Cell Calcium 61 (2017) 44–49

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Genetically encoded calcium indicators for studying long-term calcium dynamics during apoptosis

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ARTICLE INFO

Article history:
Received 5 October 2016
Received in revised form
31 December 2016
Accepted 31 December 2016
Available online 3 January 2017

Keywords:
Genetically encoded calcium indicator
GCaMP6
Fura-2
Calcium
Inositol
Apoptosis
Staurosporine

ABSTRACT

Intracellular calcium release is essential for regulating almost all cellular functions. Specific spatio-temporal patterns of cytosolic calcium elevations are critical determinants of cell fate in response to pro-apoptotic cellular stressors. As the apoptotic program can take hours or days, measurement of long-term calcium dynamics are essential for understanding the mechanistic role of calcium in apoptotic cell death. Due to the technical limitations of using calcium-sensitive dyes to measure cytosolic calcium little is known about long-term calcium dynamics in living cells after treatment with apoptosis-inducing drugs. Genetically encoded calcium indicators could potentially overcome some of the limitations of calcium-sensitive dyes. Here, we compared the performance of the genetically encoded calcium indicators GCaMP6s and GCaMP6f with the ratiometric dye Fura-2. GCaMP6s performed as well or better than Fura-2 in detecting agonist-induced calcium transients. We then examined the utility of GCaMP6s for continuously measuring apoptotic calcium release over the course of ten hours after treatment with staurosporine. We found that GCaMP6s was suitable for measuring apoptotic calcium release over long time courses and revealed significant heterogeneity in calcium release dynamics in individual cells challenged with staurosporine. Our results suggest GCaMP6s is an excellent indicator for monitoring long-term changes cytosolic calcium during apoptosis.

1. Introduction

Calcium is a second messenger that is widely involved in almost all cellular processes, including orchestrating apoptotic signaling [1–3]. To investigate the spatial and temporal regulation of intracellular calcium signals, many fluorescent calcium indicators have been developed. The first fluorescent calcium indicators were based on the highly selective calcium chelator 1,2-bis(o-aminophenoxy)ethane-*N*,*N*,-*N*',*N*'-tetraacetic acid (BAPTA) [4]. Identification of derivatives that exhibit a shift in excitation wavelength upon binding calcium was advantageous since taking the ratio of fluorescence intensity at each excitation wavelength corrected for differences in dye loading and allowed for more quantitative measurements. Many of such "ratiometric" calcium indicators have been developed, including Fura-2, Indo-1 and Fura Red, each with different affinities for calcium and excitation/emission wavelengths [5–7]. Some of the major limitations of ratiometric indicators include the low temporal resolution resulting from the requirement to monitor at two excitation or emission wavelengths and relatively small dynamic ranges [7]. To resolve these problems, single-wavelength indicators, including fluo-3 and 4, rhod-2 and Oregon Green BAPTA, were developed [8,9]. These indicators show large changes in fluorescence intensity upon binding of calcium and exhibit a wide variety of kinetic and spectral properties.

To study calcium transients in distinct cell types or in subcellular organelles, genetically encoded fluorescent calcium indicators have distinct advantages over other methods. Some of these indicators are based upon conferring calcium sensitivity to green fluorescent protein (GFP) variants using either Förster resonance energy transfer (FRET) pairs or circularly permutated GFP mutants [10–13]. Alternatively, the intrinsically calcium-sensitive bioluminescent protein aequorin is also widely used as a genetically encoded indicator [14]. GCaMPs are among the most widely used GFP-based genetically encoded indicators and consist of circular permutated green fluorescent protein, calmodulin, and a calmodulin binding motif derived from skeletal muscle myosin light chain kinase

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http://dx.doi.org/10.1016/j.ceca.2016.12.010
0143-4160/

(M13 peptide) [12,15]. Once bound to calcium, GCaMP undergoes a conformational change that leads to an increase in fluorescence intensity [12]. There have been several iterations of GCaMP to optimize the sensitivity and kinetics [16–18]. Among the most recent GCaMP derivatives are GCaMP6s, 6m and 6f (for slow, medium and fast kinetics), all of which have greatly improved brightness and larger dynamic ranges compared to their predecessors [19,20].

Given the wide variety of calcium indicators it is important to choose the most suitable indicators based on the magnitude and duration of calcium transients, which depends heavily on the type of cell and the stimuli presented. Many factors, including affinity, kinetics, sensitivity and signal-to-noise ratio contribute to the choice of calcium indicator for a specific study. As an example, high affinity indicators tend to buffer calcium transients and show a slow rise to peak and a prolonged signal. Therefore, to detect rapid calcium transients such as those in neurons, indicators with faster kinetics and lower affinity to calcium are preferred [21] such as afforded by GCaMP6f [20]. However, indicators with higher sensitivity and brighter fluorescence such as GCaMP6s may have more utility in detecting small changes in calcium or in those cases where fast kinetics are not necessary such as during the relatively slow process of apoptotic calcium release [22]. The timing from the initiation of apoptosis to completion spans over several hours [22–27]. Therefore, long-term imaging is required to monitor calcium release during apoptosis. To perform long-term imaging using synthetic calcium dyes, previous studies had to sequentially image multiple coverslips every 30 min to account for photobleaching and artifacts arising from UV illumination of living cells [22,27]. In theory, genetically encoded calcium indicators would overcome most of these limitations and facilitate monitoring of intracellular calcium over protracted time courses.

