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A comparative assessment of fluo Ca²⁺ indicators in rat ventricular myocytes

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Summary

The fluo family of indicators is frequently used in studying Ca²⁺ physiology; however, choosing which fluo indicator to use is not obvious. Indicator properties are typically determined in well-defined aqueous solutions. Inside cells, however, the properties can change markedly. We have characterized each of three fluo variants (fluo-2MA, fluo-3 and fluo-4) in two forms—the acetoxymethyl (AM) ester and the K⁺ salt. We loaded indicators into rat ventricular myocytes and used confocal microscopy to monitor depolarization-induced fluorescence changes and fractional shortening. Myocytes loaded with the indicator AM esters showed significantly different Ca²⁺ transients and fractional shortening kinetics. Loading the K⁺ salts via whole-cell patch-pipette eliminated differences between fluo-3 and fluo-4, but not fluo-2. Cells loaded with different indicator AM esters showed different staining patterns—suggesting differential loading into organelles. Ca²⁺ dissociation constants ($K_{d,Ca}$), measured in protein-rich buffers mimicking the cytosol were significantly higher than values determined in simple buffers. This increase in $K_{d,Ca}$ (decrease in Ca²⁺ affinity) was greatest for fluo-3 and fluo-4, and least for fluo-2. We conclude that the structurally-similar fluo variants differ with respect to cellular loading, subcellular compartmentalization, and intracellular Ca²⁺ affinity. Therefore, judicious choice of fluo indicator and loading procedure is advisable when designing experiments.

1. Introduction

Transient increases in cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) are essential in a variety of cellular processes ranging from fertilization to muscle contraction. Substantial research and development have focused on the creation of fluorescent indicators that can quantify [Ca²⁺]_i without disrupting normal physiology. The ideal nonratiometric fluorescent Ca²⁺ indicator should show maximal change in quantum efficiency upon binding Ca²⁺ in the normal physiological range [1].* Although *in vitro* measurement of the Ca²⁺ dissociation constant (K_d) of fluorescent indicators can serve as an index of indicator performance, the behavior

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*For a *nonratiometric* indicator, binding of Ca²⁺ causes a change in the amount of fluorescence emitted by the indicator, but does not change the *shapes* of the excitation and emission spectra. That is, upon binding Ca²⁺, a nonratiometric indicator changes its quantum efficiency of fluorescence but its optimal wavelengths of excitation and emission are essentially unchanged. In contrast, for a *ratiometric* indicator, binding of Ca²⁺ causes a change in the shape of the excitation and/or emission spectrum, as well as a change in the amount of fluorescence. That is, upon binding Ca²⁺, a ratiometric indicator changes its fluorescence quantum efficiency, as well as the optimal wavelength of excitation and/or emission [1].

of fluorescent Ca^{2+} indicators can change markedly when loaded into living cells. Without proper characterization of fluorescent indicator behavior in live-cell experiments, potential disruptive effects of the indicator on cellular physiology could be overlooked.

The cellular environment can alter the fluorescent indicators' ability to monitor $[\text{Ca}^{2+}]_i$ in several ways. Many Ca^{2+} indicators bind to proteins in the cytoplasm, leading to changes in their spectra and K_d . Some studies have suggested that as much as 85% of the cytosolic Ca^{2+} indicator is protein-bound, and could increase the K_d by up to 5-fold [2, 3]. Another cause for changes in indicator behavior in the cellular environment can be unintentional loading of the indicator into the wrong compartment (e.g. in non-cytosolic compartments when cytosolic $[\text{Ca}^{2+}]_i$ is sought). This is typically observed when the indicator loading into cells is achieved by using the membrane-permeant acetoxymethyl (AM) ester form of the indicator. In cardiac myocytes loaded with the AM ester form of the indicator, for example, up to 50% of the fluorescent Ca^{2+} indicator can be found within mitochondria. This depends critically on the indicator and its physical and chemical properties. The relative importance of compartmentalization and protein-dependent K_d shift seem to depend on the specific fluorescent Ca^{2+} indicator chosen and on the type of cell under investigation.

In cardiac research, the fluo series of indicators are often favored; these have fluorescent moieties that are fluorescein analogues, with excitation and emission maxima in the visible wavelength range and relatively high fluorescence quantum efficiency. The fluo indicators share a common architecture: a BAPTA-like Ca^{2+} -binding site linked covalently to a xanthene moiety to generate a fluorescein-like fluorophore (see figure 1). Improvements in the fluo series of Ca^{2+} indicators have increased the signal-to-noise ratio, enabling more reliable quantitative $[\text{Ca}^{2+}]$ measurements. The improved fluo derivatives show increased cellular loading efficiency, reduced pH sensitivity, and excitation maxima that better match the emission wavelengths of common lasers. Nevertheless, the extent to which these indicators interfere with native intracellular Ca^{2+} homeostasis has not been systematically characterized. Here, we have examined three different fluo derivatives (fluo-2 medium affinity (fluo-2MA, also known as fluo-8), fluo-3 and fluo-4) in freshly isolated rat ventricular myocytes.

2. Material and methods

2.1 Ventricular myocyte isolation

Single ventricular myocytes were obtained from adult rat hearts by enzymatic dissociation. All animal protocols were approved by the Animal Use and Care Committee of the University of Maryland School of Medicine. Animals were killed by intraperitoneal injection of pentobarbitol (300 mg/kg). Hearts were excised and perfused through the coronary arteries on a Langendorff system with a Ca^{2+} -free physiological saline followed by a Ca^{2+} /enzyme-containing saline mixture. Single isolated cells were separated by mechanical agitation and cellular debris was removed by filtration through a nylon mesh with 200- μm porosity [4]. Fine cellular debris was minimized as healthy myocytes were allowed to sediment by gravity, and supernatant solution was removed, at the end of successive stepwise increases of $[\text{Ca}^{2+}]$ to reach 1.8 mM.

