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Optically Selective Two-Photon Uncaging of Glutamate at 900 nm

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Supporting Information

ABSTRACT: We have synthesized a 7-diethylaminocoumarin (DEAC) derivative that allows wavelength-selective two-photon uncaging at 900 nm versus 720 nm. This new caging chromophore, called DEAC450, has an extended π-electron moiety at the 3-position that shifts the absorption spectrum maximum of DEAC from 375 to 450 nm. Two-photon excitation at 900 nm was more than 60-fold greater than at 720 nm. Two-photon uncaging of DEAC450-Glu at 900 nm at spine heads on pyramidal neurons in acutely isolated brain slices generated postsynaptic responses that were similar to spontaneous postsynaptic excitatory miniature currents, whereas significantly higher energies at 720 nm evoked no currents. Since many nitroaromatic caged compounds are two-photon active at 720 nm, optically selective uncaging of DEAC450-caged biomolecules at 900 nm may allow facile two-color optical interrogation of bimodal signaling pathways in living tissue with high resolution for the first time.

The use and recording of color in a scientific context is now a fundamental part of what we do in biomedical research.[1] Thus, it is difficult to imagine using confocal fluorescent imaging if it were still monochrome.[2] Fortunately, many technological advances have been combined to allow us to use fluorescence imaging to monitor many aspects of neuronal activity in real time in spectrally separate channels.[3] In contrast, our ability to manipulate cell function with comparable chromatic diversity lags behind imaging and seriously limits our ability to study multiple signaling pathways simultaneously.[4]

Neuroscience is a field in which optical actuation of cell function has been widely used. For example, four methods have been developed for photocontrol of neuronal membrane potential: (1) neurotransmitter uncaging to activate endogenous ligand-gated ion channels;[5,6] (2) chemical modification of mutated ion channels with optical switches;[7] (3) photochemical stimulation of genetically targeted alien ion channels;[8] and (4) excitation of genetically delivered photoregulated ion pumps and channels.[9−12] Each of these methods has advantages and disadvantages. The first is powerful because it directly activates native receptors, so is useful for understanding the details of cellular physiology in vitro.[13] A striking feature of the second and fourth methods is the wavelength selectivity that is inherent in or engineered into the chromophores, allowing two colors of light to be used orthogonally for different purposes.

Starting in 1978, hundreds of biological studies have been reported using photolysis of nitrobenzyl-caged compounds at short wavelengths of light, specifically, 350−400 nm for one-photon[14−19] and 720−740 nm for two-photon[18,20,21] photolysis. Caging chromophores that absorb at longer wavelengths than these compounds have only recently been developed and thus have been applied to relatively few biological questions. In particular, several substituted organic laser-dye-based 7-aminocoumarins[22] and organic−inorganic hybrid chromophores based on the ruthenium−bipyridyl[23,24] (RuBi) scaffold are effectively photolyzed at wavelengths longer than 400 nm for one-photon or 740 nm for two-photon photolysis. Caged neurotransmitters using such chromophores are important additions to the optical arsenal available to neurobiologists, but their absorption spectra lack pronounced minima at short wavelengths (Figure 1). Here we introduce a new caged-glutamate compound, called DEAC450-Glu (Figure 1a), that is relatively photoinactive at short wavelengths (e.g., 720 nm) and undergoes maximal two-photon excitation at 900 nm. This significant bathochromic shift thus extends the color palette of two-photon photolysis to a region that it is optically complementary to those of many other caged compounds.

The synthesis of DEAC450-Glu (Figure 1a) began with protection of the known alcohol DEAC-OH[25] to give **1**. The coumarin 3-position was functionalized with *N*-bromosuccinimide (NBS)[26] to give **2**, and subsequent Heck coupling[27] of *tert*-butylacrylate with **2** gave **3**. Deprotection of the *tert*-butyl group to obtain acid **4** was followed by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling of di-*tert*-butyl-D-Asp to give **5**. Selective removal of the silyl group afforded alcohol **6**, which was coupled to the acid side chain of L-glutamate to give fully protected DEAC450-Glu. Finally, the remaining protecting groups were removed, and the product was purified by HPLC to give DEAC450-Glu (**7**). DEAC450-Glu was found to be soluble (up to 7.5 mM) and quite stable at pH 7.4. Solutions showed no hydrolysis over 60 days at −20 °C or 5 h at room temperature, and at 37 °C only 2% was hydrolyzed in 2 h. Irradiation of DEAC450-Glu and RuBi-Glu[24]

