DOI: 10.1002/asia.201100156

In Vivo Imaging of Near-Membrane Calcium Ions with Two-Photon Probes

Chang Su Lim, Min Young Kang, Ji Hee Han, Isravel Antony Danish, and Bong Rae Cho*[a]

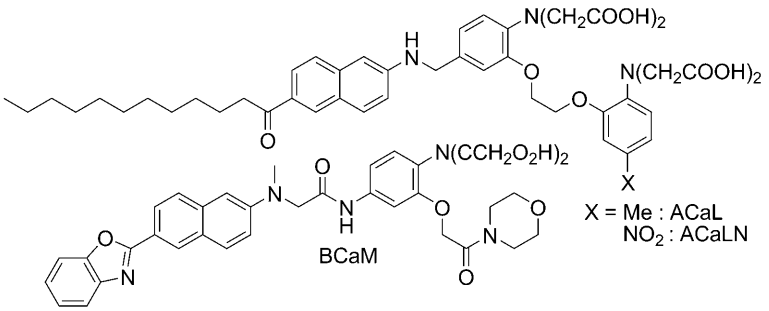
Dedicated to Professor Eun Lee on the occasion of his retirement and 65th birthday

Abstract: We report a two-photon (TP) probe (ACaLN) for near-membrane Ca^{2+} that shows a 13-fold TP excited fluorescence (TPEF) enhancement in response to Ca^{2+}, dissociation constants (K_d^{TP}) of (1.9±0.2) μM, pH-insensitivity at the biologically relevant pH, and can detect near-membrane Ca^{2+} in live cells for more than 1500 s and in living tissues at 120 μm depth without interference from other metal ions. Comparison with existing probes provides a useful strategy for the design of efficient TP probes for the near-membrane metal ions.

Keywords: calcium · fluorescence · nonlinear spectroscopy · microscopy · two-photon probe

Introduction

Calcium ions (Ca^{2+}) play a pivotal role in the physiology and biochemistry of organisms and cells.[1,2] In mammals, calcium levels are tightly regulated with bone acting as the major mineral storage site. Ca^{2+} is released from bone into the bloodstream under controlled conditions. There is a very large transmembrane electrochemical gradient of Ca^{2+} driving the entry of Ca^{2+} into cells.[1] As the cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) must be kept low, the excess Ca^{2+} is extruded out of the cells.[1] Transport of the Ca^{2+} into and out of the cells functions as a signal for numerous cellular processes such as fertilization, cell death, sensory transduction, muscle contraction, and fluid secretion.[1,2] Also, many physiological functions, including exocytosis, control of membrane K^+ and Ca^{2+} permeability, and enzyme activity, are regulated by the near-membrane Ca^{2+} concentration ($[Ca^{2+}]_m$). To understand the biological functions of $[Ca^{2+}]_m$, it is crucial to monitor the translocation of Ca^{2+} across the plasma membrane. For this purpose, we have developed a few two-photon (TP) probes (ACaL, ACaCL, and BCaM,).[3] These



probes were far superior to the existing one-photon (OP) fluorescent probes, such as C_{18}-Fura-2 and Calcium Green C_{18}, for application in two-photon microscopy (TPM).[4] TPM, which utilizes two photons of lower energy for the excitation, has become a vital tool in biology and medicine owing to the capability of imaging deep inside tissue with tightly localized emission.[5] However, the dissociation constants (K_d) of ACaL and ACaCL are in the order of 0.04–0.06 μM, whereas that of BCaM is 90 μM.[3] As $[Ca^{2+}]_m$ varies

[a] C. S. Lim,[+] M. Y. Kang,[+] J. H. Han, Dr. I. A. Danish, Prof. Dr. B. R. Cho
Department of Chemistry
Korea University
1-Anamdong, Seoul, 136-701 (Korea)
Fax: (+82) 2-3290-3544
E-mail: chobr@korea.ac.kr

[+] These two authors contributed equally to this work.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201100156.

depending on the cell types and cell functions, there is a need to develop TP probes with a variety of K_d values.

In this context, we have developed a new TP probe for near-membrane Ca^{2+} derived from 2-amino-6-dodecanoylnaphthalene (L) as the reporter (ACaLN), and *p*-nitro derivative of *O,O′*-bis(2-aminophenyl)ethyleneglycol-*N,N,N′,N′*-tetraacetic acid (NBAPTA) as the Ca^{2+} receptor. NBAPTA is a well-known Ca^{2+} chelator that has been employed in one-photon fluorescent probes, such as Calcium Green-5N and Orgon Green 488 BAPTA-5N,[6] while L is a TP polarity probe that has been widely used as a membrane probe.[7] Herein, we report that ACaLN shows a moderate K_d value (2 μM) and can detect near-membrane Ca^{2+} in live cells and tissues at >100 μm depth by TPM without interference from competing metal ions, pH, and mistargeting and photobleaching problems.

