Direct Measurement of Changes in Intracellular Calcium Transients During Hypoxia, Ischemia, and Reperfusion of the Intact Mammalian Heart

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In studies of ischemia and reperfusion, a major experimental problem has been the inability to measure intracellular ionized calcium ([Ca\textsuperscript{2+}]) in the intact heart. We have developed a new approach in which the bioluminescent calcium indicator aequorin is used to measure [Ca\textsuperscript{2+}], in the isolated, coronary-perfused ferret heart. Aequorin is loaded into subepicardial myocytes of the left ventricle, and the signals are recorded simultaneously along with isovolumic left ventricular (LV) pressure at a constant pacing rate. This system shows 1) no attenuation or change of time course of LV pressure development or coronary perfusion pressure after aequorin loading; 2) consistent responses to physiological interventions and drugs; 3) individual aequorin and pressure signals that do not require signal averaging for analysis; and 4) [Ca\textsuperscript{2+}], levels comparable with those reported in tissue or isolated myocyte cell preparations. During 5 minutes of hypoxia, diastolic [Ca\textsuperscript{2+}], and LV diastolic pressure increased while the systolic values of both [Ca\textsuperscript{2+}], and pressure decreased. The peak-to-peak systolic [Ca\textsuperscript{2+}], versus LV isovolumic pressure relation remained close to the control curve. In contrast, during 3 minutes of global ischemia, LV systolic and diastolic pressures fell rapidly, while [Ca\textsuperscript{2+}], increased substantially. The [Ca\textsuperscript{2+}], versus pressure relations for both systole and diastole shifted to the right, indicating desensitization of the contractile apparatus to [Ca\textsuperscript{2+}],. These results provide evidence that different primary mechanisms determine the systolic and diastolic responses to acute hypoxia versus ischemia. During hypoxia, changes in [Ca\textsuperscript{2+}], handling probably play a major role, while during ischemia, changes in the Ca\textsuperscript{2+} sensitivity of the myofilaments appear to be of primary importance in the modulation of contractile dysfunction. (Circulation Research 1989;65:1029–1044)

An increase in the concentration of the intracellular calcium ion ([Ca\textsuperscript{2+}]) has been proposed by several investigators as the explanation for the pathological changes that occur during acute ischemia and reperfusion.\textsuperscript{1–3} A striking rise in [Ca\textsuperscript{2+}], during acute ischemia has been directly documented recently by several different approaches, including nuclear magnetic resonance (NMR)\textsuperscript{4,5} and methods that use the fluorescent Ca\textsuperscript{2+} indicators.\textsuperscript{6–8} These experiments have raised some additional questions, which include 1) what is the primary cause of abnormal [Ca\textsuperscript{2+}], regulation during ischemia? and 2) how does the rise in [Ca\textsuperscript{2+}], relate to the mechanical dysfunction that occurs during periods of acute ischemia or after reperfusion?

We have developed a new system in which [Ca\textsuperscript{2+}], transients are obtained reproducibly with the bioluminescent protein aequorin in isolated, coronary-perfused ferret hearts.\textsuperscript{9,10} This system permits recording of changes in [Ca\textsuperscript{2+}], and the corresponding ventricular mechanical parameters on a beat-to-beat basis. By means of this system, we delineate the effects of ischemia and hypoxia on intracellular Ca\textsuperscript{2+} handling and the responses of the contractile elements to Ca\textsuperscript{2+}, which can be correlated with acute changes in ventricular systolic and diastolic function. Our results provide evidence that different
subcellular mechanisms predominate during ischemia versus hypoxia. Moreover, our technique with aequorin for recording $\left[Ca^{2+}\right]_i$ during pharmacological and physiological interventions also provides data that are both complementary and supplementary to other techniques currently available for the study of changes in $\left[Ca^{2+}\right]_i$ in the intact heart. 7-11

**Materials and Methods**

**Preparation of Isolated Perfused Heart**

Hearts were removed from 10- to 14-week-old male ferrets that were heparinized (5,000 units i.p.) and then anesthetized with chloroform. The heart was washed in a standard physiological salt solution, then mounted on a cannula that was inserted into the ascending aorta at the level of the right innominate artery and tied securely. Coronary perfusion was started immediately with the solution at 30° C via a constant flow pump (Masterflex model 7553-30, Cole-Parmer Instrument, Chicago, Illinois), which was initially set to achieve a pressure of 75 mm Hg. Aortic pressure, which in this model is equivalent to coronary perfusion pressure, was measured via a Statham P23Db pressure transducer (Gould, Cleveland, Ohio) connected to a sidearm of the infusion cannula. The pericardium was removed, and the pulmonary artery was cannulated and drained to keep the right ventricle decompressed. A thin latex balloon (Carter-Wallace, Cranbury, New Jersey) tied to the end of a 10-cm polyethylene tube (PE 160, Becton-Dickinson, Rutherford, New Jersey) was passed into the left ventricle (LV) through the mitral valve and connected to another Statham P23Db pressure transducer. 12

After the insertion of another polyethylene tube into the apex of the LV to drain thebesian venous return, the balloon was filled with saline to an end-diastolic pressure of 8-10 mm Hg and then kept isovolumic throughout the experiment. The damping ratio and the accuracy of pressure measurement in this system have been described by Wexler et al. 12

A thermistor probe (YSI model 400, Yellow Springs Instrument, Yellow Springs, Ohio) and a platinum pacemaker wire, which was attached to a Grass Model 5888 stimulator via a Grass Model SIU5 stimulus isolation unit (Grass Instrument, Quincy, Massachusetts) were inserted into the right ventricle through a right atrial incision. The standard solution was composed of (mM) NaCl 118, KCl 4.7, KH$_2$PO$_4$ 1.2, CaCl$_2$ 1.0, MgCl$_2$ 1.2, NaHCO$_3$ 23, dextrose 5.5, and lactate 1.0. Lactic acid was neutralized with NaOH before being added to the buffer. The solution was gassed with 95% O$_2$-5% CO$_2$ to adjust the pH to 7.40±0.02. During the run-in period, the flow rate was adjusted to 10-18 ml/min to provide a mean coronary perfusion pressure of 90 mm Hg. 13 The heart was positioned in a glass organ bath and submerged in solution delivered from the same bottle as the coronary perfusate.

