Fluorescence measurement of calcium transients in perfused rabbit heart using rhod 2

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Fluorescence measurement of calcium transients in perfused rabbit heart using rhod 2. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H728–H741, 1998.—Surface fluorescence spectroscopy of the beating heart to measure cytosolic calcium has been limited by the need to use ultraviolet excitation light for many of the commonly used calcium indicators. Ultraviolet light in the heart produces a high level of background fluorescence and is highly absorbed, limiting tissue penetration. Visible wavelength fluorescence dyes such as rhod 2 are available; however, the lack of spectral shift with calcium binding precludes the use of ratio techniques to account for changes in cytosolic dye concentration. We have developed a method for in vivo quantitation of cytosolic rhod 2 concentration that in conjunction with calcium-dependent fluorescence measurements permits estimation of cytosolic calcium levels in perfused rabbit hearts. Reflective absorbance of excitation light by rhod 2 loaded into myocardium was used as an index of dye concentration and the ratio of fluorescence intensity to absorbance as a measure of cytosolic calcium concentration. Endothelial cell loading of rhod 2 was found to be minimal (<5%), and dye leak rate out of the cytosol was slow, with ~5% loss of dye fluorescence occurring between 10 and 30 min after dye loading. Rhod 2 loading into subcellular compartments, determined by manganese quenching, was also minimal (<5%). The dissociation constant of rhod 2 for calcium was measured in vitro to be 500 nM, and this value increased to 710 nM in the presence of 0.5 mM myoglobin. On the basis of this value and in vivo fluorescence measurements, cytosolic calcium concentration in the rabbit heart was found to be 229 ± 90 nM at end diastole and 930 ± 130 nM at peak systole, with peak fluorescence preceding peak ventricular pressure by ~40 ms. This technique should facilitate detailed analysis of calcium transients from the whole heart.

cytosolic calcium; reflectance spectroscopy; surface fluorometry; calcium indicators

Fluorometry of biological tissues to measure cytosolic calcium (Ca$_i$) has been greatly facilitated by the introduction of fluorescent dyes with greater quantum efficiency and by the development of simpler techniques for introducing the dyes into the cytosol (15). The higher quantum efficiency has permitted studies of Ca$_i$ with relatively low intracellular dye concentrations so that there is little if any calcium-buffering effects. Quantitation of free Ca$_i$ concentrations has also been facilitated by the fact that many of these dyes exhibit either an excitation or an emission spectral shift when binding calcium. Thus Ca$_i$ can be estimated independent of intracellular dye concentration using ratio techniques (29).

Whole tissue surface fluorometry to measure Ca$_i$ has been substantially more difficult than single cell because several additional factors can affect fluorescence measurements that are independent of calcium concentration. This is particularly true in the heart. Some of these factors include the following: 1) inner filter effects caused by the tissue that can change depending on the physiological status of the heart, particularly when using excitation light in the ultraviolet region (14, 20); 2) changes in excitation light or detection efficiency due to scattering or motion associated with the contractile action of the heart (motion artifact) (20); 3) the heterogeneous nature of heart tissue can lead to loading of dye into cells other than myocytes, such as endothelial or smooth muscle cells (26); and 4) background fluorescence from endogenous fluorophores such as pyridine nucleotides can be large and can change depending on the physiological status of the heart.

The two most commonly used calcium-sensitive fluorescent dyes, fura 2 and indo 1, require excitation light in the ultraviolet range (340–380 nm), and particularly with indo 1, endothelial cell loading can account for as much as 30% of the detected surface fluorescence (25). Various techniques have been developed to minimize the other potential problems with these dyes in whole organ fluorometry, including the use of isosbestic wavelength for the heart for fluorescence detection to correct for oxygenation-dependent changes in tissue filtration (14, 20), immobilization of the heart and/or use of a calcium-insensitive fluorescence reference to suppress motion artifact (20), and hypothermic (30°C) perfusion with or without anion transport inhibitors to decrease the rate of dye leakage out of the cell (7, 29). Even with these measures, limited tissue penetration of the ultraviolet excitation light restricts calcium-
sensitive fluorescence detection to the outermost surface layer of the heart.

Recently, calcium-sensitive fluorescent dyes that absorb light substantially farther into the visible range have been developed. These fluorescent dyes, including Calcium Green and rhod 2 (12, 38, 39), have a substantial advantage over indo 1 and fura 2 in that the excitation light required is at visible wavelengths between 500 and 600 nm, where filtering of excitation or emitted light by the tissue and autofluorescence is minimal. The changes in fluorescence upon calcium binding are high. For example, there is a 100-fold increase in fluorescence when rhod 2 goes from its calcium-free form to its calcium-bound form. Therefore, the fluorescence signal-to-autofluorescence ratio can be very favorable. The one disadvantage of these dyes, however, is the distinct lack of a significant spectral shift in either excitation or fluorescence spectra with calcium binding. This feature precludes the use of ratio techniques as they are usually performed. To solve this problem, we have developed a method for in vivo quantitation of cytosolic rhod 2 concentration in conjunction with calcium-dependent fluorescence measurements which permits the estimation of [Ca] levels using the dissociation constant (Kd) of rhod 2 measured in vitro. The idea is to measure the absorbance of the dye along with the fluorescence. In the case of rhod 2, the absorbance spectrum is independent of calcium concentration, whereas the fluorescence emission increases with increasing calcium. Therefore, the rhod 2 fluorescence quantum efficiency changes upon binding calcium. Determination of fluorescence and absorbance from the heart is a measure of quantum efficiency. The ratio of fluorescence to absorbance should account for changes in dye concentration. Whole tissue reflectance spectroscopy was used as a measure of the amount of dye loading in the cytosol, and also to minimize motion artifact. Isosbestic wavelengths for the heart were used for both excitation and emission detection to minimize any changes in tissue filtration due to oxygenation state.