Results and Discussion

Synthesis of ACaLN is summarized in Scheme 1. Compound **1** was prepared according to the literature method[8] and 2-amino-6-dodecanoylnaphthalene was obtained using the same method as previously reported.[3a] Compound **2** was prepared by formylation of **1** and subsequent nitration. ACaLN was prepared in 46% yield by the reductive amination between **2** and 2-amino-6-dodecanoylnaphthalene followed by hydrolysis.

The solubility of ACaLN in 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer solution (30 mM, pH 7.2) was greater than 15 μM, which is sufficient to stain the cells (Figure S1, see the Supporting Information). The absorption and emission spectra of ACaLN show gradual bathochromic shifts with the solvent polarity in the order, 1,4-dioxane < DMF < EtOH < MOPS buffer (Figure S2 and Table S1 in the Supporting Information) and the shift is greater in the emission (64 nm) than in the absorption spectra (4 nm), thus indicating the utility of ACaLN as a polarity probe.

When Ca^{2+} was added to ACaLN in MOPS buffer solution (30 mM, pH 7.2), the fluorescence intensity increased gradually with the metal ion concentration without affecting the absorption spectra (Figures 1 a, S3, and S4 in the Supporting Information), presumably because of the blocking of the photo-induced electron transfer (PeT) process by the metal ion complexation.[5] The fluorescence enhancement

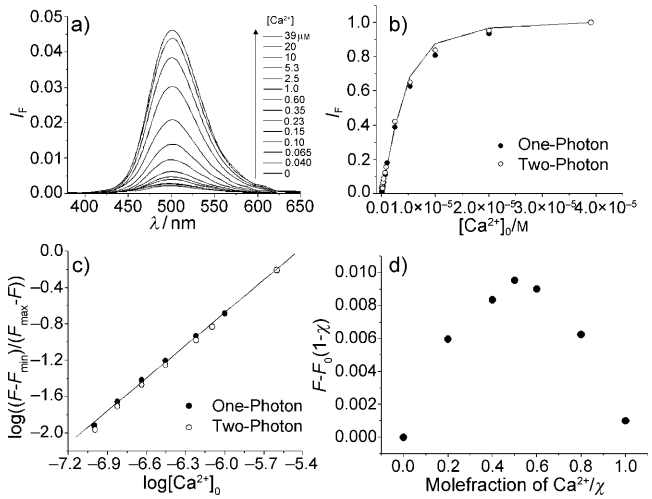
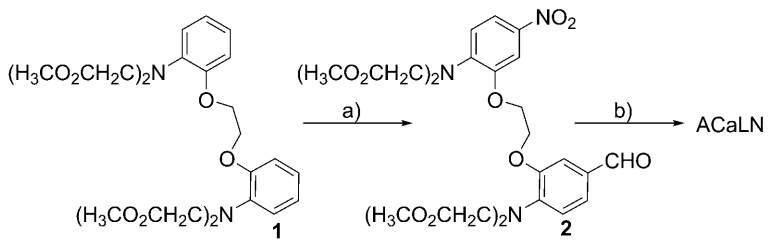


Figure 1. One-photon emission spectra (a) and one-(●) and two-photon (○) fluorescence titration curves (b) of ACaLN (3 μM) in the presence of various concentrations of free Ca^{2+} ions (0–39 μM). c) Hill plots for the complexation of ACaLN with Ca^{2+} (0–39 μM) measured by one- (●) and two-photon (○) spectroscopy. d) Job plot for determination of the stoichiometry of ACaLN-Ca^{2+} in MOPS buffer (30 mM, pH 7.2). The total concentration of Ca^{2+} was 10 μM. These data were measured in 30 mM MOPS, 100 mM KCl, 10 mM EGTA, pH 7.2. The excitation wavelengths for one- and two-photon processes are 365 and 750 nm, respectively.

factor [FEF = $(F-F_{min})/F_{min}$] of ACaLN, measured by the one- and two-photon titration curves, is 13 in the presence of 39 μM Ca^{2+}, indicating a high sensitivity to the change in the Ca^{2+} concentration (Figure 1 a). Moreover, the titration curve fitted well with the 1:1 binding model (Figure 1 b), the Hill plots were linear with a slope of 1.0 (Figure 1 c), and the Job plot exhibited a maximum point at a mole fraction of 0.50 (Figure 1 d), thereby indicating 1:1 complexation between the probe and Ca^{2+}.[9]

