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QUANTITATION OF INTRACELLULAR FREE CALCIUM IN SINGLE ADULT CARDIOMYOCYTES BY FURA-2 FLUORESCENCE MICROSCOPY: CALIBRATION OF FURA-2 RATIOS

QIAN LI, RUTH A. ALTSCHULD, AND BRADFORD T. STOKES

Departments of Physiology and Physiological Chemistry, Division of Cardiology, and The Biophysics Program, The Ohio State University, Columbus, Ohio 43210

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Isolated rat myocytes incubated with the acetoxy methyl ester of fura-2 contained partially hydrolyzed esters, necessitating in vivo calibration of the signals obtained by fluorescence microscopy for calculation of pCa. Ionophores did not produce reliable R'_{max} and R'_{min} values in respiring myocytes, and elevated free calcium caused individual cells to hypercontract and burst. These difficulties were overcome by superfusion with a glucose-free buffer containing an inhibitor and an uncoupler of oxidative phosphorylation. R'_{max} and R'_{min} values obtained by ionophore treatment of deenergized myocytes were normalized to an in vitro calibration curve. Resting pCa derived from the individual curves averaged 6.9 for calcium-tolerant rod-shaped myocytes.

The advent of calcium-sensitive fluorescent dyes, which can be loaded into intact isolated cells as permeant acetoxy methyl esters (1), has revolutionized our ability to quantify intracellular free calcium and monitor its fluctuations during cellular activity. However, the inevitable heterogeneity in a population of isolated cells and the need to correct for signals arising from extracellular dye complicates interpretation of data obtained on suspensions of cells in cuvettes. These difficulties can be mostly eliminated by using single cell fluorescence microscopy and ratio techniques with the highly fluorescent calcium probe fura-2 (2). Unfortunately, the ratioed fura-2 signals, although vastly superior to single wavelength measurements (i.e. quin-2), have not yielded reliable

The following abbreviations were used in the text: EGTA, ETHYLENE GLYCOL- bis(b-AMINO-ETHYL ETHER) N,N,N',N'-TETRA-ACETIC ACID; MOPS, MORPHOLINOPROPANESULFONIC ACID; HEPES, N-2-HYDROXYETHYLPIPERAZINE-N'-2-ETHANESULFONIC ACID; CCCP, CARBONYL CYANIDE m-CHLOROPHENYL HYDRAZONE.

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quantitative data for a number of cell types; intracellular ratios at apparently saturating free calcium do not agree with in vitro values for free acid (3). This problem with fura-2 standardization appears to involve incomplete deesterification of the acetoxy methyl form, leaving strongly fluorescent but calcium-insensitive species trapped within the cells (4). Hence, it becomes necessary to calibrate the calcium-sensitive signal of the dye in vivo rather than rely on ratio values obtained during aqueous buffer calibration. With isolated cardiac myocytes, however, simply increasing calcium permeability with an ionophore (e.g. ionomycin or Bromo-A23187) is not sufficient to produce reliable R'_{max} (calcium-saturated) and R'_{min} (calcium-free) ratios. In addition, successfully obtaining calcium saturation can cause myocytes to hypercontract into a disorganized round form and burst, spilling cellular fura-2 into the superfusate.

Here we describe a procedure that eliminates the above difficulties associated with the calibration of fura-2 fluorescence ratios in isolated adult cardiac myocytes, and which should be applicable to those other cell types where calibration has also posed technical problems.

MATERIALS AND METHODS

Calcium-tolerant adult rat heart myocytes were isolated as previously described (5). The cells were diluted to approximately 50,000/ml in a HEPES buffer and incubated 40 minutes at room temperature with 1.5 uM fura-2/AM (the acetoxy methyl ester of fura-2). The cells were washed with the above buffer and allowed to postincubate for 2 hours at room temperature to facilitate hydrolysis of the intracellular dye. The myocytes were placed in a 0.3 ml plexiglas perfusion chamber fitted with a coverslip. The cells settled onto and became loosely attached to the coverslip, and could be superfused at about 1.3 ml/min without being dislodged.

The myocytes were examined with a Zeiss IM microscope and a 63X PLAN NEOFLUAR objective. Fluorescence excitation was provided from a mercury source through a dichroic mirror (Zeiss FT395) and wavelengths (350 and 380 nm) were selected by computer-controlled (IBM-PC/XT) movement of two specially-designed filters (POMFRET and OMEGA OPTICAL) into the light path. Emission was collected (420-620 nm) by a Hammamatsu PMT/IBM system. The entire optical path to and from the cells (with the exception of the coverslip at the bottom of the experimental chamber) was through quartz elements to maximize UV transmission and avoid shifts of excitation spectra to higher wavelengths.

