that strong contractions recorded by the balloon (top arrow) coincide with weak contractions recorded by the strain gauge (bottom arrow). This result implies that alternans develops independently in different regions of the ventricle and that the pattern recorded by the intracavity balloon represents a net imbalance in the summated activity of different regions.

**Figure 8.** Recordings of effects of ischemia on the calcium transient (bottom trace) and monophasic action potential (top trace) in the same heart. A 90-second episode of ischemia (right) causes reduction of the resting potential, broadening of the action potential, and slowing of conduction, which is indicated by an increase in the time from stimulus to action potential onset. Ischemia also increases both the peak systolic and end-diastolic level of the calcium transient and causes broadening of the peak. Dotted line indicates the original monophasic action potential resting potential before ischemia.
Consecutive action potentials are uniform during the 1st minute of ischemia. However, in the 2nd or 3rd minute, a pattern of nonuniformity often develops in which action potential duration alternates from beat to beat (Figure 9A, top trace; and Figure 9B). When action potentials and calcium transients are recorded from adjacent sites, the broader action potentials coincide with the larger calcium transients (Figure 9A, bottom trace). Changes in the action potential are most prominent during repolarization and are minimal during the upstroke (Figure 9B). There is substantial variation in the upstroke of the calcium transients (first 80 msec), even though the corresponding action potentials are superimposable during this time (Figure 9B). This observation shows that the changing amplitude of the calcium transients is not due to fluctuations in excitability or conduction. Alternation of the transients is more likely to result from variations in intracellular calcium release, which is the primary source of the calcium that gives rise to the transients.26

A further implication of Figure 9 is that alternation of action potential duration can occur independently in discrete regions of the ischemic heart so that the pattern of alternation in a particular region can either be in phase or out of phase with some other region. We have confirmed this inference directly in two ischemic hearts where simultaneous monophasic action potential recordings were obtained at two different sites. Localization of monophasic action potential alternans to specific regions of the myocardium has also been reported in the ischemic pig heart24 and is consistent with the behavior we observed for the calcium transient and contraction (Figure 7B).

**Verapamil Blocks Effects of Ischemia on Calcium Transients**

The role of [Ca$^{2+}$] in the physiology of ischemia could be tested further if it were possible to block the ischemia-induced [Ca$^{2+}$] increase. Electrophysiological manifestations of ischemia are prevented by drugs that reduce calcium influx, and it has been postulated that reduction of [Ca$^{2+}$] is the basis for this action.3 Figure 10A shows the effect of a 5-minute infusion of verapamil (0.25 μM) on the ischemia-induced increase in the F$_{400}$/F$_{550}$ ratio. The top two plots (circles) show the fluorescence ratio during an ischemic trial before verapamil, whereas the bottom two plots (triangles) depict a second trial performed after verapamil infusion. Verapamil reduces the net amplitude of the calcium transient before ischemia (leftmost triangles) and reduces the end-diastolic level from which the transients arise (open triangle at left). Elevation of the calcium transients during ischemia is nearly abolished by verapamil so that the effect of verapamil on the F$_{400}$/F$_{550}$ ratio is larger during ischemia than in the preschemic period. Comparable results have been obtained in six ischemic hearts, for which mean values are listed in Table 1. The increase in the fluorescence ratio during ischemia is significantly reduced by verapamil (Table 1, first and second rows from top) as is the net amplitude of the calcium transients (Table 1, third and fourth rows from top). The bottom row of Table 1 gives the ischemia-induced increase in the calcium transient, which is calculated by subtracting the net amplitude of the transient before ischemia from the net amplitude at 1 minute of ischemia. The increase in the transient is threefold smaller after verapamil infusion. The above effects were partly reversible in four hearts where a third ischemic trial was performed after washout of verapamil for 15 minutes.

Figure 10B shows the effect of a higher concentration of verapamil (3.0 μM) in five ischemic hearts. This concentration abolishes the calcium transients and contractions but does not abolish the monopha-
FiguRe 10. Plots of effect of verapamil on calcium transients during ischemia. Panel A shows data from a single heart in which two 90-second ischemic trials have been performed, one before and one after infusion of 0.25 μM verapamil. Peak systolic values of the F400/F550 ratio are plotted as solid symbols, whereas end-diastolic values are plotted as open symbols. The first ischemic trial (circles) increases the net amplitude of the calcium transients and elevates the end-diastolic level from which they arise. These effects are reversed by reperfusion. Verapamil infusion begins 5 minutes later and continues for an additional 5 minutes. Verapamil reduces the net amplitude of the calcium transients (triangles), and reduces the end-diastolic level as well. Ischemia has little effect on the calcium transients after verapamil infusion, but the effect of ischemia is partly restored in a third trial performed after washout of verapamil for 15 minutes (not shown). Panel B shows relative changes in the F400/F550 ratio in five ischemic hearts pretreated with 3 μM verapamil. For each heart, the F400/F550 ratio is measured at 15-second intervals and is expressed as a percentage of the end-diastolic value just before verapamil infusion. 3 μM verapamil causes profound inhibition of the calcium transients so that the systolic peaks cannot be measured. Verapamil reduces the diastolic fluorescence ratio below the control value and inhibits the increase in the ratio during ischemia (open triangles). Compared with untreated hearts (Figure 6), verapamil reduces the fluorescence ratio at every point in time. An overall significance of p<0.001 was obtained by analysis of variance for repeated measures. p values for each of the 11 time points were less than 0.05 by the method of Bonferroni. End-diastolic ratios in Figure 6 were used for this analysis.