**Light-Collecting System for Aequorin Luminescence**

For collection of the aequorin light signal from the surface of the LV (described later), a part of the

**FIGURE 1.** Diagrammatic cross section of experimental apparatus. A part of coronary perfusion system and an organ bath for isolated heart were placed in light-tight box surrounded by photographic bellows. Bottom of organ bath was connected to a photomultiplier tube (PMT) and its housing via an ellipsoidal reflector. Heating system, proximal part of coronary perfusion system, and signal recording apparatus are not illustrated. RV, right ventricular; LV, left ventricular.
coronary perfusion system was positioned in a light-tight box and connected to an apparatus originally designed for aequorin experiments by Blinks. As shown in Figure 1, the coronary perfusion line distal to the pressure control reservoir (warming coil and cannula for the aorta) was positioned within a space surrounded by a light-tight photographic bellows. The transducers monitoring coronary perfusion and LV pressures were also positioned inside the light-tight box at the level of the LV. The reservoir (20 ml total capacity) was positioned just outside the light-tight box, and the capacity of the line distally was minimized to 15 ml. The drainage lines were exteriorized through the baseboard of the light-tight box, and electrical wires were connected via light-tight junctions at the base. The bath surrounding the heart (water-jacketed glass bath, internal capacity 30 ml) had a concavity (2.5 cm diameter, 1.5 cm depth) at its bottom. With this design, the inferoapical surface of the LV where aequorin would be loaded was positioned at the center of the concavity just in contact with the glass wall. To maximize the efficiency of light collection, we connected the bottom of the organ bath and the photomultiplier tube (9635QA, Thorn EMI, Fairfield, New Jersey) via an ellipsoidal reflector; the center of the concavity (lowest part of the bath) projecting into the reflector was positioned at one point of focus of the reflector, and the cathode of the photomultiplier tube was positioned at the other focal point. The bellows-enclosed portion of this device was checked regularly for light leaks, and none could be detected at the voltages we consistently used to charge our photomultiplier tube during experiments.

The effect of motion on the light signal in this system was quantified by use of a light standard (thin plastic plates painted with ^{8}Ra on one side) at a voltage at which the photomultiplier tube maintained essentially linear responses to the intensity of light impinging on the cathode. First, the light standard (1.0x0.8 mm) was shifted in position from the center to the peripheral portion of the bottom of the bath during measurement of the amount of light collected by the photomultiplier tube. Second, the light standard (2.5x2.5 mm) was sutured onto the inferoapical surface of the LV where aequorin would be loaded, and the heart was positioned at the center of the bath. Figure 2A shows that, compared with when the light source was located in the center of the bath, peripheral repositioning did not significantly affect the amount of light collected (<3% within 2 mm displacement from the center). The amount of light collected by the photomultiplier tube was maximally reduced by 2±1% (n=4) at the peak of LV pressure development compared with quiescent levels. On the other hand, Figure 2B shows a representative recording obtained during postextrasystolic potentiation. Note that cardiac motion did not significantly change the signal recorded from the standard although a pronounced increase in contractility occurred during the potentiated beat. Therefore, this light collection system is relatively insensitive to movement of the source. Nevertheless, motion artifact must be kept in mind as a possible confounding variable when interpreting small changes in [Ca^{2+}].

Loading Procedure for Aequorin

We recently developed a modification of our chemical approach for loading of bioluminescent Ca^{2+} indicator aequorin into myocardial cells of papillary muscle preparations. The essential differences of this modification from the chemical procedure that we described previously are 1) a lower concentration of Ca^{2+} chelator is present, and 2) aequorin-containing solution is injected into the interstitium (macroinjection), in contrast with intracellular microinjection or immersion. The macroinjection approach provides [Ca^{2+}] transients and levels that are qualitatively and quantitatively similar to those obtained with standard intracellular microinjection as well as immersion approaches to loading. In the present study, we applied our macroinjection method to the isolated, perfused whole heart preparation, as described below.

After a 20-minute stabilization period, the coronary perfusate was replaced with a low Ca^{2+} solution composed of (mM) NaCl 118, KCl 4.7, KH_{2}PO_{4} 1.2, MgCl_{2} 5.0, NaHCO_{3} 23, dextrose 20, and pyruvate 5.0. The pH was 7.30±0.02 when saturated by a gaseous mixture of 95% O_{2}-5% CO_{2} at 20°C. Within 3 minutes the heart became completely quiescent and coronary resistance fell. The coronary perfusion rate was then reduced to 5–8 ml/min for maintenance of a perfusion pressure of 25–30 mm Hg. Subsequently, the heart was temporarily raised out of the organ bath, and an aequorin-loading solution composed of aequorin 1.0 mg/ml, NaCl 154 mM, KCl 5.4 mM, MgCl_{2} 1.0 mM, HEPES 12 mM, dextrose 11 mM, and EDTA 0.1 mM, (pH 7.40±0.02) was injected into the interstitium of the epicardium, just beneath the epimysium, with a low-resistance glass micropipette (inner diameter of the tip, 25–38 μm) under the control of a micromanipulator (model 520-137, E. Leitz, Rockleigh, New Jersey). In toto, 3–5 μl of the aequorin-loading solution was injected within 3 minutes into a localized region of approximately 3-mm diameter. Since it would be difficult to penetrate cell membranes of viable cells with these large-diameter micropipettes, we did not monitor electrical potentials at the tip during this procedure. However, damage to cells adjacent to the path of micropipette entry could occur. Therefore, care was taken not to inject an excessive amount of the solution, which could produce tissue dissection and retention of an isolated pocket of aequorin solution within the myocardium. The micromanipulator helped to keep the tip of the micropipette in position during the pressure injection. These maneuvers were used for reduction of tissue injury during the loading proce-
FIGURE 2. Variation in amount of light collected with position of light source in bath (panel A) and with motion artifact of heart (panel B). In panel A, abscissa shows distance of light source from center of bath (illustrated in lower part of panel). In panel B, the other light source was sutured on inferoapical surface of left ventricle, and signal was recorded while heart was positioned in bath and stimulated by right ventricular pacer. Fourth to seventh beats were made by extrasystolic stimuli for induction of a postextrasystolic potentiation (eighth beat). Light sources were thin plastic plates painted with $^{85}$Ra (1.0 x 0.8 mm in panel A, 2.5 x 2.5 mm in panel B). Photomultiplier tube showed linear responses for light input at these settings. LV, left ventricular.

