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Imaging Ca^{2+} with a fluorescent rhodol

Alisha A. Contractor[†] and Evan W. Miller^{†,§,||,*}

[†]Department of Chemistry, University of California, Berkeley, CA 94720

[§]Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

^{||}Helen Wills Neuroscience Institute, University of California, Berkeley, CA 94720

Abstract

Ca^{2+} mediates a host of biochemical and biophysical signaling processes in cells. The development of synthetic, Ca^{2+} -sensitive fluorophores has played an instrumental role in understanding of the temporal and spatial dynamics of Ca^{2+} . Coupling Ca^{2+} -selective ligands to fluorescent reporters has provided a wealth of excellent indicators that span the visible excitation and emission spectrum and possess Ca^{2+} affinities suited to a variety of cellular contexts. One under-developed area is the use of hybrid rhodamine/fluorescein fluorophores, or rhodols, in the context of Ca^{2+} sensing. Rhodols are bright, photostable, and have good two-photon absorption cross-sections (σ_{TPA}), making them excellent candidates for incorporation into Ca^{2+} -sensing scaffolds. Here, we present the design, synthesis, and application of rhodol Ca^{2+} sensor- 1 (RCS-1), a chlorinated pyrrolidine-based rhodol. RCS-1 possesses a Ca^{2+} binding constant of 240 nM and a 10-fold turn-response to Ca^{2+} . RCS-1 effectively absorbs infrared light and has a σ_{TPA} of 76 GM at 840 nm, 3-fold greater than its fluorescein-based counterpart. The acetoxymethyl ester of RCS-1 stains the cytosol of live cells, enabling observation of Ca^{2+} fluctuations in cell lines and cultured neurons using both one- and two-photon illumination. Together, these results demonstrate the utility of rhodol-based scaffolds for Ca^{2+} sensing using two-photon illumination in neurons.

Graphical Abstract

*Corresponding Author: evanwmiller@berkeley.edu.

Supporting Information

Synthetic, spectroscopic and imaging data. The Supporting Information is available free of charge on the ACS Publications website.

The development of synthetic fluorescent indicators for Ca^{2+} ions is one of the success stories of organic chemistry and molecular recognition. The majority of fluorescent Ca^{2+} indicators utilize BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) as the key Ca^{2+} -recognizing domain and couple Ca^{2+} binding to changes in fluorescence intensity or wavelength.¹ First reported in 1980 by Roger Tsien,² BAPTA solved dual problems of slow Ca^{2+} -binding kinetics and pH dependence associated with the state of the art Ca^{2+} -selective chelator of the day, EGTA (ethyleneglycol-*N,N,N',N'*-tetraacetic acid). Importantly BAPTA maintains selectivity for Ca^{2+} over Mg^{2+} , which is present in concentrations exceeding cytosolic Ca^{2+} by 4 to 5 orders of magnitude. Widely used Ca^{2+} indicators include fura-2,³ which provides a ratiometric read-out of Ca^{2+} concentration using UV excitation; the fluo- and rhod-family of indicators that report on fluctuations in Ca^{2+} concentration via changes in fluorescence intensity at around 520 and 580 nm, respectively;⁴ and OregonGreen-BAPTA (OGB)⁵ and CalciumGreen-BAPTA (CGB)⁶ derivatives that employ fluorinated and chlorinated fluorescein as an optical reporter. More recently, the use of modern xanthene scaffolds has enabled access to Ca^{2+} sensing in the orange (Cal-590,⁷ CaRuby-Nano⁸) to far-red (CaTM2⁹ or Cal-630) region of the electromagnetic spectrum by employing halogenated carbofluorescein¹⁰ or silicon-fluorescein, respectively.