The purpose of this study was to evaluate this method of whole tissue surface spectroscopy using the calcium-sensitive dye rhod 2 and to detect beat-to-beat Ca transients in an isolated rabbit heart preparation during inotropic manipulation of the contractile state.

MATERIALS AND METHODS

Isolated perfused heart preparation. Hearts from adult (1–2yr old) New Zealand White rabbits (heart weight 6–7g) were obtained after the animals had been anesthetized with ketamine (50mg/kg) and given heparin sodium (200 U/kg) intravenously or intraperitoneally. Once the hearts were excised, they were placed in a 10°C perfusate bath, and the aorta was cannulated for coronary artery perfusion with a 1.5-mm metal cannula. Hearts were then perfused with Krebs-Ringer solution at 37°C, pH 7.4, containing (in mM) 115 NaCl, 1.25 CaCl2, 4.7 KCl, 11.0 glucose, 1.8 MgCl2, 1.8 KHPO4, 26 NaHCO3, 10 U/l regular insulin; the solution was gassed with 95% O2–5% CO2. Hearts were perfused via the aortic cannula in a retrograde manner for a 30-min stabilization period at an aortic root pressure of 80 mmHg. A latex balloon was inserted into the left ventricular cavity and connected to a catheter-tipped pressure transducer (Millar). The mitral valve was incised to render it incompetent, and a balloon flange was fixed to the valve to prevent herniation or extrusion. Developed pressure was measured and acquired simultaneously with the fluorescence measurements to permit comparisons of phase relationships between these two parameters. The amplified signal from the pressure transducer was connected to an auxiliary port on the SLM 8000 fluorescence spectrometer (SLM Aminco, Springfield, IL), digitized at 1,000 Hz, and stored on magnetic disks using the SLM data acquisition software.

Rhod 2 loading. Rhod 2 was loaded into cells by perfusing the heart at 37°C, with the cell-permeant acetoxymethyl ester (AM) form (Molecular Probes, Eugene, OR) dissolved in dimethyl sulfoxide (DMSO) in a recirculating manner for 5 min followed by a washout period to eliminate any hydrolyzed dye. One milligram of rhod 2 dissolved in 1 ml anhydrous DMSO was used as a stock solution for dye loading, and aliquots of dye (0.3–1 mg) were added to 100 ml of perfusate to make up the recirculating circuit. This procedure led to rhod 2 fluorescence that was 10- to 15-fold higher than background. Similar loading was observed with rhod 2 dissolved in ethanol. In later experiments, the aliquot of dye was infused over 15 min without recirculation, and an equivalent result was obtained. Developed pressure was monitored during the loading and washout period. Typically, there was a 30–50% decline in developed pressure during rhod 2 loading, followed by complete recovery within 5 min of switching off the recirculating circuit with all the dye concentrations used, except for the highest dose tested (1 mg rhod 2), where there was 80–90% recovery after 5 min.

Internal fluorescence standard loading. To quantify the effects of contractile motion (motion artifact) on the fluorescence emission signal and determine the phase relationship between motion artifact and the intracavitary pressure measurements, an internal fluorescence standard was used in a separate group of hearts (20). 5(6)-Carboxy-2′,7′-dichlorofluorescein (CICF; Molecular Probes), a fluorescent dye whose fluorescence does not change under physiological conditions, was loaded into hearts using the cell-permeant form with a recirculating circuit in a similar method as that of rhod 2 loading. After a 15-min washout period, fluorescence and reflectance measurements were made using the same excitation and emission wavelengths as with rhod 2. The same perfusion protocol used in the rhod 2 experiments was also used for the internal standard studies.

Spectroscopic measurements: optical perfusion chamber. For all spectroscopic measurements, the hearts were perfused in a fluid-sealed chamber specially designed to 1) minimize movement artifacts, 2) abate the curvature of the epicardium, and 3) maintain physiological conditions (13, 34). The chamber was built in house (at the machine shop of the University of Pittsburgh, Department of Cell Biology and Physiology); it consisted of a front frame equipped with a 1-mm-thick optical window (sapphire, Oriel, Stratford, CT) that made contact with the left ventricular free wall (34). The perfusion chamber was equipped with ports for a perfusion cannula, an intracavitary balloon, and pacing wires. Small plungers that fix the heart in three directions against the optical window minimized heart motion. NADH surface fluorescence to detect ischemia in hearts fixed inside the chamber in this manner was done. Immobilization of the heart against the optical window, as described, yielded no detectable change in NADH-dependent fluorescence emission (467 nm) intensity with 340-nm excitation light, indicating that the left ventricular surface was not made ischemic by this maneuver.
The excitation light beam was focused on the surface of the heart by a biconvex lens to provide a 5-mm excitation spot. A large beam of excitation light (5 mm) was used to permit integration over a large surface area of the heart. The emission fluorescence light was collected with a biconvex lens positioned at 90° to the incident excitation light beam. The emission wavelength was selected by a motorized monochromator and detected by a cooled photomultiplier tube (Fig. 1A, PMT-A) (Aminco SLM 8000).

Reflected excitation light was collected by a third biconvex lens focused onto a flexible liquid light guide (Oriel) coupled to a second photomultiplier tube (PMT-C). The light guide and focusing lens were positioned at 45° on a vertical plane, from the optical window and plane of incident excitation light (see Fig. 1B). A vertical plane was chosen to minimize the signal from the light reflected by the air-optical window and window-tissue interface (spectral reflection) and therefore increase the relative signal from the excitation light backscattered by the heart tissue itself. This backscatter reflectance signal was used to quantify dye loading into the myocardium and to correct for the effects of motion. Similarly, the perfusion chamber with optical window was positioned with a 30° angle of incidence (horizontal plane) to the excitation beam (see Fig. 1A). This angle for the perfusion chamber minimized the reflected excitation light from the air-window and window-tissue interfaces that was seen by the fluorescence emission detector (PMT-C). We have found that positioning of the perfusion chamber and light collectors to minimize the optical window spectral reflectivity was important for obtaining adequate fluorescence and tissue back-scatter signals.

