Calibration of Indo-1 and Resting Intracellular $[Ca]_i$ in Intact Rabbit Cardiac Myocytes

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ABSTRACT Fluorescent Ca indicators have been extremely valuable in understanding intracellular [Ca] ($[Ca]_i$) regulation in many cell types. The calibration of these indicators in the intracellular environment, however, has been a continuous challenge. We performed in vivo calibrations of indo-1 in isolated rabbit ventricular myocytes loaded with the acetoxymethylester form of indo-1 and used the perforated patch variation of whole cell voltage clamp. Voltage, [Na], and [K] gradients were eliminated to approach equilibrium. We also took advantage of the powerful Na/Ca exchange in cardiac myocytes so that $[Ca]_i$ would be equilibrated with $[Ca]_o$ (because there was no [Na] or voltage gradient). The equilibration of [Na] and [Ca] across the membrane was tested by measuring the reversal potential of Na current and poking the cell to test for changes in $[Ca]_i$-dependent fluorescence ratio. The apparent dissociation constant, K_d for indo-1 in the cellular environment was 844 nM, which is ~2–3 times higher than that in aqueous solutions. In a separate series of experiments, a null point approach was used to determine the $[Ca]_i$ in intact cells at rest for very long periods (82 ± 6 nM). This is lower than that measured 15 s after a train of steady-state twitches ($[Ca]_i$ = 294 ± 53 nM). These experiments also allowed the direct assessment of the shortening versus $[Ca]_i$ relationship in intact cells.

INTRODUCTION

In cardiac muscle, Ca is responsible for activation of contraction, which is modulated in large part by the amplitude and time course of the intracellular Ca (Ca_i) transient. The $[Ca]_i$ in cardiac myocytes is in a dynamic balance and is influenced by an array of Ca transport systems and binding sites (Bers, 1991; Bassani et al., 1992). The quantitative evaluation of $[Ca]_i$ is important in evaluating the interplay of these various systems.

The ubiquitous nature of Ca_i as a second messenger in different cell types has led to development of several approaches to measure free $[Ca]_i$ in living cells (Ca microelectrodes, aequorin, metallochromic dyes, and several generations of fluorescent indicators). All of these methods have advantages and disadvantages, but the ratiometric fluorescent indicators indo-1 and fura-2 have been particularly popular and useful in measurements of $[Ca]_i$ (because the ratio, in principle, allows intrinsic correction for [dye] and movement artifacts). Indo-1 is especially useful in cells with relatively rapid Ca_i transient such as ventricular myocytes, because the two wavelengths and ratio can be recorded simultaneously.

However, accurate calibration of $[Ca]_i$ remains difficult for several reasons (and these hold for many indicators). First, intracellular compartmentalization during loading using acetoxymethylester forms (AM) can be significant. Indeed, in cardiac myocytes where mitochondria occupy 20–36% of cell volume, indicator loading into this organelle can be substantial (Spurgeon et al., 1990). Although this can be measured and minimized or accounted for, it is difficult to abolish unless cells are loaded with the free acid form of indo-1 via patch pipettes or microinjection. Second, partial hydrolysis of the AM groups can lead to artifactual calcium-insensitive fluorescence and, consequently, underestimation of $[Ca]_i$ (Morgan, 1993). Third, at the wavelengths required cell autofluorescence (e.g., NADH) can be substantial (Ince et al., 1992; Brandes et al., 1993b). Fourth, even with pipette loading, indo-1 binds to proteins, altering both the spectral properties of the indicator as well as its affinity for Ca (Konishi et al., 1988; Blatter and Wier 1990; Hove-Madsen and Bers, 1992; Brandes et al., 1993b).

The impact of these limitations can be minimized by in vivo calibrations, but these have also been difficult because it is challenging to control $[Ca]_i$ during the calibration procedure. Therefore, most in vivo calibrations have been restricted to estimating the in vivo values of minimum and maximum fluorescence ratios (R_{min} and R_{max}) and using an in vitro dissociation constant (K_d, e.g., Li et al., 1989). Although this can yield useful results, it is not ideal.

In the present study, we present a novel equilibrium method to improve in vivo indo-1 calibration in voltage-clamped cardiac myocytes. In a separate series of experiments, we use a null point technique to measure the resting $[Ca]_i$ in intact cardiac myocytes. The calibration method allows direct estimation of the in vivo K_d and also allows direct determination of the shortening-$[Ca]_i$ relationship in intact, isolated cardiac myocytes.

MATERIALS AND METHODS

Cardiac myocyte isolation

The procedure for isolation of ventricular myocytes was described previously (Bassani et al., 1992). Briefly, hearts were excised from adult male

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rabbits anesthetized with pentobarbital sodium (70 mg kg^{-1} i.v.). The hearts were mounted in a Langendorff perfusion apparatus and perfused with nominally Ca-free Tyrode's solution for 6 min at 37°C. Perfusion was then switched to the same solution containing 1 mg/ml collagenase (Type B, Boehringer Mannheim, Mannheim, Germany) and 0.12 mg/ml pronase (Boehringer Mannheim). Perfusion continued until the heart became flaccid (~15–30 min). Then the ventricles were dispersed and filtered. The cell suspension was rinsed several times, with a gradual increase in the Ca concentration up to 2 mM. The cells were plated on Plexiglass superfusion chambers (0.15 ml) in which the bottoms were formed by glass coverslips treated with laminin (Life Technologies, Grand Island, NY) to enhance cell adhesion.

Cell shortening and fluorescence measurements

The chamber was placed on an inverted microscope (Nikon Diaphot) adapted for epifluorescence measurements. The cells were superfused with normal Tyrode's solution at room temperature (22–23°C) and field-stimulated (square waves, amplitude 20–50% above threshold, 0.5 Hz) through a pair of platinum electrodes.

