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Cytosolic calcium transients from the beating mammalian heart

(cardiac muscle/ischemia/excitation–contraction coupling/indo-1)

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ABSTRACT **To elucidate the role of cytosolic calcium, $[Ca^{2+}]_i$, in the physiology of the normal and ischemic heart, we have developed a method for recording $[Ca^{2+}]_i$ transients from the epicardial surface of the rabbit ventricle after arterial perfusion with the cell-permeant cytosolic calcium indicator indo-1 AM. Hearts were illuminated at 360 nm, and fluorescence was recorded simultaneously at 400 and 550 nm. The F_{400}/F_{550} fluorescence ratio was calculated by an analog circuit that allowed cancelation of small movement artifacts that were present at single wavelengths. Clear $[Ca^{2+}]_i$ transients were present in the F_{400}/F_{550} signal and were remarkable for their slow decay. Slow decay of the transients was not due to buffering of $[Ca^{2+}]_i$ by indo-1, since there was no associated impairment of contraction or relaxation. The peak amplitude of the $[Ca^{2+}]_i$ transients was increased by ouabain, adrenaline, postextrasystolic potentiation, and acetylcholine. The extent to which the transients decayed diminished with shortening of the interbeat interval, but decay of the transients could be further diminished by acetylcholine or caffeine. A major advantage of the intact heart over isolated myocytes is the ability to measure changes in $[Ca^{2+}]_i$ during ischemia. Ischemia produced a marked increase in both peak systolic and end-diastolic $[Ca^{2+}]_i$, which was most rapid during the first 30 sec, and approached a plateau value after 90 sec. This increase in $[Ca^{2+}]_i$ was associated with a characteristic broadening of the peak of the transient. The increase in $[Ca^{2+}]_i$ during ischemia is consistent with a proposed causative role of $[Ca^{2+}]_i$ in mediating early electrophysiological abnormalities.**

Rapid fluctuations in cytosolic calcium, $[Ca^{2+}]_i$, are known to regulate the electrical and mechanical activity of the heart on a beat-to-beat basis and to mediate its responses to hormones, physiological interventions, and disease states. Direct $[Ca^{2+}]_i$ measurement techniques have advanced considerably but still face important limitations (1). These limitations include (*i*) the relative insensitivity of certain indicators (e.g., aequorin) at low levels of $[Ca^{2+}]_i$ (*ii*) the requirement for signal averaging, and (*iii*) the need to use isolated cells or fibers, where conditions differ from those that prevail *in vivo*. Newly synthesized fluorescent calcium indicators offer potential advantages because of their brightness, ease of introduction, and greater sensitivity at diastolic values of $[Ca^{2+}]_i$ (2). Initial studies in cardiac myocytes have shown that the second-generation indicators, fura-2 (3, 4) and indo-1 (5–8), can be loaded into the myofilament space as the cell-permeant acetoxymethylester derivatives to give $[Ca^{2+}]_i$-dependent fluorescence transients. Of these compounds, indo-1 is of particular interest because it permits cancelation of movement artifact by comparison of fluorescence emissions at two wavelengths (2, 5–7). In the present study, we have utilized this strategy to record high-resolution $[Ca^{2+}]_i$ transients from the surface of actively beating rabbit hearts following arterial perfusion with the cell-permeant acetoxymethylester indo-1 AM. We know of no previous instance in which physiological $[Ca^{2+}]_i$ transients have been recorded from an intact, functioning organ. Our recordings show that removal of $[Ca^{2+}]_i$ between beats is unexpectedly slow. Thus, the level of $[Ca^{2+}]_i$ at end-diastole—which was previously thought to be constant—can, in fact, be modulated by neurotransmitters and other factors. In addition, we show that myocardial ischemia produces a rapid and drastic increase in $[Ca^{2+}]_i$, which had been predicted on physiological grounds but could never previously be observed.