2.2 Confocal microscopy and cell loading

Fluo indicators were loaded into ventricular myocytes in one of two ways: 1) incubation with 2.5 μM of the AM ester form for 15 min followed by 10 min of additional incubation in the absence of the AM ester to allow intracellular de-esterification; 2) introducing the K^+ salt of the indicator through a whole-cell pipette. Cells were excited with 488-nm light from an argon ion laser and the measured fluorescence (>505 nm) was imaged using a laser-scanning confocal microscope (LSM 510 NLO, Zeiss, Jena, Germany). Loading of

indicators (both methods) and experimental recording were all performed at room temperature.

In order to minimize the effect of potential variations in Ca^{2+} transients from preparation to preparation, all three indicators were loaded into myocytes isolated from the same heart, whenever possible. Typically, successive 1-ml aliquots of cell suspension (at 15,000–30,000 cell/ml, by hemocytometer) were loaded and experimented on for the same amount of time. In order to minimize potential variation due to differences in elapsed time since cell isolation, indicator loading sequence was circularly permuted for successive preparations of cells (e.g., preparation 1: fluo-4, fluo-3, then fluo-2; preparation 2: fluo-3, -2, then -4; preparation 3: fluo-2, -4, then -3). In patch-clamp experiments, sequentially loading was not always possible. However, there were no isolation-dependent differences in cell health between groups as judged by action potential duration (defined here as elapsed time between 10% rise and 90% decay, APD₉₀; 38.6 ± 2.0 ms for fluo-4-loaded cells, 39.2 ± 1.6 ms for fluo-3, and 37.0 ± 1.0 ms for fluo-2; $p > 0.25$ for all). In non-patched cells, differences in action potential duration could not be assessed, but planar cell areas were similar in each group ($2,560 \pm 63 \mu\text{m}^2$ for fluo-4, $2,500 \pm 99 \mu\text{m}^2$ for fluo-3, $2,530 \pm 130 \mu\text{m}^2$ for fluo-2; $p > 0.5$ for all).

2.3 Ca^{2+} transient activation and fractional shortening measurements

Myocytes were stimulated by either field stimulation (25–30 V, 2.0 ms) through platinum electrodes (4.5 mm separation) or by current injection (2 nA, 2.0 ms) using the whole-cell current clamp configuration of the patch clamp technique. Patch pipettes (1.5–2.5 M Ω) were fabricated from borosilicate glass (World Precision Instruments, Sarasota, FL) on a Flaming-Brown P97 micropipette puller (Sutter Instrument Co., Novato, CA). Voltage and current control was accomplished using an Axopatch 200B amplifier (Molecular Devices, Union City, CA). Data were digitized at 5 kHz (Axon 3422 digitizer, Molecular Devices), smoothed using a tunable active filter (Frequency Devices), and acquired on a PC using pClamp 8 software (Molecular Devices).

Fractional shortening profiles were measured offline using fluorescence line-scan images; the transmitted-light line-scan images were used for verification. Briefly, transmitted and fluorescence recordings were compared; experiments where the edge of myocytes lifted out of the focus plane in the fluorescence images were eliminated. Fluorescence images were then smoothed and processed to remove all fluorescence information except background to yield black and white line-scan images that display the longitudinal edges of the myocytes plotted against time. Length—vs-time profiles were taken and normalized using measurements of resting length and maximum shortening during the first contraction.

2.4 Permeabilization, dye concentration and in vitro K_d measurement

In some experiments cells were permeabilized with 0.007% saponin for 1 min. Dye concentration was measured in cardiac myocytes and Jurkat cells (supplemental Figure 3). Briefly, a known number of cells (assessed by hemocytometer) were loaded with AM esters, washed with HBSS and lysed with digitonin (30 μM). After cellular debris was removed by centrifugation, indicator concentration was measured on a spectrofluorometer using the standard-addition method. Cellular dye content was estimated through volumetric calculation using average myocyte volume of 36.8 pL/cell and cytosolic volume fraction of 0.65 [5] and average Jurkat volume of 0.765 pL/cell [6]. *In vitro* K_d was determined using various solutions of known $[\text{Ca}^{2+}]$, created by mixing 4 mM EGTA and 4 mM Ca-EGTA in various ratios [7, 8].

2.5 Solutions, drugs

Experiments were performed in normal Tyrode's solution containing (in mM): 140 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, and 10 glucose at pH 7.4. Pipette solution for current clamp experiments contained (in mM): 120 K-aspartate, 8 KCl, 10 NaCl, 5 MgATP, 0.3 NaGTP, 10 HEPES and 0.05 indicator (K⁺ salt) at pH 7.2. Solution for permeabilization experiments contained (in mM): 100 K-aspartate, 20 KCl, 3 MgATP, 0.5 MgCl₂, 0.5 EGTA, 10 phosphocreatine, 20 HEPES, 5 U/mL creatine phosphokinase, 1.5% PVP at pH 7.2. Probenecid, caffeine, thapsigargin, and 6-carboxyfluorescein diacetate were purchased from Sigma-Aldrich Co. Both salt and AM forms of fluo-3 and fluo-4, and Oregon Green 488 BAPTA-1 K⁺ salt were purchased from Invitrogen (Carlsbad, CA); salt and AM forms of fluo-2MA and Fluo-2 Mg were purchased from Teflabs (Austin, TX).

3. Results

3.1 Ca²⁺ and fractional shortening measurements using acetoxymethyl (AM) esters of fluo indicators

To compare the performance of the three fluo indicators, freshly isolated rat ventricular myocytes were examined after loading with the individual AM indicators (2.5 μM, with 0.0125% Pluronic F127 and 0.125% DMSO final) for 15 minutes followed by 10 minutes of de-esterification. Myocytes were then paced by field stimulation at three different frequencies (0.5, 1, and 2 Hz; for 12 seconds), while fluorescence change along the longitudinal axis of the myocytes was monitored by line-scan confocal microscopy. Fractional shortening was also concurrently monitored by edge detection using both transmission and fluorescence images (see Methods).