The instrumentation for cell shortening and fluorescence measurement was a modified version of the PTI (Photon Technology International, Monmouth Junction, NJ) Alphascan system described elsewhere (Bassani et al., 1992, 1993a). Excitation wavelength was 365 nm, and fluorescence emitted by the cell was recorded as photon counts per second at 405 and 485 nm. The illumination field was restricted to a circular spot 30 μm in diameter. The emission field was restricted to a single cell with the aid of an adjustable window. The background fluorescence recorded from a field of the same size at both wavelengths was subtracted from the signal recorded from the cell before the fluorescence ratio (405/485) was calculated. Measurements were not corrected for intracellular indo-1 binding and compartmentalization or cell autofluorescence (<5% of total fluorescence). Cells were also *trans*-illuminated with red light to allow simultaneous measurement of fluorescence and shortening. A red reflective filter (Corion, Holliston, MA) was placed in the emission light path to reflect the cell image to a video camera (model VDC 3800, Sanyo) connected to a video-edge detection circuit (Crescent Electronics, Sandy, UT), and the output was acquired using the PTI Alphascan system.

Cells were loaded with indo-1 by incubation with 10 μM indo-1 AM (Molecular Probes Inc., Eugene, OR) for 15 min at room temperature. After loading, the cells were superfused with normal Tyrode's solution for at least 1 h to washout extracellular dye and allow intracellular indo-1 de-esterification. Electrically stimulated contractions were not significantly affected by the loading procedure.

In preliminary experiments designed to determine the extent of indo-1 compartmentalization in mitochondria, there was no detectable cellular fluorescence remaining after quenching cytosolic indo-1 with 100 μM extracellular Mn, in contrast to results of Miyata et al. (1991) in rat myocytes. Furthermore, virtually all of our cellular fluorescence could be abolished by dialysis by a patch pipette lacking indo-1. We believe that the loading procedure (time and temperature) and species differences may explain the different results. Loading for 40 min at 22°C (vs. our usual 15 min) produced some apparent compartmentalization in rat, but not in rabbit, myocytes. At this high level of indo-1 loading, the cells would also display significant changes in contractile activity (e.g., increase of time-to-peak and duration of shortening) and were not useful in our experiments.

Fluorescence ratios ($R = F_{405}/F_{485}$) were converted to free $[Ca]_i$ according to the equation (Grynkiewicz et al., 1985): $[Ca]_i = K_d \cdot \beta[(R - R_{min})/(R_{max} - R)]$, using either an in vitro K_d of 250 nM or the K_d measured in vivo. The β value (ratio of the free/bound indo-1 fluorescence at 485 nm) was 3.0.

Electrophysiology

The perforated patch technique (Korn et al., 1991) was used to minimize disruption of endogenous cellular proteins and Ca buffering mechanisms and allow voltage control in an intact cell, while allowing reproducible measurement of Na and Ca currents for up to 2 h (3 times more than the duration of the experiments). Small cells (15 × 60 μm) were used in this work to minimize cell capacitance and allow better voltage control. The recording setup was composed of an Axopatch 1C amplifier using pCLAMP software (version 5.7.2) and a TL1 interface (Axon Instruments, Foster City, CA). Data were digitized at 7.5 kHz and filtered at 5 kHz. Capacitive pulses were subtracted using P/8 subpulse routine. Pipettes were fabricated from TW-150–3 capillary tubing (World Precision Instruments, Inc., Sarasota, FL), using a Model P-97 Flaming-Brown pipette puller (Sutter Instrument Co., Novato, CA). Pipettes were typically 0.8–1.2 MΩ in resistance. The liquid junction potentials between the two pipette solutions and the bath solution were measured (3–5 mV) and corrected for. To minimize electrode capacitance, the tips of the electrodes were coated with hydrophobic material (Pap Pen, Daido Sangyo Co., Japan).

An amphotericin B stock solution (100 mg/ml) in dimethylsufoxide (DMSO) was prepared within 2 weeks of use and was kept at −20°C in the dark. The stock solution was diluted in the pipette solution to give a final concentration of 240 μg/ml. The final amphotericin B solution was sonicated and kept near 0°C in the dark (Zhou and Neher, 1993). The same solution could be used for as long as 10 days without apparently affecting the amphotericin B perforating activity. After gigaseal formation, the perforated patch configuration was complete in about 10–15 min, during which the pipette potential was set to −60 mV, and voltage pulses (5 mV, 20 ms) were delivered at 100 Hz to monitor the reduction in access resistance due to amphotericin B pore formation. Typically, access resistance and cell capacitance were 10–15 MΩ and 80–150 pF, respectively. In some experiments, $CaCl_2$ (1 mM) was added to the pipette solution to test the perforated patch technique, and $[Ca]_i$ did not rise during patch recording (as evaluated by indo-1 fluorescence).

Because amphotericin B is light-sensitive, the fluorescence measurements were done intermittently in brief periods no longer than 15–20 s. Continuous illumination for as little as 60 s could induce a marked increase in access resistance, suggesting a possible photodegradation of amphotericin B molecules. In this case, voltage control could be restored in only 10% of the cells after about 5–10 min without illumination.

In the perforated patch configuration, we measured Na currents to evaluate the reversal potential. A brief (200 ms) hyperpolarization to −120 mV was followed by steps (15-ms duration) to voltages ranging from −90 to +50 mV to determine the I-V relationship. The brief, regular hyperpolarization allowed recovery of some, but not all, Na channels from the inactivated state. Ca currents (not shown) were measured at the beginning of the experiments to evaluate the cell condition.

Pipette solutions

In addition to 240 μg/ml amphotericin B, the pipette solution contained (in mM): 135 KCl, 15 NaCl, 1.5 $MgCl_2$, HEPES, pH-adjusted to 7.3 with KOH at 22°C. In some cells, K was replaced by Cs to minimize K currents and measure Na currents more accurately. Ca contamination in these solutions was not a problem because Ca could not cross the pores formed by amphotericin B. We did not find any difference by adding 0.5 mM EGTA to the pipette solution. Some experiments were also done with SO_4 replacing most of the pipette Cl to minimize possible Donnan potentials (Horn and Marty, 1988). Similar results were obtained for I_{Na} reversal potential, suggesting that such effects are small here.