dure; there was no evidence of damage on histologic examination of the loaded region (see "Results"). Our papillary muscle studies have shown that the quantitative values for resting [Ca$^{2+}$] are similar with macroinjection and microinjection. Because it is known that injured myocytes retain significantly higher cytosolic Ca$^{2+}$ levels, these data suggest that tissue injury by the macroinjection method is small and can provide values for [Ca$^{2+}$] that are comparable with those obtained with the microinjection technique.

After loading, the heart was repositioned in the concavity at the bottom of the organ bath and the aequorin-loaded region of the LV was directed toward the cathode of the photomultiplier tube. The Ca$^{2+}$ concentration of the coronary perfusate was slowly increased in a stepwise fashion every 15 minutes over the period of 1 hour for prevention of calcium paradox. When the Ca$^{2+}$ concentration in the perfusate reached 0.3 mM, the perfusion rate and the temperature were gradually increased over the course of 15 minutes to attain the levels present before loading. During this period, the heart showed an initiation of weak and sporadic contractions.

After replacement of coronary perfusate by the standard solution at the previous perfusion rate at 30° C, the LV isovolumic pressure and coronary perfusion pressure recovered within 3 minutes, and steady state was achieved within 20 minutes.

The amount of aequorin actually loaded into myocytes (estimated as $I_{max}$; see "Calibration of Aequorin Light Signals" below) appeared to be a very small fraction of the total amount of aequorin macroinjected into the interstitium. This conclusion is based on a comparison of $I_{max}$ to the total light emission occurring from 3–5 μl of aequorin-loading solution at 30° C in vitro. Assuming that the photomultiplier tube maintained a nearly linear response over this range, the mean value of $I_{max}$ was 0.24±0.09% of the total aequorin activity (n = 8). The remainder of the aequorin was either washed out of the tissue during perfusion or remained in the interstitial space to be consumed rapidly when the Ca$^{2+}$ concentration in the perfusate was increased to 1 mM.

The precise cellular mechanism of loading remains uncertain. It is unlikely that the aequorin molecule can passively diffuse across normal cell membranes. It is also unlikely that the myocytes can activate a significant degree of energy-dependent membrane transport (i.e., pinocytosis) at 20° C. Moreover, since the solution that was perfusing the heart tissue when the loading solution was macroinjected had a Ca$^{2+}$ contamination of 6.0±0.9 μM (total amount of Ca$^{2+}$ measured by atomic absorption spectrophotometer, model 303, Perkin-Elmer, Eden Prairie, Minnesota) and had only weak Ca$^{2+}$ buffering capacity via the bicarbonate and phosphate anions it contained, this solution in itself should not cause significant membrane breakdown.

We speculate that the low [Ca$^{2+}$] in the loading solution may modify the cell membrane transiently and reversibly and allow intracellular entry of aequorin molecules surrounding the cells. Assuming that this is the case, we have to consider the possibility that the loading solution may affect spe-
cific portions of the cell membrane, such as the maculae and fasciae adherens regions of the intercalated disks, which could subsequently lead to inhomogeneous distribution of aequorin within the cell. Against this are our findings in papillary muscle preparations, where we obtained comparable quantitative signals with standard microinjection and with the macroinjection approach. We also found that the aequorin signals recorded after macroinjection could demonstrate $[\text{Ca}^{2+}]_i$ transients mainly operated by sarcolemmal Na$^+$/Ca$^{2+}$ exchange mechanisms in ryanodine-treated papillary muscle preparations, which is consistent with data previously observed in single cells loaded with fura-2. We believe these widely varied observations would not be seen if aequorin were localized in specific subcellular compartments.

**Signal Recording**

Aequorin light signals were recorded from the photomultiplier as anodal current. We set the zero level at the mean of the dark current. After passing through a current discriminator, the light signal was filtered through an analog low-pass window ($-6$ dB at 25 Hz; 8-pole Bessel, UAF-41, Burr-Brown, Tucson, Arizona). The light signal, $LV$ isovolumic pressure, coronary perfusion pressure, and right ventricular stimulus artifact were then simultaneously recorded both on a magnetic tape recorder (model 3964A, Hewlett-Packard, Palo Alto, California) and a 4-channel chart strip recorder (model 2400S, Gould). The tape recording was continued without interruption from the beginning of the protocols until the end of the experiment. Therefore, all light signals emitted by aequorin were recorded except during periodic brief periods ($<10$ seconds) when the light shutter was closed for a check of the stability of the baseline.

**Calibration of Aequorin Light Signals**

A calibration procedure similar to that described by Allen and Blinks was used for conversion of light signals to quantitative $\text{Ca}^{2+}$ concentrations. In brief, the procedure involves 1) normalization of the aequorin light at each phase of the experiment by the amount of active aequorin in the preparation, and 2) conversion of the normalized light signal of a $[\text{Ca}^{2+}]_i$ value by use of an in vitro calibration curve.