METHODS

Albino male New Zealand rabbits weighing between 1.8 and 2.2 kg were sacrificed by cervical fracture. The heart was rapidly excised and perfused with a saline solution at a constant aortic flow rate of 20–30 ml/min. The perfusate contained 115 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 0.7 mM $MgCl_2$, 28 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 20 mM glucose, 10 units of insulin per liter, and 0.1% fetal calf serum adjusted to pH 7.4, equilibrated with 95% O_2/5% CO_2, and heated to maintain epicardial temperature at 30°C ± 1°C. Contractions of the left ventricle were measured with an intracavitary latex balloon that contained a fiber-optic pressure transducer (Camino Laboratories) or with an epicardial strain gauge that had the advantage of monitoring wall stress near the site at which fluorescence was measured. The left ventricle was vented by a small polyethylene catheter that drained thebesian vein flow. Some hearts were paced at 180/min by an epicardial plunge electrode.

Fluorescence excitation was provided by a 100-W Hg vapor lamp. Illumination was filtered at 360 ± 5 nm and directed through a silica fiber-optic cable onto a circular region of epicardium 1 cm in diameter. Movement artifact was minimized by attachment of the fiber-optic cables to the heart using a plastic hub and rubber girdle. Fluorescence was collected by a ring of eight coaxial fiber-optic cables, divided by a beam splitter, and then filtered at 400 ± 12.5 nm and 550 ± 20 nm before reaching photomultiplier tubes (Hamamatsu). The above emission wavelengths were found to give clearer fluorescence transients than the *in vitro* fluorescence maxima of indo-1 (Ca^{2+} bound = 405 nm; Ca^{2+} free = 480 nm). Our chosen emission wavelengths allow greater rejection of the noncalcium-dependent fluorescence of unhydrolyzed indo-1 AM (fluorescence maximum = 450 nm), which is retained in tissue after loading (9). Photomultiplier output at the two emission wavelengths was entered into an electronic ratio circuit and the fluorescence ratio, F_{400}/F_{550}, was displayed on a strip-chart recorder.

Indo-1 AM was solubilized in a mixture of dimethyl sulfoxide/pluronic F-127 (25%, wt/vol), 1 ml of which was added to 400 ml of Tyrodes solution containing 5% fetal calf serum. The final concentration of indo-1 AM was 2.5 μM. Perfusion with indo-1 AM continued for 30 min; this was followed by a 30-min washout. This method of indicator loading produced a 5- to 12-fold increase in fluorescence at

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Abbreviation: $[Ca^{2+}]_i$, cytosolic calcium.

both emission wavelengths. Movement artifact in the loaded hearts could be assessed by recording fluorescence emissions at the isosbestic wavelength (7). In isolated hearts, movement artifact was generally minor in comparison to the $[Ca^{2+}]_i$-dependent fluorescence changes. When significant artifact was present, it did not noticeably distort the fluorescence transients observed in the ratio mode.

RESULTS

Characteristics of the Fluorescence Transients. Hearts loaded with indo-1 exhibit clear $[Ca^{2+}]_i$ transients in their fluorescence signals (Fig. 1). The $[Ca^{2+}]_i$ transient slightly precedes the onset of contraction, rises more steeply and decays more slowly, often continuing to decay after mechanical relaxation is ostensibly complete (Fig. 2). $[Ca^{2+}]_i$ transients are most clearly evident in the F_{550} signal, where the systolic increase in $[Ca^{2+}]_i$ produces a transient decrease in fluorescence (Fig. 1, bottom trace). This fluorescence decrease contributes to the concomitant increase in the F_{400}/F_{550} ratio (middle trace). A transient increase in the F_{400} signal (top trace) is seen less clearly, since this wavelength is nearer to the isosbestic point (cf. refs. 2 and 7) and also exhibits greater overlap with the fluorescence of incompletely hydrolyzed indo-1 AM (F_{Peak} = 450 nm; ref. 9).

The $[Ca^{2+}]_i$ transients are similar in time course to those observed with tetracarboxylate indicators [quin-2 (10), fura-2 (4), and indo-1 (5–8)] in isolated cardiac cells. A principal feature of the $[Ca^{2+}]_i$ transients is their slow decay, which can continue for more than one second, so that the level of $[Ca^{2+}]_i$ from which each transient arises varies as a function of beat frequency (Figs. 1 and 2*B*). The slow decay of the indo-1 fluorescence transients is not due to buffering of intracellular calcium by the indicator, because there is no corresponding delay in the upstroke or falling phase of the contraction (Fig. 2*A*, left panel). The peak systolic pressure of >100 mm Hg (1 mm Hg = 133 Pa) in indo-1-loaded hearts does not differ significantly from that observed in hearts perfused for 60 min