All fluo indicators were able to report [Ca²⁺]_i transients (CaT) accompanied by fractional shortening (FS) during field stimulations (Figure 2). The intensity of the diastolic (basal) fluorescence was significantly higher in fluo-4 AM loaded myocytes than in fluo-3 AM loaded myocytes ($p < 0.005$, $n = 28$). Fluo-2MA AM loaded myocytes showed a brightness intermediary to fluo-4 and fluo-3 ($p > 0.5$ when compared to fluo-3 and fluo-4, $n = 29$). Quantitative analysis of Ca²⁺ sparks showed that the basal frequency, rise time, full-width at half maximum (FWHM) and decay time were not significantly different among the three fluo indicators (see supplemental data). The most striking differences between the indicators were observed during the first CaT after a prolonged (>60s) diastolic period when the SR has attained a higher Ca²⁺ content, and were in the time required for CaT to decay to baseline (Figure 2D, left). Averaged fluorescence profiles of the CaTs in a train were used to determine the t_{90-10} , the time required for the CaT to decay from 90% to 10% of peak amplitude. The t_{90-10} was significantly shorter in fluo-3 AM loaded cells than in fluo-4 AM loaded cells (415 ± 24 ms, $n = 12$, vs. 599 ± 42 ms, $n = 15$; $p < 0.001$), and dramatically shorter when compared with t_{90-10} in fluo-2MA AM loaded cells (777 ± 40 , $n = 8$, $p < 0.0001$). Examining the last CaT in the train, when SR release is in steady state (i.e., when Ca²⁺ transients are of constant amplitude), showed that CaTs reported by fluo-3 decayed faster even after steady state was reached— t_{90-10} values were 426 ± 24 ms (fluo-3, $n = 12$), 631 ± 29 (fluo-4, $n = 15$), and 682 ± 40 (fluo-2MA, $n = 8$), with $p < 0.0001$ for both comparisons (Figure 2D, right). A consequence of the slower CaT recovery in fluo-2MA and fluo-4 loaded myocytes was an elevation of the fluorescence baseline at higher stimulation rates; notably, this shift was not observed with fluo-3 (at 1 Hz, Figure 4D). In contrast to the CaT decay times, CaT rise times were not different between indicators throughout the entire field stimulation train (Figure 4C, $p > 0.5$ for all). The indicator responses differed in one additional respect: the first CaT in a train was larger in myocytes loaded with fluo-2MA AM and fluo-4 AM than in fluo-3 AM loaded myocytes—peak $\Delta F/F_0$ values were 12.2 ± 0.6 for fluo-2MA and 11.6 ± 0.8 for fluo-4, compared with 9.4 ± 0.5

for fluo-3 ($p < 0.005$ and $p < 0.05$ respectively); this difference was not apparent for subsequent CaTs in the train ($p > 0.1$, Figure 4B).

In addition to effects on CaTs, the three indicators also had different effects on fractional shortening (FS). Alterations in FS kinetics are best observed by examining the normalized averaged traces of contractions evoked by individual field stimulation pulses. Figure 2E compares the normalized averaged FS transients of the first and last field stimulations (at 0.5 Hz) from myocytes loaded with the AM esters of the fluo indicators. Strikingly, fluo-2MA AM loaded myocytes showed significantly prolonged FS profiles than myocytes loaded with fluo-3 AM or fluo-4 AM: full widths at 25% of maximum were 586 ± 34 ms, (fluo-2MA, $n = 8$), 269 ± 14 ms, (fluo-3, $n = 13$) and 285 ± 14 ms (fluo-4, $n = 13$) ($p < 0.0001$). This difference was observed even at a steady state (Figure 2E & 4E). The prolonged FS in fluo-2MA AM loaded myocytes was associated with a slower time-to-peak (382 ± 11 ms vs. 217 ± 10 ms for fluo-3, $p < 0.0001$) and slower decay (t_{90-10} of 223 ± 13 ms vs. 138 ± 18 ms for fluo-3, $p < 0.005$). Relative to fluo-3 AM loaded myocytes, cells loaded with fluo-4 AM also displayed slower FS decay ($t_{90-10} = 229 \pm 17$ ms, $p < 0.005$). The strikingly apparent difference in the CaT and FS between fluo indicators was surprising. To identify possible mechanisms for these differences, experiments were performed where cytosolic indicator concentration was set to a known value.

3.2 CaT and FS measurements using the potassium salts of fluo indicators

Previous studies have identified potentially negative attributes of the AM forms of various indicators, such as the possibility of over-loading the cells with the indicators and undesired subcellular compartmentalization [9]. In order to examine if these processes underlie the differences observed between fluo indicators, further experiments were conducted using the salt forms of the fluo indicators. The K^+ salt of each fluo indicator was loaded into myocytes *via* a patch pipette; $[Ca^{2+}]_i$ and fractional shortening were recorded during action potentials induced by current injection.

Figure 3 shows representative line-scan recordings of three different myocytes loaded respectively with the K^+ salt forms of the three fluo indicators, along with normalized averaged traces of the first and last CaT and FS. Suprathreshold current injection (2 nA) triggered a Ca^{2+} transient and a corresponding shortening. Similar to the results obtained with the AM forms, fluo-3 again showed the lowest basal and peak fluorescence, while fluo-2MA and fluo-4 were similarly brighter. Using the K^+ salt form of the indicators eliminated many, but not all, of the differences observed with use of the various AM esters. Averaged normalized traces from myocytes loaded with fluo-3 K^+ and fluo-4 K^+ were nearly indistinguishable (Figure 3D&E) and the kinetic parameters of both the CaT and FS were not significantly different (Figure 4). CaT and FS kinetics were faster in fluo-2MA K^+ loaded myocytes than in fluo-2MA AM loaded myocytes, but were still significantly slower than those in myocytes loaded with fluo-3 K^+ or fluo-4 K^+ . Figure 4 summarizes the kinetic parameters for all of the fluo indicators tested in cells stimulated at 0.5 Hz. Similar results were also found at 1 and 2 Hz (data not shown). These data suggest that Ca^{2+} transient kinetics are more perturbed when cells were AM-loaded than when cells were salt-loaded, with fluo-3 AM causing the least perturbation. The observation that using the K^+ salt form can eliminate the differences between fluo-3 and fluo-4 but not fluo-2MA, suggests that each of these indicators has different intrinsic characteristics that can delay normal Ca^{2+} cycling and that such effects may or may not depend on loading method.