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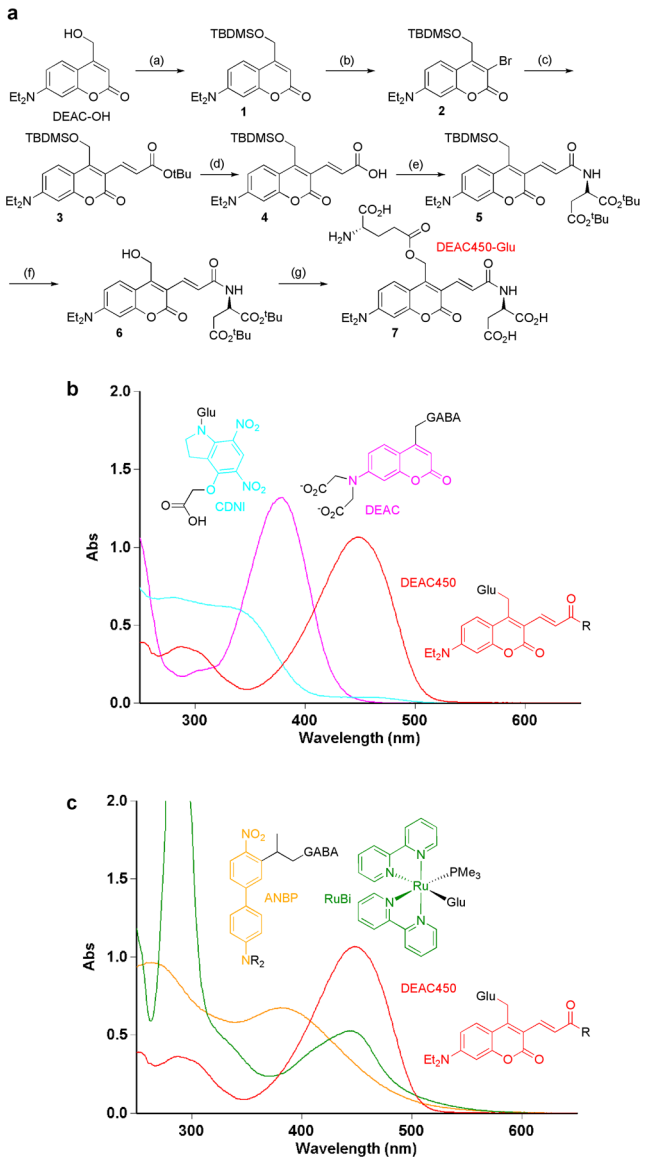

Figure 1. Synthesis and spectral properties of DEAC450-Glu. (**a**) Reagents and conditions: (a) *tert*-Butyldimethylsilyl chloride, imidazole. (b) NBS, NaOAc. (c) *tert*-Butylacrylate, $Pd(OAc)_2$, LiCl, $NaHCO_3$, Bu_4NCl. (d) TFA. (e) Di-*tert*-butyl-D-Asp, EDC. (f) TBAF. (g) *tert*-Butyl-BOC-L-glutamate, EDC followed by TFA. (**b**) Absorption spectra of CDNI-Glu (cyan), *N*-DCAC-GABA (DEAC core in pink), and DEAC450-Glu (red). (**c**) Absorption spectra of RuBi-Glu (green), ANBP-GABA (orange), and DEAC450-Glu (red).