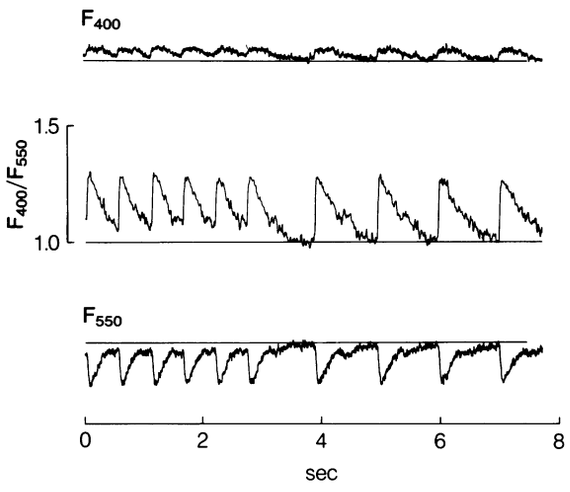

FIG. 1. Calcium-dependent fluorescence changes in rabbit hearts loaded with indo-1. Each beat is accompanied by a phasic $[Ca^{2+}]_i$ transient, in which fluorescence increases at 400 ± 12.5 nm (top trace) and decreases at 550 ± 20 nm (bottom trace). The middle trace displays the fluorescence ratio, F_{400}/F_{550}, which is calculated by an analog circuit. This ratio is a monotonic function of $[Ca^{2+}]_i$ (2) and is not distorted by motion artifacts. The upstroke of the $[Ca^{2+}]_i$ transients is rapid, with a 50% rise-time of about 20 msec; but decay of the transients is slow and continues throughout the interval between beats. The first six beats are driven by electrical stimulation of the right ventricle, whereas the last four occur spontaneously after cessation of pacing. Decay of the $[Ca^{2+}]_i$ transients between beats is substantially greater in spontaneous rhythm than during rapid pacing.