TABLE 1. Effects of Verapamil and Ischemia on [Ca2+]i-Dependent Fluorescence Transients in Six Hearts

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<th></th>
<th>Control trial</th>
<th>Verapamil trial</th>
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<tr>
<td>Ischemia-induced increase in diastolic fluorescence ratio</td>
<td>0.12 ± 0.03</td>
<td>0.04 ± 0.02*</td>
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<tr>
<td>Ischemia-induced increase in systolic fluorescence ratio</td>
<td>0.15 ± 0.03</td>
<td>0.05 ± 0.02†</td>
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<td>Net amplitude of [Ca2+]i transient before ischemia</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.01†</td>
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<tr>
<td>Net amplitude of [Ca2+]i transient at 1 minute of ischemia</td>
<td>0.15 ± 0.01§</td>
<td>0.10 ± 0.01‡</td>
</tr>
<tr>
<td>Ischemia-induced increase in [Ca2+]i transient</td>
<td>0.03 ± 0.01</td>
<td>0.01 ± 0.01*</td>
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Values are mean±SEM. Values are calculated from normalized ratios for which the end-diastolic ratio at the beginning of the control trial (before ischemia) is defined as 1.00. 0.25 μM verapamil was administered; ischemia lasted for 60 seconds.

*p<0.05, †p<0.005, §p<0.01 compared with control trial; §p<0.005 compared with preischemic value.

3.0 μM verapamil compared with values from untreated hearts (Figure 6, p<0.001), but the reduction is not appreciably greater than that produced by 0.25 μM verapamil. Although the monophasic action potentials remain normal after 3.0 μM verapamil, the effects of ischemia, such as reduction of the resting potential, broadening of the action potential, and alternation of action potential duration are prevented or greatly delayed.

Elevation of CO2 Simulates Effects of Ischemia on Calcium Transients

The mechanism by which calcium transients are elevated during ischemia is uncertain but probably involves impairment of calcium sequestration. Calcium sequestration can be impaired by acid metabolites such as CO2, which accumulates rapidly during ischemia27 and is known to increase [Ca2+]i in barnacle muscle fibers28 and in isolated cardiac fibers.29,30 As shown in Figure 11, infusion of hypercarbic saline increases the F400/F550 ratio during both systole and diastole and causes broadening of the calcium transients similar to that seen in ischemia. This effect develops within 1 minute (Figure 11B) and recovers fully during reperfusion with saline containing normal CO2. The effects shown in Figure 11 have been observed in a total of five hearts for which the pH values of the infused saline ranged from 6.3 to 6.6. A similar result has been obtained in three hearts in which lactic acid (15–20 mM) was added to the saline solution, producing pH values of 6.3, 6.4, and 6.9. The effect of lactic acid on [Ca2+]i-dependent fluorescence is not ascribable to the lactate anion because infusion of a 20-mM lactate solution that had been neutralized to pH 7.30 had no effect on the F400/F550 fluorescence ratio in two hearts.

The response to CO2 does not represent a direct effect of reduced pH on indo 1 fluorescence. This has been established by in vitro acidification (to pH...
6.0) of three indo 1 solutions for which resulting fluorescence ratios \( \frac{F_{400}}{F_{550}} \) are plotted in Figure 12. Acidification has no significant effect on the \( \frac{F_{400}}{F_{550}} \) ratio, irrespective of whether the indo 1 solution is saturated with calcium (top plot), free of calcium (bottom plot), or partially saturated with calcium (middle plot). The increase in the \( \frac{F_{400}}{F_{550}} \) ratio during in vivo acidification must therefore be ascribed to an increase in \([\text{Ca}^{2+}]_i\).