The dissociation constants (K_d) were calculated from the fluorescence titration curves (Figure 1 b).[10,11] The K_d values of ACaLN for Ca^{2+} in MOPS buffer solution as determined by one- (K_d^{OP}) and two-photon (K_d^{TP}) processes are 2.1 ± 0.4 and 1.9 ± 0.2 μM, respectively. For comparison, the K_d^{TP} value of ACaL is 0.041.[3a] The larger K_d value for ACaLN can be attributed to the nitro group in the BAPTA which may reduce the binding ability. Moreover, the K_d^{TP} value of BCaM is 90 μM.[3c] Therefore, we now have a series of near-membrane TP probes for Ca^{2+} with K_d^{TP} values ranging from 0.05 to 90 μM. This allows the detection of near-membrane Ca^{2+} concentrations ranging from sub-μM to μM to sub-mM.

ACaLN showed a high selectivity for Ca^{2+}, as revealed by the negligible fluorescence intensity in the presence of 2 mM concentrations of Mg^{2+}, 100 μM of Fe^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, and Zn^{2+} and a large en-



Scheme 1. Synthesis of ACaLN. a) 1) $POCl_3$, DMF; 2) HNO_3, AcOH, Ac_2O; b) 1) 2-amino-6-dodecanoylnaphthalene, $NaBH(OAc)_3$, EtOAc, CH_2Cl_2; 2) KOH, EtOH.

hancement in the fluorescence intensity upon addition of 39 μM of Ca^{2+} (Figure 2a). Hence, this probe can detect the intracellular free Ca^{2+} with minimum interference from other competing metal ions. Moreover, it was pH-insensitive at pH > 6.5 (Figure 2b).

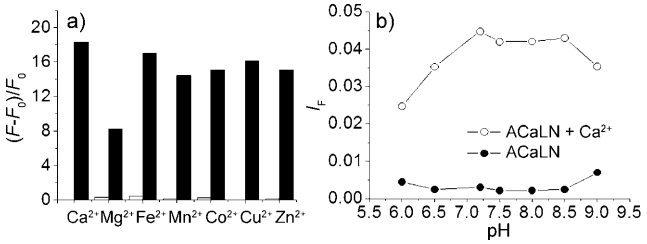


Figure 2. a) The relative fluorescence intensity of 5 μM ACaLN in the presence of 2 mM for Mg^{2+}; 100 μM for Fe^{2+}, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+} (empty bars) followed by addition of 39 μM Ca^{2+} (filled bar). b) Effect of the pH on the fluorescence intensity of 3 μM ACaLN in the presence of 0 (●) and 39 μM (○) of Ca^{2+}. These data were measured in 30 mM MOPS buffer (100 mM KCl, 10 mM EGTA, pH 7.2).

The TP action spectrum of the Ca^{2+} complexes with ACaLN in buffer solution indicated a two-photon action cross section ($\Phi\delta$) value of 20 GM at 750 nm (Table 1). The value is smaller than that of ACaCL ($\Phi\delta = 90$ GM), apparently because of the smaller Φ value for ACaLN-Ca^{2+} (0.043 vs. 0.018). The nitro group in the receptor appears to have quenched the fluorescence significantly. Nevertheless, the TPM images of cells and tissues labeled with ACaLN were bright enough to detect near-membrane Ca^{2+} by TPM (Figure 3).

To demonstrate the utility of this probe, we obtained the TPM image of cultured HT22 cells (clonal mouse hippocampal cell) labeled with 3 μM ACaLN (Figure 3a). The image was bright and revealed the Ca^{2+} distribution in the plasma membrane. We then monitored the TPEF intensity in the ACaLN-labeled HT22 cells after addition of histamine, an agonist that stimulates the cells to release $[Ca^{2+}]_c$ from intracellular stores, such as the endoplasmic reticulum (ER).[12] It was expected that the excess $[Ca^{2+}]_c$ liberated by histamine would be extruded out of the cell through the cell membrane to maintain the Ca^{2+} homeostasis, thereby increasing the $[Ca^{2+}]_m$.[12] Indeed, the TPEF intensities in the cell membrane began to rise after addition of histamine (100 mM), reached a peak value after 150 s, and returned to the baseline level after 300 s. When the cells were treated with 2 mM $CaCl_2$, the $[Ca^{2+}]_m$ increased immediately, reached a maximum intensity after 50 s, and then decreased to the baseline level after 10 min (Figure 3b).[12] Hence, ACaLN is clearly capable of monitoring the changes in $[Ca^{2+}]_m$ in live cells over a long time period.