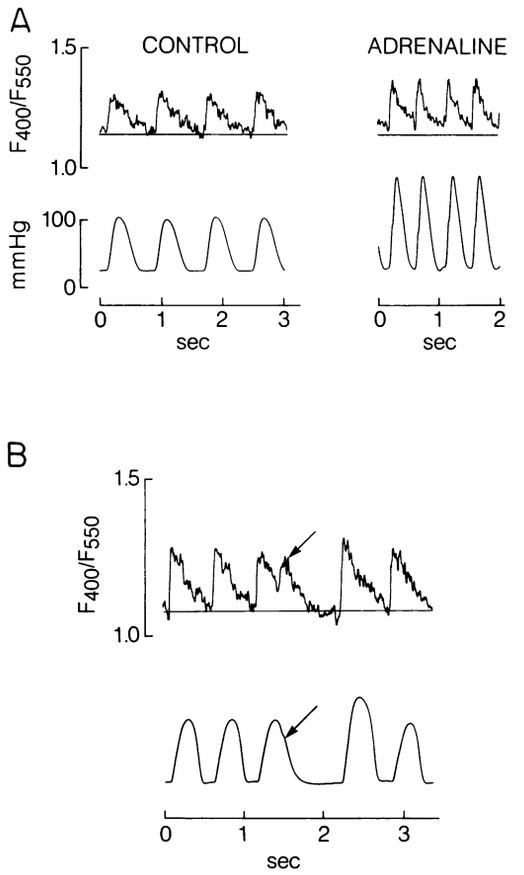

FIG. 2. (*A*) Effects of adrenaline on $[Ca^{2+}]_i$ transients (top traces) and left ventricular contraction (bottom traces). Isovolumic left ventricular pressure is measured by introducing a double-cannulated latex balloon by way of the mitral valve orifice. One cannula is connected to a syringe filled with deaerated saline for inflation of the balloon. The other cannula is used for insertion of an intracavitary fiber-optic pressure transducer, which records a systolic pressure of >100 mm Hg. $[Ca^{2+}]_i$ transients precede the onset of contraction by about 20 msec. Decay of $[Ca^{2+}]_i$ transients is slow and continues beyond the apparent completion of mechanical relaxation. Infusion of adrenaline (1 μg/ml for 90 sec) produces a prompt and reversible increase in the $[Ca^{2+}]_i$ transients (right column), which is accompanied by an increase in peak systolic pressure. Mechanical relaxation and decay of the $[Ca^{2+}]_i$ transients are both accelerated by adrenaline. (*B*) Effect of a premature beat on $[Ca^{2+}]_i$ and contraction. Contraction of the left ventricular free wall is monitored by a strain-gauge transducer placed about 4 mm from the site at which $[Ca^{2+}]_i$ transients are measured. A spontaneous premature beat (arrows) produces a diminished $[Ca^{2+}]_i$ transient (top trace) and a weaker contraction (bottom trace) that is fused with the previous beat. This is followed by a postextrasystolic compensatory pause during which $[Ca^{2+}]_i$ continues to decay throughout diastole. The ensuing beat demonstrates postextrasystolic potentiation in both signals.

with saline alone. The absence of a demonstrable buffering effect is a potential advantage of indo-1 over the "first-generation" indicators quin-2 and 5,5′-difluorobis[*o*-aminophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, both of which impair contraction when they are loaded into intact hearts (11, 12).

Several observations indicate that the fluorescence transients do not include appreciable motion artifact. The clearest indication of this is the reciprocal variation in fluorescence at 400 and 550 nm. Reciprocal fluorescence changes continue in late diastole, when mechanical relaxation is apparently complete (Fig. 2). Ability of the fluorescence ratio to cancel

motion can also be demonstrated by vibration of the apparatus or by rapid inflation of the intraventricular balloon.

$[Ca^{2+}]_i$ Transients Do Not Saturate Indo-1. Indo-1 fluorescence changes appropriately in response to ionic conditions that elevate $[Ca^{2+}]_i$. Simultaneous elevation of extracellular K^+ (to 75 mM) and Ca^{2+} (to 15 mM) produces a sustained increase in the F_{400}/F_{550} ratio that is 2- to 4-fold larger than the systolic transients and is fully reversible. Exposure to 100 mM Ca^{2+} in the presence of a calcium ionophore (ionomycin, 1.5 μM) produces a larger and possibly saturating increase in the fluorescence ratio (Fig. 3). Although our measurements do not permit computation of $[Ca^{2+}]_i$, it is clear that intracellular indo-1 does not become saturated during the peak of the transients. This observation excludes very large values of $[Ca^{2+}]_i$ (e.g., 10^4 nM) and is consistent with estimates obtained in isolated myocytes containing indo-1 or fura-2 [systolic $[Ca^{2+}]_i$ = 600–900 nM; end-diastolic $[Ca^{2+}]_i$ = 100–300 nM; refs. 4–8].

Correlation of Fluorescence Transients with Contraction. To demonstrate further the physiologic relevance of the $[Ca^{2+}]_i$ transients, we performed maneuvers that alter the strength of contraction in a predictable manner. Infusion of adrenaline (Fig. 2*A*; 1 μg/ml; n = eight infusions) or ouabain (1 μM; n = three infusions) causes a prompt, reversible increase in the peak of the $[Ca^{2+}]_i$ transients. Conversely, reduction of extracellular calcium to 0.1 mM abolishes contraction, reduces the peak of the fluorescence transients, and causes a parallel reduction in the end-diastolic $[Ca^{2+}]_i$. In contrast, increases in preload, achieved by inflation of the intracavitary latex balloon, increase contraction strength without altering either systolic or diastolic $[Ca^{2+}]_i$.

Changes in cardiac rhythm also affect the transients, often within one cardiac cycle. As noted above, changes in interbeat interval alter the end-diastolic $[Ca^{2+}]_i$ from which the subsequent transient arises (Fig. 1). In addition, single premature beats can produce postextrasystolic potentiation of the $[Ca^{2+}]_i$ transients. A spontaneous premature beat (Fig. 2*B*, arrows) causes a diminished $[Ca^{2+}]_i$ transient and a weaker contraction, which is fused with the preceding beat. During the ensuing pause, $[Ca^{2+}]_i$ decays below the baseline, presumably due to greater uptake of calcium by the sarcoplasmic reticulum. The following beat is potentiated and is accompanied by a larger and more long-lasting $[Ca^{2+}]_i$ transient. These changes in $[Ca^{2+}]_i$ have long been postulated to be the basis of postextrasystolic potentiation (13) but have never been observable directly.