Bath solutions

The solutions for R_{min} and R_{max} determination (K-buffer) contained (mM): 10 NaCl, 130 KCl, 1 $MgCl_2$, and 5 HEPES. To this solution, either 10 mM $CaCl_2$ or 5 mM EGTA (in nominally Ca-free solution) was added, and the pH was adjusted to 7.2 at 22°C. The control Tyrode's solution (NT) had the following composition (mM): 140 NaCl, 6 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 5 HEPES.

Thapsigargin (Calbiochem, La Jolla, CA) stock solution (10 mM) was prepared in DMSO and diluted 2000-fold in NT (NT-TG) immediately

before use. Caffeine was added to NT as a solid. Ouabain (Sigma) stock solution (15 mM) was prepared in water. Dilution from stock solutions was performed immediately before use.

The Ca-EGTA buffers were prepared and calibrated according to Bers et al. (1994) and contained (mM): 135 KCl (or CsCl), 15 NaCl, 1.5 $MgCl_2$, 10 HEPES, and 5 EGTA, pH 7.3 at 22°C with KOH or CsOH. Ouabain (1 mM) was added to block Na-K pump. The [Ca] in these buffers was calculated (Max Chelator version 6.52, Bers et al., 1994) and verified with a Ca electrode (Hove-Madsen and Bers, 1992) to obtain accurate free [Ca] standard solutions (30–2000 nM).

Determination of R_{min} and R_{max} for indo-1 in vivo

For in vivo measurement of R_{min} and R_{max}, indo-1-loaded cells were exposed to 10 mM caffeine twice, to empty SR Ca stores, and superfused with 0Ca solution for 15 min. Bath solution was then switched to K-buffer containing 5 mM EGTA. After 20 min perfusion, 3 μM carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone (FCCP, Sigma) and 10 mM 2-deoxyglucose were added to achieve metabolic inhibition and to limit hypercontracture upon further increase in [Ca]. Cells developed rigor 5–10 min after these inhibitors were added. To obtain R_{min}, 10 μM nonfluorescent Ca ionophore Br-A23187 (Calbiochem) was added to the solution and measurements were taken after the fluorescence at both wavelengths reached stable values. Then, for R_{max} determination, bath solution was EGTA-free and contained 10 mM Ca (Br-A23187 was present throughout the determinations).

Equilibrium $[Ca]_i$ calibration method: theory

We took advantage of the powerful Na/Ca exchange system in rabbit cardiac myocytes to equilibrate Ca across the sarcolemma and set the system at electrochemical equilibrium so that $\Delta\mu Ca = n\Delta\mu Na$, where $\Delta\mu Ca$ and $\Delta\mu Na$ are the electrochemical gradients for Ca and Na, respectively, and n is the stoichiometry of the exchange. Substituting the usual expressions for electrochemical driving force and rearranging some terms the equilibrium condition is

$$\frac{[Ca]_i}{[Ca]_o} = \left(\frac{[Na_i}{[Na]_o}\right)^n \cdot \exp\left\{\frac{(n-2)\cdot E_m F}{RT}\right\}, \quad (1)$$

where E_m is the membrane potential and R, T, and F are the universal gas constant, temperature, and Faraday constant, respectively. Under our experimental conditions where $[Na]_i = [Na]_o$ and $E_m = 0$, $[Ca]_i$ should be the same as $[Ca]_o$ (regardless of the value of n). This assumes that Ca transport via Na/Ca exchange is dominant in our experiments such that it, rather than any other transport system controls $[Ca]_i$. The SR Ca-pump was inhibited with thapsigargin, and the SR Ca content was released by caffeine exposure. Ion channels cannot build up a [Ca] gradient at $E_m = 0$ and would tend to help dissipate deviations from equilibrium. Only a sarcolemmal Ca-pump that might transport Ca at rates comparable with Na/Ca exchange could make $[Ca]_i$ lower than $[Ca]_o$. This is unlikely because Ca transport via Na/Ca exchange is overwhelmingly stronger than the sarcolemmal Ca-pump both at rest and during elevated $[Ca]_i$ in rabbit ventricular myocytes (Bers et al., 1989, 1993; Bassani et al., 1992, 1994). An exception might occur when $[Ca]_i$ becomes very low. In this case, the Ca transport via Na/Ca exchange may be kinetically limited (i.e., much below V_{max}). In this case, a system with a lower V_{max}, but higher affinity (such as the sarcolemmal Ca-ATPase) might keep $[Ca]_i$ below the equilibrium for the Na/Ca exchange (the equilibrium method was not used at very low [Ca]). In any event, the assumptions that $[Na]_i = [Na]_o$ and $[Ca]_i = [Ca]_o$ were tested experimentally.

Equilibrium $[Ca]_i$ calibration method: procedure

As soon as the indo-1 AM loading procedure was completed, the cells were treated with thapsigargin (TG, 5 μM) to inhibit completely and irreversibly the SR Ca uptake (Bassani et al., 1993b). The solution flow was interrupted, and the bath solution was replaced by NT-TG solution that was kept in contact with the cells for 1.5–2 min. After this period, TG was washed out and the SR Ca content was depleted by fast application of caffeine (10 mM, 30-s duration) in the presence of Na (Bassani et al., 1992). A second caffeine application after a train of field-stimulated twitches showed no Ca release because the SR had been depleted of Ca and the SR Ca uptake was blocked by TG. The electrodes for field stimulation were removed from the bath, and the cells were then equilibrated in NT for 10–20 min before initiating the patch-clamp procedure.