Fluorescence measurements. After dye loading, a washout period of nonrecirculating perfusion was done for up to 1 h to establish the time required to achieve relative steady state for unhydrolyzed dye washout and to determine the rate of dye leak out of the tissue. Excitation light at 524 nm with emission light at 589 nm was used for the fluorescence measurements. Although maximal light absorption by rhod 2 is at 554 nm, these two wavelengths were chosen for the tissue fluorescence measurements since they have been shown to be isosbestic for heart absorbance and therefore tissue filtration effects due to changes in oxygenation state are minimized (14). Instantaneous ratio of the fluorescence emission (589 nm, PMT-A) over the reflected excitation light (524 nm, PMT-C) was done electronically in an effort to correct the fluorescence signal for any changes due to heart motion. The fluorescence emission and reflectance signals were averaged over 40 ms, digitized, and stored continuously for periods up to 40 s. Averaging the fluorescence signal for 40 ms was done to optimize signal-to-dark noise ratio. With an average heart rate of 2 Hz, the 40-ms acquisition rate provided ~12 data points during a single cardiac cycle. Averaging the fluorescence signal for shorter periods (10–30 ms) can be done to obtain more data points during the cardiac cycle with a lower signal-to-dark noise ratio. The background photon count obtained from the heart before dye loading was subtracted from the dye-loaded fluorescence measurements. Calibration of the maximum fluorescence emission signal was done by perfusing the heart with 10 µM A-23187 or 10 µM digitonin.

Reflectance measurements. Because rhod 2 has no spectral shift when it binds calcium, to quantify calcium, we must account for changes in the dye concentration in the tissue. To do this in the heart, back-scattered excitation light was collected and measured by a separate PMT (PMT-C) as described above (see Fig. 1). Relative dye concentration in the heart tissue was determined by measuring the relative change in reflected light at two wavelengths (524 and 589 nm, both isosbestic for the heart), before and after dye loading. The excitation light wavelength was scanned from 500 to 600 nm (1 nm/s), and the reflected light was collected by PMT-C. The 524-nm excitation light was chosen because rhod 2 absorbs light effectively at this wavelength, and 589 nm was chosen as a reference wavelength because rhod 2 does not absorb light in that region and reflectance at 589 nm is not affected by the oxygenation state of the heart. The ratio of reflected light at 524 nm to 589 nm was used as an index of dye concentration in the heart tissue. Calibration of the reflectance signal was done by subtracting the reflectance spectra of the heart without rhod 2 from the spectra of the rhod 2-loaded heart.

Dye absorbance in whole heart ($A_{\text{rhod 2}}$) was calculated using the formula

$$A_{\text{rhod 2}} = \log \left( \frac{I_{524} I_{589 \text{o}}}{I_{524} I_{589 \text{rhod 2}}} \right)$$

where $I_{524}$ and $I_{589}$ is the reflectance intensity at 524 and 589 nm excitation of the heart, respectively, before (subscript o) and after (subscript rhod 2) dye loading. Reflectance spectra were obtained every 5 min after dye loading, and additional spectra were obtained between interventions described in the perfusion protocol. In a separate group of hearts, tissue
reflectance spectra were obtained every 5 min in hearts perfused with buffer alone, without dye, to determine the effects of the perfusion protocol on the native reflectance characteristics of the heart tissue.

Calibration of the dye. To calculate the in vitro $K_d$ for rhod 2 and calcium, fluorescence measurements of standard concentrations of calcium were made using the SLM 8000 spectrometer. Eleven samples of varying calcium concentration (0–39.8 µM) and containing rhod 2 (10 µM) were prepared from two stock solutions of known calcium concentration (calcium calibration buffer kit, Molecular Probes) using the method of Tsien and Pozzan (37). A second set of identical samples, but with the addition of 0.5 mM myoglobin (Sigma, St. Louis, MO), was prepared, and fluorescence measurements were obtained. Myoglobin was added to mimic some of the absorbance characteristics of myocardium and the effects of proteins on the affinity of the dye for calcium. Fluorescence emission was collected from 540 to 600 nm, exciting at 524 nm.

Rhod 2 absorbance measurements were also performed in the SLM 8000 spectrometer by scanning excitation light from 500 to 600 nm across a 3-mm quartz cuvette containing 10 µM rhod 2 with and without calcium. The transmitted light was collected directly across the cuvette by a biconvex lens focused on a liquid light guide and detected by PMT-C. These spectra were then compared with spectra obtained by reflectance measurements from the surface of the heart tissue.

Calibration of the fluorescence emission signal from hearts was done by obtaining fully calcium-bound fluorescence by perfusing with 10 µM A-23187 or 10 µM digitonin. Fluorescence minimum was assumed to be equal to background based on the very low fluorescence of calcium-free rhod 2 observed in the in vitro experiments.

Calculation of $C_{ai}$ concentration in whole heart. $C_{ai}$ was calculated using the following equation (22)

$$
C_{ai} = K_d(F_t - F_o)/(F_{max} - F_t)
$$

where $K_d$ is the dissociation coefficient for rhod 2 and calcium (obtained from in vitro calibration with rhod 2 + myoglobin) and is equal to 710 nM, $F_t$ is the fluorescence of the rhod 2-loaded heart at time $t$, $F_{max}$ is maximum fluorescence of the rhod 2-loaded heart in the presence of 10 µM ionomycin or digitonin, and $F_o = F_b + a(F_{max} - F_b)$, where $F_b$ is the background counts from the heart before dye loading and $a$ is rhod 2 fluorescence in the absence of calcium/rhod 2 fluorescence in the absence saturating calcium. For rhod 2, the value of $a$ is approximately zero (see Fig. 2); thus for rhod 2, $F_o$ was assumed to be equal to $F_b$.