We further investigated the utility of this probe in tissue imaging. TPM images were obtained from a section of a fresh rat hippocampal slice incubated with 30 μM ACaLN for 30 min at 37 °C. The bright-field image reveals the CA1 and CA3 regions as well as the dentate gyrus (DG, Figure 3c). As the structure of the brain tissue is known to be inhomogeneous in its entire depth, we accumulated 10 TPM images at depths of 90–180 μm to visualize the distributions of the $[Ca^{2+}]_m$. The accumulated TPM image shows that the $[Ca^{2+}]_m$ is more abundant in the CA1 than in the DG and CA3 regions (Figure 3d). The TPM image obtained at a higher magnification clearly reveals Ca^{2+} distribution in the cell membrane at 120 μm depth in live tissue (Figure 3e). Moreover, when ethylene diamine tetraacetic acid (EDTA), a membrane-permeable Ca^{2+} chelator that can effectively remove Ca^{2+}, was added to the imaging solution, the TPEF intensity decreased (Figure 3h). Further, the TPM images of the slices labeled with ACaLN revealed the $[Ca^{2+}]_m$ distribution in the given *xy* plane at 90–180 μm depth, indicating the sectioning capability of TPM (Figure S7 in the Supporting Information). These findings demonstrate that ACaLN is capable of detecting membrane Ca^{2+} at 120 μm depth in live tissues using TPM.

It is noteworthy that the TPM image of the ACaLN-labeled tissue reveals the distribution of $[Ca^{2+}]_m$ over the entire cell membranes deep inside intact tissue, while those labeled with ACaCL and BCaM show intense and dim regions, respectively. This outcome suggests that the K_d value of ACaLN is similar to $[Ca^{2+}]_m$, whereas that of ACaCL is too small and emits strong TPEF in the regions where $[Ca^{2+}]_m \gg 0.05$ μM, and that of BCaM is too large and emits little TPEF in the region where $[Ca^{2+}]_m \ll 90$ μM. Notably, low and high affinity probes can highlight the $[Ca^{2+}]_m$-rich regions. This result underlines the importance of developing TP probes for $[Ca^{2+}]_m$ with a variety of Ca^{2+} ion affinities. Moreover, the image with BCaM is not as clear as those with ACaL and ACaLN and reveals blurred domains where the probes appear to be aggregated, indicating that a long chain hydrocarbon is crucial for the staining the cell membranes deep inside intact tissue.

Table 1. Photophysical data for ACaL, ACaLN, and BCaM.[a]

Compoundd	$\lambda_{max}^{(1)}/\lambda_{max}^{fl}$ [b]	Φ[c]	K_d^{OP}/K_d^{TP}[d]	$\lambda_{max}^{(2)}$ [e]	$\Phi\delta$[f]
ACaL[g]	369/500	0.0037		nd[g]	nd[h]
ACaL + Ca^{2+}[g]	372/502	0.043	0.045/0.041	780	90
ACaLN	364/498	0.0013		nd[g]	nd[h]
ACaLN + Ca^{2+}	354/497	0.018	2.1/1.9	750	20
BCaM[i]	360/470	0.070		nd[g]	nd[h]
BCaM + Ca^{2+}[i]	360/470	0.98	89/78	780	150

[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^4s/photon (GM). The uncertainty is ±15%. [g] Ref. [3a]. [h] Not determined because TPEF intensity was too weak to measure the TP action cross section accurately. [i] Ref. [3c].