**Discussion**

These experiments show that it is practical to study qualitatively the effects of ischemia on cytosolic calcium activity with the fluorescent calcium indicator indo 1. This is the first study in which calcium transients, action potentials, and contractions have been directly correlated in the intact heart. Indo 1 and the related compound fura-2 have been used previously in isolated myocytes from both embryonic and adult hearts.12-14,26,31 Studies with myocytes show that the acetoxymethyl ester form of these indicators can be loaded selectively into the myofilament space and can give high-quality calcium transients at concentrations that do not produce significant buffering.13,30 The calcium transients recorded from the rabbit heart are similar in time course to those obtained in isolated cells, and they respond appropriately to interventions with known effects on \([\text{Ca}^{2+}]_i\). Absence of buffering is confirmed by the normal strength and duration of contractions in the indo 1-loaded hearts (Figure 2).

A particularly important feature of indo 1 is that motion artifact can be cancelled by obtaining the ratio of signals at two emission wavelengths. This strategy has been used previously in isolated cardiac myocytes.13 Several observations show that the fluorescence ratio in indo 1-loaded rabbit hearts is free of motion artifact. First, calcium transients always precede the onset of contraction (Figure 2) and have an upstroke velocity that is faster than the rise in force. Second, the fluorescence transients at 400 and 550 nm are opposite to one another, and are often monophasic, with no common mode deflection at any point in the cycle (e.g., Figure 1). Hearts with monophasic transients at 400 and 550 nm produce no transients at the isosbestic wavelength of indo 1.11-13 In other hearts, motion does produce a common mode deflection in the 400- and 550-nm signals, but these deflections cancel in the ratio, leaving monophasic calcium transients comparable to those in Figure 1.

The major limitation of the indo 1 method, as presently used, is the absence of a satisfactory calibration procedure. Calibration is known to be hindered by incomplete deesterification of the indo 1-AM,17 which clearly occurs based on the results.
of the manganese quenching studies. Changes in autofluorescence may also complicate the calibration of indo 1 recordings, although these effects are largely cancelled in the ratio, and can be corrected more precisely by subtracting the autofluorescence at each wavelength.

Once it is possible to record calcium transients in the intact heart, it is then possible to study the effects of ischemia. Ischemia produces striking abnormalities in the calcium transient that appear 5–10 seconds after cessation of flow. These abnormalities include 1) marked elevation of the systolic peak, 2) marked elevation of the end-diastolic level, 3) broadening of the systolic peak, and 4) an increase in the net amplitude of the transients. All of these changes can be observed in the individual fluorescence signals (Figure 5) as well as in the \( \frac{F_{400}}{F_{550}} \) ratio.

The above observations indicate that fluctuations in cytosolic calcium become larger during the first 90 seconds of ischemia in the rapidly paced rabbit heart. We presume that the calcium-dependent fluorescence ratio, \( \frac{F_{400}}{F_{550}} \), is a monotonic function of cytoplasmic free calcium, \([Ca^{2+}]_f\). Granted that this is so, our recordings show not only an increase in the peak \([Ca^{2+}]_f\) that is achieved during systole but also an increase in the end-diastolic level from which the transients arise. Changes in end-diastolic \([Ca^{2+}]_f\) are a common finding with tetracarboxylate indicators, owing to the fact that at physiological heart rates each transient arises before the preceding transient has fully decayed. End-diastolic \([Ca^{2+}]_f\) is strikingly dependent upon the interbeat interval and can be altered by pharmacological agents that either accelerate or impair the uptake of calcium by the sarcoplasmic reticulum.\(^{32}\) It is, of course, possible that the fluorescence ratio is affected by some other factor, such as accumulation of calcium within a noncytosolic compartment that contains indo 1. However, our cell fractionation studies (see “Materials and Methods”) show that most of the calcium-sensitive indo 1 fluorescence is in the soluble fraction and that very little calcium-sensitive fluorescence is associated with the mitochondria, which would be the most likely compartment to accumulate calcium under conditions of metabolic stress.

In addition to the overall elevation of \([Ca^{2+}]_f\), ischemia produces striking nonuniformities in the calcium transient, which are associated with similar nonuniformities in electrical and mechanical activities. The above abnormalities may explain various physiological correlates of ischemia, such as the dispersion of electrical refractoriness that leads to arrhythmias in the first few minutes of ischemia.

**Mechanism of Ischemia-Induced \([Ca^{2+}]_f\) Increase**

The role of abnormal calcium handling in cardiac ischemia has been discussed for many years and is especially well documented in models where protracted ischemia is followed by reperfusion.\(^{33,34}\) Whether or not \([Ca^{2+}]_f\) increases during brief ischemic trials is less certain for two reasons. First, the rapid decline in contractile force during the first 2 minutes of ischemia might be regarded as evidence against a \([Ca^{2+}]_f\) increase. It is now clear that the sensitivity of the myofilaments to calcium is depressed during ischemia and that contractions may therefore cease, even in the face of a brisk \([Ca^{2+}]_f\) increase (Figure 2). Second, studies with aequorin-loaded cardiac fibers have shown that extended periods of hypoxia or metabolic inhibition\(^{20}\) often fail to increase \([Ca^{2+}]_f\). Possible reasons for this discrepancy include 1) failure of isolated preparations to simulate conditions in the intact heart (e.g., inability of isolated fibers to sustain physiological work loads and stimulus rates), 2) failure of energy deprivation to reproduce the conditions of ischemia, and 3) differences in the behavior of various calcium indicators.