To account for changes in dye concentration during fluorescence measurements, Eq. 2 was modified to include changes in absorbance due to dye leakage, $A_{rhod_2}$ (see Eq. 1). Thus Eq. 2 becomes

$$
C_{ai} = K_d \times [(F_t - F_o)/A_t]/[(F_{max} - F_o)/A_{max}]
$$

(3)

where $A_t$ is the dye absorbance, $A_{rhod_2}$, in the dye-loaded heart at time $t$, and $A_{max}$ is the dye absorbance, $A_{rhod_2}$, in the heart measured just before ionomycin or digitonin administration. Equation 3 can be further simplified to yield

$$
C_{ai} = K_d \times (F_t - F_o)/(A_t/A_{max})(F_{max} - F_o) - (F_t - F_o)
$$

(4)

Determination of dye loading into subcellular organelles. To determine the extent of uptake of rhod 2 by subcellular organelles, quenching of dye fluorescence by manganese was used (28). Hearts loaded with rhod 2 were perfused with Krebs buffer containing 100 µM MnCl$_2$ for 30 min. The ionophore A-23187 (10 µM) was then added to the perfusate containing manganese (200 µM), and perfusion was continued for an additional 30 min. To determine whether dye was still present in the myocardium, a bolus of CaCl$_2$ (10% solution) containing A-23187 was then infused into the heart, and perfusion was stopped. In an additional three hearts, ruthenium red (24 µM) was added to the perfusate for 15 min before the manganese infusion and was continued throughout the ionophore and calcium infusions. Dye-dependent fluorescence and left ventricular pressure were recorded simultaneously during the entire infusion period in both groups of hearts.
The extent of fluorescence quenching of rhod 2 by manganese was determined in vitro in a cuvette containing a solution of 10 μM rhod 2 (free acid), 150 mM KCl, 30 mM NaCl, 1 mM MgSO₄, and 48 mM CaCl₂ at 37°C. Manganese concentrations were varied from 0 to 15 mM, maintaining the same final volume of 3 ml.

Distribution of rhod 2 in myocytes. Guinea pigs of either sex (250–450 g) were injected with heparin sodium (1,000 U ip) and Nembutal (300 mg/kg). Once the animals were anesthetized, the heart was removed and perfused with oxygenated N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-buffered saline solution (HBSS) containing (in mM) 135 NaCl, 1 MgCl₂, 10 HEPES, 4 KCl, and 0 CaCl₂ at 37°C. Manganese was determined in vitro in a cuvette containing 10 µM rhod 2 (free acid), 150 mM KCl, 30 mM NaCl, 1 mM MgSO₄, and 48 mM CaCl₂ at 37°C. Manganese concentrations were varied from 0 to 15 mM, maintaining the same final volume of 3 ml.

The in vitro absorbance spectra for rhod 2 in an aqueous solution is shown in Fig. 4 in the presence of saturating calcium (1 mM) and myoglobin (0.5 mM). We measured the effects of calcium concentration on absorbance of rhod 2 and found that there was no change in the absorbance spectra of the dye in the presence or absence of calcium. However, the addition of 0.5 mM myoglobin to the cuvette did affect the spectra significantly, particularly in the region of 500–550 nm, where myoglobin absorption is greater. Spectrum A in Fig. 4 is the spectrum C in Fig. 3 is the spectrum obtained from the rhod 2-loaded heart. Excitation was at 524 nm. The results are shown in Fig. 2A. The presence of myoglobin resulted in a small red shift in fluorescence, and the Kₐ for rhod 2 and calcium was calculated to be 710 nM. This latter value was used to quantitate calcium in the heart.

The in vitro absorbance spectra for rhod 2 in an aqueous solution is shown in Fig. 4 in the presence of saturating calcium (1 mM) and myoglobin (0.5 mM). We measured the effects of calcium concentration on absorbance of rhod 2 and found that there was no change in the absorbance spectra of the dye in the presence or absence of calcium. However, the addition of 0.5 mM myoglobin to the cuvette did affect the spectra significantly, particularly in the region of 500–550 nm, where myoglobin absorption is greater. Spectrum A in Fig. 4 is the spectrum obtained from the rhod 2-loaded heart. Excitation was at 524 nm.
the absorption spectrum of the dye plus myoglobin solution (spectrum B), however, we were able to obtain an absorption spectrum that was identical to that of dye alone (spectrum C). This is evidence that the myoglobin effects on the fluorescence and absorbance spectra are due to an inner filter effect.

To determine the effects of wavelength on tissue penetration in the heart, the optical density (OD) of a perfused rabbit right ventricular free wall (~2 mm thick) was measured. At 340 and 380 nm (i.e., indo 1 and fura 2 excitation), the OD was found to be 3.86 and 3.92, respectively. At 480 nm, the OD was 3.2. At the rhod 2 excitation wavelength (520 nm), OD was 3.0, and at 585 nm, OD was 2.8.

Reflectance spectra obtained from the heart with and without rhod 2 were obtained by scanning excitation wavelength and collecting the back-scattered light from the tissue (Fig. 5, top). Corrected reflective absorbance of light by rhod 2 loaded into a heart is shown in Fig. 5, bottom (spectrum B). This spectrum was obtained by plotting log I_o/I_{rhod 2}, where I_o is the reflectance spectrum of the heart without dye and I_{rhod 2} is the spectrum of the same heart after dye loading and 15 min of dye washout. The absorption spectrum of rhod 2 alone in a cuvette is superimposed for purposes of comparison (Fig. 5, bottom, spectrum A). Note that in the heart, there is a red shift in peak dye absorption and increased absorption at 520 nm when compared with dye in a cuvette. We did not observe any further changes in the in vivo absorbance spectra (I_o/I_{rhod 2}) during the entire washout or perfusion protocol period.

