A two-photon fluorescent probe for near-membrane calcium ions in live cells and tissues†

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Received (in Cambridge, UK) 10th June 2009, Accepted 1st July 2009
First published as an Advance Article on the web 29th July 2009
DOI: 10.1039/b911337a

A two-photon fluorescent probe (ACaL) is reported that can be excited by 780 nm fs pulses, shows high photostability and negligible toxicity, and can visualize near-membrane Ca^{2+} in live cells and deep inside live tissues by two-photon microscopy.

Calcium is a versatile intracellular signal messenger controlling numerous cellular functions; it plays vital roles in many cellular processes such as fertilization, cell death, sensory transduction, muscle contraction, motility, exocytosis, and fluid secretion.[1] Intracellular free Ca^{2+} levels ($[Ca^{2+}]_i$) are maintained by the coordinated actions of various calcium pumps, ion channels, and calcium-buffering proteins. When stimulated with agonists, cytosolic Ca^{2+} levels are rapidly elevated either by Ca^{2+}-influx from the extracellular space or by Ca^{2+}-release from internal Ca^{2+} stores such as endoplasmic reticulum (ER). In resting cells, which must maintain low $[Ca^{2+}]_i$, the excess Ca^{2+} must be extruded to the outside *via* plasma membrane Ca^{2+} ATPase (PMCA) or transported back into the ER through sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA).[2]

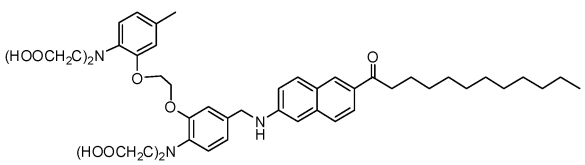
To investigate the changes in near-membrane Ca^{2+} concentration and the translocation of Ca^{2+} across the plasma membrane, C_{18}-Fura-2 and Calcium Green C_{18} have been developed.[3] However, use of these probes with one-photon microscopy (OPM) results in blurred cell images. Moreover, tissue imaging was not possible due to the short excitation wavelength (<500 nm). To visualize the near-membrane Ca^{2+} deep inside live tissues, it is crucial to use two-photon microscopy (TPM). TPM, which employs two lower energy, near-infrared photons as the excitation source, has the advantages of increased penetration depth (>500 μm), localized excitation, and prolonged observation time.[4–6] Recently, we reported a series of two-photon (TP) probes for calcium ion (ACa1–ACa3)[7] that can visualize the calcium waves in live cells and tissues at >100 μm depth by TPM for a long period of time with minimum interference from tissue preparation artifacts, self-absorption, auto-fluorescence, photobleaching, and photodamage.

As an extension to these studies, we have now developed a novel TP probe for near-membrane Ca^{2+} (ACaL; Scheme 1) that is capable of imaging the near-membrane Ca^{2+} in live cells and tissues at >100 μm depth by TPM. Our strategy was to link the 5-methyl derivative of *O*,*O*′-bis(2-aminophenyl)-ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA-5-Me) with 2-dimethylamino-6-laurylnaphthalene (laurdan) as the TP chromophore. It was expected that ACaL would be predominantly located in the plasma membrane with the orientation of the dodecanoyl group in the membrane interior and the BAPTA-5-Me moiety lying near the membrane surface and thereby able to trap the Ca^{2+} extruded from the cytoplasm, causing fluorescence. It is to be noted that BAPTA-5-Me is a well known Ca^{2+} chelator that has been widely employed in various one-photon fluorescent probes such as Fura-2, Fluo-3, Rod-2,[8] as well as in a TP probe (ACa2),[7] whereas laurdan is a TP polarity probe that has been used to detect the lipid rafts in the cell membrane.[9]

The preparation of ACaL is described in the ESI.† The absorption and emission spectra of ACaL showed gradual red shifts with the solvent polarity in the order 1,4-dioxane < DMF < EtOH < EtOH–H_2O (1 : 1) (Fig. S1 and Table S1, ESI†). The effect was greater for the emission (54 nm) than absorption spectra (13 nm), thus indicating the utility of this compound as a polarity probe.