Ca^{2+} indicators are widely used in neurobiology, where fluorescent indicators provide a convenient method to measure the relative activity of neurons. OGB, in particular, continues to be used for *in vivo* applications,¹¹ despite the recent emergence of excellent genetically-encoded Ca^{2+} -sensitive fluorescent proteins,¹² on account of its well-tuned K_d (170 nM) and fast Ca^{2+} -binding kinetics.¹³ The use of fluorescent Ca^{2+} indicators, whether synthetically-derived or genetically-encoded, is frequently paired with two-photon microscopy, which allows greater tissue penetration on account of the use of high energy, pulsed lasers in the infrared regime. Despite the critical dependence on the use of two-photon illumination to image in thick tissues, most widely-used Ca^{2+} indicators show only modest ability to effectively absorb two-photon (2P) illumination.¹⁴ CGB has a two photon excitation cross section of approximately 30 GM (when saturated with Ca^{2+}).¹⁵ Alternatively, indicators based on rhodamines, with larger 2P cross sections in the 50 to 100 GM range (for example, Calcium Orange or Calcium Crimson),¹⁵ often localize to cellular compartments rather than cytosol.¹⁶ To surmount the problems of low 2P cross section while maintaining good Ca^{2+} affinity, sensitivity, and cellular localization, we hypothesized that rhodol-based fluorophores¹⁷ would enable Ca^{2+} sensing under 2P illumination in live cells. Rhodols have intermediate to strong 2P cross sections, at around 120 GM,^{18, 19} and show good cellular localization.^{20, 21} Despite these promising characteristics, rhodols have never been combined with BAPTA to create a Ca^{2+} sensor. We here report the design, synthesis, characterization and application of rhodol Ca^{2+} -sensor 1, a fluorescent Ca^{2+} indicator with good 2P excitation cross section, sub-micromolar dissociation constants for Ca^{2+} , and the ability to report on $[\text{Ca}^{2+}]$ fluxes in live cells and neurons under 2P illumination.

Rhodol Ca^{2+} sensor 1 is synthesized in a single step from the corresponding 6'-carboxy-dichloropyrrolidylrhodol (**1**) and the AM-ester of 5-aminoBAPTA (**2**) (Scheme 1). Synthesis of **1** proceeds in 4 steps from carboxy-dichlorofluorescein. Construction of isomerically pure 6'-carboxy-dichlorofluorescein is achieved via recrystallization from acetic anhydride/

pyridine to give the 6' isomer in gram quantities.²² Protection of the pendant carboxylate as the *t*-butyl ester²³ enables selective protection of a single phenolic oxygen as the methoxymethyl (MOM) ether²⁴ and subsequent conversion of the remaining oxygen to the triflate. Conversion of the fluorescein to rhodol proceeds via Pd-catalyzed C-N bond formation with pyrrolidine followed by removal of the *t*-butyl ester to give compound **1** in 30% yield over two steps. Formation of amide bond between rhodol **1** and the tetra-acetoxymethyl ester of 5'-amino-BAPTA **2**⁹ provides the acetoxymethyl ester of rhodol Ca²⁺ sensor **1** (RCS-1 AM) in 15% overall yield following purification. Saponification of this material provides the hexapotassium salt of RCS (RCS-1 K) for *in vitro* spectroscopic characterization.

RCS-1 shows excitation and emission profiles characteristic of substituted rhodols, with a λ_{max} absorption at 515 nm and emission maximum centered at 551 nm (Figure 1a). Addition of Ca²⁺ results in an increase in fluorescence intensity (Figure 1a). The quantum yield for RCS-1 is Ca²⁺-dependent. In the absence of Ca²⁺, Φ_{551} is 0.073; in the presence of saturating levels of Ca²⁺ (39 μM), Φ_{551} is 0.73. There is no Ca²⁺-dependent change in absorption (Figure 1a). Titration with Ca²⁺ reveals a dissociation constant of 240 nM (± 6 nM, S.E.M., $n = 3$ separate determinations), which is well-matched to the K_d reported for CGB-1 (190 nM)^{6, 13} (Figure 1b, Figure S1). In our hands, we determined a K_d of 180 nM for CGB-1 (± 5 nM, S.E.M., $n = 3$ separate determinations), indicating a good match between RCS-1 and CGB-1. RCS-1 possesses a large dynamic range in response to Ca²⁺, exhibiting a 10-fold increase (± 1 , S.E.M., $n = 3$) in fluorescence intensity upon Ca²⁺ binding (Figure 1b), again, well-matched to the dynamic range for CGB-1 (which we measured as a 13-fold increase).⁶ We measured the two-photon excitation spectrum of RCS-1. Using rhodamine B as a standard, we determined the two-photon absorption cross-section (σ_{TPA}) of RCS-1.^{15, 19} RCS-1 displays a σ_{TPA} maximum at 840 nm, with a value of 72 GM. This is 3-fold larger than the 24 GM σ_{TPA} value we determined for CGB-1 (approximately 24 GM at 840 nm).¹⁵