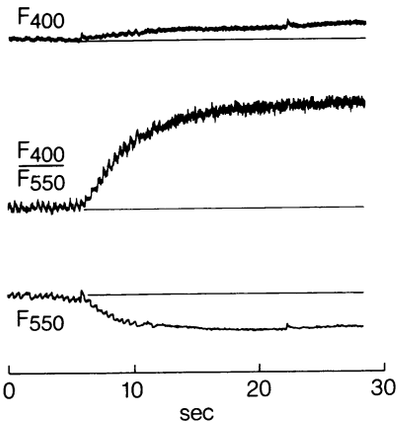

FIG. 3. Fluorescence recordings obtained during infusion of high calcium (100 mM) and ionomycin (1.5 μM), which begins 6 sec after the start of the recording. Fluorescence emissions change reciprocally, producing a sustained increase in the F_{400}/F_{550} ratio, which is substantially larger than the spontaneous $[Ca^{2+}]_i$ transients. The increase in the fluorescence ratio is invariably larger than that produced by KCl infusion and may reflect saturation of intracellular indo-1 by calcium. Transients are abolished under these conditions, and the heart is in contracture.

Effects of Neurotransmitters and Caffeine. Another means of altering the time course of the $[Ca^{2+}]_i$ transient is by infusion of drugs that alter calcium transport. Indo-1 may be especially suited for studying the reuptake of calcium by the sarcoplasmic reticulum, owing to its particular sensitivity at diastolic levels of $[Ca^{2+}]_i$. Infusion of adrenaline causes clear acceleration in the decay of the $[Ca^{2+}]_i$ transient, along with faster mechanical relaxation (Fig. 2*A*). Adrenaline is believed to accelerate calcium reuptake by the sarcoplasmic reticulum through the intracellular messenger cyclic AMP.

In contrast to adrenaline, caffeine is believed to inhibit relaxation through impairment of calcium sequestration (14). Fig. 4 shows the effect of an abrupt infusion of 20 mM caffeine. Caffeine causes an increase in systolic and diastolic $[Ca^{2+}]_i$, with progressive impairment of mechanical relaxation (bottom trace). The increase in diastolic $[Ca^{2+}]_i$ is dependent upon beat frequency. Caffeine always slows the relaxation of tension and $[Ca^{2+}]_i$, but the continuous elevation of the signals seen in Fig. 4 only occurs when beat frequency is rapid. Our findings are consistent with the effects of caffeine on quin-2-loaded myocyte suspensions (15, 16) but contradict results in aequorin-loaded cardiac fibers, where caffeine suppresses $[Ca^{2+}]_i$-dependent luminescence (17). One explanation for this disagreement would be dependence of aequorin signals upon gradients of $[Ca^{2+}]_i$ generated by the sarcoplasmic reticulum (18). By collapsing these gradients, caffeine could mask a $[Ca^{2+}]_i$ increase in cells containing aequorin but not in cells containing quin-2 or indo-1.

A second drug that may potentiate contraction by impairment of calcium reuptake is acetylcholine. High concentrations (>1 μM) of acetylcholine are known to increase the contractility of cardiac muscle (19), while increasing the intracellular concentration of inositol 1,4,5-triphosphate, which retards sequestration of calcium by intracellular organelles (20). As shown in Fig. 5, when 0.01 mM acetylcholine is administered for 2 min (center panel) there is a marked reduction in beat frequency and an increase in the peak amplitude and end-diastolic value of the $[Ca^{2+}]_i$ transients. Compared to the control recording, the decay of the transients from any given level is slower after acetylcholine than during the normal contractions. This observation is not explained by the reduction in beat frequency, since cessation of pacing in Fig. 1 reduces end-diastolic $[Ca^{2+}]_i$ without increasing the peak systolic value. The effects of acetylcholine are nearly reversed in the right-hand panel of Fig. 5. These observations suggest that acetylcholine retards calcium reuptake by the sarcoplasmic reticulum, which could explain, at least in part, why acetylcholine potentiates contraction.