3.3 Sub-cellular localization of fluo indicators

The most important characteristic of the AM ester of an indicator is its ability to enter the cell by crossing the cell membrane. Hydrolysis of the AM ester by cellular esterases

liberates the salt form, which, being membrane-impermeant, is trapped and accumulated within the cell. Esterase activity is not exclusively present in the cytosol, however, but can be expressed in other subcellular compartments as well. Therefore, incubation with the AM ester not only loads indicator into the cytosol, but can result in loading of the indicator into various organelles. This behavior has been used to advantage to bias loading of fluo-5N AM into the SR and rhod-2 AM into mitochondria. Thus, although the dyes tested here are considered “cytosolic” indicators, there is a high likelihood that the dye is also compartmentalized inside organelles. Differing extents of compartmentalization may be an explanation for differences observed in the Ca^{2+} and contractile transients reported by the various AM indicator tested here. Therefore, high resolution confocal microscopy was used to assess compartmentalization.

Since the indicators are Ca^{2+} -sensitive, with K_d 's comparable to the values of cytosolic $[\text{Ca}^{2+}]$, it stands to reason that indicator compartmentalization is best observed through identification of the SR. The high Ca^{2+} content of the SR implies that $[\text{Ca}^{2+}] \gg K_d$; this ensures that the vast majority of indicator molecules compartmentalized in the SR are Ca^{2+} -bound and thus contribute maximal fluorescence intensity. Sixteen 512×512 bit images were averaged and used to identify SR staining patterns in ventricular myocytes loaded with the individual fluo-AM esters exactly as for transient recordings. Figure 5 shows representative images of the staining pattern observed for the three fluo AM indicators. The SR pattern is very apparent in the fluo-4 AM loaded myocytes but less discernible in fluo-3 AM loaded myocytes (Figure 5A). The SR staining pattern was essentially absent in myocytes loaded with salt versions of indicators (Figure 6). To examine SR compartmentalization more closely, regions of interest were selected and fluorescent intensities were averaged in the transverse direction and plotted against positions along the longitudinal axis (Figure 5B). In the resulting fluorescence profiles the average peak-to-peak distance was $1.80 \pm 0.02 \mu\text{m}$ (for fluo-4, $n=11$), consistent with SR periodicity, and did not vary with the indicator used ($1.82 \pm 0.01 \mu\text{m}$ for fluo-3, $n=11$ and $1.83 \pm 0.02 \mu\text{m}$ for fluo-2MA, $n=14$ both, $p > 0.1$ for all). The average intensity difference from peak to valley was significantly different between most indicators ($\Delta F/F_0$ values of 0.135 ± 0.007 (fluo-4 AM, $n = 11$), 0.114 ± 0.007 (fluo-3 AM, $n = 11$), and 0.100 ± 0.008 (fluo-2MA AM, $n = 14$); Figure 5D). The peak to valley intensity fluctuations were abolished when AM-loaded cells were depleted of SR Ca^{2+} by treatment with thapsigargin in combination with caffeine (Figure 6). We also compared indicator fluorescence intensity in the nuclear region to that outside the nucleus: in resting myocytes, the nuclear/non-nuclear intensity ratio values were 1.30 ± 0.11 (fluo-4 AM; $n=14$), 1.52 ± 0.15 (fluo-3 AM; $n=14$), and 1.13 ± 0.11 (fluo-2MA AM; $n=14$); these results are summarized in Figure 5E.

When myocytes loaded with fluo-4 AM were permeabilized with saponin and the cytosol was displaced with intracellular solution, the SR staining pattern could still be observed (Figure 7). Application of caffeine to deplete SR Ca^{2+} in the same cell attenuated the SR pattern, while the pattern was restored upon caffeine washout (Figure 7). Our results suggest that loading with AM esters leads to significant organellar compartmentalization of the indicator. Compartmentalization seems to be greater with fluo-4 AM when compared to fluo-3 AM; this difference could underlie the differences seen in the kinetics of the Ca^{2+} and contractile transients observed between fluo-3 AM and fluo-4 AM. Notably, however, our findings suggest that compartmentalization cannot account for the aberrantly long calcium and contractile transients recorded in myocytes loaded with fluo-2MA AM.

3.4 Dose-dependent effects of fluo-2MA on CaT and FS

Dose-response experiments were conducted to insure that fluo-2MA was directly responsible for slowing of the CaT and FS. Decreasing the concentration of fluo-2MA AM from $2.5 \mu\text{M}$ to $0.75 \mu\text{M}$ accelerated CaT and FS kinetics significantly (Figure 8A&B).

Further reducing the concentration of fluo-2MA AM to 0.25 μM resulted in CaT and FS that were very similar in kinetics to those recorded in fluo-3 AM loaded myocytes. Reducing fluo-2MA AM concentrations was also associated with decreased transient peak and basal fluorescence intensities. In order to acquire reasonable signal to noise and to keep the laser exposure equal in all experiments, using low concentration of fluo-2MA required opening the detector pinhole. Even with the pinhole opened, the peak ($\Delta F/F_0$) of the CaT recorded with low concentrations of fluo-2MA was significantly smaller than the CaT peak with 2.5 μM . These results suggest that fluo-2MA causes a dose-dependent slowing of Ca^{2+} -mediated contractions.