When Ca^{2+} was added to ACaL in 3-(*N*-morpholino)-propanesulfonic acid (MOPS) buffer solution (30 mM, 100 mM KCl, 10 mM EGTA, pH 7.2), the fluorescence intensity increased dramatically as a function of metal ion concentration (Fig. 1(a)), probably due to the blocking of the photo-induced electron transfer (PeT) process by the complexation with the metal ions. A nearly identical result was observed in the two-photon process (Fig. S2a in ESI†). The fluorescence enhancement factors $[(F - F_{min})/F_{min}]$ of ACaL determined for the one- and two-photon processes were 12 and 10, respectively, in the presence of 39 μM Ca^{2+} (Table 1 and Fig. S2a in ESI†).[10] The values are similar to that (14) reported for Calcium-Green.[3a] Moreover, linear Hill plots determined for Ca^{2+} binding with slopes of 1.0 indicated 1 : 1 complexation between ACaL and Ca^{2+} (Fig. S2c in ESI†).[11]

ACaL showed a modest response toward Zn^{2+} and Mn^{2+}, a much weaker response toward Mg^{2+}, and no response



Scheme 1 The structure of ACaL.

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† Electronic supplementary information (ESI) available: Experimental details for the synthesis, photophysical studies, and cell and tissue imaging. See DOI: 10.1039/b911337a

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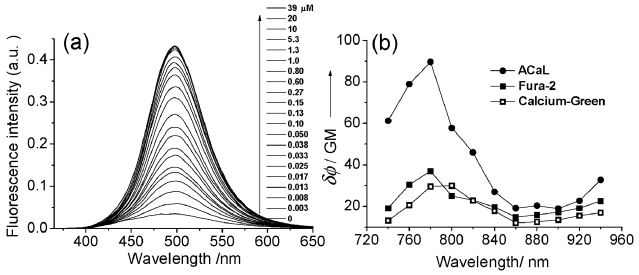

Fig. 1 (a) One-photon fluorescence spectra of 2 μM ACaL in MOPS buffer in the presence of free Ca^{2+} (0–39 μM). (b) Two-photon action spectra of ACaL (●), Fura-2 (■) and Calcium-Green (□) in MOPS buffer in the presence of 39 μM free Ca^{2+}.

toward Fe^{2+}, Cu^{2+}, Co^{2+}, Ba^{2+}, and Sr^{2+} (Fig. S3a in ESI†). As the intracellular free-ion concentration of Mn^{2+} is negligible, this probe can detect the intracellular Ca^{2+} concentration in the regions in which the chelatable Zn^{2+} concentration is much lower than $K_d^{TP}(Ca^{2+})$. Furthermore, it was pH-insensitive at pH >6.5 (Fig. S3b in ESI†).

To assess whether ACaL can detect near-membrane Ca^{2+}, the K_d^{OP} values were determined in vesicles composed of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)/40 mol% cholesterol (CHL), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and DOPC–sphingomyelin–CHL (1 : 1 : 1, raft mixture). It is well established that the cell membrane is composed of liquid-ordered (l_o) and liquid-disordered (l_d) domains, and DPPC/CHL, DOPC, and raft mixture are good models for the l_o and l_d domains and the cell membrane, respectively.[9,12] The K_d^{OP} values measured in these vesicles are 0.082 ± 0.008, 0.11 ± 0.02 and 0.097 ± 0.010 μM, respectively (see footnotes for Table 1 and Fig. S4b, S4e and S4h in ESI†).[10] The values are ~2-fold larger than that measured in MOPS buffer and comparable to K_d^{OP} = (0.062 ± 0.007) μM reported for Calcium Green C_{18} in DOPC.[3a] The larger K_d^{OP} value of ACaL than that of ACa2 can be attributed to the lauryl group, which would stabilize the ACaL–Ca^{2+} complex by inducing a more hydrophobic environment around BAPTA. Moreover, the emission spectra of the ACaL–Ca^{2+} complex show λ_{max} at 435 and 462 nm in DPPC/CHL and DOPC, whereas that from the raft mixture shows λ_{max} at 439 nm with a shoulder, which could be fitted to two Gaussian functions centered at 432 and 468 nm, respectively (Fig. S5 in ESI†). This indicates that the emission from the AcaL–Ca^{2+} complex can reasonably reflect both of the l_o and l_d domains in the raft mixture. Therefore, the K_d^{OP} values measured in the vesicles would better reflect its binding ability in the cell membrane compared with that measured in the MOPS buffer; ACaL can detect near-membrane Ca^{2+} in the submicromolar range.

