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A comparative assessment of fluo Ca^{2+} indicators in rat ventricular myocytes

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ABSTRACT

The fluo family of indicators is frequently used in studying Ca^{2+} 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 Ca^{2+} 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-2MA. Cells loaded with different indicator AM esters showed different staining patterns—suggesting differential loading into organelles. Ca^{2+} 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 Ca^{2+} affinity) was greatest for fluo-3 and fluo-4, and least for fluo-2MA. We conclude that the structurally-similar fluo variants differ with respect to cellular loading, subcellular compartmentalization, and intracellular Ca^{2+} affinity. Therefore, judicious choice of fluo indicator and loading procedure is advisable when designing experiments.

1. Introduction

Transient increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) 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 $[Ca^{2+}]_i$ without disrupting normal physiology. The ideal nonratiometric fluorescent Ca^{2+} indicator should show maximal change in quantum efficiency upon binding Ca^{2+} in the normal physiological range [1].[1] Although *in vitro* measurement of the Ca^{2+} dissociation constant (K_d) of fluorescent indicators can serve as an index of indicator performance, the behavior 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 $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ 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

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[1] For a *nonratiometric* indicator, binding of Ca^{2+} 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 Ca^{2+}, a nonratiometic 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 Ca^{2+} 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 Ca^{2+}, a ratiometric indicator changes its fluorescence quantum efficiency, as well as the optimal wavelength of excitation and/or emission [1].

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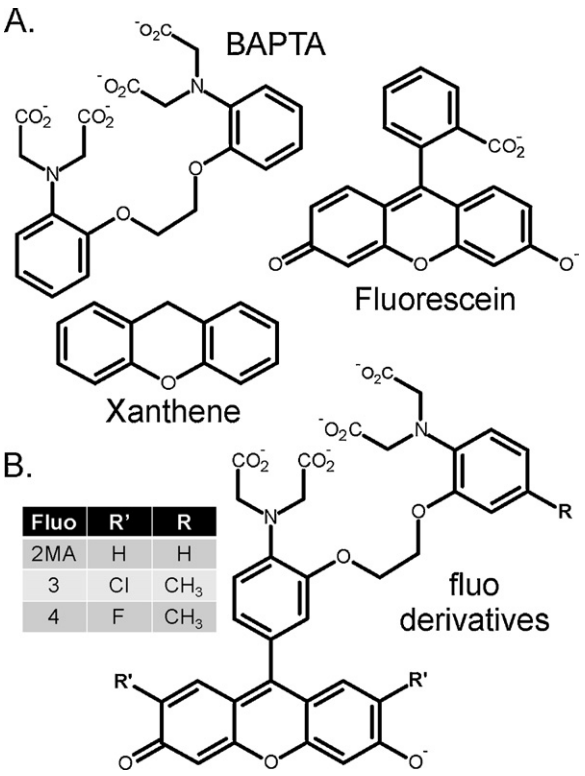

Fig. 1. Structure of fluo derivatives and molecules with common architecture. The structure of BAPTA, fluorescein and xanthene are shown in panel A; while the shared structure of the fluo analogs is shown in B. It can be appreciated that the indicators basically comprise a BAPTA molecule fused with a fluorescein-like fluorophore. The indicators tested differ only by the absence or presence of 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 = CH_3. Fluo-2MA is also known as fluo-8).

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 Fig. 1). Improvements in the fluo series of Ca^{2+} indicators have increased the signal-to-noise ratio, enabling more reliable quantitative [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 [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-2MA; preparation 2: fluo-3, -2MA, then -4; preparation 3: fluo-2MA, -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-2MA; $p > 0.25$ for all). In non-patched cells, differences in action potential duration could not be assessed, but planar cell areas were similar in each group (2560 ± 63 μm^2 for fluo-4, 2500 ± 99 μm^2 for fluo-3, 2530 ± 130 μm^2 for fluo-2MA; $p > 0.5$ 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 MΩ) 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 (See Supplement Fig. 2).