CHEMISTRY
AN ASIAN JOURNAL

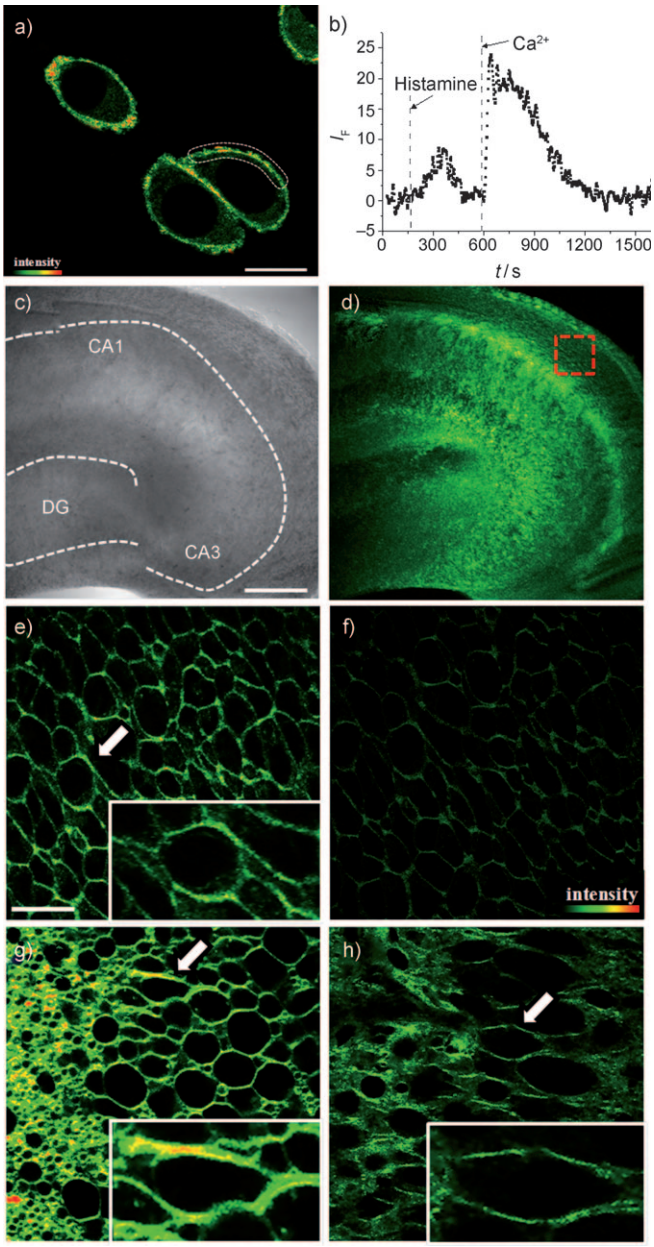


Figure 3. a) Pseudo colored two-photon microscopy (TPM) images of HT22 cells labeled with 3 μM ACaLN. b) Time course of two-photon excited fluorescence (TPEF) at the position marked with a dotted line in (a) after stimulation with 100 mM histamine in nominally calcium-ion-free buffer, followed by addition of 2 mM $CaCl_2$ to the imaging solution. TPEF was collected at 360–620 nm upon excitation at 750 nm. c–f) Images of a fresh rat hippocampal slice labeled with 30 μM ACaLN. c) 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. d) 10 TPM images collected in (c) along the *z* direction at the depths of 90–180 μm were accumulated. e) TPM images of the red labeled region at a depth of ~120 μm by 100× magnification. f) TPM images of the same region after addition of 200 μM EDTA to (e). g,h) TPM images of fresh rat hippocampal slices labeled with 30 μM ACaCL and BCaM, respectively. Image (g) was taken from Ref. [3a], and images (d–f) and (h) were collected at 360–620 nm upon excitation at 750 and 780 nm, respectively, with fs pulses. The images in the white boxes are the enlarged images of the cells indicated by the white arrows. Scale bar, 15 (a), 30 (e, f, g, h), and 300 μm (c, d), respectively.

Conclusions

In conclusion, we have developed a TP probe (ACaLN) that shows a 13-fold TPEF enhancement in response to Ca^{2+}, dissociation constants (K_d^{TP}) of (1.9±0.2) μM, pH insensitivity in the biologically relevant pH, and can detect near-membrane Ca^{2+} in live cells for more than 1500 s and in living tissues at 120 μm depth without interference from other metal ions. These results provide a useful strategy for the design of efficient TP probes for the near-membrane metal ions.

Experimental Section

Synthetic Materials and Methods

Silica gel P60 (SiliCycle) was used for column chromatography. All other chemicals were purchased from Sigma–Aldrich and were used as received.

*Compound **2***

A mixture of $POCl_3$ (3.2 mL, 34 mmol) and *N,N*-dimethylformamide (DMF; 6 mL) was stirred at 0 °C for 1 h. This mixture was added to the solution of **1** (6.0 g, 11 mmol) in DMF (30 mL) at 0 °C and heated to 60 °C for 30 min. The crude product was cooled, transferred to a beaker, neutralized with $NaHCO_3$ (aq), and extracted with ethyl acetate. The solvent was evaporated and the crude product was purified by column chromatography using hexane/EtOAc (1:1) as the eluent. Yield: 4.8 g (76 %); m.p.: 139 °C; IR(KBr): $\tilde{\nu}$=2955, 1751, 1686 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ=9.78 (1 H, s), 7.41–7.37 (2 H, m), 6.91–6.80 (4 H, m), 6.78 (1 H, d, *J*=8.4 Hz), 4.28 (4 H, s), 4.24 (4 H, s), 4.15 (4 H, s), 3.55 (6 H, s), 3.54 ppm (6 H, s). This compound (0.50 g, 0.89 mmol) was dissolved in acetic anhydride (30 mL) and stirred under ice cold conditions. A solution of 70 % nitric acid (0.063 mL, 0.98 mmol) in acetic acid (5 mL) was slowly added for 3 h. After the addition was over, the mixture was further stirred for 1 h and the reaction was quenched by dilute $NaHCO_3$ (aq). The product was extracted with ethyl acetate, dried over $MgSO_4$, and purified by column chromatography using hexane/EtOAc/$CHCl_3$ (1:1:1) as the eluent. Yield: 0.24 g (45 %); m.p.: 134 °C; IR(KBr): $\tilde{\nu}$= 2955, 1755, 1683, 1520 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ=9.82 ppm (1 H, s), δ=7.88 (1 H, dd, *J*=9.0, 2.4 Hz), 7.74 (1 H, d, *J*=2.4 Hz), 7.43 (1 H, dd, *J*=7.8, 2.1 Hz), 7.39 (1 H, d, *J*=2.1 Hz,), 6.80 (1 H, d, *J*= 7.8 Hz), 6.71 (1 H, d, *J*=9.0 Hz), 4.33 (4 H, s), 4.23 (8 H, s), 3.63 (6 H, s), 3.60 ppm (6 H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ=190.4, 171.2, 170.9, 145.2, 145.1, 142.9, 142.6, 140.9, 129.9, 127.2, 118.5, 116.7, 116.0, 110.5, 107.8, 67.4 ,66.8, 53.6, 53.5, 52.1, 52.0 ppm.