The TP action spectra of the Ca^{2+} complex with ACaL in buffer solution indicated a $\Phi\delta$ value of 90 GM at 780 nm for ACaL–Ca^{2+}, 2.5-fold larger than those of Calcium-Green-Ca^{2+} and Fura-2-Ca^{2+} (Fig. 1(b) and Table 1). Since the TP action cross section ($\Phi\delta$) of C_{18}-Fura-2 and Calcium Green C_{18} are expected to be similar to those of Calcium-Green-Ca^{2+} and Fura-2-Ca^{2+}, TPM images for samples stained with ACaL would be much brighter than those stained with the existing probes.

The pseudo-colored TPM image of cultured ROS cells [rat osteoblast-like osteosarcoma cells (ROS 17/2.8)] labeled with 5 μM ACaL clearly reveals the Ca^{2+} distribution in the plasma membrane. The TPEF spectrum from the plasma membrane (red curve) was unsymmetrical and could be fitted to two Gaussian functions with peak maxima at 435 nm (blue curve) and 475 nm (green curve), respectively (Fig. 2(b)). Moreover, the spectrum is very similar to that measured in the raft mixture with similar peak maxima of the dissected Gaussian functions (Fig. S5 in ESI†). Hence, the TPM image can most reasonably be attributed to the AcaL–Ca^{2+} complexes associated with l_o and l_d domains in the plasma membrane. Further, the TPM images collected at 360–460 and 500–620 nm ranges are almost the same except for the brightness (Fig. 2(c) and (d)). This indicates that Ca^{2+} is almost evenly distributed in both domains. Therefore, we have detected the near-membrane Ca^{2+} with ACaL by TPM by using the detection window at 360–620 nm.

To demonstrate the utility of this probe, we have monitored TPEF intensity of the ACaL-labeled ROS cells after addition of parathyroid hormone (PTH), a hormone that stimulates the cells to release free Ca^{2+} ($[Ca^{2+}]_i$) from intracellular stores.[13] Because $[Ca^{2+}]_i$ must be restored to the basal level, the excess $[Ca^{2+}]_i$ must be extruded from the cell *via* PMCA and/or transported to ER through SERCA.[2,8] Hence, the near-membrane Ca^{2+} concentration is expected to increase upon treatment with PTH and then decrease with time. Indeed, the TPEF intensities in the cell membrane began to rise at 50–100 s after addition of PTH (100 nM), reached at the maxima after 400–500 s, and then decreased to the basal levels after ~1200 s. When PTH was added after treating the cells for 10 min with thapsigarin (5 μM), an inhibitor that blocks the re-entry of Ca^{2+} into intracellular store formed by

Table 1 Photophysical data for ACaL, Calcium-green and Fura-2[a]

Compd.	$\lambda_{max}^{(1)}/\lambda_{max}^{fl}$[b]	Φ[c]	K_d^{OP}/K_d^{TP}[d]	$\lambda_{max}^{(2)}$[e]	$\Phi\delta$[f]
ACaL	369/500	0.0037		nd[g]	nd[g,h]
ACaL + Ca^{2+}	372/502	0.043	0.045[i]/0.041	780	90
Calcium Green C_{18} + Ca^{2+}	494/523[j]	0.75[l]	0.17[j]/nd[g]	800[k]	37[k]
C_{18}-Fura-2 + Ca^{2+}	335/505[l]	0.49[l]	0.14[l]/nd[g]	780[m]	36[m]

[a] All data were measured in 30 mM MOPS buffer in the absence and presence (39 μM) of free Ca^{2+}. [b] λ_{max} of the one-photon absorption and emission spectra in nm. [c] Fluorescence quantum yield. The uncertainty is ±10%. [d] Dissociation constants for Ca^{2+} in μM measured by one- (K_d^{OP}) and two-photon (K_d^{TP}) processes. The uncertainty is ±12%. [e] λ_{max} of the two-photon excitation spectra in nm. [f] The two-photon action cross section in 10^{-50} cm^4 s $photon^{-1}$ (GM). The uncertainty is ±15%. [g] Not determined. [h] TPEF intensity was too weak to measure the TP action cross section accurately. [i] K_d^{OP} values measured in DPPC/CHL, DOPC, and raft mixture are 0.082 ± 0.008, 0.11 ± 0.02, and 0.097 ± 0.010 μM, respectively. [j] Ref. 3*a* [k] Measured by using Calcium Green. [l] Ref. 3*b* [m] Measured by using Fura-2.