Effects of Ischemia. A primary reason for recording $[Ca^{2+}]_i$ transients in the intact heart is to determine the effects of myocardial ischemia, which cannot occur in superfused cells or fibers. Fig. 6 shows the effects of a 90-sec episode of global ischemia on the F_{400}/F_{550} ratio in five hearts paced at 180 beats per min. Ischemia produces a prompt increase in the peak amplitude of the $[Ca^{2+}]_i$ transients along with a parallel increase in end-diastolic $[Ca^{2+}]_i$. The overall excursion of the $[Ca^{2+}]_i$ transient also increases and there is a characteristic change in shape, with broadening of the peak, and acceleration of the terminal decay (right-hand *Inset* of Fig. 6). These changes could reflect the nonlinear relation between the fluorescence ratio and $[Ca^{2+}]_i$, which compresses the ratio as $[Ca^{2+}]_i$ increases. Alternatively, the transients could be broadened by a specific effect of ischemia on calcium transport. The effects of ischemia are fully reversed by 1 min of reperfusion, are reproducible during a series of trials, and

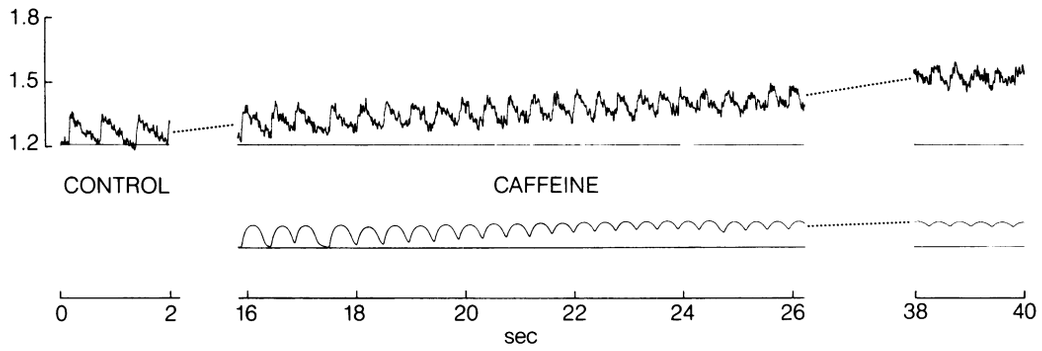

FIG. 4. Effects of a 30-sec infusion of caffeine (20 mM) on the F_{400}/F_{550} ratio (top trace) and left ventricular contraction (bottom trace). Caffeine infusion (middle and right columns) causes a marked elevation of diastolic and systolic $[Ca^{2+}]_i$ due to impaired calcium uptake by the sarcoplasmic reticulum. This is accompanied by a sustained contracture. Comparable results were obtained in five infusion trials in three hearts.

are similar in every heart that has been studied. The effects of ischemia on indo-1 fluorescence are not due to changes in epicardial temperature, changes in autofluorescence (which was measured in the absence of indo-1), or changes in cytosolic pH. Acidification of indo-1 *in vitro* produces a small decrease in its calcium sensitivity, which would have effects opposite to those produced by ischemia. An early increase in $[Ca^{2+}]_i$ during ischemia has been predicted from electrophysiological observations but has never been directly observed.

DISCUSSION

Our results show that it is practical to record $[Ca^{2+}]_i$-dependent fluorescence transients in the rabbit heart using the tetracarboxylate indicator indo-1. To our knowledge, physiological $[Ca^{2+}]_i$ transients recorded from an intact organ have not been reported previously. Other investigators have recorded much slower $[Ca^{2+}]_i$ fluctuations from mammalian hearts loaded with quin-2 (11) or the NMR probe 5,5′-difluorobis[*o*-aminophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (12), but neither of these methods permits the resolution of $[Ca^{2+}]_i$ transients, and both indicators appear to produce severe buffering of $[Ca^{2+}]_i$, leading to impairment of mechanical contraction and relaxation.