An important feature of ischemia, which is not duplicated by hypoxia or metabolic inhibition, is accumulation of acid metabolites, such as CO\(_2\). Acidification\(^{35}\) and CO\(_2\) accumulation\(^{27}\) both occur within seconds of coronary occlusion. Myocardial acidification is accelerated by pacing and diminished by verapamil.\(^{35}\) Maneuvers that modulate acidification may have parallel effects on \([Ca^{2+}]_f\).

Acidification of isolated cardiac fibers increases the peak amplitude of \([Ca^{2+}]_f\), transients recorded with aequorin (Orchard\(^{36}\); Figure 1). A corresponding increase in diastolic \([Ca^{2+}]_f\) has not been resolved with aequorin but can be observed in quiescent fibers with ion-selective electrodes. Bers and Ellis\(^{39}\) find that cytoplasmic acidification can elevate resting \([Ca^{2+}]_f\) to 2 \(\mu\)M, which is greater than the level that is normally achieved during the peak of the \([Ca^{2+}]_f\), transient. This observation corroborates our finding that infusion of CO\(_2\) into isolated hearts can elevate end-diastolic \([Ca^{2+}]_f\), to the normal systolic level (Figure 11).

Our experiments with indo 1 partly confirm results obtained with the calcium-sensitive nuclear magnetic resonance probe, 5,5'-difluorobis(o-aminophenoxy) ethane-N,N',N'-tetraacetic acid (5F-BAPTA).\(^{36,37}\) In these studies, ischemia produces a threefold increase in time averaged \([Ca^{2+}]_f\), after 20 minutes, but no increase is observed during the first 5 minutes. A possible reason for this discrepancy is the greater buffering that 5F-BAPTA produces, which inhibits contraction at physiological concentrations of extracellular calcium.

**Correlation of \([Ca^{2+}]_f\) With Electrophysiological Derangements**

A principal reason why abnormalities in \([Ca^{2+}]_f\) could be important during ischemia lies in the ability of cytosolic calcium to regulate membrane potential. Increased \([Ca^{2+}]_f\) can depolarize cardiac cells by means of a calcium-activated inward current. Such current can flow either through sodium-calcium exchange\(^{7}\) or through calcium-activated cation channels\(^{8}\) that have been described in cardiac cells. Activation of these currents by a sustained
rise in [Ca\textsuperscript{2+}], could contribute to the reduction in resting potential that occurs during ischemia.

A major arrhythmogenic effect of ischemia is dispersion of the ventricular refractory period, which allows premature beats to initiate ventricular fibrillation.\textsuperscript{2} Recordings obtained in ischemic dog hearts show that the onset of ventricular fibrillation is preceded by the appearance of an alternans pattern in the T wave of the epicardial electrogram.\textsuperscript{38} Recordings obtained with suction electrodes in the ischemic pig heart reveal beat-to-beat alternations of action potential duration, which do not occur in simultaneous recordings obtained from the border zone or normal myocardium.\textsuperscript{24} We have observed similar alternation of action potential duration in the globally ischemic rabbit heart (Figure 9) and have shown that there are associated variations in the amplitude of the intracellular calcium transient. These variations in the calcium transient are not caused by conduction block, or impaired excitability, because the time course of the action potential does not vary during the first 80 msec, when the calcium transient rises to its peak (Figure 9B). Variations in the calcium transient can occur independently in localized regions of the heart. This can be shown by rapid relocation of the fiber-optic probe, which can reverse the parity of the alternans during continuous recordings. Because membrane currents initiated by the calcium transient are known to have important effects on the action potential plateau,\textsuperscript{6-10} it seems quite possible that primary abnormalities of cytosolic calcium might be responsible for the nonuniformities of repolarization that occur in the ischemic heart.

An important implication of our findings is that the ability of various drugs to limit the electrophysiological consequences of ischemia may reflect prevention of concurrent abnormalities of [Ca\textsuperscript{2+}]. In our experiments, concentrations of verapamil that inhibit the effects of ischemia on the calcium transient also prevent the abnormalities of the monophasic action potential that are illustrated in Figures 8 and 9. Both verapamil and diltiazem retard the occurrence of ventricular fibrillation in vivo\textsuperscript{39,40} and prevent the decline in the threshold for production of fibrillation by application of pulse trains during the T wave.\textsuperscript{41} The latter finding suggests that the uniformity of the ventricular refractory period is somehow preserved. Our experiments show that preservation of normal [Ca\textsuperscript{2+}] levels could be the basis for these beneficial actions.

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