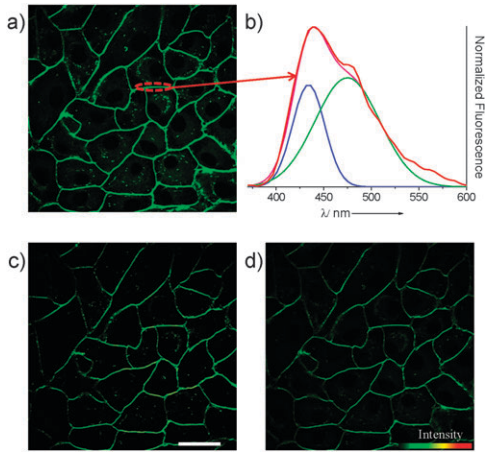

Fig. 2 Pseudo-colored TPM images of ACaL-labeled (5 μM) ROS cells collected at 360–620 nm (a), 360–460 nm (c), and 500–620 nm (d). (b) Two-photon excited fluorescence spectrum from the plasma membrane (red curve). The sky blue and pink curves represent the dissected Gaussian functions. The excitation wavelength was 780 nm. Cells shown are representative images from replicate experiments (n = 5). Scale bar, 30 μm.

endoplasmic reticulum (ER), [14] the TPEF intensities were more enhanced and the decay rates were slower (Fig. 3(d)). Further, the TPEF intensity decreased upon treatment with ethylene glycol tetraacetic acid (EGTA), a membrane-permeable Ca^{2+} chelator that can effectively remove Ca^{2+} (Fig. S6 in ESI†). It is to be noted that the TPM image shown in Fig. 3 is much clearer and brighter than the OPM image obtained with Calcium Green C_{18} after addition of external 1.2 mM Ca^{2+}. Hence, ACaL is far superior to the existing probes and is clearly capable of detecting near-membrane Ca^{2+} in live cells for longer than 1500 s.

We further investigated the utility of this probe in tissue imaging. TPM images were obtained from a part of fresh rat hippocampal slice incubated with 10 μM ACaL for 30 min at

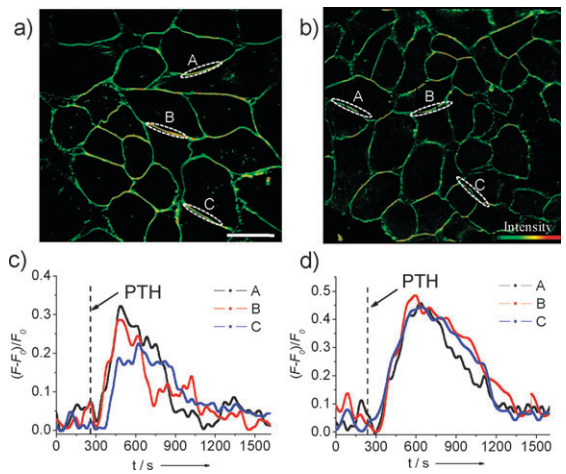

Fig. 3 Pseudo-colored TPM images of ROS cells labeled with ACaL (5 μM) in the absence (a) and presence of thapsigargin (5 μM) (b). (c, d) Time courses of TPEF at each designated position for samples a (c) and b (b) after stimulation with PTH (100 nM). Images were collected at 360–620 nm with 1.6 s intervals using an excitation wavelength at 780 nm. Scale bar, 30 μm.

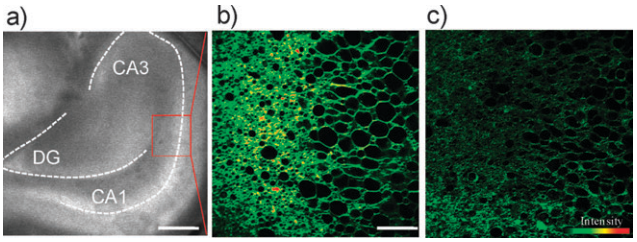

Fig. 4 Images of a fresh rat hippocampal slice labeled with 10 μM ACaL. (a) Bright-field images showing the CA1 and CA3 regions as well as dentate gyrus (DG) upon 10× magnification. White dotted lines indicate the pyramidal neuron layers. (b) TPM images of the red labeled region at a depth of ~120 μm by 100× magnification. (c) TPM images of the same region after addition of 200 μM EGTA to (b). Images were collected at 360–620 nm upon excitation at 780 nm with fs pulses. Scale bar, 300 (a) and 30 (b, c) μm, respectively.