After the perforated patch configuration was established and Ca current checked, the bath solution was changed to one of the Ca-EGTA buffers and E_m was set to 0 mV. After a few (2–5) seconds, fluorescence and shortening were measured (10- to 15-s acquisition time) and the bath solution was changed to a new Ca-EGTA buffer with different $[Ca]_o$. Fluorescence ratio and cell length were measured in each solution.

The assumption that $[Na]_i = [Na]_o$ was tested by measuring the reversal potential of Na current as described above. To confirm that $[Ca]_i = [Ca]_o$ during our calibration experiments, we removed the electrode from the cell after the presumed equilibrium and then the cell was poked aggressively using another electrode (2- to 3-μm diameter) filled with the same bath solution (same $[Ca]_o$). The cell damage produced by this procedure did not cause a significant loss of Ca indicator, but should induce rapid equilibration of $[Ca]_i$ and $[Ca]_o$. Neither the withdrawal of the perforated patch pipette nor the poking damage produced any change in the fluorescence ratio (whereas in mM $[Ca]_o$ both procedures cause a very rapid rise in $[Ca]_i$ and hypercontracture). This indicates the validity of our assumption of [Ca] equilibration before removal of the perforated patch electrode.

Null point method to assess resting $[Ca]_i$ in intact cells

We also measured resting free $[Ca]_i$ in intact cells, where the cell was allowed to control its own $[Ca]_i$ and E_m. For this different series of experiments, we used a null point approach. Resting $[Ca]_i$ must be defined explicitly, because diastolic $[Ca]_i$ between contractions is higher than after several seconds of rest and $[Ca]_i$ continues to decline slowly during prolonged rest. We chose to study intact cells that had been resting for a very long period of time (>1 h) in NT to best approximate a stable steady state.

These cells were then superfused with Ca-EGTA buffers with $[Ca]_o$ comparable with anticipated $[Ca]_i$ (i.e., 50–200 nM). After superfusion with one of these solutions for 5–15 s, the cell was poked to damage the cell membrane, to determine the $[Ca]_o$ that did not result in change in fluorescence ratio (i.e., the null point where $[Ca]_o = [Ca]_i$). Of course, there was normally some change in fluorescence ratio (ΔR) when the $[Ca]_o$ was not identical to $[Ca]_i$. To find the null point, we assumed that the amplitude of ΔR was linearly related to the difference between $[Ca]_o$ and $[Ca]_i$ for values near the null point. Thus the extrapolated $[Ca]_o$ at which $\Delta R = 0$ should correspond to the resting $[Ca]_i$.

Statistical analysis

Data were expressed as means ± SEM. Least-squares linear regression and nonlinear curve fitting were used (GraphPad software, version 4.03) when appropriate. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Controlling $[Ca]_i$ and cell shortening at micromolar levels of $[Ca]_o$

Fig. 1 shows raw data for a typical equilibrium calibration experiment in which shortening (Fig. 1 *A*) and fluorescence ratio (Fig. 1 *B*) are measured simultaneously as a function of $[Ca]_o$ in a voltage-clamped cell at $E_m = 0$. Both $[Ca]_i$ and cell length reached stable levels within 5–10 s of the solution changes. After $[Ca]_i$ and cell shortening were elevated to high

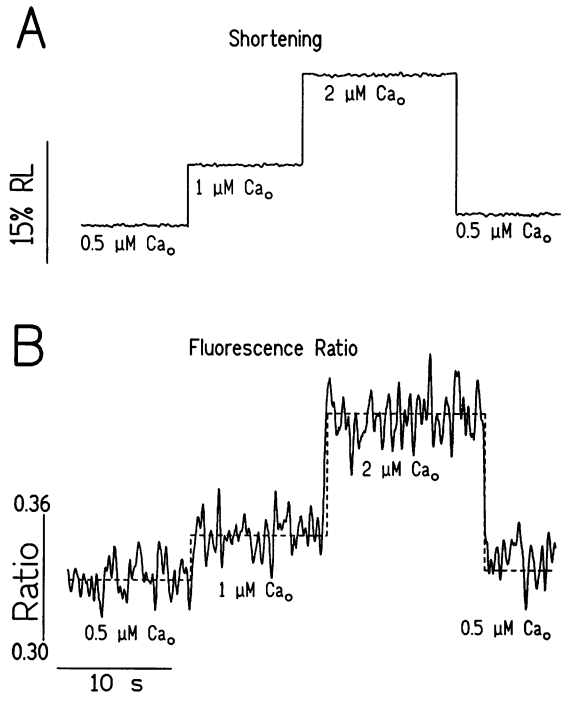

FIGURE 1 Measurement of cell shortening (*A*) as % of resting length (RL) and fluorescence ratio (*B*) during equilibrium $[Ca]_i$ calibration protocol for different $[Ca]_o$. Isolated rabbit myocytes were voltage-clamped using the perforated-patch configuration (E_m = 0 mV, see text for details). At each solution change, there was a 3- to 5-s break in the record, although the lines were connected. Dashed line indicates average level of the fluorescence ratio.