3.5 Cytosolic indicator loading achieved through AM esters

We can infer from the chemical structures that fluo-2MA is the least hydrophobic of the three indicators we tested. Since indicator loading through incubation with an AM ester depends critically on the aqueous solubility of the AM ester, we conjectured that the lower hydrophobicity of fluo-2MA AM could cause greater intracellular accumulation of fluo-2MA. Therefore, experiments were performed to quantify cytosolic concentrations of the indicators achieved through AM loading. A known number of cardiac myocytes were loaded with the various AM indicators for 15 min followed by 15 min of de-esterification in medium containing no AM ester. Sufficient digitonin was then added to permeabilize plasma membranes and release cytosolic indicator. Cell debris was removed by centrifugation, and indicator concentration in the clear lysate was measured on a spectrofluorometer using the standard addition method. The results clearly show that after AM loading, fluo-2MA reaches a higher cytosolic concentration than fluo-3 and fluo-4 (Figure 8D). These results were reconfirmed in Jurkat cells, whose homogeneity and known intracellular volume make them ideal for loading studies. In Jurkat lymphocytes, increasing the extracellular AM concentration from 2.5 μM to 10 μM further increased cytosolic loading of fluo-2MA, but not of fluo-3 and fluo-4 (supplemental Figure 3). Greater cytosolic accumulation of fluo-2MA after AM loading can partly explain the larger perturbation of CaT and FS kinetics caused by fluo-2MA. This is not the whole explanation, however, because differences in observed kinetics were not completely eliminated even when myocytes were loaded with the salt forms of the indicators at identical concentrations through the whole-cell patch pipette. Although it is remotely conceivable that fluo-3 and fluo-4 are more efficiently extruded than fluo-2MA by organic anion transporters and thus perturb CaT and FS less in the whole-cell configuration, this possibility was ruled out when the whole-cell experiments were conducted in the presence of probenecid to block organic anion transporters [10]. The kinetic differences persisted in the presence of probenecid (data not shown).

3.6 Magnesium sensitive fluo-2 FS measurements

One possible explanation for the effects of fluo-2MA on CaT and FS is that the molecule itself physically interacts, and thus inhibits, the contractile machinery. To investigate this, experiments were performed using fluo-2 Mg, a fluorescent dye that has the same fluorophore as fluo-2MA but whose ability to bind Ca^{2+} is weaker by ~ 2 orders of magnitude. Fluo-2 Mg also has $K_{d,\text{Mg}} = 1.9 \text{ mM}$. Thus, Fluo-2 Mg should minimally perturb intracellular Ca^{2+} signaling and thus allow any fluorophore-dependent effects to be examined. When myocytes loaded with Fluo-2 Mg AM were paced with field stimulation, the resultant FS were significantly faster than those recorded in fluo-2MA AM loaded myocytes ($p < 0.0001$ comparing the full width at 25 % FS maximum for the first pulse and $p < 0.001$ for the steady state pulse). Indeed, the FS were nearly kinetically indistinguishable from those recorded in fluo-3 AM loaded myocytes ($p > 0.1$ for all kinetic parameters measured and for both first and steady-state pulses, except full width at 25% of maximum on the first pulse only where $p < 0.001$). These results suggest that the slowing of the CaT

and FS caused by fluo-2MA is a consequence of the Ca^{2+} -chelating ability of fluo-2MA. This surmise can only be true if the Ca^{2+} affinity of fluo-2MA differs markedly from that of fluo-3 and fluo-4.

3.7 Estimates of fluo indicators' intracellular Ca^{2+} affinity

The *in vitro* Ca^{2+} affinities of the three indicators are similar: the K_d being 390, 390, and 350 nM for fluo-2MA, fluo-3 and fluo-4, respectively. Remarkably, previous studies have shown that loading fluo-3 and fluo-4 into cells weakens their Ca^{2+} affinity significantly (K_d of 800 and 1,400 nM for fluo-3 and fluo-4, respectively). We therefore asked if fluo-2MA's effects on CaT and FS could be the result of its Ca^{2+} affinity becoming significantly different from those of fluo-3 and fluo-4 in the cytosol. To examine this possibility, the Ca^{2+} affinity of each indicator was measured in physiological buffer and in a simulated intracellular milieu (physiological solution containing high concentrations of proteins). The presence of protein caused a rightward shift in the K_d curves for all of three fluo indicators (Figure 9AD). As hypothesized, the protein-dependent shift in the K_d was modest for fluo-2MA (from 432 ± 16 nM to 610 ± 120 nM, $p > 0.1$, $n = 4$) but large for fluo-3 (from 447 ± 20 nM to 1130 ± 160 nM, $p < 0.005$, $n = 4$) and fluo-4 (from 418 ± 18 nM to 891 ± 30 nM, $p < 0.0001$, $n = 4$). These results suggest that the *in vivo* K_d for fluo-2MA is much lower than that of fluo-3. One may infer that the rate of Ca^{2+} dissociation from the indicator is slower for fluo-2MA than fluo-3.

To confirm that fluo-2MA effects on CaT and FS are due to a lower *in vivo* K_d , CaT and FS were measure in myocytes loaded with a Ca^{2+} indicator whose reported *in vivo* K_d is close to the K_d of fluo-2MA in the presence of high protein concentrations. Oregon Green 488 BAPTA-1 (OGB-1) is also a BAPTA-based indicator with a reported *in vivo* K_d of 480 nM [3]. When myocytes loaded with OGB-1 through the patch pipette were stimulated electrically, the observed CaT and FS profiles were very similar to those in fluo-2MA loaded cells (Figure 8E). These results, taken together with the foregoing, suggest that fluo-2MA has a lower *in vivo* K_d than fluo-3 and fluo-4, and thus releases Ca^{2+} at a slower rate leading to prolongation of the CaT and FS.

4. Discussion

The three fluo indicators tested are remarkably similar in structure (Figure 1), and have almost identical *in vitro* K_d s. Inside cardiac myocytes, however, these indicators can perturb CaT and FS to different degrees. These indicators apparently disrupt CaT and FS by different mechanisms. Fluo-4 AM appears to disturb Ca^{2+} handling due to its relatively higher tendency to compartmentalize, while fluo-2MA seems to exert its effect by retaining a relatively high Ca^{2+} affinity inside the myocyte. How these perturbations could lead to experimental errors is discussed below.