at 473 nm at pH 7.4 revealed that the former was photolyzed 3 times faster, corresponding to a photolysis quantum yield of 0.39. HPLC analysis of the reaction mixture showed that DEAC450-OH was cleanly released. The new chromophore has an extinction coefficient of 43 000 M^{-1} cm^{-1}. These data taken together show that DEAC450-Glu is photochemically one of the most efficient caged Glu probes that has been developed (see Table 1). Importantly, the absorption maximum at 450 nm is significantly red-shifted compared to simple DEAC derivatives such as *N*-DCAC-GABA[28] (Figure 1b). Furthermore, the absorption minimum of DEAC450 is at the maximum for the CDNI chromophore[28,29] (Figure 1b). The relative absorption at λ_{min} is 9% of that at λ_{max}; no such distinct minima exist for other caged compounds that have been recently shown to be two-photon-sensitive (Figure 1c).[24,30,31] This relative difference in linear absorption is further enhanced in the two-photon domain; the DEAC450 chromophore is >60 times more fluorescent at 900 nm than at 720 nm. This significant difference in two-photon excitation led us to test comparative uncaging of DEAC450-Glu on single spine heads in acutely isolated brain slices at these wavelengths.

Two-photon photolysis of a solution of DEAC450-Glu (local perfusion from a nearby pipet at 0.25 mM) at 900 nm (10 mW, 0.5 ms) induced excitatory postsynaptic currents at an isolated spine head (Figure 2a) that were similar in size and duration to

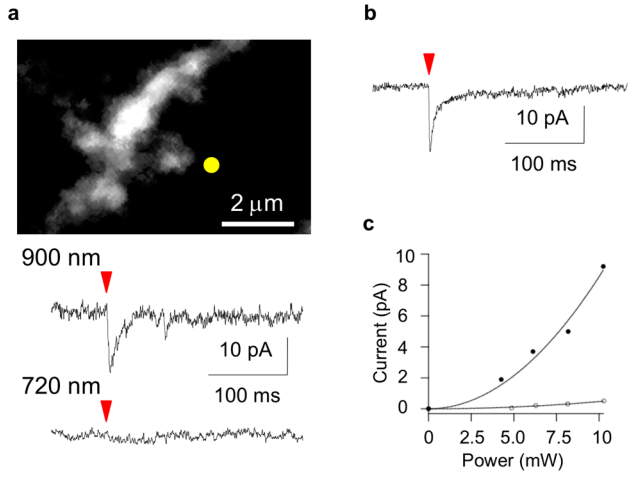

Figure 2. Optically selective two-photon uncaging of glutamate at 900 nm. Caged neurotransmitters were topically applied to pyramidal cells through a puffer pipet positioned just above the surface of an acutely isolated mouse brain slice. Excitation was performed with a mode-locked Ti:sapphire laser tuned to the specified wavelength. Power was measured at the exit of the microscope objective. Currents were measured at the cell soma using whole-cell voltage-clamp recordings. (**a**) Two-photon fluorescence image of a dendritic segment on a pyramidal neuron filled with Alexa-594 (top panel). DEAC450-Glu, applied at 0.25 mM, was irradiated with 900 or 720 nm light for a period of 0.5 ms. The photolysis beam (yellow dot) was positioned next to a spine head, and the postsynaptic current induced at a laser power of 10 mW at 900 nm was similar to that evoked by synaptic release. Irradiation at the same position at a power of 12 mW at 720 nm evoked no current. (**b**) MNI-Glu, applied at 10 mM, was irradiated with 720 nm light for a period of 0.5 ms and evoked a postsynaptic current similar to that of synaptic release. Importantly, nitroindolinyl-caged glutamate is 38-fold less two-photon-active at 830 nm.[28] (**c**) Power-dosage curve of evoked postsynaptic current showing a two-photon excitation effect on the postsynaptic current for DEAC450-Glu at 900 nm (solid points). A much weaker response was evoked at 720 nm (open circles).

spontaneous miniature excitatory postsynaptic potentials.[32] A comparable amount of energy at 720 nm (144%) evoked no response (Figure 2a). This latter energy dose can be used to photolyze nitroindolinyl-caged neurotransmitters such as MNI-Glu[32−35] (Figure 2b) and CDNI-GABA.[29] It should be noted that DEAC cages and other fluorophores do not interfere with nitroaromatic photolysis at 720 nm.[18,28] Power response curves implied that DEAC450-Glu is uncaged by two-photon excitation at 900 nm (Figure 2c), as are MNI-Glu and NDBF-EGTA at 720 nm.[32,36] Similar to other caged Glu and GABA probes,[24,29,30] DEAC450-Glu had off-target pharmacological side effects upon GABA-A receptor currents. We found that the half-maximal effective concentration (EC_{50}) for

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blocking of evoked GABA-A receptor currents on layer 2/3 pyramidal neurons was ~33 μM. In comparison, two commercially available caged neurotransmitters, MNI-Glu and RuBi-Glu, have EC_{50} values of 105 and 7.7 μM, respectively (see the Supporting Information).