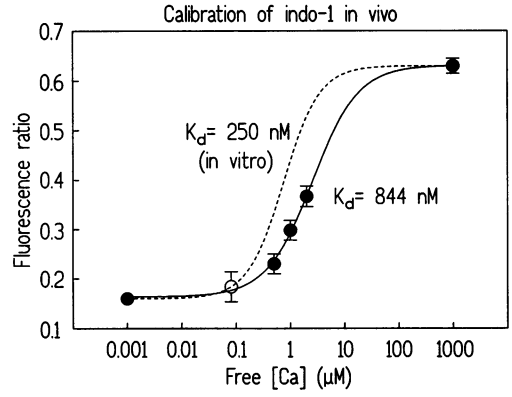

FIGURE 2 Calibration curve of indo-1 in vivo. Relationship between fluorescence ratio and $[Ca]_o$ from experiments as shown in Fig. 1 (including R_{min} and R_{max} measurements, ●, N = 6). Dashed line represents the theoretical curve calculated with K_d= 250 nM. Resting ratio (*open symbol*) was obtained using the null point approach (see text and Fig. 4 for details). The solid line is a curve fit to Ratio = $\{(R_{max} - R_{min})/(1 + (K_d\beta/[Ca])^n)\} + R_{min}$ for the data shown as circles in the figure. (K_d = 844 nM, Hill coefficient n = 1.02).

levels in 2 μM $[Ca]_o$, the cell usually did not recover fully to the initial length or fluorescence ratio. Thus, measurements for further analysis were restricted to one sequence of increasing $[Ca]_o$ steps up to 2 μM. In some cells, Na currents were also studied or the cell was poked with a second microelectrode to confirm that [Ca] was equilibrated across the membrane.

Fig. 2 shows the relationship between fluorescence ratio and $[Ca]_o$ from experiments like that shown in Fig. 1 (*filled circles, solid line*). The R_{min} (0.16 ± 0.012) and R_{max} (0.63 ± 0.015) values are those obtained in our standard in vivo calibration (see Materials and Methods). The resting ratio (~0.1 μM) was obtained by using the null point approach (see Materials and Methods and Fig. 4 for details). The data were fit with a sigmoidal curve allowing determination of an in vivo K_d (0.84 μM) and Hill coefficient (1.02). The dashed line represents the theoretical curve obtained with K_d = 0.25 μM from in vitro calibrations, but still using in vivo R_{min} and R_{max}. The Hill coefficient fit to the data in Fig. 2 is that expected for a simple 1:1 stoichiometry, even though there might be reasons to expect otherwise (e.g., because of Ca-dependent indo-1 binding to cell proteins, Hove-Madsen and Bers, 1992).

Are the conditions for equilibrium established?

The results described above assume that both [Na] and [Ca] gradients are abolished at the point when the fluorescence and shortening are measured. For [Na] this was confirmed by measuring the reversal potential for Na current. Fig. 3 *A* shows time course of typical Na currents for pulses to the indicated E_m values from a brief hyperpolarization (200 ms, −120 mV). The peak current-voltage relationship (Fig. 3 *B*) indicates that the reversal potential was very close to 0 mV, which is as expected if there is no transsarcolemmal [Na] gradient. In current-voltage relationships with smaller E_m steps around 0 mV (but with smaller E_m range than in Fig. 3), the reversal potential is typically within 0.5 mV of 0. It

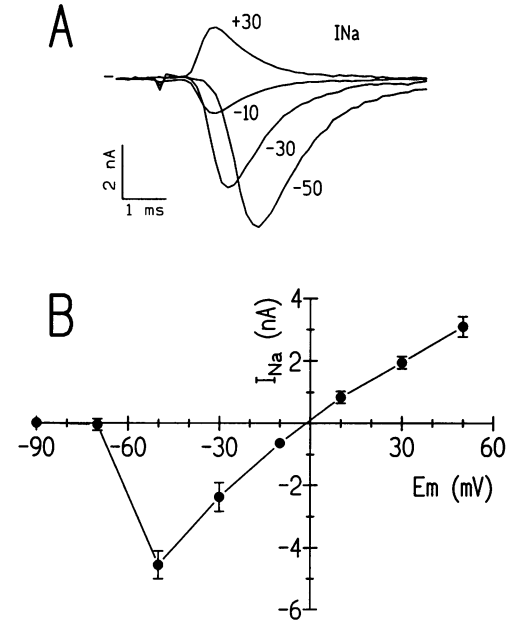

FIGURE 3 Testing for [Na] equilibrium across the sarcolemma during calibration procedure. Time course of typical Na currents (*A*) for pulses from a brief hyperpolarization (200 ms, −120 mV) to indicated E_m. I_{Na}-voltage relationship (*B*) shows that the reversal potential was very close to 0 mV (N = 5).

should be noted that the even this small error in predicted $[Na]_i$ would imply that the [Ca] gradient would be 1.057 rather than 1 (according to Eq. 1). Thus, the more critical and central test is whether there is any [Ca] gradient.

To test whether there was any [Ca] gradient at the end of experiments like that shown in Fig. 1, the electrode was removed and the cell was poked by a second electrode-containing solution identical to the bath solution. No significant change in fluorescence ratio or cell length were observed during at least 15 s. The cell damage, of course, led to gradual loss of indicator, but this loss was very slow compared with the measurement period (but confirmed cell damage). Using ratiometric measurements also minimizes the impact of eventual dye leakage.

Null point measurement of resting $[Ca]_i$ in intact myocytes

A separate series of experiments was carried out to estimate the resting $[Ca]_i$ in intact cells in NT (without voltage clamp). An important distinction is that in this case we allow the intact cell to regulate $[Ca]_i$ itself at its normal resting E_m (rather than trying to abolish [Na], [Ca], and E_m gradients as in the equilibrium method shown in Fig. 1).

First, the fluorescence ratio was measured after a very long rest period in NT (>1 h). The extracellular solution was then switched to one where $[Ca]_o$ was in the range anticipated for $[Ca]_i$ (at the time indicated by the arrow in Fig. 4 *A*). As shown in Fig. 4 *A*, a probing microelectrode containing the same Ca-EGTA solution as the bath was used to poke the cell abruptly and repeatedly (at the bar). The fluorescence ratio increased when $[Ca]_o$ was 100 nM but decreased when $[Ca]_o$ = 50 nM, indicating that the $[Ca]_o$ that would produce no fluorescence change was between these two values. Pooled data are shown in Fig. 4 *B* along with the linear regression to estimate the null point. With this method, we estimate that the $[Ca]_i$ in these cells after long periods of rest in NT and then briefly in a low $[Ca]_o$ solution was 81.8 ± 6.4 nM. This value is used in the in vivo calibration curve in Fig. 2 (*open circle*).