4.1 AM loading

Fluorescent Ca^{2+} indicators anionic polycarboxylates cannot readily cross biomembranes. The carboxylates can be masked as AM esters which, being membrane-permeant, can enter cells readily. Once inside, cellular esterases cleave the labile AM esters to regenerate the polycarboxylate form which, being membrane-impermeant, is trapped and accumulated inside the cell [11]. Although fluorescent indicators are all loaded into the cytosol by the same basic mechanism, the rates of loading can vary significantly. The loading rate appears to depend more on the molecular weight (MW) of the indicator (inversely proportional), with little dependence on number of AM esters per indicator molecule [9]. In this study, we compared the cellular loading of three fluo AM derivatives (Figure 8). Similar to other reports, the fluo derivative with the lowest molecular weight, fluo-2MA AM, was able to

accumulate to significantly higher intracellular concentrations. Interestingly, there was no significant difference in intracellular accumulation between fluo-3 and fluo-4, even though the MW difference between the two is comparable to the difference between fluo-4 and fluo-2MA. These results confirm an earlier suggestion that the loading efficiency depends on the hydrophobicity of the AM form of the indicator. Fluo-2MA, with no methyl group and no halogen substitutes, is much less hydrophobic than fluo-3 and fluo-4. This means fluo-2MA AM can dissolve at higher concentrations in aqueous solutions, allowing a greater flux of AM ester to enter the cell and be hydrolyzed by esterases [9]. This observation suggests that fluo-3 and fluo-4 could have much better loading with the absence of methyl group; however, this modification would lead to a molecule with much lower affinity for Ca^{2+} due to the loss of the electron sharing methyl from the Ca^{2+} chelating group. Similarly, addition of a methyl group to the Ca^{2+} chelating moiety can increase Ca^{2+} affinity as seen with fluo-2 high affinity, where the hydrogen on the Ca^{2+} chelator end is replaced with a methyl (same position as fluo-3 and fluo-4). Although, the actual contribution of different groups to overall hydrophobicity and Ca^{2+} affinity is difficult to predict by structure alone, especially in the intracellular milieu; making studies such as the one presented here appreciably indispensable.

4.2 Compartmentalization of fluorescent indicators

Because it is technically simple and noninvasive, incubation with AM ester has become the most popular method for loading fluorescent indicators into cells. The major drawback of this approach is that AM esters can enter organelles and be de-esterified therein to accumulate indicator within organelles. The majority of studies have suggested that mitochondria are major compartmentalizing organelle in cardiac myocytes [12, 13]. In the current study we have shown that fluo AM derivatives can also compartmentalize into the SR to varying degrees (Figure 5). This was confirmed by removal of the SR staining pattern with the application of drugs that cause SR Ca^{2+} depletion. When SR Ca^{2+} depletion was done in permeabilized myocytes, evidence of mitochondrial compartmentalization also became observable (Figure 7C). Indicator compartmentalization in the SR is not novel; indeed, several studies have made use of indicator loading into the SR to study SR Ca^{2+} dynamics [14–16]. What was surprising was that such a striking SR pattern could be observed after loading with such a low concentration of fluo-4 AM (2.5 μM) for only a short time (15 min) and at room temperature (22°C). Previous studies aiming to take advantage of compartmentalization to study intraorganellar ion dynamics usually biased conditions to favor organellar loading by using higher concentrations of AM esters (5 μM), longer loading times (30 minutes) and increased temperatures (30°C).

In this study, SR compartmentalization was found to be higher in fluo-4 AM loaded myocytes than in myocytes loaded with fluo-2MA and fluo-3 AM. This was somewhat surprising since the greater fluo-2MA AM loading into the cytosol might have led one to expect higher organellar loading as well. Empirically, however, fluo-2MA actually showed the lowest SR compartmentalization. This could be attributed to the higher aqueous solubility of fluo-2MA AM and thus increased access to cytosolic esterases, and/or relative lower transport of the polycarboxylate form into the SR anionic transporters, which have been postulated to mediate compartmentalization in some cell types [10]. Irrespective of the mechanism, fluo-4 compartmentalizes into the SR of rat ventricular myocytes and this can slightly slow CaT and FS; the slowing effect is abolished when fluo-4 is loaded through a patch pipette. Most likely fluo-4 compartmentalization causes an increase in intra-SR Ca^{2+} buffering, which can not only slow the recovery rates of CaT, but also substantially increase the peak of CaT and SR load [17]. Although in these studies SR Ca^{2+} buffering was altered with chelators that had K_{d} s much larger than that of fluo-4 (K_{d} s of 0.2–11 mM), the effects were most pronounced with chelators of higher affinity (0.2 mM). The effect of the presence

of fluo-4 within the SR would be presumably minor, as fluo-4's K_d is below the suspected physiological SR cycling Ca^{2+} range [15]. Even though during normal excitation-contraction coupling, SR-compartmentalized fluo-4 would mostly be in the Ca^{2+} -bound form, its presence could slow intra-SR Ca^{2+} diffusion, leading to a slight delay in SR Ca recovery. From these results and the study by [17], it could be suggested that normal CaT and FS are delayed by the presence of an intra-SR Ca^{2+} indicator such as fluo-5N, although no studies to date have acknowledged this possibility explicitly. Also interesting is the fact that the fluo-4 SR staining pattern could be decreased by the application of caffeine in permeabilized cells or the combination of thapsigargin and caffeine in intact cells, suggesting that SR Ca^{2+} concentrations fall to levels near the *in vivo* K_d of fluo-4 during prolonged (<20 sec) caffeine application. This interpretation is predicated on the assumption that the intra-SR K_d for fluo-4 is similar to the intracellular K_d . This assumption is based on studies that have calibrated intra-SR Ca^{2+} indicator K_d s *in vivo* and *in vitro* at a protein concentration of 50 mg/ml [18] —the same concentration of protein reported in the cytosol and used in this study.