The combination of large turn-on response to Ca²⁺ binding, nanomolar dissociation constant, and strong two-photon absorption cross-section suggests RCS-1 will be useful for monitoring Ca²⁺ transients in living cells. To investigate the ability of RCS-1 to report on Ca²⁺ fluxes in live cells, we utilized the tetra-AM ester version of RCS-1. Masking polar carboxylates with AM esters enable uptake anionic BAPTA-containing indicators into cells.²⁵ Cellular esterases remove the AM esters, liberating the Ca²⁺-responsive fluorophore. Bath application of RCS-1 to HeLa cells (1.7 μM , with Pluronic F-127, 0.01%) resulted in bright intracellular fluorescence, as determined by laser scanning confocal microscopy (Figure 2a). Stimulation of RCS-1-loaded cells with histamine (5 μM) resulted in the induction of periodic Ca²⁺ oscillations that were readily observable with RCS-1 fluorescence (Figure 2b). Gratifyingly, Ca²⁺ transients could be monitored using infrared, two-photon microscopy, with excitation provided at 840 nm (Figure 2c and d). Under both one- and two-photon illumination, RCS-1 displays photostability comparable to CGB-1.

Building on these observations, we next determined whether RCS-1 could report on neuronal activity in cultured mammalian hippocampal neurons. Incubation of RCS-1 (1.7 μM , with Pluronic F-127, 0.01%) with cultured rat hippocampal neurons resulted in bright

cellular fluorescence, as determined by widefield epifluorescence microscopy (Figure 3a). RCS-1 can report on spontaneous activity in hippocampal cultures (Figure 3b). Action potentials evoked by field stimulation give clear increases in fluorescence (Figure S2). Importantly, RCS-1 can also report on spontaneous activity in cultured neurons when imaged under 2P illumination, using 840 nm excitation light (Figure 3c and d).

In summary, we present the design, synthesis, and application of RCS-1, a rhodol-based fluorescent indicator for Ca^{2+} . The large turn-on response to Ca^{2+} , high Ca^{2+} affinity, and good two-photon cross-section of RCS-1, coupled with the ability to monitor spontaneous activity in cultured neurons, make it a promising candidate for monitoring $[\text{Ca}^{2+}]$ transients in the context of neurobiology and beyond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Spectroscopy characterization of rhodol Ca^{2+} sensor 1. a) Absorbance spectrum of rhodol Ca^{2+} sensor 1 (10 μM) in the absence (black dotted line) and presence (black solid line) of 39 μM Ca^{2+} . Fluorescence emission spectra of rhodol Ca^{2+} sensor 1 (10 μM) in the absence (green dotted line) and presence (green solid line) of 39 μM Ca^{2+} . Excitation provided at 515 nm. b) Ca^{2+} titration of rhodol Ca^{2+} sensor 1. The fluorescence emission spectra of rhodol Ca^{2+} sensor 1 was recorded at increasing concentrations of Ca^{2+} from 0 μM to 39 μM , with intermediate concentrations of 17 nM, 38 nM, 65 nM, 100 nM, 150 nM, 225 nM, 351 nM, 602 nM, and 1.35 μM . Excitation provided at 515 nm. c) Two-photon absorption spectra of rhodol Ca^{2+} sensor 1 with Ca^{2+} (39 μM). Error bars are $\pm$ standard deviation for $n = 3$ separate determinations.

Figure 2.

Live-cell imaging of histamine-evoked Ca^{2+} fluctuations with rhodol Ca^{2+} sensor 1. a) Confocal fluorescence microscopy images (one-photon) of HeLa cells incubated with rhodol Ca^{2+} sensor tetra-AM (1.7 μM). Scale bar is 20 μm . b) Quantification of intracellular $[\text{Ca}^{2+}]$ fluctuations measured in response to stimulation with histamine (5 μM). c) Two photon laser scanning fluorescence microscopy images of HeLa cells incubated with rhodol Ca^{2+} sensor tetra-AM (1.7 μM). Scale bar is 20 μm . d) Quantification of intracellular $[\text{Ca}^{2+}]$ fluctuations measured in response to stimulation with histamine (5 μM).

Figure 3.

Visualization of Ca^{2+} transients in rat hippocampal neurons using rhodol Ca^{2+} sensor 1. a) Widefield fluorescence micrograph of cultured rat hippocampal neurons incubated with rhodol Ca^{2+} sensor 1 tetra AM ester (1.7 μM). Scale bar is 20 μm . b) Relative changes rhodol Ca^{2+} sensor 1 fluorescence vs. time for neurons in panel a. c) Two-photon laser scanning microscopy image of rat hippocampal neuron stained with rhodol Ca^{2+} sensor 1 tetra AM ester (1.7 μM). Scale bar is 20 μm . d) Intracellular Ca^{2+} transients recorded as relative changes in rhodol Ca^{2+} sensor 1 fluorescence vs. time for the neuron in panel c.

Scheme 1.
Synthesis of rhodol Ca^{2+} sensor 1.