DEAC450-Glu has a uniquely powerful set of properties in comparison with the many other caged glutamates. Table 1 shows a summary of the properties of a range of widely used and recently developed caged-glutamate probes. It can be seen that DEAC450-Glu is highly efficient for uncaging with visible light. For example, in comparison to the two commercially available probes[24,32] in Table 1, DEAC450-Glu is ~23 times more active than RuBi-Glu at 450 nm and ~317 times more active than MNI-Glu at 405 nm. The latter wavelength corresponds to excitation with a purple laser that is widely deployed on confocal microscopes.[37] The 11-fold difference in excitation at 350 versus 450 nm may permit two-color uncaging experiments in the linear excitation domain when DEAC450 is paired with regular nitrobenzyl-caged compounds. However, we believe that the real strength of our new caging chromophore for two-color uncaging may be seen when two-photon excitation is used. In this modality, the relative ability to excite DEAC450 at 900 nm versus 720 nm is >60 times higher. Such optical selectivity allowed us to induce currents at single spine heads in acutely isolated brain slices, that were similar in size and kinetics to the currents produced by synaptic events,[32] by uncaging at 900 nm (Figure 2a). Importantly, we found that a higher-energy dosage at a shorter wavelength (720 nm) evoked no significant currents (Figure 2a). This short wavelength is the one that has been widely used for uncaging of nitroindolinyl-caged transmitters (e.g., MNI and CDNI compounds;[29,32,35] Table 1). Thus, DEAC450 and such chromophores could form a near-perfect pair of optically complementary cages for two-color, two-photon uncaging. Several other reports of two-color uncaging have appeared,[38−42] but all of these approaches are constrained in some important way. Some require complete photolysis at the longer wavelength before the application of uncaging at the shorter wavelength.[38−40] Others require the relative concentrations of the caged molecules to be set in such a way that the compounds have hugely different concentration ratios.[41] Finally, some chromophores require uncaging with light that is not compatible with modern microscope glass or amenable to single-synapse stimulation.[42] In contrast, our new caging chromophore, DEAC450, enables for the first time two-photon uncaging of a biomolecule at long wavelengths (900 nm) with almost complete optical selectivity versus shorter wavelengths (720 nm). For synaptic physiology, this technological advance is an important breakthrough, as it may allow the study of how excitatory and inhibitory transmitters sculpt dendritic integration with single-synapse resolution.[43]

Table 1. Summary of the Properties of Caged Glutamate Probes[a]

cage	ε/M^{-1} cm^{-1} (λ_{max}, nm)	QY	$\varepsilon \times$ QY/ M^{-1} cm^{-1}	2PuCS/GM (λ/nm)
MNI[32,44]	4500 (336)	0.085	383	0.06 (740)
RuBi[24]	5600 (450)	0.13	728	0.14 (800)
PMNB[45]	9900 (317)	0.1	990	0.45 (800)
CDNI[35]	6400 (330)	0.5	3200	0.06 (720)
PNEB[46]	9900 (317)	0.1	990	3.2 (740)
DEAC450	43000 (450)	0.39	16800	0.5[b] (900)

[a]Symbols and abbreviations: ε, extinction coefficient; λ_{max}, absorption maximum; QY, quantum yield; 2PuCS, two-photon-uncaging cross section. [b]Estimated from Figure 2.

■ ASSOCIATED CONTENT

Supporting Information

Chemical and physiological experimental details and analytical data for chemical synthesis. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ NOTE ADDED AFTER ASAP PUBLICATION

Table entry MNI Lamba max 4500 (436) was corrected to (336) and reposted April 24, 2013.