4.3 Dissociation constants and Ca^{2+} buffering

Cardiac myocytes loaded with fluo-2MA either by AM incubation or through a patch pipette displayed prolonged CaT and FS. This study suggests that inside cells, fluo-2MA has a higher Ca^{2+} affinity than fluo-3 and fluo-4, and this causes a slowing of the normal Ca^{2+} release and subsequent contraction. Studies on myocytes have shown that a Ca^{2+} indicator with lower K_d (higher affinity) reports slower CaT kinetics than an indicator with higher K_d [19, 20]. Interestingly in the study on cardiac myocytes, a large difference was also observed in the CaT amplitude and rise time. While the differences in CaT amplitude could partially be explained by *in vitro* calibration parameters, the slowing of CaT time course was attributed to the presence of a higher affinity Ca^{2+} indicator. In this study, both the peak amplitude and rise time of the CaT were not significantly different; this was not surprising because the fluo indicators compared here had similar K_d (~2-fold difference between fluo-2MA and fluo-3; Figure 9), unlike the previous studies where K_d s differed by 250-fold. In fluo-2MA loaded myocytes there was a slowing of the CaT during the decay period but no change in the peak and rise time of CaT and sparks (see supplemental), suggesting that resting $[\text{Ca}^{2+}]_i$, SR Ca^{2+} release and termination occurred normally. This is unlike previous studies that have suggested that increasing Ca^{2+} buffering by addition of a high affinity Ca^{2+} indicator can disrupt such elements of ECC [21–23]. Here it is suggested that fluo-2MA displays slower CaT and FS because it interacts less with native proteins in cardiac myocytes compared to fluo-3 and fluo-4.

4.4 Ca^{2+} indicator-protein interaction

The K_d of fluorescent ion indicators is dependent on a number of environmental factors, including pH, temperature, ionic strength, viscosity, the presence of competing ions, and protein binding. Usually the K_d values measured inside living cells are significantly higher than those measured *in vitro*. The fluo indicators are no exception: in HeLa cells, the K_d s of fluo-3 and fluo-4 both increase from those determined *in vitro* (390 nM to 810 nM and 345 nM to 1000 nM, respectively) [3]. In this study, similar shifts in the K_d s of fluo-3 and fluo-4 were observed when high concentrations of protein were in the simulated intracellular solution. Efforts to determine the *in vivo* K_d s of the indicator were unsuccessful owing to technical limitations. Of the three indicators tested in the simulated intracellular milieu, the K_d of fluo-2MA was the least affected. It was also noticed that nuclei staining was less pronounced in fluo-2MA loaded myocytes (Figure 5A&E). Pores in the nuclear membrane have an exclusion limit of ~40 kD [24, 25]; thus small solutes like Ca^{2+} ions and Ca^{2+} indicators can freely equilibrate between the nucleus and cytosol. In light of this, the increased staining of nuclei by fluo-3 and fluo-4 cannot be due to a nuclear-cytosolic $[\text{Ca}^{2+}]$

gradient, but rather is due to variation of indicator properties (e.g., K_d , quantum efficiencies of the Ca^{2+} -free and -bound forms, etc.) in various organelles [3]. The differences in intra-organelle K_d likely arise from indicator-protein interactions or possible variation in viscosity. In any event, fluo-2MA appears less sensitive to these factors, and may thus be a good indicator for comparing cytosolic and nuclear $[\text{Ca}^{2+}]$.

Successful use of fluorescent indicators requires them to report physiological fluctuations in cytosolic ion concentration without disrupting homeostasis. As a conservative rule of thumb, an indicator can accurately report concentrations that differ from its K_d by a factor of 10. Thus an indicator useful for studying cardiac $[\text{Ca}^{2+}]_i$ fluctuations should have an intracellular K_d closer to 1 μM to minimize slowing of CaT and FS recovery kinetics. While fluo-2MA appears not to be well suited for cardiac research owing to its relatively low intracellular K_d , rapid and efficient loading into cells may recommend its use in other cell types or possibly whole organ studies. Fluo-3 and fluo-4 both have a more desirable intracellular K_d , but compartmentalization of fluo-4 AM can result in a slight slowing of the recovery rate of the CaT and consequently increased baseline at high simulation rates (1Hz). Therefore, if pipette loading is not possible, fluo-3 AM appears to be the best fluo indicator for studying cytosolic Ca^{2+} transients in cardiac myocytes. The comparative advantages and disadvantages of all the indicators tested are summarized in Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Structure of fluo derivatives and molecules with common architecture. The structure of BAPTA, fluorescein and xanthene are shown in panel A; while the shared structure of the fluo analogs are shown in B. It can be appreciated that the indicators basically comprise a BAPTA molecule fused with a fluorescein-like fluorophore. The indicators tested differ only by the absence or presence halogen/methyl substituents at the R'/R location, respectively. [26][†]

[†]The original fluo-2 indicator created by Minta *et al.* [26] (later renamed fluo-2 high-affinity, or fluo-2HA) has R' = H and R = CH₃. Fluo-2MA is also known as fluo-8.

Figure 2.

Ca^{2+} transients and fractional shortenings from AM loaded cardiomyocytes. Typical recording of fluorescence and bright-field information during 0.5 Hz field stimulation in the line-scan mode (longitudinal axis) from the three different AM loaded indicators (A–C). Panel D shows the average normalized Ca^{2+} transient (CaT) profile from the first and last (steady state) pulse of the stimulation train. The averaged normalized fractional shortening (FS) profiles from the first and last pulse are shown in E. The CaT and FS recovery rates differ between the indicators.

Figure 3.

Ca²⁺ transients and fractional shortenings from pipette loaded cardiomyocytes. Recording of fluorescence and bright-field responses during current injections (0.5 Hz) from the three different pipette loaded indicators is shown in A–C. The averaged normalized CaT profile from the first and last pulse of the stimulation train are shown in D. Panel E shows the averaged FS profile from the first and steady state pulse. The differences observed between fluo-3 and fluo-4 with AM loading is not apparent with pipette loading, while fluo-2MA still displays slower kinetics when compared to fluo-3 regardless of loading method.

Figure 4.