Fluorescence ratios at varying pCa with fura-2 free acid were obtained with the above system, and the characteristics of the calibrating solutions were established on a Perkin-Elmer MPF444 recording spectrofluorimeter. The calibration solution contained (in mM) 115 KCl, 20 NaCl, 3 $MgCl_2$, 2 EGTA and 10 MOPS. The absolute EGTA concentration was estimated and the contaminant calcium of the stock solution measured by atomic absorption

spectroscopy as in Johnson et. al.(6). The amount of calcium needed to achieve a given pCa was calculated from the computer program of Perrin and Sayce (7) and the pH was held constant at 7.00 +/- 0.02 by addition of 45% KOH as needed.

RESULTS AND DISCUSSION

The relationship between calculated pCa and the ratio of emitted light during excitation at 350 and 380 nm for fura-2 free acid as measured in the chamber is illustrated in Figure 1. The curve was constructed from a least squares analysis program (Graph PAD, 1987) for non-linear data (n=5, M +/- SE) run on an IBM/PC. It is clear from the figure that the fluorescence ratios change little after a pCa below 4.5 or above 8.0. For an in vivo calibration, it would therefore seem unnecessary to achieve free calcium greater than ca. 50 uM for R'_{max} or less than 10 nM for R'_{min}.

It is surprisingly difficult to cause a significant increase in free calcium in respiring and normally elongated (rod-shaped) cardiac myocytes using a calcium ionophore. As shown in Table 1, the rate of increase in free calcium varies considerably among individual myocytes from an apparently homogeneous population. Under conditions where an increase in the 350/380 nm ratio is observed, the cells are converted from a normally elongated shape into a disorganized round form. While this morphologic

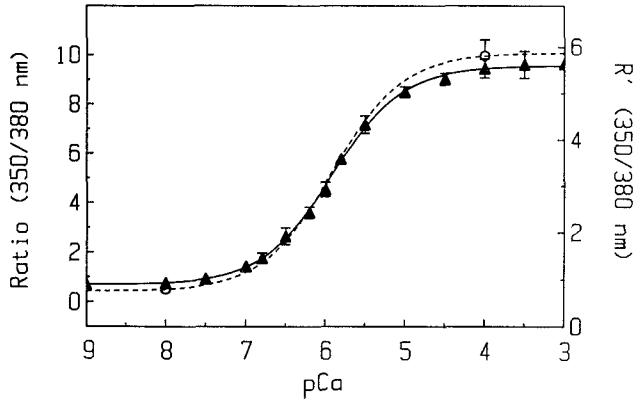

Figure 1. Ratio measurements (mean +/- SE) of five separate determinations (solid triangles) of different calcium concentrations in our standard aqueous buffer (RATIO 350/380 nm). The solid line was fitted (Hill coeff. = .97; EC50= -5.9) by a non-linear curve fit subroutine of the Graph PAD IBM program. The dashed line is a normalized plot using R'_{max} = 5.81 +/- .35, R'_{min} = 0.70 +/- .02 with the same Hill coefficient and EC50.

Table 1. Heterogeneity of ratio alterations in the presence of ionophore (BrA23187) in energized cells

	cell #			
	1	2	3	4
control	0.96 (rod)	1.00 (rod)	1.07 (rod)	1.12 (rod)
add BrA23187/Ca				
+ 5 min.	1.25 (rod)	1.29 (rod)	3.27 (rnd)	5.31 (rnd)
+30 min.	1.25 (rod)	1.95 (rod)	7.25 (rnd)	7.22 (rnd)
+60 min.	1.89 (rod)	5.89 (rnd)	6.98 (rnd)	6.29 (rnd)

The responses of four individual cells from the same preparation to an ionophore that is presumed to dramatically and uniformly increase the intracellular calcium concentration in the presence of 4.0 mM calcium chloride. As the ratio increases, normally elongated cells (rod) are converted into a form (rnd) without sarcomeric organization.

change may not interfere directly with the fluorescence measurements, the round or hypercontracted myocyte is characterized by large superficial blebs that are mechanically fragile and easily burst. Moreover, studies with quin-2-loaded myocytes in suspension (8) and with digitonin-permeabilized myocytes incubated in varying pCa buffers have established that hypercontracture occurs as free calcium approaches 1 μM (9). Thus, there is no assurance that a saturating intracellular calcium concentration has been reached, even in cells that become rounded.

There is ample evidence that hypercontracture is an energy-dependent process in isolated myocytes, whereas the conversion of a normally elongated cell to a "square" but striated form denotes a drastic decline in cellular ATP (10). It has also been established that an ATP-depleted myocyte cannot hypercontract without restoration of a minimal level of cytosolic ATP (11). Figure 2 illustrates the response of ATP-depleted myocytes to a calcium ionophore. In this experiment, the myocytes were superfused with a glucose-free buffer containing 3.3 mM amytal to inhibit NADH dehydrogenase and 2 uM CCCP to collapse the mitochondrial membrane potential and stimulate ATPase activity. After the cells assumed a square shape indicative of ATP depletion, the addition of ionomycin produced a rapid increase in free calcium whereas the removal of extracellular calcium caused a rapid decline. Squared myocytes that leak fura-2 (<20%), as noted

