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Genetically Encoded Calcium Indicators For Studying Long-Term Calcium Dynamics During Apoptosis

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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.

Keywords

genetically encoded calcium indicator; GCaMP6; Fura-2; calcium; inositol; apoptosis; staurosporine

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Author contributions

Data was collected and analyzed by M.I.G and J.J.C. Manuscript was written by M.I.G, J.J.C. and D.F.B. All authors have read and approved the published manuscript.

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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 permuted 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 circularly permuted green fluorescent protein, calmodulin, and a calmodulin binding motif derived from skeletal muscle myosin light chain kinase (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 hours. 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 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% CO₂. 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 2µg per 400,000 cells. Experiments were carried out 24 hrs 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, 107mM NaCl, 20 mM HEPES, 2.5 mM MgCl₂, 7.5 mM KCl, 11.5mM glucose, and 1 mM CaCl₂) 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 0.1µM, .5µM, 1µM, and 10µM of histamine were recorded via excitation at 340 nm, 380 nm (Fura-2 AM) and 480 nm (GCaMPs) in 3 seconds intervals during continuous recording. GCaMP6 calcium traces are expressed as the ratio relative to resting fluorescence (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 seconds on a Nikon TiS inverted microscope with a 40x 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 hrs after treatment with 10 μ M staurosporine (or DMSO for control). A calcium release event was defined as a 1.5-fold rise in fluorescence intensity over the baseline ($F/F_0 > 1.5$).

2.4. Active caspase-3 assays

HeLa cells were treated with 10 μ M staurosporine or DMSO in L-15 media with 10% FBS and 1% penicillin/streptomycin at room temperature (25°C) for 2–12 hrs to recapitulate the conditions used to image long-term calcium. Cell lysates were collected every 2 hours 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 $\pm$ 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 μ M to 10 μ M. 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 μ M) 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 μ M and 1 μ M 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 a saturating histamine (10 μ M). In those cells that responded to histamine, Fura-2 detected larger release events compared to both GCaMP6 proteins. GCaMP6s performed similarly to Fura-2 at all concentrations except 0.1 μ M. The indicator GCaMP6f performed significantly worse than the other two indicators at reporting peak release of calcium. These data indicate that GCaMP6s performs similarly to Fura-2 at detecting agonist-induced calcium release, and may outperform Fura-2 at detecting small release events at subsaturating concentrations of agonist.

3.2. Long-term imaging of apoptotic calcium release induced by staurosporine

To study calcium dynamics during apoptosis, we induced cell death in HeLa cells by using the broad-spectrum kinase inhibitor staurosporine (STS) [29, 30]. First we determined the conditions under which STS-treated cells show elevated caspase activity, a relatively late event in apoptosis, while the control cells did not undergo cell death. Using air-buffered Leibovitz's L-15 media at room temperature, STS-treated cells showed a significant increase in caspase activity, as well as cleavage of α -fodrin, starting from 6hr to 12hrs, whereas there was no significant caspase activity in control cells (Fig. 2A–B). Therefore, we chose to image calcium release in HeLa cells continuously for 10 hours after STS treatment, a time at which the majority of STS-treated cells had undergone apoptosis but no cell death was observed in control cells.

Based upon the performance of GCaMP6s in Figure 1, we performed long-term calcium imaging using this indicator in control and STS-treated cells. In control cells treated with DMSO, we found that 78% of the cells ($n=42$) did not undergo morphological changes and had infrequent low amplitude spontaneous intracellular calcium transients during the 10 hour imaging period (Fig. 2B,D; Supplementary video 1). In STS-treated cells, over 90% of the cells went through distinct morphological changes, including cell shrinkage and blebbing at the cell surface (Supplementary video 2) [31]. In 76% of STS-treated cells ($n=35$), there were significant calcium release events including oscillations and slow waves induced by STS (Fig. 2C,D). Interestingly, we observed extensive variability in the frequency, duration and timing of calcium release in each individual cell following STS treatment. To better characterize such variability, we categorized the pattern of calcium release into three types: oscillations, gradual increase (no significant oscillatory behavior but increased basal calcium), and a combination of both oscillations and gradual increase. We found that 41.1% of the STS-treated responders show oscillations only, 7.4% showed gradual increase, and 51.5% showed a combination of both patterns (Fig. 2E). Previous studies using Fura-2 and averaging traces from multiple cells demonstrated that STS-treated cells showed gradual increases in basal calcium levels that correlates with cell death [22, 27]. To assess whether we observe a similar trend using GCaMP6s, we calculated the average of all cells in one representative experiment and found a similar trend of increased calcium levels in STS-treated cells, while no increase was observed in control cells (Fig. 2F). Additionally, we also observed similar patterns of calcium release in HEK293T cells treated with STS (Supplementary Figure 1).