The effects of the washout period on rhod 2 fluorescence, absorbance, and fluorescence/absorbance in a whole heart are shown in Fig. 6. Fluorescence emission (F) was obtained by exciting at 524 nm and detecting emission at 589 nm. Corrected reflective absorbance (dye absorbance, A) was calculated by obtaining the ratio of reflected light at 524 nm (dye sensitive) over 589 nm (dye insensitive) before and after dye loading. Figure 6 shows A_{rhod 2} (see Eq. 1) plotted as a function of washout time after initial dye loading (note that the scale shown is truncated). The rate of decline of dye-dependent absorbance reached a steady rate by 10–15 min of dye-free Krebs-Ringer perfusion of the heart. Fluorescence intensity declined in a parallel manner to absorbance during the washout period. From 10 min after loading to 30 min, there was a 20% drop in fluorescence and a 12% drop in absorbance. Calculating the ratio of fluorescence over absorbance (F/A), we observed approximately a 5% change in this value from 10 min after dye loading to 30 min. On the basis of these observations, interventions aimed at altering Ca2+, described in the perfusion protocol, were started after 15 min of dye washout.

To determine optimal dye-loading concentration techniques for the whole heart, experiments were done loading rhod 2-AM into isolated nonworking rabbit hearts (n = 3) using either a 150-ml recirculating circuit containing buffered perfusate with dye or the esterified form of the dye was dissolved in DMSO and...
injected as a bolus over 1 min directly into the perfusion tubing without recirculation. We found no difference between the two dye-loading techniques with respect to decline in developed pressure during loading or recovery after 5 min of washout. There was also no difference in the increase of fluorescence (10–15 times autofluorescence) or the morphology of the calcium transients with equivalent dye loads. To further ascertain the optimal dose of esterified dye, incremental boluses of rhod 2-AM were administered to the heart ranging from 0.3 to 1 mg, measuring the increase in fluorescence vs. dye-dependent absorption as well as recovery of contractile function after 15 min of dye washout. We found that between 0.3 and 0.7 mg of esterified dye bolus, there was a linear relationship between the rise in fluorescence and absorption, A_rhod 2 (data not shown). However, when loading the heart with larger doses of dye (>0.7 mg), developed pressure did not recover with washout to preloading levels, and the relationship between fluorescence and absorbance was no longer linear. This change could be due to a change in Ca from buffering by rhod 2, loading of subcellular compartments in the myocytes with the larger dye bolus, or the dye concentration reached a level where Beer’s law no longer holds. Therefore, a loading dose of 0.5–0.7 mg dye ester per rabbit heart (6–7 g) was used for the perfusion protocol experiments.

To quantify the effects of contractile motion (motion artifact), the fluorescence emission signal was acquired over several cardiac cycles from hearts loaded with the internal fluorescence standard CI6F (Fig. 7). Surface fluorescence signal (top) was superimposed on the simultaneous left ventricular pressure signal obtained from the intracavitary balloon (bottom). The effects of immobilization of the heart by the perfusion chamber are demonstrated when the heart was allowed to rotate free and away from the optical window (Fig. 7A) and lightly pressed against the window (Fig. 7B). Note that with the heart moving freely, fluorescence changes are gradual, and peak fluorescence occurs exactly at mid-diastole (180° out of phase) with intracavitary pressure. With the heart immobilized against the optical window, there was a marked decrease in the amplitude of the fluorescence signal during the cardiac cycle from 12 to 4% of total fluorescence, and these fluctuations became random with no obvious phase correlation with the left ventricular pressure signal.

Fluorescence quenching of rhod 2 by manganese measured in vitro is shown in a representative titration experiment (Fig. 8C). Increasing concentrations of manganese dropped fluorescence to 23 ± 3% (n = 4) of the intensity obtained from rhod 2 in the presence of CaCl2. Manganese was therefore unable to completely quench rhod 2 fluorescence in vitro. In the heart, the extent of rhod 2 uptake by subcellular organelles was determined by manganese infusion (100 µM) followed by the addition of the ionophore A-23187. Within 5 min of the start of the MnCl2 infusion, fluorescence from the heart fell to <50% of the premanganese fluorescence, and the

Fig. 6. Effects of dye washout with Krebs buffer on reflective absorbance and fluorescence emission in a heart given a bolus with rhod 2-AM (0.5 mg) at time 0. F, fluorescence emission (excitation, 524 nm; emission, 589 nm); A, reflective absorbance (reflected light at 524 nm/589 nm before and after dye loading); F/A, ratio of fluorescence intensity over reflective absorbance obtained at same time point. (All values expressed as percentage of value obtained 3 min after dye bolus. Note that scale is truncated.)

Fig. 7. Fluorescence emission (top trace) and left ventricular pressure (bottom trace) obtained simultaneously from hearts loaded with internal fluorescence standard CI6F in the presence of CaCl2. Manganese was therefore unable to completely quench rhod 2 fluorescence in vitro. In the heart, the extent of rhod 2 uptake by subcellular organelles was determined by manganese infusion (100 µM) followed by the addition of the ionophore A-23187. Within 5 min of the start of the MnCl2 infusion, fluorescence from the heart fell to <50% of the premanganese fluorescence, and the
amplitude of the fluorescence transient decreased to the levels seen before loading rhod 2, i.e., autofluorescence (Fig. 8A). Simultaneous left ventricular pressure remained essentially unchanged during the rapid fall in fluorescence, indicating that the manganese infusion had little effect on cardiac function (Fig. 8B). By 30 min of manganese infusion, fluorescence had fallen to $36 \pm 3\%$ ($n = 3$) of premanganese fluorescence. The addition of 10 µM A-23187 to the perfusate containing 200 µM MnCl$_2$ had no significant effect on fluorescence emission from the heart ($35 \pm 2\%$ of premanganese fluorescence). The bolus of CaCl$_2$ (10% solution) given after 30 min of infusion with manganese and the ionophore caused a threefold rise in fluorescence from the heart, indicating that rhod 2 was still present in the myocardium (data not shown). Ruthenium red, due to its absorption of excitation light, caused a significant drop in rhod 2 fluorescence before manganese infusion but had no further effects on the relative change in fluorescence with manganese or A-23187.