**Normalization of aequorin light signals.** At the end of each experiment, the heart was perfused with 4% Triton X-100 (Fischer Scientific, Orangeburg, New York) in 50 mM CaCl$_2$ at a constant flow rate and 30°C. Within 30 seconds the myocytes were rendered hyperpermeable and discharged all their remaining aequorin, which quickly reacted with the saturating $[\text{Ca}^{2+}]_i$ in the perfusate. The completion of light emission was assumed when no further light was detected during a 5-minute period. The rapidity of this response was much faster than previously reported by Smith and Allen. For estimation of the amount of active aequorin remaining in the preparation ($L_{\text{max}}$ in vivo) at a certain period during the experiment, the aequorin signals recorded on a magnetic tape were played back on the chart strip through a filter with a 1.0-second time constant. Then the integral of the signals from the period of interest to the end of the experiment was added to the integral of light emitted during Triton X-100 lysis, which was corrected by a rate constant (discussed later) to obtain the value of $L_{\text{max}}$.

Time-dependent consumption of aequorin could be a serious problem in estimation of $[\text{Ca}^{2+}]_i$ values during a single continuous intervention. The rate of aequorin consumption in each preparation was 8–30% of initial values of $L_{\text{max}}$ depending on the particular protocol to which the heart was exposed. Especially in an ischemia experiment (results presented later), aequorin consumption may reach 3% of initial values of $L_{\text{max}}$ after 3 minutes. To compensate for this problem, we repeated the procedure for calibration at frequent intervals during the period of interest, as noted in some figures by multiple calibration bars.

**Conversion of fractional luminescence $L/L_{\text{max}}$ to estimated $[\text{Ca}^{2+}]_i$.** For conversion of fractional luminescence ($L/L_{\text{max}}$) into a quantitative $[\text{Ca}^{2+}]_i$, a calibration curve (Figure 3) was determined in vitro. Aequorin was preincubated with 1 mM Mg$^{2+}$ and prepared according to the methods of
The minimum detectable level of \([Ca^{2+}]_i\) depends on

by use of a nonlinear regression analysis with iteration methodology, we obtained the values of two constants: \(K_a = 4.50 \times 10^6/M \) and \(K_r = 130.0\). This formula was used for correction of fractional luminescence to \([Ca^{2+}]_i\) throughout the present study.

The rate constant that we measured for consumption of aequorin was 32% higher than that reported recently by Smith and Allen\(^{25}\) but within the expected range.\(^{26,27,29,30,32,33,34}\) In the lower ranges of \(Ca^{2+}\) (i.e., \(<3 \times 10^{-7}\) M), our calibration curve suggests less aequorin luminescence than that reported by Smith and Allen,\(^{25}\) but more than that reported by Yue and Wier.\(^{35,36}\) The upward shift of our curve from that obtained by Yue and Wier,\(^{35,36}\) could be related to the difference in \([Mg^{2+}]\) in the buffer solutions, which was 2 mM in their determinations and 1 mM in ours (for rationale see Reference 37); however, this would not explain the differences between our curve and that of Smith and Allen.\(^{25}\) Small differences in pH (7.1 in our determination; 7.0 in those of Smith and Allen\(^{25}\) are unlikely to be the cause. Since the aequorin used in of these determinations consisted of a heterogeneous mixture of several different isomers with different \(Ca^{2+}\) sensitivities,\(^{3,8,26}\) we feel that the most likely explanation for these differences is related to differences in isomeric composition of the batches of aequorin tested. This interpretation underlines the importance of performing a calibration curve for each different batch of aequorin before attempting quantitative measurement of \(Ca^{2+}\).

As indicated by the calibration curve, aequorin is less sensitive to \(Ca^{2+}\) in the lower concentration range. Therefore, some investigators hesitate to estimate \([Ca^{2+}]\) during periods of rest or diastole. The minimum detectable level of \([Ca^{2+}]\), depends on the amount of aequorin present in the cell (i.e., \(L_{max}\)), the dark current of the photomultiplier tube, the \(Ca^{2+}\)-independent luminescence of aequorin (basal glow), and the steepness of the calibration curve in the range of pCa 6.5-7.0.\(^{32}\) Table 1 shows that, in our experiments, diastolic \([Ca^{2+}]_i\) (0.33 \(\mu\)M) was experimentally measured as an anodal current of 0.28 nA (mean values). In these experiments, the dark current of the photomultiplier tube (880 volts) was 0.07 nA. As described above, we set zero at the mean level of the dark current while regularly compensating for any drift of the preamplifier. The current induced by basal glow\(^{31}\) of aequorin (log \(L_{max} = -6.30\) by our calibration curve) was 0.02 nA, which is approximately 50% less than that which would be estimated from the Smith and Allen\(^{25}\) calibration curve. Moreover, over the range of pCa change from 7.0 to 6.5, our intensity of current increased 340% compared with an increase in current of 220% from Smith and Allen’s\(^{25}\) calibration. Taken together, the differences in calibration curves enhances our ability to detect the levels of \(Ca^{2+}\) that are present in resting muscle and during diastole. We estimate that the minimum current that we could reliably detect is 0.05 nA, which

| \(L_{max}\) (\(\mu\)A) | 42.8±23.7 | 10 |
| Peak of \(Ca^{2+}\) transient | 1.96±1.39 |
| \(L_{max}\) (10^-5) | 4.83±1.42 |
| \([Ca^{2+}]_i\) (\(\mu\)M) | 0.86±0.11 |
| Diastolic \(Ca^{2+}\) level | 0.28±0.15 |
| \(L_{max}\) (10^-5) | 0.68±0.16 |
| \([Ca^{2+}]_i\) (\(\mu\)M) | 0.33±0.04 |

Data are expressed as mean±SD. In each experiment, peak \([Ca^{2+}]_i\) and resting \([Ca^{2+}]_i\) values were estimated by averaging 10 serial beats at steady state while heart was paced at 120±5 beats/min at 30° C. Perfusate contained 1 mM \(Ca^{2+}\). Experiment was terminated by perfusion with 4% Triton X-100 in 50 mM \(CaCl_2\). In vitro calibration curve is shown in Figure 3. \(L_{max}\), integration of light signals from the time \([Ca^{2+}]_i\), was estimated until end of experiment. \(L_{max}\), aequorin light emission.