It would have been ideal to perform the null point test after brief rest periods or during diastole in cells in NT, but that was not practical because $[Ca]_i$ continues to decline slowly at rest. However, measurements of the resting fluorescence ratio in NT (15 s after a steady-state train of contractions at 0.5 Hz) indicates resting free $[Ca]_i$ to be 294 ± 53 nM (using the calibration in Fig. 2, n = 8).

Shortening-$[Ca]_i$ relationship in isolated cardiac myocytes

The equilibrium calibration approach in Fig. 1 with simultaneous cell length measurement allowed us to evaluate the steady-state shortening versus $[Ca]_i$ relationship in intact, isolated rabbit myocytes. In Fig. 5 the filled circles correspond to shortening (expressed as % of the resting cell length). This curve is steeper and shifted to lower [Ca] than

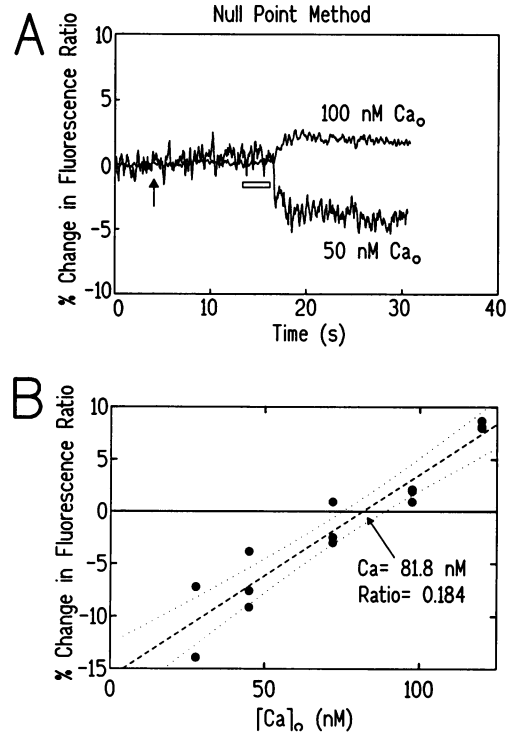

FIGURE 4 Null point approach to test [Ca] gradient. (*A*) Change in fluorescence ratio induced by cell membrane rupture by poking the cell with a pipette containing the bath solution. Two example are shown in solutions containing different $[Ca]_o$. Cells were kept at rest for about 1 h to attain steady-state resting $[Ca]_i$. Arrow indicates the solution switch from NT to the Ca-EGTA buffer (100 or 50 nM Ca). Bar indicates the period during which the cell membrane was poked 3–4 times with a large tip diameter microelectrode (2–3 μm). Pooled data and linear regression line are shown in *B*. The estimated null point for these cells was 81.8 ± 6.42 nM, N = 14.

is typically observed in force-[Ca] curves in skinned cardiac muscle (Bers, 1991), but is similar to force-$[Ca]_i$ curves measured in intact ventricular muscle using aequorin and fura-2 (Yue et al., 1986; Gao et al., 1994).

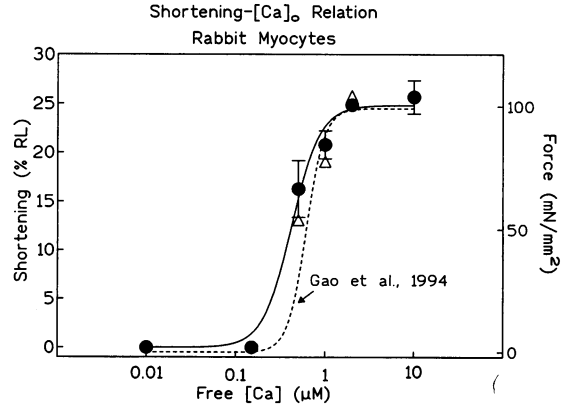

FIGURE 5 Shortening versus $[Ca]_o$ relationship in intact, isolated rabbit myocytes. Data points are obtained from experiments like that in Fig. 1 (●, ——) and from "pseudo-tetanus" in thapsigargin-treated cells (△). The dashed line is a calculated force-[Ca] relationship using parameters obtained by Gao et al. (1994) from experiments in intact rat ventricular muscle.

We also attempted to tetanize individual cells after treatment with TG and depletion of the SR Ca content. By varying the stimulatory frequency (up to 1.5–2 Hz) in the presence of high $[Ca]_o$ (8 mM), different stable levels of shortening (pseudo-tetanus) and fluorescence ratio could be obtained. Free [Ca] was then calculated using the in vivo calibration in Fig. 2. The open triangles in Fig. 5 are data from a cell using this procedure. The stable levels of contraction and $[Ca]_i$ were not entirely fused as in classic skeletal muscle tetani. However, the cells normally could not be driven fast enough for full fusion. Similar shortening-$[Ca]_i$ relationships were obtained during equilibrium calibration in voltage-clamped cells (*filled circles*) and during tetanizing activation in intact cells (*triangles*). Thus, the intact cell in a more physiologic condition seems to respond to increases in $[Ca]_i$ in a manner similar to that observed in perforated patch experiments.

Ca_i transients in isolated rabbit ventricular myocytes

Fig. 6 shows Ca_i transients obtained in intact rabbit cardiac myocytes during electrical stimulation using both the in vitro (left) or in vivo K_d. Steady-state twitches (0.5 Hz) are followed by a rapid caffeine application. After another period of steady-state stimulation (not shown), a second caffeine application was performed in 0Na,0Ca solution. This typical experiment illustrates the impact of K_d on calibrated Ca_i transients in vivo. In this cell, the calculated values for resting $[Ca]_i$ varied from ~145 (resting in the absence of Ca_o) to ~250 nM (diastolic Ca at 0.5 Hz) when K_d = 844 nM, and from 50 to 70 nM when K_d = 250 nM.