To test whether GCaMP6s can be used to monitor calcium release in cell death induced by other apoptosis-inducing drugs, we treated HeLa cells with the anthracycline antibiotic doxorubicin (DOX), a chemotherapeutic drug known to alter calcium homeostasis [32, 33]. We found that DOX-treated cells had markedly different kinetics of calcium release compared to STS. In contrast to STS treated cells, calcium elevations initiated after 6–7 hours of recording and continued to rise slowly for another 5 hours (Supplementary Figure 2). Concomitantly, caspase-3 activation was delayed compared to STS treated cells and was not readily apparent until 12 hours after treatment (Supplementary Figure 2A). These results indicate that different apoptosis-inducing drugs can have dramatically different apoptotic calcium release kinetics.

4. Discussion

Investigating calcium over extended time periods such as during cell death requires specialized approaches. Ideally, a fluorescence calcium indicator needs to have high sensitivity and be extremely photostable over a period of hours or even days. In the present study we found that GCaMP6s exhibited improved performance over Fura-2 when used to monitor calcium transients induced by histamine and during apoptosis induced by staurosporine. The expression pattern of GCaMP6s is well distributed throughout the cell and can readily be used *in vitro* or *in vivo* [20]. Compared to GCaMP6f, GCaMP6s has a higher affinity to calcium and slower kinetics, but with higher brightness (Table 1) [20]. Both GCaMP6 proteins are significantly slower than Fura-2 (Table 1), which may limit their utility for imaging fast events.

Imaging calcium during cell death can be technically demanding. Previous approaches used by our group and others required loading and imaging sequential coverslips or taking static measurements every few hours or days after stimulation [22, 25–27]. These limitations precluded the possibility of following calcium dynamics in a single cell throughout the entire apoptotic process. We found remarkable heterogeneity in the calcium responses after STS stimulation which were previously uncharacterized. Previous studies have shown that cytochrome c release is coordinated after STS stimulation [34, 35], and this process is likely mediated by calcium [22, 36]. In HeLa cells under similar conditions reported here, this occurs between 6 and 8 hours [22, 34]. We find that there is an increase in oscillatory activity around 6–8 hours in some cells (Figure 2C), however this is clearly not observed in all cells. Thus, we conclude there must be another factor which functions alongside with calcium to induce coordinated cytochrome c release during cell death. Future studies examining RFP-cytochrome c release simultaneously with GCaMP6s imaging would help to resolve this apparent discrepancy and to further interrogate the role of calcium as a coordinating factor driving cytochrome c release during cell death.

Using GCaMP6s, we have found remarkable heterogeneity in apoptotic calcium release in both cultures of a single cell type such as HeLa cells, but also marked differences between cell types in response to STS. Also notable is that single cell apoptotic calcium release kinetics are strongly dependent upon the type of stimulus being presented. This raises an important question: if the endpoint of apoptotic signaling is death, why is there such heterogeneity in the responses? One technical explanation is that these cultures, although derived from the same tissue, have become heterogeneous through repeated passaging. Also, since the cultures have not been synchronized, each cell is potentially in a different stage of the cell cycle with significant implications for the response to staurosporine. Alternatively, the heterogeneous calcium release response to apoptotic stimuli is an intrinsic and evolutionarily selected property of the apoptotic calcium release machinery. This property would be advantageous for having a reversible step in the apoptotic process so that damaged cells can be repaired and spared unnecessary death. We have shown previously that this is an intrinsic property of the Fas death receptor pathway, with the irreversible step encompassing cytochrome c binding to the IP₃R [25]. Using a blocking peptide that inhibits cytochrome c to the IP₃R, we have shown similar findings in STS-induced cell death of HeLa cells [37]. Future work using live cell imaging of the apoptotic process using a reporter along with

calcium release in single cells will greatly facilitate the mechanistic underpinnings why the apoptotic calcium release process is heterogeneous.

Although we have highlighted the advantages of GCaMP6 proteins in this report, there are certain advantages to using dye-based reporters. Fura-2 is a ratiometric dye, which controls for heterogeneity in indicator loading and facilitates relatively easy quantification of absolute calcium levels [5]. Using specific loading techniques designed to intentionally cause compartmentalization of AM ester dyes, organelle calcium dynamics can also be interrogated using dye-based reporters [38]. Genetically encoded indicators rely upon transfection or viral transduction of plasmid DNA into the cell type to be investigated. Some cell types, and in particular primary cells such as adult cardiomyocytes and neurons, are refractory to transfection/transduction and thus dye-based methods are preferred. As mentioned previously, GCaMP6s and GCaMP6f are much slower than dye-based approaches, and thus would not be preferred for doing high-speed imaging. Indeed, the wide variety of calcium indicators with different spectra, affinities, quantum yield, and other characteristics allows for tailoring the dye to the exact experiment preparation and functional readout.