To further address the possibility that calcium transients measured with rhod 2-loaded hearts might originate from the sum of mitochondrial and cytosolic Ca$^{2+}$ transients occurring on a beat-to-beat basis, isolated myocytes loaded with rhod 2-AM or TMRE were imaged by confocal microscopy, and the distribution of fluorescence was compared (Fig. 9). Confocal images of dye distribution in myocytes showed that rhod 2 (loaded at either 23 or 37°C) is primarily located in the cytosol, and the distribution of rhod 2 fluorescence is different from that produced by the mitochondrial-specific dye TMRE.

Figure 9, top panel, illustrates the fluorescence of a myocyte bathed with TMRE in the absence (left) and presence of FCCP (right). TMRE rapidly accumulated in the mitochondria (10–15 min) and exhibited the typical punctate pattern associated with mitochondria aligned in myocytes below the surface membrane (8). The collapse of transmembrane potential ($\Delta\psi$) resulted in a dramatic decrease in TMRE fluorescence ($n = 8$) as previously reported by Loew et al. (23). Figure 9, middle, illustrates the fluorescence images obtained from myocytes loaded with rhod 2 at 23°C. Rhod 2-loaded myocytes produced a very different dye distribution (Fig. 9, middle) as the punctate pattern of mitochondria was not apparent. The subsequent addition of FCCP had no effect on rhod 2 fluorescence ($n = 8$).

To exclude the possibility that some rhod 2 diffused into mitochondria and the high fluorescence intensity of the dye in the cytosol masked the presence of dye in the mitochondria, rhod 2-loaded myocytes were exposed to low concentrations of the detergent digitonin. As illustrated in Fig. 9, bottom, rhod 2 loading again did not produce the punctate appearance typical of mitochondrial stains (next to last bottom panel). The extracellular free calcium concentration was reduced from 1 mM to 2 µM using calcium-ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N' -tetraacetic acid-buffered solutions to ensure that the mitochondria would not be...
exposed to high calcium levels which might collapse the mitochondrial potential. Digitonin (20 µM) was then added to permeabilize the surface membrane (but not the mitochondrial membrane). As expected, rhod 2 trapped in the cytosol diffused out of the myocyte, and there was no detectable level of rhod 2 after digitonin treatment (n = 6). Furthermore, the cytosol and, by inference, the mitochondria contained high levels of calcium (2 µM) such that any rhod 2 trapped in the mitochondria and nondiffusible rhod 2 in the cytosol would fluoresce strongly and would be readily detected as in the control panels.

Bradykinin has been shown to raise Ca$^2+$ specifically in endothelial cells and not in myocytes in whole heart.
preparations (25, 26). To determine the degree of endothelial cell loading of rhod 2, the effects of a bradykinin bolus (0.1 nmol) on left ventricular pressure and rhod 2 fluorescence were measured. The change in mean fluorescence was 2.1% with bradykinin (Table 1 and Fig. 10A). In contrast, the 0.25-mg calcium chloride bolus caused a marked rise in systolic pressure (40–50 mmHg) and fluorescence (Table 1 and Fig. 10B). This rise was followed by a gradual decline with ventricular pressure recovering before the fluorescence. The effects of 10 µM digitonin at the end of the perfusion protocol are shown in Fig. 10C. Immediate contracture of the heart was seen with an approximately fivefold rise in fluorescence. Similar fluorescence rise was seen in hearts infused with the calcium ionophore A-23187. A plateau in the fluorescence was seen in most hearts after 30 s, and this value was used as the peak fluorescence for the calibration calculations.

With the use of the fluorescence maxima value obtained with A-23187, Ca2+ concentration was determined in a group of hearts loaded with rhod 2. Diastolic Ca2+ was calculated to be 229 ± 90 nM and systolic level of 930 ± 130 nM (n = 4). This value was obtained using fluorescence and absorbance measurements made 15 min after dye loading. Figure 11 shows a calibrated calcium fluorescence signal obtained from a beating heart loaded with rhod 2 (A) with the corresponding intracavitary pressure signal (B). Note the rapid up-stroke of the calcium-dependent fluorescence during systole with a gradual decline or shoulder during diastole. Peak fluorescence preceded peak-developed pressure by ~40 ms.