DISCUSSION

In the present work, we have developed a novel equilibrium method for indo-1 calibration in isolated cardiac myocytes voltage-clamped with the perforated patch method. We have also assessed resting $[Ca]_i$ in intact cells using a null point approach. An important point in the calibration method is the possibility of improving the determination of K_d in vivo without disrupting the cellular environment. The method can also be useful for those interested in more detailed studies of the shortening-$[Ca]_o$ relationship in intact cardiac cells.

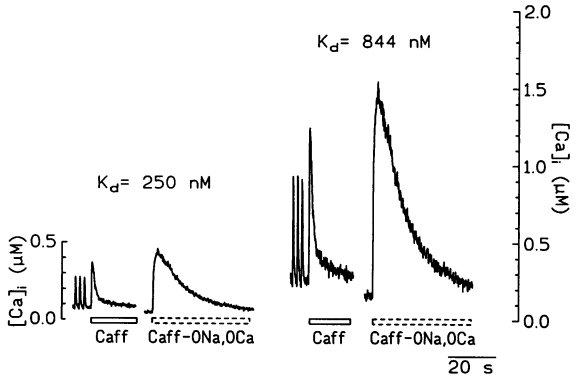

FIGURE 6 Ca_i transients calculated with different values of K_d. The series of Ca_i transients was obtained in rabbit ventricular myocytes without thapsigargin during the following protocol: electrical stimulation at 0.5 Hz, application of 10 mM caffeine (Caff, *solid open bar*) and after steady-state stimulation (not shown), caffeine was reapplied in 0Na,0Ca solution (Caff-0Na,0Ca, *dashed bar*). The same fluorescence signals were converted to $[Ca]_i$ using our in vivo R_{min} and R_{max} values, and indo-1 K_d of 250 (*left*) or 844 nM (*right*).

In vivo $[Ca]_i$ calibration

Although virtually all attempts at in vivo calibrations have intended to establish equilibration of $[Ca]_i$ with $[Ca]_o$, this has neither been easily achieved nor easily tested (Li et al., 1989; Ikenouchi et al., 1991). Thus, the precise value of $[Ca]_i$ corresponding to a given fluorescence ratio is usually not known. This has led most investigators to limit calibrations to estimating R_{min} and R_{max} in vivo, but using in vitro value for K_d, not necessarily obtained under the same conditions. Ikenouchi et al. (1991) tested one intermediate [Ca] (500 nM) using the ionophore Br-A23187 and concluded that the in vivo K_d of indo-1 was the same as the in vitro value (250 nM).

However, there has been increasing evidence that indo-1 and fura-2 bind extensively to intracellular proteins, which alters the fluorescence spectrum and reduces the Ca affinity of the indicators (Konishi et al., 1988; Blatter and Wier 1990; Uto et al., 1991; Hove-Madsen and Bers, 1992). Indeed, because ~75% of indo-1 is expected to be bound to cellular constituents in mammalian cardiac myocytes, there is reason to expect an increased K_d in the intracellular environment. Hove-Madsen and Bers (1992) measured the characteristics of indo-1 in suspensions of digitonin-permeabilized rabbit ventricular myocytes at high protein concentration and estimated that the K_d for indo-1 in the cellular environment would be expected to be ~1000 nM. The present study using single intact rabbit myocytes is in reasonable agreement with this value (K_d ~ 844 nM). This is about twice the K_d value we obtain for aqueous buffers on the same microscope stage (350–400 nM in several different series at 22°C).

The present method of calibration has the advantage that it is done in vivo in the native cellular environment, and we have verified the $[Ca]_i$ associated with a given fluorescence ratio. Thus, it should intrinsically account for protein binding and spectral alterations, which are a consequence of that environment. In principle, it should also account for autofluorescence and incompletely de-esterified forms of the indicator (as long as those factors do not change appreciably over the course of an experiment). These factors, however, may contribute to the lower dynamic range typically observed with AM loading (i.e., R_{max}/R_{min} = 4–5, present results; Ikenouchi et al., 1991) versus free acid loading (9–14, Balke et al., 1994) or aqueous solution cuvette and microscope stage measurements (15–21, Grynkewicz et al., 1985; Bers et al., 1990; Mattiazzi et al., 1994).

Intracellular compartmentalization is a notorious problem, especially in cardiac myocytes where mitochondrial volume is large (Spurgeon et al., 1990). We were fortunate to obtain loading conditions here that appear to minimize this problem.

The present calibration would not be expected to overcome this problem. Although we might raise [Ca] in the mitochondria ($[Ca]_m$) as well as the cytosol, the two concentrations might not be the same (especially given the large negative intramitochondrial potential). Thus, Ca_i transients that are to be calibrated would be likely to differ greatly in the $[Ca]_i/[Ca]_m$ ratio compared with the relative steady state during calibration. Consequently, the calibration would not allow accurate prediction of $[Ca]_i$.

There may even be a kinetic limitation with the free acid form of indo-1 localized to the cytosol (in addition to the intrinsic Ca-binding kinetics). This is because the binding of the indicator to cellular constituents is greater at high [Ca] (Hove-Madsen and Bers, 1992). Thus, in the steady-state calibration, there may be a decrease in the apparent Ca sensitivity of indo-1 at high [Ca] (due to higher protein binding and, thus, lower Ca affinity). This might not occur during a rapid Ca_i transient (i.e., the protein-bound fraction of indo-1 may not change that rapidly). This may then represent another instance where the distribution of the indicator during calibration may not match that during the Ca_i transients of interest.

This calibration approach should also be applicable in cells using the ruptured patch configuration where dialysis by the patch pipette alters the intracellular environment. Such an approach allows introduction of indicator in the free acid form, but it may also dialyze away normal cellular constituents, including soluble proteins, which are responsible for binding much of the cellular indo-1 (Hove-Madsen and Bers, 1992). Again, measurement of the Ca calibration under the steady-state conditions expected during the experiment would be most appropriate. Thus, in these dialyzed cells the K_d for indo-1 might be closer to the in vitro value.