As indicated by the calibration curve, aequorin is less sensitive to \(Ca^{2+}\) in the lower concentration range. Therefore, some investigators hesitate to estimate \([Ca^{2+}]_i\) during periods of rest or diastole. The minimum detectable level of \([Ca^{2+}]_i\), depends on

<table>
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<tr>
<th>Table 2. Comparisons of Hemodynamic Parameters at Steady State Before and After Aequorin-Loading Procedure</th>
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<tr>
<td>(n)</td>
</tr>
<tr>
<td>Paciing interval (msec)</td>
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<tr>
<td>Mean perfusion pressure (mm Hg)</td>
</tr>
<tr>
<td>Peak LV pressure (mm Hg)</td>
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<tr>
<td>End-diastolic LV pressure (mm Hg)</td>
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<tr>
<td>Time to peak pressure (msec)</td>
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<td>Time to 50% pressure regression (msec)</td>
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Data are expressed as mean±SD. Heart was paced via a right ventricular catheter at equivalent rates before and after loading. Left ventricular (LV) volume and coronary perfusion rate were kept constant before and after loading. Temperature was 30° C; perfusate contained 1 mM \(Ca^{2+}\).
TABLE 3. CPK Leakage Before, During, and After Aequorin-Loading Procedure

<table>
<thead>
<tr>
<th>Before procedure</th>
<th>15 minutes after beginning</th>
<th>15 minutes after end</th>
<th>5 minutes after end</th>
<th>30 minutes after end</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>30±0.4</td>
<td>20±0.8</td>
<td>25±0.8</td>
<td>30±0.4</td>
</tr>
<tr>
<td>Perfusion pressure (mm Hg)</td>
<td>89±17</td>
<td>25±10</td>
<td>52±18</td>
<td>96±24</td>
</tr>
<tr>
<td>[Ca²⁺] in perfusate (mM)</td>
<td>1.0</td>
<td>0.01</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>CPK (IU/min)</td>
<td>0.063±0.028</td>
<td>0</td>
<td>0.101±0.053</td>
<td>0.055±0.022</td>
</tr>
<tr>
<td>CPK/total CPK (10⁻²/min)</td>
<td>2.6±1.3</td>
<td>0</td>
<td>4.1±1.9</td>
<td>2.3±1.1</td>
</tr>
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Data are expressed as mean±SD. Total CPK (950±65 IU/heart weight [g]) was obtained by Triton X-100 lysis at end of experiment. During loading procedure there was no detectable CPK leakage in coronary venous drainage in any experiments (indicated by zero). Minimum sensitivity of nicotinamide adenine dinucleotide reduction reaction was 5 IU/l; thus, the zero corresponds to values less than 0.04 IU/min in these settings (n=4). CPK, creatine phosphokinase.

Results

Effects of the Loading Procedure

Table 2 shows hemodynamic indexes compared before and after the aequorin-loading procedure. The volume of the LV balloon was kept constant throughout the experiment; the coronary perfusion flow and right ventricular pacing rates were also kept constant before and after loading. There were no significant differences in the LV peak and end-diastolic pressures or in the time course of contraction before and after the loading procedure. The equivalent levels of perfusion pressure before and after loading indicate that the coronary vasculature remained intact. This implies that during the loading procedure (i.e., exposure to low Ca²⁺, low flow, and low temperature), the myocardial tissue and coronary vasculature do not suffer significant injury. To confirm the lack of damage to our preparations, we continuously monitored cardiac creatine phosphokinase (CPK) leakage by collecting the effluent from the pulmonary arterial drain throughout the loading procedure. Measurements were performed by Bioran Medical Laboratories, Boston, by means of nicotinamide adenine dinucleotide (NAD) reduction reaction (A-gent Reagents, Abbott Laboratories, Dallas, Texas); the data are presented in Table 3. In these experiments, complete cell lysis was obtained by 4% Triton X-100 perfusion, which was started 30 minutes after the end of the loading procedure. The integral of the CPK value obtained during 20 minutes of Triton perfusion was defined as "total CPK." There was a small but consistent increase in CPK at the point of transition from the quiescent stage to active contraction; however, the magnitude of this increase was small and did not suggest a significant amount of cell necrosis. Because of the sensitivity of this method (as reflected by the ratio of CPK versus total CPK), we hypothesized that if mechanical or chemical injury was caused by the local injection of aequorin-loading solution, it should be reflected in the CPK values obtained during the early phases of loading. However, no such CPK leak was observed. To further rule out local damage that could occur by the injection of aequorin-loading solution into the apical subepicardium of the LV, we examined this region in five preparations under light microscopy. After the aequorin-loading procedure, transmural tissue blocks (5×5 mm) were obtained from both the apical, aequorin-loaded region and the lateral region of the LV (control). After treatment with embedding compound (Tissue-Tek optimum cutting temperature compound No. 4583, Miles, Kankakee, Illinois), the tissue block was rapidly frozen at −30°C, then mounted on an ultracytomicrotome (model 4550, Ames, Waltham, Massachusetts). Sections 5 μm thick were made in parallel with the epicardial surface. The Wright-stained preparation was examined at magnifications of ×40 to ×1,000 by independent pathologists without information as to whether the preparation was obtained from the aequorin-loaded region or the control region. Particular attention was directed toward identifying the types of myocardial tissue damage known to occur during Ca²⁺ paradox or during myocardial reperfusion after prolonged ischemia, which involve contraction band necrosis, bleb formation, or cell vacuolation; however, except for slight interstitial edema, no such abnormal findings were observed in any preparation. The muscle fibers showed regular striations with an interval of 1.75 μm (mean) that were evenly distributed in the cytosolic space. There was no alteration between control and aequorin-loaded regions, and the two regions were indistinguishable from each other.

