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Development of practical red fluorescent probe for cytoplasmic calcium ions with greatly improved cell-membrane permeability

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ABSTRACT

Fluorescence imaging of calcium ions (Ca^{2+}) has become an essential technique for investigation of signaling pathways involving Ca^{2+} as a second messenger. But, Ca^{2+} signaling is involved in many biological phenomena, and therefore simultaneous visualization of Ca^{2+} and other biomolecules (multicolor imaging) would be particularly informative. For this purpose, we set out to develop a fluorescent probe for Ca^{2+} that would operate in a different color region (red) from that of probes for other molecules, many of which show green fluorescence, as exemplified by green fluorescent protein (GFP). We previously developed a red fluorescent probe for monitoring cytoplasmic Ca^{2+} concentration, based on our established red fluorophore, TokyoMagenta (TM), but there remained room for improvement, especially as regards efficiency of introduction into cells. We considered that this issue was probably mainly due to limited water solubility of the probe. So, we designed and synthesized a red-fluorescent probe with improved water solubility. We confirmed that this Ca^{2+} red-fluorescent probe showed high cell-membrane permeability with bright fluorescence. It was successfully applied to fluorescence imaging of not only live cells, but also brain slices, and should be practically useful for multicolor imaging studies of biological mechanisms.

1. Introduction

The development of sophisticated fluorescent probes has contributed to elucidation of the molecular mechanisms of many complex biological phenomena [1–4]. In particular, fluorescence imaging of Ca^{2+} has become an essential technique for investigation of signaling pathways involving Ca^{2+} as a second messenger. For example, changes in the intracellular Ca^{2+} concentration are related to physiological responses in obesity, as well as immune responses and pathological responses in Alzheimer's disease [5–10]. Because Ca^{2+} signaling is involved in so many biological phenomena [11,12], simultaneous visualization of Ca^{2+} and other biomolecules, that is, multicolor imaging, would be particularly informative for detailed study of the mechanisms.

Fluorescent Ca^{2+} indicators can be categorized into two main classes: those based on genetically encoded fluorescent proteins [13,14] and those based on fluorescent small organic molecules [5]. Both types of indicators have characteristic advantages and disadvantages; for example, small-molecular probes have the particular advantage that their AM ester form (cell-permeable acetoxymethyl ester derivative) can be readily bulk-loaded into live cells with no need for transfection. Most currently used small-molecular fluorescent probes for Ca^{2+} are fluorescein derivatives, such as Fluo-3, Fluo-4, Calcium Green-1, and Oregon Green 488 BAPTA-1, and emit green fluorescence (ca. 527 nm) [15–17]. There are also some red-emitting fluorescent probes for Ca^{2+}, such as Rhod-2 (ca. 576 nm), which is a rhodamine derivative [15]. These red-emitting fluorescent probes for Ca^{2+}, including Rhod-2, are widely used for

Abbreviations: TM, TokyoMagenta; AM, acetoxymethyl ester.

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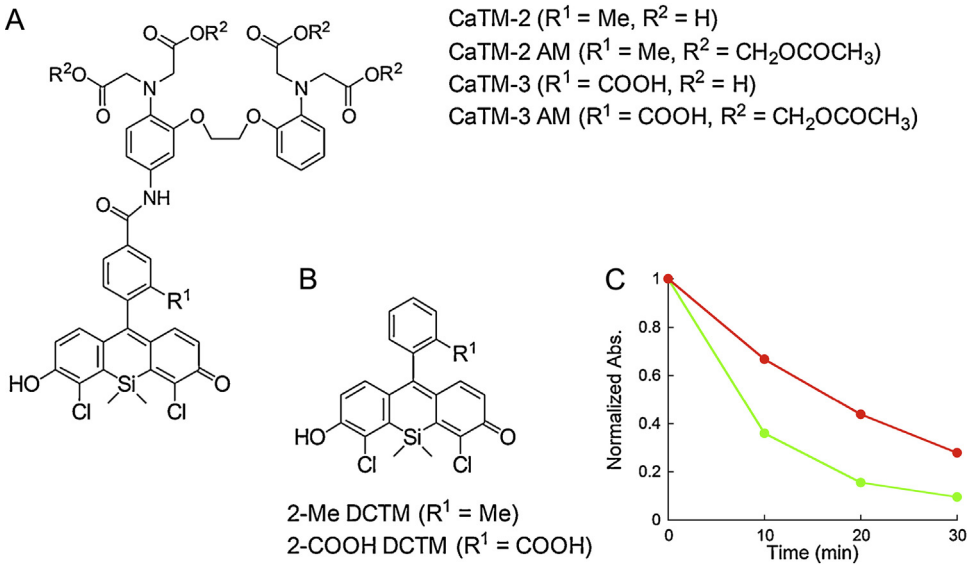