ACaLN

A mixture of 2-amino-6-dodecanoylnaphthalene (0.10 g, 0.16 mmol) and **2** (53 mg, 0.16 mmol) in anhydrous EtOAc (5 mL) was refluxed overnight. The solvent was evaporated, and $NaBH(OAc)_3$ (70 mg, 0.32 mmol) in CH_2Cl_2 (5 mL) was added, and the mixture was refluxed overnight. The product was isolated by evaporation and purified by column chromatography using hexane/EtOAc (5:1) as the eluent. Yield: 89 mg (61 %). Ethanol (2.5 mL) and KOH in ethanol (1.0 M, 0.97 mL) were added to this compound (89 mg, 0.097 mmol). After stirring overnight, the volume was reduced by half, and dilute HCl (aq) was added slowly until pH 3–4 to obtain the product as an orange solid, which was washed with distilled water. Yield: 62 mg (75 %); m.p.: 159 °C; IR(KBr): $\tilde{\nu}$=3412, 1718, 1618, 1512 cm^{-1}; ^1H NMR (300 MHz, [D_4]MeOH): δ=8.26 (1 H, d, *J*=1.5 Hz), 7.78–7.70 (2 H, m), 7.64 (1 H, d, *J*=9.0 Hz), 7.64 (1 H, d, *J*=2.4 Hz), 7.48 (1 H, d, *J*=9.0 Hz), 7.10–7.00 (2 H, m), 6.92 (1 H, dd, *J*=8.1, 1.5 Hz), 6.83 (1 H, d, *J*=8.1 Hz), 6.73 (1 H, d, *J*=1.8 Hz), 6.71 (1 H, d, *J*=9.0 Hz), 4.32 (2 H, s), 4.25 (4 H, s), 4.20 (4 H, s), 4.06 (4 H, s), 3.00 (2 H, t, *J*=7.5 Hz), 1.69 (2 H, quin, *J*=7.5 Hz), 1.26 (16 H, m), 0.88 ppm (3 H, t, *J*=6.9 Hz); ^{13}C NMR (100 MHz, [D_4]MeOH): δ=201.6, 174.6, 173.4, 150.5, 149.3,

148.4, 145.5, 140.4, 138.5, 138.1, 133.6, 130.6, 130.2, 130.0, 129.7, 125.9, 125.7, 124.1, 120.2, 118.8, 118.0, 115.2, 112.8, 108.1, 103.2, 67.7, 66.7, 54.4, 46.9, 46.7, 37.9, 35.3, 33.8, 31.9, 29.6, 29.3, 26.9, 25.7, 24.9, 22.6 ,13.3 ppm. HR-MS calculated for $[M+H]^+$: 859.3767; found: 859.3765.

Spectroscopic Measurements

Absorption spectra were recorded on a Hewlett–Packard 8453 diode array spectrophotometer and fluorescence spectra were obtained with Aminco-Bowman series 2 luminescence spectrometer using a 1 cm path-length standard quartz cell. The fluorescence quantum yield was determined by using Coumarin 307 as the reference using the literature method.[13]

Solubility of ACaLN in MOPS Buffer

A small amount of dye was dissolved in DMSO to prepare the stock solutions (1.0×10^{-3} M). The solution was diluted to $6.0\times10^{-3}\sim6.0\times10^{-5}$ M and added to a cuvette containing MOPS buffer (3.0 mL, pH 7.2) using a micro syringe. In all cases, the concentration of DMSO in H_2O was maintained to be 0.2%.[3] The plots of absorbance against the dye concentration were linear at low concentration and showed downward curvature at higher concentrations (Figure S1 in the Supporting Information). The maximum concentration in the linear region was taken as the solubility. The solubility of ACaLN in MOPS buffer was greater than 15 μM.