Aequorin Signal and Quantitative [Ca²⁺]i

Figure 4A shows individual nonaveraged tracings of the aequorin light signal and isovolumic LV pressure. There was minor variation in the amplitude and time course of the aequorin signal, while the LV pressure showed a steady state, probably due to fluctuation of the dark current of the photomultiplier tube. However, each aequorin signal shows a time course and temporal relation to mechanical contraction that are similar to those obtained from ferret ventricular papillary muscles at 30°C. The ability to evaluate the real time individual [Ca²⁺]i transients allowed us to study the beat-to-beat changes that occur during short-term
Intracellular Calcium

**FIGURE 4.** Panel A: Original tracing showing simultaneous recordings of aequorin light signals and isovolumic left ventricular pressure (LVP). Aequorin signal was passed through a low-pass filter (—6 dB at 25 Hz). Preparation was paced at 110 beats/min. Perfusion contained 1 mM Ca²⁺ at 30°C. Panel B: Nonaveraged recording obtained when steady state was interrupted by extrasystolic stimuli, imposed repeatedly in a random fashion. Panel C: Peak [Ca²⁺], versus peak LVP in a representative heart. Small dots show steady state peak [Ca²⁺], peak LVP obtained from each single beat during determination of extracellular Ca²⁺ concentration responses. [Ca²⁺], was varied from 0.25 mM (left, lower group) to 16.0 mM (right, upper group); LVP at 16 mM [Ca²⁺] was assumed to be the maximal response (max LVP) and was used for normalization of each LVP value. The curve was obtained by fitting these data to the Hill equation (for details, see “Results” and Table 4). Responses to extrasystolic stimuli and the corresponding postextrasystolic potentiations (extrasystolic interval was varied from 240 to 360 msec) are shown as closed and open triangles, respectively. Open circles show responses to acetylstrophanthidin (3×10⁻⁷ to 3×10⁻⁶ M). These relations lay on the curve. In contrast, norepinephrine (closed circles, 1×10⁻⁸ to 1×10⁻⁷ M) shifted relation toward right in a concentration-dependent fashion. All pacing rates were 125 beats/min.

In the steady state, when the heart was perfused with 1 mM [Ca²⁺], at 30°C and paced at 120±5 beats/min, the estimated [Ca²⁺] at the peak of the transients was 0.86±0.11 µM; when the pressure showed steady diastolic levels, the [Ca²⁺] was 0.33±0.04 µM (Table 1). This value for diastolic [Ca²⁺], is higher than that reported in quiescent myocytes; however, our value is consistent with data recorded in contracting cultured chick myocytes and in working papillary muscle preparations.

Quantitative analysis of the relation between the [Ca²⁺], transients and the corresponding LV pressure was performed by use of the peak-to-peak values while [Ca²⁺] was varied between 0.25 and 16 mM, such as is shown in Figure 4C. As shown in Table 4, these data were fitted to the Hill equation, wherein

\[
P/P_{max} = \frac{[Ca^{2+}]^n}{K_{1/2}^n + [Ca^{2+}]^n}
\]

where \(P_{max}\) is the maximal developed pressure of LV, obtained while the heart was perfused with 16

<table>
<thead>
<tr>
<th>Number of</th>
<th>(P_{max}) (mm Hg)</th>
<th>(n)</th>
<th>(K_{1/2}) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>204±19</td>
<td>5.98±0.36</td>
<td>1.03±0.11</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD. \(P_{max}\), maximal developed pressure of left ventricle, obtained while heart was perfused with 16 mM Ca²⁺; \(K_{1/2}\), at which pressure development is one half of maximal value; \(n\), Hill coefficient.
Effects of Ischemia and Hypoxia

Mechanical changes during acute hypoxia (coronary perfusion by nitrogen-saturated solution, Po2<15 mm Hg)21) consistent with those previously reported22,24,30 were observed in our whole-heart preparations (n=11); namely, a combination of reduced LV systolic pressure development and increased LV diastolic pressure (Figure 5A, middle row). After 5 minutes of hypoxia, these changes returned rapidly to the control conditions when the ventricle was reoxygenated. The simultaneously recorded [Ca2+]i transients are shown in the top row of Figure 5A. Two distinct changes in [Ca2+]i were observed consistently: 1) an immediate but gradual increase in the diastolic [Ca2+]i level and 2) a gradual decrease in the peak [Ca2+]i level. These changes in [Ca2+]i were also reversed by reoxygenation. The LV pressure-[Ca2+]i relation at 3 and 5 minutes after the induction of hypoxia demonstrates that this relation lies close to the control curve (Figure 5B).

As documented previously,22,24 systolic pressure development was more rapidly and more severely depressed during ischemia, induced by total occlusion of perfusion line at the ascending aorta (Figure 6A, n=12), than during hypoxia. Moreover, in contrast with hypoxia, the LV diastolic pressure level decreased abruptly when the perfusion line was clamped, and no increase in the LV diastolic pressure was observed during the subsequent 3
These functional changes during ischemia were associated with a more remarkable rise in the diastolic [Ca\textsuperscript{2+}] level (top row) than that seen during hypoxia. The peak systolic [Ca\textsuperscript{2+}] level also increased with ischemia, in contrast with the observation during hypoxia. The increase in the level of resting [Ca\textsuperscript{2+}], reached a maximum level around 1 µM. The force versus [Ca\textsuperscript{2+}], relation after 1 and 3 minutes of ischemia in the preparation of Figure 6A is shown in Figure 6B. Both in systole and in diastole, ischemia clearly shifted the curve to the right.

For further study of the mechanisms of contractile failure during ischemia and hypoxia, a short period of ischemia was superimposed on the hypoxia condition (Figure 712). Previous exposure to hypoxic perfusion did not inhibit the rapid decline in the LV systolic and diastolic pressures during ischemia. Furthermore, reperfusion with hypoxic perfusate after ischemia resulted in improved systolic function. These functional changes were associated with the specific [Ca\textsuperscript{2+}] changes during ischemia and reperfusion shown in Figure 6A.

Figure 8 shows the changes in the time course of the [Ca\textsuperscript{2+}], transients and LV pressure traces that occurred, respectively, during hypoxia (panel A) and ischemia (panel B).