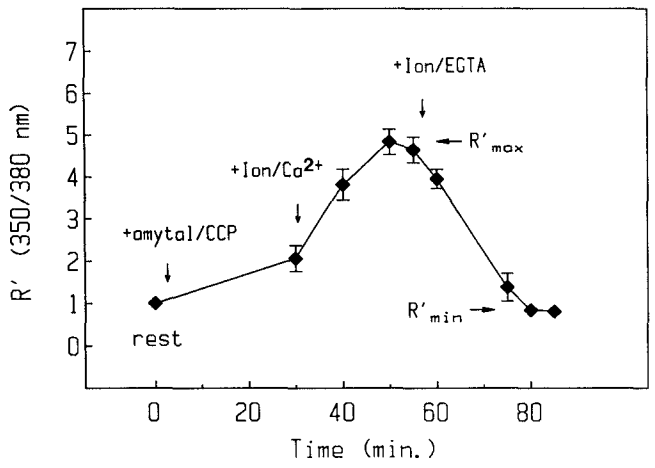

Figure 2. Determination of in vivo R'_{max} and R'_{min} in energy depleted myocytes treated with a calcium ionophore (ION). Cells became ATP depleted with 10 min. subsequent to amytal/CCP addition. R'_{max} and R'_{min} were determined after ION/saturating calcium or ION/EGTA, respectively. These new extremes for the fura-2 dye in vivo are used by the Graph PAD program to construct the normalized calibration plot (dashed line in Figure 1).

by a decrease in fluorescence intensity, are not used in the calibration paradigm. Table 2 summarizes the results of an experiment where the in vivo calibration R'_{max} and R'_{min} values were compared to those obtained for the suspending medium after an aliquot of the stock myocyte suspension was lysed with digitonin. Agreement between the two methods was quite acceptable, especially in view of the variability among individual cells in a single suspension. It should be noted that the digitonin-insoluble residue was removed by centrifugation in these experiments, and in the absence of fura-2 loading, there was no appreciable fluorescence in this fraction. These data, when compared to those in Figure 1, support the concept that the discrepancy between in vitro calibrations with the fura-2

Table 2. R'_{max} and R'_{min} of intact cells and supernate from digitonin lysed preparations

	intact	digitonin lysed
R'_{max}	5.24 +/- 0.90 (11)	5.49 +/- 0.66 (3)
R'_{min}	0.68 +/- 0.07 (5)	0.70 +/- 0.03 (3)

Multiple determinations of R'_{max} and R'_{min} from intact cells and from preparations of digitonin lysed myocytes. Data are expressed as Mean +/- SE; the number of cells/preparations is indicated in parentheses for the intact/lysed cells respectively.

Table 3. Examples of calculated $[Ca^{2+}]i$ using different pCa values for the presumed saturated Ca level of a given R'_{max}

	pCa presumed	
R'_{max} = 5.81 +/- 0.35	4.0	4.5
R'_{min} = 0.70 +/- 0.02	8.0	8.0
	$[Ca^{2+}]i$ (nM)	
R'_{rest} = 1.13 +/- 0.03	112 +/- 14	107 +/- 5
R'_{square} = 1.63 +/- 0.15	263 +/- 53	251 +/- 65

R'_{rest} and R'_{square} are ratios prior to and 15 min. after ATP depletion respectively (n=8).

free acid and *in vivo* R'_{max} and R'_{min} values are caused by calcium-insensitive fluorescent species (4).

The R'_{max} and R'_{min} values determined as shown in Figure 2 were used to create a calibration curve unique to the cells in a given experiment. We replaced the Rmax and Rmin values obtained with the free acid with those from the cells and curve fit a new calibration curve to these values using the Graph PAD program. The Hill coefficient and mid-point (EC50) of the *in vivo* curve were set equal to the *in vitro* curve based on the assumption that the acid form of fura-2 behaves the same in the cell as it does in an aqueous, protein-free solution (12). Finally, the ratio values for a given experiment were converted by the computer directly into pCa values with available sub-routines. We routinely assume that our *in vivo* R'_{max} is equal to pCa of 4.0 and R'_{min} is 8.0. As shown in Table 3, a 0.5 pCa unit error in our assumed R'_{max} would have only a minimal effect on calculated intracellular pCa.

In summary, we have developed a new method for calibrating the Ca^{2+}-sensitive fura-2 fluorescence signal in an excitable cell population. An important feature is the use of deenergizing conditions to eliminate energy-dependent Ca^{2+} extrusion and sequestering reactions, which seem to oppose the effects of the calcium ionophores, ionomycin and Bromo-A23187, on intracellular pCa. In isolated myocytes, ATP depletion also prevents the morphologic changes that can interfere with successful calibration. By

using a computer-assisted graphic method, we are able to correct for the presence of partially hydrolyzed dye intermediates and avoid the K_d assumptions associated with the arithmetic calculation of pCa from ratio values. This technique should be applicable to other cell types that accumulate dye intermediates or counter ionophore-induced Ca^{2+} fluctuations by strong homeostatic reactions.

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