DISCUSSION

In this investigation, we present a novel method for measuring Ca2+ in an isolated buffer-perfused heart preparation, using the calcium-sensitive dye rhod 2. By combining measurements of fluorescence emission corrected for dye leak by absorbance measurements, we were able to estimate Ca2+ levels that vary between

| Table 1. Rhod 2 fluorescence from the heart: effects of bradykinin and calcium |
|---------------------------------|-----------------|-----------------|
|                                 | Before Intervention | During Intervention |
| Rhod 2 fluorescence, %          |                   |                   |
| Mean                            | 100              | 103 ± 2.1        |
| Systolic                        | 111 ± 0.93       | 115 ± 3.6        |
| Diastolic                       | 89.5 ± 0.93      | 91.6 ± 3.5       |
| Pressure, mmHg                  |                   |                   |
| Systolic                        | 78.7 ± 2.4       | 74.0 ± 3.5       |
| Diastolic                       | 0                | 0                |
| Calcium chloride (0.25 mg bolus)|                   |                   |
| Rhod 2 fluorescence, %          |                   |                   |
| Mean                            | 100              | 135 ± 17*        |
| Systolic                        | 112 ± 1.5        | 145 ± 17*        |
| Diastolic                       | 83.1 ± 7         | 122 ± 13*        |
| Pressure, mmHg                  |                   |                   |
| Systolic                        | 89.7 ± 6.7       | 168 ± 17*        |
| Diastolic                       | 4.3 ± 4.3        | 6.0 ± 3.5        |

Values are means ± SE of 3 experiments per group. Rhod 2 fluorescence values are expressed as percentage of preintervention mean value. *P < 0.05 vs. before intervention.
et al. (29) using indo 1 (315 nm) at peak systole, with values reported by Mohabir et al. (19); however, they correlate less well, particularly (90 and 930 nM) during the cardiac cycle in the isolated Krebs buffer-perfused rabbit heart. It is important to note that these values were calculated using the background fluorescence from the heart before dye loading as the minimum fluorescence (F₀ in Eq. 4) instead of the emission intensity measured from the heart after manganese quenching. This is based on our finding that manganese was only able to quench 77% of rhod 2 fluorescence in vitro and 65% of the fluorescence in vivo (Fig. 8). Our calculated values correlate well with measurements of Cai of whole rat heart with a whole heart preparation where motion during systole, with values reported by Mohabir et al. (29) using indo 1 (315 ± 25 to 609 ± 29 nM).

Rhod 2 absorbs visible wavelength light in the range where tissue absorbance and autofluorescence are low (500–600 nm). This is a distinct advantage of rhod 2 over the more commonly used fluorescent dyes (indo 1 and fura 2) which require ultraviolet excitation light that is strongly absorbed by myocardium and consequently limit tissue penetration and fluorescent emission intensity. Also, because of the large change in quantum efficiency of rhod 2 upon binding calcium and the favorable Kᵣ for calcium (710 nM in the presence of 0.5 mM myoglobin), a large signal is obtained from calcium transients (30–50% of total fluorescence, Fig. 11). Thus rhod 2 provides a much larger beat-to-beat change in fluorescence than that seen with other dyes such as indo 1 and fura 2, permitting a more detailed evaluation of the different components of the calcium transient.

The signal-to-noise ratio with the ultraviolet excitation dyes can be an important problem particularly with a whole heart preparation where motion during contraction can have a profound effect on the detected signal (4). Several techniques have been employed to minimize the effects of motion on the emission signal with these dyes, including 1) physical restriction of the heart to limit its motion with respect to the light detectors, 2) using reflected (spectral) light as a reference to ratio (32) or subtract (30) from the fluorescence signal, or 3) using a fluorescence standard (20) or ligand-insensitive emission wavelength (4) as the reference signal to correct for motion. With the combination and optimization of these techniques, motion artifact intensities of 2–3% of the total fluorescence have been achieved (4). However, if the indicator fluorescence signal is weak, even this small a signal from motion can account for ~10% of the fluctuations in fluorescence. The fluorescence signal from motion would be expected to be greatest during contractile transitions. Thus, with the large signal obtained from rhod 2 fluorescence during systole, the effects of motion are significantly reduced. Fluctuations in fluorescence with cardiac contraction were in the range of 4–5% in beating hearts loaded with the internal fluorescence standard CICF (fluorescence independent of calcium), as compared with 30–50% of total fluorescence from hearts loaded with rhod 2.

Dye distribution among the different cell types in the heart also is a potential source of error when attempting to measure myocyte calcium (25). To detect the level of endothelial cell loading, bradykinin is used since it has been shown to increase Ca in endothelial cells in a transient manner. In our preparation, with rhod 2 loaded into the heart at 37°C, we did not detect a significant rise in rhod 2 fluorescence with bradykinin (Fig. 10A). In contrast, there was a 150% rise in fluorescence seen with the calcium chloride bolus (Fig. 10B). Therefore, any endothelial cell loading that may occur with rhod 2 is below our detectable level, and thus the calcium signal detected from the heart appears to come from myocytes.

The cellular distribution of rhod 2 and the lack of rhod 2 accumulation in the mitochondria is key to the interpretation of the fluorescence measurements and calculation of cytosolic free Ca²⁺. The effective quenching of rhod 2 fluorescence (~80%) by manganese implies that most of the dye is located in the cytosol (i.e., a manganese-accessible region). The residual rhod 2 fluorescence detected in the presence of manganese is not due to dye trapped in the mitochondria because rhod 2 might be trapped in other subcellular organelles and because manganese is unable to completely quench rhod 2 in the presence of calcium in the cellular milieu. Moreover, manganese effectively blocked calcium transients, implying that there is little rhod 2 in mitochondria, since mitochondrial free calcium is also expected to oscillate on a beat-to-beat basis (8, 35). More compelling evidence was provided by confocal images of myocytes loaded with rhod 2-AM. A key criteria for the discrimination of mitochondrial vs. cytosolic loading would be the colocalization of TMRE, a mitochondrial potential probe, and of rhod 2. In Fig. 9, panels 1 and 4 demonstrate that rhod 2 localization is markedly different from that of TMRE, with rhod 2 exhibiting a relatively homogeneous distribution and TMRE exhibiting the typical punctate appearance of mitochondrial
labels. The addition of the proton uncoupler FCCP abolished the potential-dependent accumulation of TMRE in the mitochondria, which indicated that the mitochondria of our myocytes maintained a large negative potential and were metabolically sound. In contrast, FCCP did not alter the rhod 2 fluorescence, as expected irrespective of where the dye is located, as long as FCCP does not produce calcium gradients across the mitochondria. Although, Fig. 9, panels 3 and 4, shows that the rhod 2 fluorescence emanates predominantly from the cytosol, the accumulation of rhod 2 in the mitochondria could not be excluded. Figure 9, panel 5, illustrates a myocyte that is moderately loaded with rhod 2 and fluoresced less brightly and less homogeneously (e.g., up to 100-fold gradient of intensity) as the cell in Fig. 9, panel 3. In this case, the appearance of fluorescence “bands” could be mistaken for mitochondrial labeling instead of dye exclusion by the myofilaments, which act as dye diffusion barriers. Treatment of such myocytes with the detergent digitonin resulted in the complete loss of rhod 2 fluorescence (100-fold decrease in intensity) and with no residual fluorescence in the cell (e.g., >3-fold shift in intensity across the cell). Thus almost all the dye could diffuse out of digitonin-treated cells, and little or no dye was trapped in the mitochondria.