**Discussion**

Before results with this new approach can be interpreted, it is important to assess whether the intracellular Ca\textsuperscript{2+} indicator aequorin or the loading procedure we utilized alters the physiological properties of the heart. We have previously shown that our chemical approaches to cellular loading of aequorin, including the macroinjection technique applied in this study, do not affect the mechanical properties of ferret papillary muscles.9,15,16 Moreover, macroinjection produces Ca\textsuperscript{2+} signals that are qualitatively and quantitatively similar to those obtained with microinjection of aequorin directly into the intracellular space.9 In the present study, a similar lack of adverse effect was noted in application of our chemical-loading methodology to the saline-perfused whole heart.

As shown in Table 2, neither aequorin nor the loading procedure significantly modifies the mechanical characteristics of LV global function or the integrity of the coronary vasculature. The level of CPK leakage shown in Table 3 indicates that our
isolated perfused heart preparation demonstrates little evidence of deterioration over the time course of the experiments. Histological examination of the region loaded with aequorin did not show any evidence of structural damage. In addition, correlations of the aequorin signals with isovolumic LV pressure (Figure 4C) indicate that our system not only shows normal physiological characteristics in the basal state but also exhibits expected responses to standard interventions. The responses to inotropic interventions were similar to those we and others have reported in aequorin-loaded papillary muscles and trabeculae carneae from animals and man. Therefore, we concluded that our system could be used to monitor subcellular responses to more complex interventions such as hypoxia and ischemia.

In addition to the aequorin method reported in the present study, which is based on recording of a bioluminescence response, fluorescence and NMR recordings of [Ca\textsuperscript{2+}] have been reported. Several of the fluorescent Ca\textsuperscript{2+} indicators have been utilized in whole-heart studies including quin 2,\textsuperscript{11} indo-1,\textsuperscript{6-8} and fura-2,\textsuperscript{59} each of which responds to a change in [Ca\textsuperscript{2+}], by altering its pattern of fluorescence. The relative properties of bioluminescent and fluorescent Ca\textsuperscript{2+} indicators have been the topic of recent reviews.\textsuperscript{57,58} In contrast with early reports,\textsuperscript{11} recent studies suggest that depressed contractility is not a necessary consequence of this approach, at least with regard to indo-1\textsuperscript{18} and fura-2.\textsuperscript{56} The fluorescence approach has been used to monitor [Ca\textsuperscript{2+}], changes in the isolated perfused heart in response to inotropic agents\textsuperscript{7} and ischemia\textsuperscript{6,8} during simultaneous recording of LV pressure and, in one study, simultaneously with action potential recordings.\textsuperscript{6} Despite the utility of these fluorescent indicators, a major limitation has been the inability of accurate quantitation of [Ca\textsuperscript{2+}]. The NMR approaches to [Ca\textsuperscript{2+}], recordings have utilized the Ca\textsuperscript{2+} indicator 5F-BAPTA, which changes its NMR spectrum when it combines with Ca\textsuperscript{2+}.\textsuperscript{4,5,59} Although quantitation of [Ca\textsuperscript{2+}], has been reported with this technique, data acquisition requires averaging over many cardiac cycles; therefore, examination of beat-to-beat changes in [Ca\textsuperscript{2+}], is not possible with this technique. In addition, the amounts of 5F-BAPTA that must be loaded into myocytes to obtain detectable signals result in significant Ca\textsuperscript{2+} buffering and depression of contractile function.\textsuperscript{4,5} Nevertheless, this technique has been fruitfully applied to studies of myocardial ischemia\textsuperscript{4,59} and provides the potential for simultaneous measurement of other intracellular ions and metabolic substrates and products of importance, including intracellular pH, ATP, phosphocreatine, and inorganic phosphate (Pi).\textsuperscript{4,59} Some of the relative advantages and disadvantages of each of these approaches at their current level of development are listed in Table 5. It is clear that the use of multiple approaches for study of similar problems has the potential to provide complementary and supplementary findings, as discussed below for hypoxia and ischemia.

The results obtained during the present study with hypoxia and reoxygenation of the whole heart
were similar to those we previously reported in ferret papillary muscles.51 During acute hypoxia, parallel changes occur in [Ca\(^{2+}\)], and isovolumic LV pressure during both systole and diastole. These results are similar to the effects of "low-flow ischemia" reported in NMR studies\(^{59}\) or to the effects of "anoxic superfusion" observed in the indo-1-loaded isolated myocyte\(^{60}\) and support the hypothesis that [Ca\(^{2+}\)], plays a primary role in the mechanical changes that occur during hypoxia. The depression of light and tension was reversed by reoxygenation. Although Figure 5B shows that hypoxia may shift the pressure versus [Ca\(^{2+}\)] relation slightly to the right of the control curve,\(^{51}\) this shift is relatively modest compared with ischemia (see below). However, in hypoxia of longer duration, the shift in this relation could modulate the contractile response to [Ca\(^{2+}\)], more profoundly in a time-dependent fashion.\(^{61}\) As we observed previously in ferret papillary muscles,\(^{51}\) the prolongation of tension that occurred during reoxygenation cannot be attributed either to changes in the time course of the Ca\(^{2+}\) transient, which becomes abbreviated, or to a decreased Ca\(^{2+}\) sensitivity of the contractile apparatus, which would be expected to abbreviate systole (Figure 8A\(^{62,63}\)). This indicates that additional, at present unknown, mechanisms must be involved in the reoxygenation phenomenon.\(^{21}\) We do not have direct data that indicate the cause of the changes in [Ca\(^{2+}\)], during acute hypoxia and reoxygenation; however, changes in membrane potentials\(^{64}\) as well as rapid inhibition of Ca\(^{2+}\) reuptake by the sarcoplasmic reticulum\(^{65,66}\) and the subsequent redistribution of Ca\(^{2+}\) in the internal
During acute ischemia, the rapid and profound decreases in LV peak and diastolic pressures contrast with the increases in the peak and resting levels of [Ca\textsuperscript{2+}]. It is generally agreed that the very rapid decline of systolic and diastolic pressure of the LV is due to a mechanical factor (loss of erectile effect of coronary vasculature perfusion\textsuperscript{12,53,73}). Decreased stretch of the myofilaments, which occurs with the cessation of coronary perfusion,\textsuperscript{26} might modify the [Ca\textsuperscript{2+}]-force relation.\textsuperscript{77} The rightward shift of this relation observed 1 minute after the onset of ischemia (Figure 6B) could be explained by this mechanical theory; however, this mechanical factor would reach its steady state within 1 minute.\textsuperscript{53} In our experiments, the [Ca\textsuperscript{2+}]-force relation continued to shift further to the right after 1 minute, which is shown as points 3 minutes after the induction of ischemia. Similar [Ca\textsuperscript{2+}] and LV pressure responses have been reported with fluorescent and NMR approaches to whole-heart studies during early ischemia\textsuperscript{4,2} and after development of contracture.\textsuperscript{6} We interpret this to mean that in contrast with hypoxia, changes in the responsiveness of myofilaments to Ca\textsuperscript{2+} primarily determine the level of force generation in the ischemic condition. This occurs in part through reduction of sarcomere preload during the early seconds of ischemia, but predominantly through metabolic changes during the subsequent period. Study of the combined effects of hypoxia and ischemia (Figure 7) not only further supports the idea that the primary mechanisms during these two conditions are different but also suggests that flow-dependent accumulation of metabolites plays the major role during ischemia since reperfusion with hypoxic perfusate restored both the [Ca\textsuperscript{2+}] and the contractile function.