37 °C. The bright field image reveals the CA1 and CA3 regions as well as the dentate gyrus (DG, Fig. 4(a)). The TPM image obtained at a higher magnification clearly reveals Ca^{2+} distribution in the cell membrane at 120 μm depth in live tissue (Fig. 4(b)). When EGTA was added to the imaging solution, the TPEF intensity decreased (Fig. 4(c)). These findings demonstrate that ACaL is capable of detecting membrane Ca^{2+} at 120 μm depth in live tissues using TPM.

To conclude, we have developed a TP probe (ACaL) that shows 10-fold TPEF enhancement in response to Ca^{2+}, dissociation constants (K_d^{TP}) of (0.041 ± 0.005) μM, pH-insensitive in the biologically relevant pH, and emit 2.5-fold stronger TPEF than Calcium-Green and Fura-2 upon complexation with Ca^{2+}. Better than the currently available probes, this probe can visualize near-membrane Ca^{2+} in live cells for more than 1500 s and living tissues at 120 μm depth without interference from other metal ions.

Notes and references

1 (*a*) R. Rizzuto and T. Pozzan, *Physiol. Rev.*, 2006, **86**, 369.
2 (*a*) M. J. Berridge, *Cell Calcium*, 2002, **32**, 235; (*b*) D. E. Clapham, *Cell*, 2007, **131**, 1047; (*c*) A. Bergner, J. Kellner, A. Tufman and R. M. Huber, *J. Exp. Clin. Cancer Res.*, 2009, **28**, 25.
3 (*a*) Q. P. Lloyd, M. A. Kuhn and C. V. Gay, *J. Biol. Chem.*, 1995, **270**, 22445; (*b*) E. F. Etter, M. A. Kuhn and F. S. Fay, *J. Biol. Chem.*, 1994, **269**, 10141; (*c*) Y. Tojyo, A. Tanimura and Y. Matsumoto, *Biochem. Biophys. Res. Commun.*, 1997, **240**, 189.
4 W. R. Zipfel, R. M. Williams and W. W. Webb, *Nat. Biotechnol.*, 2003, **21**, 1369.
5 F. Helmchen and W. Denk, *Nat. Methods*, 2005, **2**, 932.
6 R. M. Williams, W. R. Zipfel and W. W. Webb, *Curr. Opin. Chem. Biol.*, 2001, **5**, 603.
7 H. M. Kim and B. R. Cho, *Acc. Chem. Res.*, 2009, **42**, 863.
8 *The Handbooks–A Guide to Fluorescent Probes and Labeling Techonologies*, ed. R. P. Haugland, Molecular Probes, Eugene, OR, 10th edn, 2005.
9 K. Gaus, E. Gratton, E. P. Kable, A. S. Jones, I. Gelissen, L. Kritharides and W. Jessup, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 15554.
10 J. R. Long and R. S. Drago, *J. Chem. Educ.*, 1982, **59**, 1037.
11 K. A. Connors, *Binding Constant*, Wiley, New York, 1987.
12 (*a*) K. Simons and E. Ikonen, *Nature*, 1997, **387**, 569; (*b*) K. Simons and D. Toomre, *Nat. Rev. Mol. Cell Biol.*, 2000, **1**, 31.
13 (*a*) D. T. Yamaguchi, J. Green, C. R. Kleeman and S. Muallem, *Cell Calcium*, 1991, **12**, 609; (*b*) G. S. Belinsky, P. Morley, J. F. Whitfield and A. H. Tashjian, Jr, *Biochem. Biophys. Res. Commun.*, 1999, **266**, 448.
14 G. Inesi, S. Hua, C. Xu, H. Ma, M. Seth, A. M. Prasad and C. Sumbilla, *J. Bioenerg. Biomembr.*, 2005, **37**, 365.