Fig. 1. Chemical structures of fluorescent probes for Ca^{2+}. A. Chemical structures of red fluorescent Ca^{2+} probes, CaTM-2 and CaTM-3, and their cell-permeable derivatives, CaTM-2 AM and CaTM-3 AM. B. Chemical structures of 2-Me DCTM and 2-COOH DCTM [26,27]. C. Photobleaching tests. Normalized absorbance at 482 nm for 1 μM fluorescein (green) and 591 nm for 1 μM 2-COOH DCTM (red). Absorbance measurements were made at 25 °C in 100 mM sodium phosphate buffer at pH 9.0 containing 0.1% DMSO as a cosolvent after samples were exposed to light (30 mW, 470–495 nm for fluorescein, 565–585 nm for 2-COOH DCTM, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biological studies, but the cationic nature of the rhodamine scaffold generally causes Rhod-2 AM to localize into mitochondria [18]. Although this behavior is useful for monitoring Ca^{2+} dynamics in mitochondria, visualization of cytoplasmic Ca^{2+} is often much more important for research on Ca^{2+} signaling pathways. Influx of Ca^{2+} into the cytoplasm from the extracellular environment or intracellular stores, such as the endoplasmic reticulum, triggers numerous cellular responses via the interaction of Ca^{2+} with various Ca^{2+}-binding proteins, such as calmodulin and troponin C [11,12]. Fura Red is a representative near-infrared (NIR) fluorescent probe for cytoplasmic Ca^{2+} in biological research, but has the major drawback of extremely low fluorescence quantum efficiency ($\Phi_{fl} \approx 0.013$) [19]. Accordingly, the fluorescence signal is very small unless a high concentration of Fura Red or a high-powered laser is used. However, the use of a high dye concentration has a buffering effect on Ca^{2+}, and the use of a high laser power causes rapid photobleaching of the dye and phototoxicity to the cells.

The red or far-red wavelength region is especially attractive for fluorescence imaging, affording higher tissue penetration due to reduced scattering, low absorption by endogenous biomolecules, and lower phototoxicity, and also allows monitoring of Ca^{2+} in cells or tissues expressing yellow- or green-colored fluorescent proteins or labeled with other green-colored fluorophores [20–22]. Several new red-to-NIR fluorescent probes for cytoplasmic Ca^{2+}, such as Quest Rhod-4 AM, CaSiR-1 [23], KFCA [24], and Calcium Rubies [22,25] have recently been reported [20]. We developed a red (ca. 609 nm) fluorescent probe for monitoring cytoplasmic Ca^{2+}, CaTM-2 and CaTM-2 AM (Fig. 1A), based on the 2-Me DiChloro-TokyoMagenta (2-Me DCTM) scaffold (Fig. 1B) [26]. However, the cell-membrane permeability was limited, and we considered that the low water solubility of the probe was a major contributor to its relatively poor introduction efficiency into cells.

Here, we present a new red-fluorescent Ca^{2+} probe, CaTM-3 AM, with high cell-membrane permeability and bright fluorescence (Fig. 1A). We very recently reported the synthesis and characterization of 2-COOH DCTM (Fig. 1B), which has a carboxy group in place of the methyl group of 2-Me DCTM [27]. Taking account of the high water solubility of 2-COOH DCTM compared with that of 2-Me DCTM, we designed and synthesized CaTM-3 AM as a novel, highly cell-permeable candidate probe for visualization of cytoplasmic Ca^{2+}. We also evaluated its suitability for practical use by employing it for fluorescence imaging of cytoplasmic Ca^{2+} in live cells and in brain slices.

2. Methods

2.1. UV–vis and fluorescence spectra measurements

UV–vis spectra were obtained with a spectrometer (UV-1650, Shimadzu, Japan). Fluorescence spectroscopic studies were performed on a fluorescence spectrometer (F-4500, Hitachi, Japan). The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 700 V. 1 mM dyes in DMSO were used as stock solutions.

2.2. Photobleaching tests

1 μM of dye in 100 mM sodium phosphate buffer at pH 9.0 containing 0.1% DMSO as a cosolvent was poured into the cuvette. The solution was exposed to light (30 mW, 470–495 nm for fluorescein and 565–585 nm for 2-COOH DCTM) through a rod lens using a Xe light source, MAX301 (Asahi Spectra Co., Ltd.) for 30 min. After every 10 min of light irradiation, UV–vis spectra were obtained with a spectrometer (UV-1650, Shimadzu, Japan).

2.3. Relative fluorescence quantum yields

For determination of the relative fluorescence quantum efficiencies (Φ_{fl}) of CaTM-3, 2-Me TM in 0.1 M sodium phosphate buffer (pH 9.0) (Φ_{fl} = 0.42) was used as a standard [28]; calculation was done using Eq. (1):

$$\Phi_x/\Phi_{std} = [A_{std}/A_x][n_x{}^2/n_{std}{}^2][D_x/D_{std}] \quad (1)$$

where std and x represent the standard (2-Me TM) and sample, respectively. A, n, and D are the absorbance at the excitation wave-