Our findings contradict several reports that argue that the delocalized positive charge of rhod 2-AM resulted in its selective accumulation in mitochondria and was thus used to monitor mitochondrial free calcium in a variety of cells (hepatocytes, Ref. 16; Chinese hamster ovary cells, Ref. 33; rat brain astrocytes, Ref. 17; rat chromaffin cells, Ref. 2). Most studies used cell imaging to measure the cellular localization of rhod 2 but did not use more stringent criteria such as the colocalization of TMRE and rhod 2 or trapping rhod 2 in mitochondria after detergent solubilization of the cell membrane or a truly independent measurement of mitochondrial free calcium. In isolated rat myocytes, the preliminary reports with rhod 2 have been controversial. Sheu and Jou (36) indicated that rhod 2 monitored mitochondrial calcium oscillation on a beat-to-beat basis. More recently, Duchen et al. (11) argued for a more cautious interpretation of rhod 2 signals because of a redistribution of dye from mitochondria to cytosol upon the addition of FCCP. Interestingly, measurements of Ca, with other calcium indicator dyes (e.g., fura 2, indo 1, fluo 3) have now been reappraised because a substantial percentage of dye accumulates in the mitochondria of myocytes (1, 8, 35). This implies that the delocalized positive charge on rhod 2 may be considerably less important than the loading conditions, with respect to the dye's cellular distribution. Thus rhod 2 in combination with the present loading conditions may provide more accurate measurements of Ca, compared with other calcium indicators because of the small accumulation detected in subcellular organelles.

Absolute quantitation of Ca, using rhod 2 in a whole heart preparation is the one aspect of this dye that is complex. Because of the lack of spectral shift in absorbance or fluorescence with calcium binding, a measure of the cytosolic dye concentration and a measure of maximum fluorescence under saturated conditions is required to calculate the calcium concentration. Transmission of light across a tissue has been used to quantitate other dyes, including fura red in frog skeletal muscle (21). In the present study, reflected absorbance was used to correct for changes in dye concentration. Reflected light from an opaque substance contains two components; one is the specular component, or light reflected directly from the surface, and the other is the diffuse, which is a consequence of multiple internal reflections within the tissue and is, in part, a function of the absorbance characteristics of the tissue (3, 6, 31). For our purposes, we sought to minimize the specular component and optimize the back-scattered diffuse component, which contains information relating to tissue absorbance. To achieve this, the location of the reflectance detector becomes important, since the direction of spectrally reflected light is determined by the direction of the incident beam with respect to the reflective surface. Thus the reference detector should be positioned to optimize the collection of diffuse or back-scattered light (18).

In our experiments, we used the reflectance spectrum of the heart before dye loading as a reference, which was subtracted from subsequent spectra obtained after dye loading. The observation that the resultant difference spectrum closely resembles the absorbance spectrum of rhod 2 obtained from a cuvette in the presence of myoglobin (Fig. 5) supports our contention that changes in the corrected reflectance measurements are directly related to rhod 2 concentration. Furthermore, the correction factor obtained by this method appears to account for changes in dye concentration as evidenced by the observation that in a beating heart, calcium-dependent rhod 2 fluorescence measurements remained relatively stable during the 30-min observation period (Fig. 6). Exact quantitation of rhod 2 concentration in the heart, however, requires defining the path length of the reflected light (10, 18). This is most often accomplished by time-resolved spectroscopic analysis of the reflected light (9). For our purposes, the estimation of dye concentration and relative changes that occur during the experimental protocol provide a method of estimating Ca concentration in a whole heart model using a fluorescent dye that provides a sufficiently robust signal permitting detailed analysis of the calcium transients.

To obtain a measure of the maximum rhod 2 fluorescence under calcium-saturated conditions, we used A-23187 and digitonin. We found the use of digitonin troublesome, however, since digitonin can cause cytosolic proteins such as myoglobin to leak out of the cell. For this reason, we based our calculations of Ca, on maximum fluorescence obtained with an infusion of A-23187. Other techniques such as tetany of the heart in the presence of a high extracellular calcium concentration may also useful (19).

Analysis of the calcium fluorescence signal obtained from the perfused rabbit heart (Fig. 11) indicates a sharp rise in Ca, during early systole, which occurs 40
ms before the pressure rise. The subsequent fall in calcium appears to have two phases, an early rapid phase followed by a more gradual decline to presystolic levels. This second phase or shoulder in the calcium peak is probably significant and has been observed in calcium transients from single myocytes using other calcium indicators (7, 19). Furthermore, response of the calcium transients from single myocytes using other phase followed by a more gradual decline to presystolic calcium appears to have two phases, an early rapid ms before the pressure rise. The subsequent fall in calcium (Fig. 10B) demonstrates a slower rate of rise in peak calcium fluorescence than the rate of rise in peak pressure. This comparison may be useful for defining calcium sensitivity of myofibrils.

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