Summarized CaT and FS data from AM and pipette loaded myocytes. Graphs show the summarized results extracted from the raw CaT and FS profiles for fluo-4 (white), fluo-3 (light gray) and fluo-2MA (dark gray) in AM (open bars) and pipette (striped bars) loaded myocytes. The major difference observed between indicators was with the CaT recovery, measured as the time needed for the transient to decay from 90% of the peak to 10% (A). Ca^{2+} transient peak amplitude was also measured (B). There were no differences in the rise time of the Ca^{2+} transient between all of the indicators and loading methods (C). Due to the fact that the Ca^{2+} transient took so long to recover in fluo-2MA and fluo-4 AM loaded myocytes, a baseline shift could be observed at stimulation rates ≥ 1 Hz (D), and this difference was eliminated with pipette loading. A similar pattern of slowing kinetics was

also observed in the FS (E&D). In FS data a slowing time to peak was apparent in fluo-2MA loaded myocytes (G), and this was not dependent on loading method. FS rise time, measured as the time for the contraction to go from 25% to 75%, was slower in fluo-2MA and fluo-4 AM loaded myocytes when compared to fluo-3. The rise time difference between fluo-3 and fluo-4 was eliminated by pipette loading, while pipette loading did not remove the difference seen with fluo-2MA. Unpaired t-tests were performed to determine significance (summary of analyses in supplemental Figure 4).

Figure 5.

Staining pattern in AM loaded myocytes. Extent of compartmentalization was measured from multiple averaged high-resolution images of myocytes loaded with the AM form of fluo-2MA, fluo-3 or fluo-4 (A). A periodic staining pattern can be observed in myocytes loaded with fluo-4 AM, this pattern is less apparent in fluo-2MA and fluo-3 AM loaded myocytes. This can be seen better when a fluorescence profile is created (B) from a $25\ \mu\text{m} \times 2\ \mu\text{m}$ region of interest (dotted red box, A) within each myocyte and average peak-to-valley measurement are made (D). The peaks of the fluorescence fluctuations are spaced at $\sim 1.8\ \mu\text{m}$ (C). Extent of nuclei staining was also measured and found to be highest in fluo-3 AM loaded myocytes (E). NS, $p < 0.05$; #, $p < 0.05$; *, $p < 0.005$

Figure 6.

SR pattern is absent in myocytes loaded with the salt form of the indicators. When myocytes are loaded with fluo-4 through the pipette, no significant staining pattern can be observed. Multiple averaged high resolution images were acquired of myocytes loaded with fluo-4 AM or fluo-4 K⁺ salt (A). A periodic staining pattern can be observed in myocytes loaded with fluo-4 AM; this pattern is not as apparent in fluo-4 K⁺ loaded myocytes. This can be seen better when a fluorescence profile is created (B) from a region of interest (dotted red box, A) within each myocyte (and averaged peak-to-valley measurements are made, C). The pattern was diminished when the sarcoplasmic reticulum (SR) is emptied by treatment with 1 μM thapsigargin (Thaps) and 10 mM caffeine (Caff) (C). *, $p < 0.0001$

A. fluo-4 AM loaded, saponin-permeabilized - brightfield

B. fluo-4 AM loaded, saponin-permeabilized - fluorescence

C. During caffeine (10 mM) application

D. 3 min washout

Figure 7.

Plasma membrane permeabilization does not remove SR staining pattern, while emptying the SR Ca^{2+} does. Permeabilizing the sarcolemma with saponin and washing the cytosol does not remove the staining pattern observed in fluo-4 AM loaded myocytes (B). The pattern does fade when the SR Ca^{2+} is depleted by rapid application of caffeine (10 mM, C). When caffeine is applied, other cellular organelles, such as mitochondria, become noticeable (red arrowheads C). Upon caffeine washout, the staining pattern returns (D).

Figure 8.

Fluo-2MA AM loads into cells at higher concentrations and its effects are dose-dependent. Fluo-2MA AM causes a dose-dependent slowing of CaT (A) and FS (B) kinetics at both the first and steady state pulse. Experiments were performed to measure the concentration of indicators that load into myocytes by AM ester incubation. Cardiac myocytes were loaded with various AM esters at 2.5 μM for 15 minutes, followed by de-esterification for 15 minutes. Cells were lysed with digitonin (30 μM) followed by bath sonication (~10–20 s). Lysed suspension was centrifuged at 1520 $\times$ g for 10 min to pellet cell debris and yield clarified lysate. For each lysate the optimal excitation and emission wavelengths were determined. An emission spectrum was acquired at optimal excitation wavelength, and then emission spectra were recorded after additions of known amounts of the K^+ salt of the

appropriate indicator. Fluorescence intensity was plotted against added indicator concentration (C); the x-intercept was then used to calculate the average intracellular indicator concentration achieved through AM ester loading (D). Higher intracellular indicator concentration was attained by incubation with fluo-2MA AM than with fluo-3 or fluo-4 AM.

Figure 9.

The K_d of fluo-2MA is least affected by high concentrations of protein. *In vitro* K_d measurements in the presence and absence of protein (50 mg/ml BSA) were made. The presence of protein (used to mimic the intracellular milieu) significantly increased the K_d s of fluo-3 and fluo-4, while the effect on fluo-2MA's K_d was much less (A–D). A Ca^{2+} indicator that has a reported lower *in situ* K_d (similar to the projected intracellular K_d of fluo-2MA) is Oregon Green 488 BAPTA-1 (OR-Green-B1). When myocytes were pipette loaded with OGB-1 the CaT and FS during current injection was strikingly similar to those recorded in fluo-2MA loaded myocytes (E). NS, $p < 0.1$; #, $p < 0.0001$; *, $p < 0.005$.

Table 1

	Fluo-4 AM	Fluo-3 AM	Fluo-2MA AM	Fluo-4 K ⁺	Fluo-3 K ⁺	Fluo 2MA K ⁺
CaT decay	++	+++	+	+++	+++	++
FS decay	+	+++	+	+++	+++	+
CaT baseline shift	++	+++	+	+++	+++	+++
Absence of SR pattern	+	++	+++	+++	+++	+++
Nuclei/Cytosolic staining	++	+++	+	ND	ND	ND
Dye Loading	+	+	++	NA	NA	NA
K _d saline (nM)	NA	NA	NA	418 ± 18	447 ± 20	432 ± 16
K _d saline + protein (nM)	NA	NA	NA	891 ± 30	1130 ± 160	610 ± 120

+ denotes poor and +++ denotes good, ND denote Not Determined, while NA denotes Not Applicable.