Determination of Apparent Dissociation Constants

A series of solutions containing various $[Ca^{2+}]$ were prepared in the presence of 3 μM ACaLN in 30 mM MOPS buffer and they were adjusted to pH 7.2. The apparent dissociation constant (K_d) was determined using the following equation: $F-F_{min}=[Ca^{2+}](F_{max}-F_{min})/(K_d+[Ca^{2+}])$, where F is the observed fluorescence, F_{max} is the fluorescence for the Ca^{2+}-ACaLN complex, and F_{min} is the fluorescence for the free ACaLN. The K_d value that best fits the titration curve (Figures 2 and S3 in the Supporting Information) using the equation was calculated by using the Excel program as previously reported.[9,10] In order to determine the K_d^{TP} for the two-photon process, the TPEF intensity was recorded in the range of 400–620 nm with a CCD detector excited by a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at a wavelength of 750 nm and output power 1348 mW, which corresponded to approximately 200 mW average power in the focal plane.

Measurement of Two-Photon Cross Section

The two-photon cross section (δ) was determined by using the femtosecond (fs) fluorescence measurement technique as described previously.[14] ACaLN was dissolved in 30 mM MOPS buffer (pH 7.2) at concentrations of 5.0×10^{-6} M and then the two-photon induced fluorescence intensity was measured at 740–900 nm using rhodamine 6G as the reference, the two-photon property of which has been well characterized in the literature.[15] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta=\delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$: where the subscripts s and r stand for the sample and reference molecules, respectively. The intensity of the signal collected by a CCD detector was denoted as S. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as c. δ_r is the TPA cross section of the reference molecule. The TP action cross section of ACaLN was 20 GM at 750 nm.

Cell Culture and Imaging

HT22 clonal mouse hippocampal cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, WelGene) supplemented with heat-inactivated 10% fetal bovine serum (FBS, WelGene), penicillin (100 units mL^{-1}), and streptomycin (100 μg mL^{-1}). All cells were kept in a humidified atmosphere of 5:95 (v/v) of CO_2/air at 37 °C. Two days before imaging, the cells were passed and plated on glass-bottomed dishes (MatTek). For labeling, the growth medium was removed and replaced with DMEM without FBS. The cells were incubated with ACaLN (3 μM) for 5 min at 25 °C. The cells were washed three times with phosphate buffered saline (PBS; Gibco) without Ca^{2+} and Mg^{2+} and then imaged.

Two-Photon Fluorescence Microscopy

Two-photon fluorescence microscopy images of ACaLN-labeled cells and tissues were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP2) with a ×100 (NA=1.30 OIL) and ×10 (NA=0.30 DRY) objective lens, respectively. The two-photon fluorescence microscopy images were obtained with a DM IRE2 Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Coherent Chameleon, 90 MHz, 200 fs) set at wavelength of 750 nm and output power of 1348 mW, which corresponded to approximately 10 mW average power in the focal plane. To obtain images in the 360~460 nm and 500~620 nm ranges, internal PMTs were used to collect the signals in 8 bit unsigned 512×512 pixels at 400 Hz scan speed.

Detection window

The TPEF spectrum from the ACaLN-labeled cells was unsymmetrical and could be fitted to two Gaussian functions with peak maxima at 434 nm (blue curve) and 475 nm (green curve), respectively (Figure S6b in the Supporting Information). The spectra are very similar to those measured in DPPC/CHL and DOPC, which are good models for the l_o and l_d domains, respectively (Figure S5 in the Supporting Information). Moreover, the TPM images collected in the 360–460 and 500–620 nm ranges are nearly the same except for the brightness (Figure S6c, d in the Supporting Information). Therefore, we have detected near-membrane Ca^{2+} with ACaLN by using the detection window at 360–620 nm.

Preparation and Staining of Fresh Rat Hippocampal Slices

All experiments were performed in accordance with the guidelines established by the Committee of Animal Research Policy of Korea University College of Medicine. Slices were prepared from the hippocampi and the hypothalmi of 2 day-old rats (SD). Coronal slices were cut into 400 μm-thick using a vibrating-blade microtome in artificial cerebrospinal fluid (ACSF); 138.6 mM NaCl, 3.5 mM KCl, 21 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 9.9 mM D-glucose, 1 mM $CaCl_2$, and 3 mM $MgCl_2$. Slices were incubated with 30 μM ACaLN (or BCaM) in ACSF bubbled with 95% O_2 and 5% CO_2 for 30 min at 37 °C. Slices were then washed three times with ACSF and transferred to glass-bottomed dishes (MatTek) and observed in a spectral confocal multiphoton microscope. After washing three times with ACSF, the slices were transferred to glass bottomed dishes (MatTek) and observed under a spectral confocal multiphoton microscope. To observe the effect of EDTA, a solution of EDTA in MOPS buffer (200 μM) was added to this sample and the TPM image was obtained.