Influence of constants on calibration

We have obtained an in vivo K_d value of 844 nM in the present study for cells loaded using the AM form of indo-1 (95% confidence range, 707–1009 nM). This is a useful value, but it should probably not be applied directly to cells under other experimental conditions where the cellular environment may differ. Ideally, one should perform similar measurements for the specific cells and conditions under investigation.

It can be readily appreciated from the calibration formula given in Materials and Methods that the intrinsic effect of altering K_d is just a direct multiplicative factor for [Ca] (i.e., doubling K_d simply doubles the calculated [Ca]). Thus, if relative changes in $[Ca]_i$ are sufficient for one's experimental aims, then choosing the correct K_d is not essential. R_{min} is also hard to measure reliably because it is difficult to get $[Ca]_i$ to very low levels (e.g., <1 nM). Small errors in estimation of R_{min} produce disproportionately large errors at low $[Ca]_i$, thus the resting $[Ca]_i$ and the shape of the Ca_i transient can also be altered. The same problems apply for R_{max} errors and high $[Ca]_i$ (i.e., both peak $[Ca]_i$ and shape of the $[Ca]_i$ waveform can be altered). In cardiac myocytes, R_{max} is also difficult to measure reliably because massive hypercontracture at very high $[Ca]_i$ can destroy cellular integrity. The use of metabolic inhibitors to lock cells in rigor (Li et al., 1989) has been a practical way around this problem, but it might also alter the cellular environment and is therefore also not ideal.

Shortening-$[Ca]_i$ relationship in intact cells

The [Ca]-dependent regulation of the myofilaments is an important aspect in the control of cardiac muscle contraction. A large number of studies over the years have examined the myofilament Ca sensitivity in "skinned" multicellular preparations, and much useful information about modulators and mechanisms has been accrued. Typically, the [Ca] for half-maximal force has been 2–6 μM with Hill coefficients of 2–2.5 (see Bers, 1991). Estimates made in intact fibers using intracellular Ca indicators have suggested that the myofilament Ca sensitivity is greater (half-maximal force at 0.5–0.6 μM Ca) and has higher Hill coefficients (5–6, Yue et al., 1986; Gao et al., 1994). Our shortening versus $[Ca]_i$ relationship showed half-maximal contraction at 0.42 μM (Hill coefficient 2.7).

It should be noted that we are measuring shortening with no external load on the cells (aside from adhesion to the laminin-coated coverslip). Indeed, because we don't really know the mechanical load on these cells, these results should be viewed with some caution. In this context, the overall similarity for the [Ca] dependence of shortening with force measurements (Fig. 5) is a bit surprising, particularly because the myofilament Ca sensitivity decreases at shorter sarcomere lengths (Kentish et al., 1986). With the extensive shortening in high $[Ca]_i$ (~25%), we would expect sarcomere length to have decreased enough so that the myofilament Ca sensitivity is reduced by a factor of ~2 (Kentish et al., 1986). Additional data would be required to address this issue quantitatively, but it is possible that this effect contributes to the apparently lower steepness of the top versus the bottom half of the $[Ca]_i$-shortening data in Fig. 5.

Resting $[Ca]_i$ in cardiac myocytes

Harkins et al. (1993) recently reevaluated the level of resting $[Ca]_i$ in frog skeletal muscle, taking into account changes in indicator properties in the cellular environment. They found higher values than previous reports (Blatter and Blinks, 1991). Resting $[Ca]_i$ in cardiac muscle is not a simple value because it changes significantly with time. The diastolic $[Ca]_i$ between pulses, even at modest frequencies, is higher than after a 10- to 20-s rest, and $[Ca]_i$ continues to decline very slowly during rest. The continual decline in $[Ca]_i$ led us to choose very long rest periods (>1 h) for the null point experiments so that $[Ca]_i$ would not be changing while we tried to measure it. The resting $[Ca]_i$ in these cells did not change much when the low $[Ca]_o$ solutions replaced NT (see Fig. 4 *A*). Resting $[Ca]_i$ after these very long rests was ~82 nM (Fig. 4 *B*). When the null point approach was used shortly after a train of pulses, higher values were estimated (>200 nM), but timing was more critical and the fluorescence ratio

was changing with time. Using the mean fluorescence measured 15 s after steady-state stimulation at 0.5 Hz, we found a resting $[Ca]_i$ of 294 ± 53 nM. Thus, resting $[Ca]_i$ in cardiac myocytes depends greatly on the history and conditions of the myocyte.

It is also worth noting that the equilibrium $[Ca]_i$ predicted for true equilibrium in an intact cell at rest for the Na/Ca exchange system would be ~10 nM (for $[Na]_i$ = 7 mM and E_m = −80 mV in NT). This equilibrium value may never be reached because other Ca fluxes may counteract net Ca efflux, which will become progressively slower as $[Ca]_i$ declines. This slowing of the Ca transport rate via Na/Ca exchange at very low $[Ca]_i$ may make it more difficult to reach true equilibrium at very low $[Ca]_o$. Therefore, we confined the experiments in Fig. 1 to the higher $[Ca]_i$, where the Na/Ca exchange appears to reach equilibrium. Thus, the complementary data from the null point experiments and R_{min} determinations are especially useful.

In conclusion, we have described a novel method for in vivo calibration of intracellular indo-1 in cardiac myocytes, combining equilibrium and null point methods. The K_d for indo-1 in the cell was found to be 2–3 times higher than in aqueous in vitro solutions. The experiments also allowed assessment of the resting free $[Ca]_i$ and the steady-state shortening-$[Ca]_i$ relationship in cardiac myocytes, which were only perturbed by perforated patch electrodes.

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