In this study, we found that GCaMP6s and 6f perform as well as Fura-2 in reporting intracellular calcium elevations induced by histamine stimulation of HeLa cells. The indicator GCaMP6s outperformed Fura-2 in detecting release events induced by low concentrations of agonist. We next used GCaMP6s to monitor staurosporine-induced calcium transients over the course of 10 h. We were able to continuously monitor cytosolic calcium during cell death revealing the kinetics of calcium release and surprising heterogeneity in the responses to staurosporine. Our results support of the utility of GCaMP6s as a general purpose calcium indicator protein with several properties superior to dye-based methodologies, especially for long-term imaging.

2. Experimental methods

2.1. Cell culture and transfection

HeLa cells and HEK293T cells were purchased from ATCC and maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and kept in an incubated atmosphere maintained at 37 °C and 5% CO_2. Cells were plated on glass coverslips and transfected with GCaMP6s and GCaMP6f using lipofectamine 2000 (Invitrogen) following manufacturer's instructions. The total amount of cDNA transfected was 2 μg per 400,000 cells. Experiments were carried out 24 h after transfection. Plasmids pGP-CMV-GCaMP6s and 6f were a gift from Douglas Kim (Addgene plasmid numbers 40753 and 40755).

2.2. Histamine-induced calcium release measurements

HeLa cells were incubated with 5 μM Fura-2 AM (Molecular Probes) in imaging solution (1% BSA, 107 mM NaCl, 20 mM HEPES, 2.5 mM $MgCl_2$, 7.5 mM KCl, 11.5 mM glucose, and 1 mM $CaCl_2$) for 30 min at RT. The solution was replaced with imaging solution without Fura-2 AM for an additional 20 min. Images were acquired using a Nikon TiS inverted microscope as previously described [28]. After transfection, responses to 0.1 μM, 0.5 μM, 1 μM, and 10 μM of histamine were recorded via excitation at 340 nm, 380 nm (Fura-2 AM) and 480 nm (GCaMPs) in 3 s intervals during continuous recording. GCaMP6 calcium traces are expressed as the ratio relative to resting fluorescence ($\Delta F/F_0$). Fura-2 calcium traces are expressed as the ratio of fluorescence emitted at two different excitations wavelengths (340/380 nm). Percent responders and peak release was determined manually. A calcium transient was counted when the Fura-2 ratio rose 10% above the baseline ratio. Similar to Fura-2, a calcium transient was counted when GCaMP6 fluorescence rose above 5% from baseline fluorescence. All measurements are representative of a minimum of three independent experiments.

2.3. Staurosporine-induced calcium release measurements

Coverslips with GCaMP6s transfected HeLa cells or HEK293T cells were mounted in an imaging chamber and bathed in Leibovitz's L-15 media at room temperature and allowed to rest for 30 min. Images were captured every 60 s on a Nikon TiS inverted microscope with a 40× oil objective. Baseline fluorescence (F0) was determined by using the average intensity of the first 20 images before treatment. Cells were imaged for up to 10 h after treatment with 10 μM staurosporine (or DMSO for control). A calcium release event was defined as a 1.5-fold rise in fluorescence intensity over the baseline ($\Delta F/F0 > 1.5$).

2.4. Active caspase-3 assays

HeLa cells were treated with 10 μM staurosporine or DMSO in L-15 media with 10% FBS and 1% penicillin/streptomycin at room temperature (25 °C) for 2–12 h to recapitulate the conditions used to image long-term calcium. Cell lysates were collected every 2 h and enzymatic determination of DEVDase (caspase-3-like) activity was performed as described previously [25]. Additionally, cell lysates were run on SDS-PAGE gels and mouse monoclonal anti-α-fodrin (clone AA6) were used to detect full-length and caspase-3 cleaved α-fodrin.

2.5. Statistical analysis

All results are presented as means ± SEM. Statistical comparisons were performed using an unpaired *t*-test. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Histamine-induced calcium release measured using fura-2, GCaMP6s, and GCaMP6f

We first compared the performance of GCaMP6s and GCaMP6f with Fura-2 in HeLa cells. We quantified the calcium release kinetics in response to a dose response of histamine ranging from 0.1 μM to 10 μM. In general, both GCaMP6 indicator proteins performed similarly to Fura-2 at detecting calcium transients in the cytosol evoked by histamine (Fig. 1A). Quantitatively, GCaMP6s was more efficient at detecting calcium release events evoked by low (0.1 μM) concentrations of histamine in HeLa cells compared to GCaMP6f and Fura-2 (Fig. 1B–D). Fura-2 has moderately better performance at detecting events evoked by 0.5 μM and 1 μM histamine (Fig. 1B–D). The indicator GCaMP6f was the least sensitive reporter at detecting release events at all concentrations of histamine. To compare peak calcium release induced by histamine stimulation between the three indicators, we normalized calcium release at subsaturating concentrations of histamine as a percentage of the response to