Acknowledgements

This work was supported by the National Research Foundation (NRF) grants funded by the Korean Government (No. 2010-0018921) and Priority Research Centers Program through the NRF funded by the Ministry of Education, Science and Technology (2010-0020209). C. S. Lim, M. Y. Kang, J. H. Han, and I. A. Danish were supported by BK21 scholarship.

[1] E. Carafoli, *Physiol. Rev.* **1991**, *71*, 129–153.
[2] a) M. D. Bootman, M. J. Berridge, *Cell* **1995**, *83*, 675–678; b) M. D. Bootman, M. J. Berridge, P. Lipp, *Cell* **1997**, *91*, 367–373; c) M. D. Bootman, M. J. Berridge, P. Lipp, *Nature* **1998**, *395*, 645–648; d) D. E. Clapham, *Cell* **1995**, *80*, 259–268.
[3] a) P. S. Mohan, C. S. Lim, Y. S. Tian, W. Y. Roh, J. H. Lee, B. R. Cho, *Chem. Commun.* **2009**, 5365–5367; b) Y. N. Shin, C. S. Lim, Y. S. Tian, W. Y. Rho, B. R. Cho, *Bull. Korean Chem. Soc.* **2010**, *31*, 599–605; c) H. J. Kim, J. H. Han, M. J. Kim, C. S. Lim, H. M. Kim, B. R. Cho, *Angew. Chem.* **2010**, *122*, 6938–6941; *Angew. Chem. Int. Ed.* **2010**, *49*, 6786–6789.

[4] a) Q. P. Lloyd, M. A. Kuhn, C. V. Gay, *J. Biol. Chem.* **1995**, *270*, 22445–22451; b) E. F. Etter, M. A. Kuhn, F. S. Fay, *J. Biol. Chem.* **1994**, *269*, 10141–10149.
[5] a) H. M. Kim, B. R. Cho, *Acc. Chem. Res.* **2009**, *42*, 863–872; b) H. M. Kim, B. R. Cho, *Chem. Asian J.* **2011**, *6*, 58–69; c) S. Sumalekshmy, C. J. Fahrni, *Chem. Mater.* **2011**, *23*, 483–500.
[6] *The Handbooks–A Guide to Fluorescent Probes and Labeling Technologies*, *10th ed.* (Ed.: R. P. Haugland), Molecular Probes, Eugene, **2005**.
[7] K. Gaus, E. Gratton, E. P. Kable, A. S. Jones, I. Gelissen, L. Kritharides, W. Jessup, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 15554–15559.
[8] R. Y. Tsien, *Biochemistry* **1980**, *19*, 2396–2404.
[9] K. A. Connors, *Binding Constants*, Wiley, New York, **1987**.
[10] a) R. Y. Tsien, T. Pozzan, T. J. Rink, *J. Cell Biol.* **1982**, *94*, 325; b) R. Y. Tsien, T. Pozzan, *Methods Enzymol.* **1989**, *172*, 230–261; c) A. Takahashi, P. Camacho, J. D. Lechleiter, B. Herman, *Physiol. Rev.* **1999**, *79*, 1089–1125.
[11] a) J. R. Long, R. S. Drago, *J. Chem. Educ.* **1982**, *59*, 1037–1039; b) K. J. Hirose, *Incl. Phenom. Macrocycl. Chem.* **2001**, *39*, 193–209.
[12] a) S. Lin, K. A. Fagan, K. X. Li, P. W. Shaul, D. M. Cooper, D. M. Rodman, *J. Biol. Chem.* **2000**, *275*, 17979–17985; b) T. Nagai, S. Yamada, T. Tominaga, M. Ichikawa, A. Miyawaki, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 10554–10559.
[13] J. N. Demas, G. A. Crosby, *J. Phys. Chem.* **1971**, *75*, 991–1024.
[14] S. K. Lee, W. J. Yang, J. J. Choi, C. H. Kim, S. J. Jeon, B. R. Cho, *Org. Lett.* **2005**, *7*, 323–326.
[15] C. Xu, W. W. Webb, *J. Opt. Soc. Am. B* **1996**, *13*, 481–491.

Received: February 15, 2011
Published online: June 17, 2011