Finally we would like to highlight a technical note about potential artifacts associated with long-term imaging. We found that the choice of buffer and incubation conditions have dramatic effects on the viability of cells mounted on the microscope stage. We found that imaging in Leibovitz's L-15 media was highly suitable for controlling pH when imaging in air and preserved cellular integrity for up to 12 hours (Fig. 2A–B). Importantly, long-term calcium imaging with GCaMP6s demonstrated that it overcame the limitations that conventional calcium indicators have such as poor photostability and leakage and is ideal for recording calcium dynamics during apoptosis for hours and possibly days. It should be noted that GFP-based indicators are sensitive to environmental factors such as pH. It is well known that intracellular acidification of 0.3–0.4 pH units is a common feature of apoptotic cell death [39, 40]. Calcium-bound GCaMP6s fluorescence is stable from pH 6.5–9.5 [41], suggesting that GCaMP6s performance would not be appreciably affected by pH during cell death. Furthermore, our data does not indicate any significant loss of GCaMP6s fluorescence over the time courses being investigated in our model system. Regardless, this is a very important consideration when using GCaMPs or indeed any fluorescent calcium reporter to measure apoptotic calcium release.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary movies 1 and 2 associated with this article can be found in the online version.

Highlights

- Genetically encoded GCaMP6s indicator performed as well or better than Fura-2.
- Cytosolic calcium could be measured continuously for hours using GCaMP6s.
- Apoptotic calcium release induced by staurosporine was highly heterogeneous.
- GCaMP6s is an excellent general-purpose calcium indicator protein.

Fig. 1. Comparison of Fura-2 to GCaMP6s and GCaMP6f in measuring agonist-induced calcium release

A. Representative single cell traces showing calcium changes in HeLa cells incubated with Fura-2 or expressing GCaMP6f/GCaMP6s in response to increasing concentrations of histamine (0.1 μM, 0.5 μM, 1 μM and 10 μM). Fluorescence signals are scaled as F/F_0 (GCaMP6 proteins) and Fura-2 fluorescence signals are presented as the 340/380nm ratio.

B–D. Bar graphs show percent of cells that show a change in fluorescence in response to increasing concentrations of histamine (% responders). The total number of cells quantified is as follows: Fura-2 (n=92), GCaMP6f (n=103) and GCaMP6s (n=179). **E–G.** Peak release in response to different concentration of histamine (0.1 μM, 0.5 μM and 1 μM) was analyzed for each calcium indicator. Peak release is reported as percent of saturating dose of histamine (10 μM histamine). All data are shown as mean ± SEM, *p < 0.05 vs Fura-2 at each concentration.

Fig. 2. Changes in cytosolic calcium during staurosporine-induced cell death

A. Caspase-3-like activity (DEVDase activity) in HeLa cells treated with 0.1% DMSO (control) and 10 μM staurosporine (STS) were measured every 2 hours from zero to 12 hours post treatment. **B.** α -fodrin cleavage in response to DMSO or STS treatment in HeLa cells. Full-length α -fodrin (265kD) is indicated by a black arrowhead and the 145/150kD cleaved product is indicated by a grey arrowhead. **C–D.** GCaMP6s transfected HeLa cells were treated with DMSO or 10 μM STS and imaged for 10 hours. Traces of GCaMP6s fluorescence in individual cells are shown ($n=9$ for one experiment). Cells treated with DMSO showed rare low amplitude changes in cytosolic calcium level throughout the experiment, with individual events not obvious at this scale (C). **E.** Bar graph showing percent responders in control ($n=42$) and STS-treated cells ($n=35$), * $p < 0.05$. **F.** Bar graph showing the frequency of different types of calcium signals in individual STS-treated cells. Cells with release events that go above baseline and have ending calcium levels return to baseline are categorized as an *oscillation*. Cells with no rapid and obvious release events above baseline but show a gradual increase towards the end of 10 hours are categorized as *gradual increase*. Cells showing both oscillation and gradual increase patterns are categorized as *both*. **G.** Traces of the average fluorescence of all cells in one representative experiment ($n=10$).

Table 1

Calcium binding characteristics of Fura-2, GCaMP6s and GCaMP6f

Indicator	K _d (nM)	Quantum Yield	K _{on} (x 10 ⁷ M ⁻¹ s ⁻¹)	K _{off} (s ⁻¹)
Fura-2 [5, 42]	135–224	0.49	10	23
GCaMP6s [20]	144±4	0.61	0.78	1.12
GCaMP6f [20]	375±14	0.59	1.05	3.93