Alterations in cation homeostasis during and after myocardial ischemia may account for some of the changes in contractile function.\textsuperscript{78} It is important to note that the Mg\textsuperscript{2+} ion modulates the interaction of aequorin with Ca\textsuperscript{2+},\textsuperscript{20,28,30,32} In the present study, we assumed intracellular ionized magnesium ([Mg\textsuperscript{2+}]) under control conditions was 1 mM.\textsuperscript{37} During prolonged hypoxia or ischemia, [Mg\textsuperscript{2+}] would be expected to increase, since [Mg\textsuperscript{2+}] should rise inversely with the decrease in intracellular ATP ([ATP]).\textsuperscript{79} Since our calibration method assumes that [Mg\textsuperscript{2+}] remains constant (see "Materials and Methods"), this could interfere with our ability to detect changes in [Ca\textsuperscript{2+}]. To minimize these problems, we limited the duration of exposure to hypoxia and ischemia to short periods during which significant shifts in ATP concentrations would not be expected to occur.\textsuperscript{13}

Since an increase in intracellular inorganic phosphate ([P\textsubscript{i}]) decreases both the number of cross-bridges in the force-generating state and the actomyosin ATPase activity,\textsuperscript{80} it is conceivable that differences in the [P\textsubscript{i}] accumulation rate could be responsible for differences in force generation between ischemia and hypoxia. Accumulation of [P\textsubscript{i}] has been implicated as a contributor to contractile failure in hypoxia\textsuperscript{61}; even more rapid accumulation could occur in ischemia where metabolites cannot be removed by perfusion. Alternatively, a significant accumulation of intracellular ionized hydrogen ([H\textsuperscript{+}]), which has been documented in the early phase of ischemia but which is minimal with acute hypoxia,\textsuperscript{81,82} could be a cause of these changes.\textsuperscript{83} It is known that acidic pH inhibits Ca\textsuperscript{2+} binding to myofibrillar troponin C,\textsuperscript{84} thereby reducing actomyosin ATPase activation and force generation per unit [Ca\textsuperscript{2+}].\textsuperscript{85} An increase in resting [Ca\textsuperscript{2+}], has been observed during induction of acidosis in cardiac muscle.\textsuperscript{86} Of more interest, although the direct effect of acidosis on the sarcoplasmic reticulum is decrease of Ca\textsuperscript{2+} uptake and its subsequent release,\textsuperscript{85} in the presence of increased [Ca\textsuperscript{2+}], acidosis instead increases the magnitude of the [Ca\textsuperscript{2+}] transients and exacerbates the spontaneous oscillatory release of Ca\textsuperscript{2+}.\textsuperscript{87} With acidosis, therefore, the amount of sarcoplasmic reticulum Ca\textsuperscript{2+} release seems to be determined by the severity of Ca\textsuperscript{2+} redistribution in the cell. So far as we know, therefore, a substantial and rapid increase in [H\textsuperscript{+}], is the most reasonable explanation for the desensitization of

<table>
<thead>
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<th>Method</th>
<th>Depression of contractility*</th>
<th>Beat-to-beat recording of [Ca\textsuperscript{2+}]</th>
<th>Quantitation of [Ca\textsuperscript{2+}]</th>
<th>Simultaneous measurements of other [ions i]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear magnetic resonance (SF-BAPTA)</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>Fluorescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(quin2, indo-1, fura-2)</td>
<td>Yes/No†</td>
<td>Yes</td>
<td>Not†</td>
<td>No</td>
</tr>
<tr>
<td>Bioluminescence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(aequorin)</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

*In minimum necessary concentrations for recording of [Ca\textsuperscript{2+}].
†Yes for quin2, no for indo-1 and fura-2, steady-state levels only.
‡Theoretically possible.
the myofilaments observed in the acute phases of ischemia, in contrast with the findings in acute hypoxia.

In conclusion, we have described a new method that uses aequorin for recording of [Ca\(^{2+}\)], in the isolated perfused whole heart. Our approach should be useful in the study of the effects of pathophysiological states and pharmacological agents on LV contractile function. The results of studies with ischemia and hypoxia show that different primary mechanisms determine the mechanical responses in these two conditions. During hypoxia changes in [Ca\(^{2+}\)] handling probably play a major role, while during ischemia changes in the Ca\(^{2+}\) sensitivity of the contractile elements appear to be of primary importance in modulation of contractile function.

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Aequorin was purchased from the laboratory of Dr. J.R. Blinks, Mayo Clinic, Rochester, Minnesota.

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*KEY WORDS*: calcium • aequorin • ischemia • reperfusion • hypoxia
Direct measurement of changes in intracellular calcium transients during hypoxia, ischemia, and reperfusion of the intact mammalian heart.
Y Kihara, W Grossman and J P Morgan

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