A major finding of our study is that the decay of the fluorescence transients continues throughout diastole and that a variety of pharmacologic and physiologic maneuvers that alter contractile force produces parallel changes in systolic and end-diastolic $[Ca^{2+}]_i$. This behavior contrasts with that of aequorin transients, which decay fully during the twitch and show no variation in luminescence at end-diastole (21–23). Four considerations indicate that the continued decay of indo-1 transients in diastole reflects physiologically meaningful changes in $[Ca^{2+}]_i$. (*i*) Measurements of the *in vitro* kinetics of indo-1 show that the dissociation rate constant (130 sec^{-1}) is at least an order of magnitude faster than the decay of the fluorescence transients we observe (24). (*ii*) When cardiac myocytes are loaded with either indo-1 or fura-2 in the acetoxymethylester form, trapping of the indicator appears to be confined to the myofilament space (4, 7). (*iii*) Decay of the $[Ca^{2+}]_i$ transients is prevented by caffeine, which prevents reuptake of calcium by the sarcoplasmic reticulum (14). Decay of the transients is also slowed by acetylcholine, which could affect sarcoplasmic reticulum function through generation of inositol 1,4,5-triphosphate. (*iv*) A slow component of relaxation has been inferred from recordings of cell edge movement in isolated myocytes,

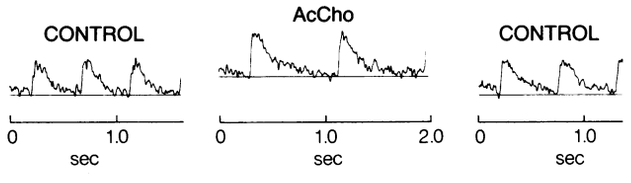

FIG. 5. Effects of acetylcholine (0.01 mM) on $[Ca^{2+}]_i$ transients in a spontaneously beating rabbit heart. The left panel shows transients obtained under control conditions; the center panel shows transients recorded from the same heart after a 2-min infusion of acetylcholine (AcCho). Acetylcholine slows the heart rate, increases the peak amplitude of the $[Ca^{2+}]_i$ transient, and slows the decay of the transient following the peak. The upward shift of the transients during acetylcholine infusion contrasts with the effect of changes in heart rate produced by electrical pacing (Fig. 1). In the latter case, transients decay to a lower level at slow heart rates, which is the opposite of the behavior produced by acetylcholine. The right-hand panel is obtained 5 min after cessation of acetylcholine infusion and shows substantial recovery of the heart rate and the rate of relaxation of the transient.

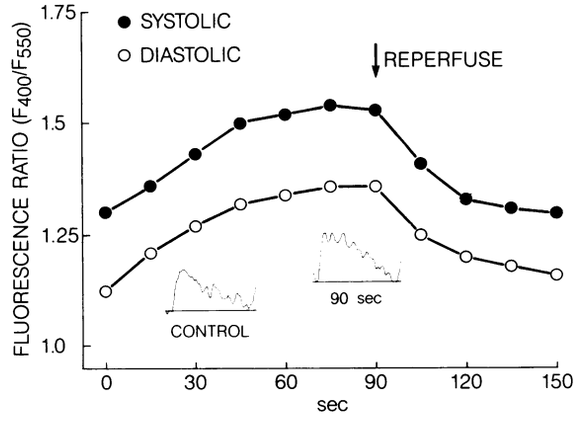

FIG. 6. Effects of global ischemia on $[Ca^{2+}]_i$-dependent fluorescence transients. Ischemia is produced by cessation of coronary perfusion for 90 sec while the heart is paced at (180 beats per min) by an epicardial plunge electrode. (*Insets*) Change in the $[Ca^{2+}]_i$ transient produced by ischemia. The left-hand transient is obtained prior to ischemia, whereas the right-hand transient is after 90 sec of ischemia. Ischemia elevates systolic and diastolic $[Ca^{2+}]_i$ while broadening the peak of the transient. Contraction is rapidly inhibited during this period, despite the inferred increase in $[Ca^{2+}]_i$. The data points show mean values of the F_{400}/F_{550} ratio during the systolic peak (●) and at end-diastole (○) in five hearts. A progressive increase in the fluorescence ratio begins promptly after the onset of ischemia, reaching a steady state after 90 sec. Ischemia also causes a consistent increase in the net amplitude of the transients. $[Ca^{2+}]_i$ transients decay smoothly to their baseline values during reperfusion. The diastolic ratio (mean ± SD) is 1.15 ± 0.08 at onset of ischemia vs. 1.36 ± 0.12 at 90 sec of ischemia ($P = 0.01$). Corresponding systolic values are 1.30 ± 0.07 vs. 1.53 ± 0.16 ($P = 0.01$). The overall effects of ischemia and reperfusion are highly significant by the Friedman test ($\chi^2 = 47.1$, df = 10, $P < 10^{-6}$ for systolic values; $\chi^2 = 47.4$, df = 10, $P < 10^{-6}$ for diastolic values).

which bear no mechanical load (25–27). It has been proposed that $[Ca^{2+}]_i$-dependent relaxation continues throughout diastole in all cardiac cells but that the restraining effect of connective tissue on the myocytes prevents this relaxation from being observed in intact muscle (26). This interpretation is confirmed by simultaneous comparison of $[Ca^{2+}]_i$ with contraction in the intact heart. Dissociated myocytes from the rabbit heart have $[Ca^{2+}]_i$ transients that are similar to those that we obtain in the intact heart (6); however, whereas isolated myocytes continue to relengthen for hundreds of milliseconds (6, 28) relaxation of the intact ventricle reaches an abrupt completion while $[Ca^{2+}]_i$ is still declining (Fig. 2).

Effects of Ischemia. The second major finding of our study is the ability of myocardial ischemia to rapidly and drastically increase $[Ca^{2+}]_i$ during all phases of the cardiac cycle. The possibility of a $[Ca^{2+}]_i$ increase has been suspected for several years based on the ability of calcium channel blockers and related drugs to diminish the consequences of ischemia (29, 30). We presume that ischemia can liberate calcium from intracellular stores, since influx through the slow channel is thought to be inhibited (31).

The time course of the $[Ca^{2+}]_i$ increase during ischemia has previously been uncertain. Measurements of $[Ca^{2+}]_i$ obtained in isolated fibers or myocytes often fail to show an increase during the first few minutes of hypoxia or metabolic inhibition (32, 33). These results may reflect limitations of the measurement technique (18) or failure of the experimental model to reflect accurately conditions in the ischemic heart. Recently Marban *et al.* (12) have used NMR spectroscopy to measure $[Ca^{2+}]_i$ in intact ferret hearts containing 5,5′-difluorobis[*o*-aminophenoxy]ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid. With this technique, ischemia produces a 3-fold increase in the time averaged $[Ca^{2+}]_i$ after 20 min, but there is no increase during the first 5 min. In these experiments $[Ca^{2+}]_i$ was severely buffered by the indicator, which may account for the slow time course of the $[Ca^{2+}]_i$ increase during ischemia.

The timing of an ischemia-induced $[Ca^{2+}]_i$ increase is particularly crucial in determining whether $[Ca^{2+}]_i$ could potentially mediate changes in electrical activity that produce lethal ventricular arrhythmias in the acutely ischemic heart (29, 34). Ischemia produces a rapid reduction in resting membrane potential, which is initially independent of extracellular K^+ accumulation (35). K^+-independent depolarization is most prominent during the first 30–60 sec of ischemia. It is therefore possible that the $[Ca^{2+}]_i$ increase that we observe directly alters the resting potential of ischemic heart cells by means of a calcium-activated inward current (28). Such a current could flow either through the sodium–calcium exchange or through calcium-activated cation channels that are known to occur in the heart (36, 37). The finding that ischemia elevates diastolic $[Ca^{2+}]_i$ beyond the normal systolic level strongly supports the above view.

In addition to electrophysiological abnormalities, the first 2 min of ischemia are characterized by a rapid reduction in contraction strength. Previous workers have shown that suppression of contraction by hypoxia involves an impaired response of the myofilaments to calcium rather than reduction of the $[Ca^{2+}]_i$ transients (32, 38). The experiment in Fig. 6 shows that a similar mechanism operates in ischemia. What is less clear from previous work is whether the disappearance of contractions during ischemia conceals from the experimenter an early increase in $[Ca^{2+}]_i$ (18, 32, 33). The experiment described here should be definitive, since it involves a highly sensitive $[Ca^{2+}]_i$ indicator, a truly ischemic preparation, and close simulation of *in vivo* conditions.

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