JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

pubs.acs.org/JACS

Spectral Evolution of a Photochemical Protecting Group for Orthogonal Two-Color Uncaging with Visible Light

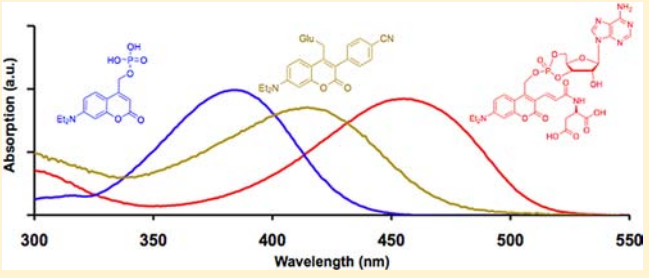
Jeremy P. Olson,[†,§] Matthew R. Banghart,[‡,§] Bernardo L. Sabatini,[‡] and Graham C. R. Ellis-Davies*[,†]

[†]Department of Neuroscience, Mount Sinai School of Medicine, New York, New York 10029, United States

[‡]Department of Neurobiology, Howard Hughes Medical Institute, Harvard Medical School, Boston, Massachusetts 02115, United States

Supporting Information

ABSTRACT: Caged compounds are molecules rendered functionally inert by derivatization with a photochemical protecting group. We describe the design logic behind the development of a diethylaminocoumarin (DEAC) caging chromophore, DEAC450, that absorbs blue light strongly (ε_{450} = 43,000 M^{-1} cm^{-1}) and violet light 11-fold more weakly. The absorption minimum is in the wavelength range (340−360 nm) that is traditionally used for photolysis of many widely used nitroaromatic caged compounds (e.g., 4-carboxymethoxy-5,7-dinitroindolinyl(CDNI)-GABA). We used this chromophore to synthesize DEAC450-caged cAMP and found this probe was very stable toward aqueous hydrolysis in the electronic ground state but was photolyzed with a quantum efficiency of 0.78. When DEAC450-cAMP and CDNI-GABA where co-applied to striatal cholinergic interneurons, the caged compounds were photolyzed in an chromatically orthogonal manner using blue and violet light so as to modulate the neuronal firing rate in a bidirectional way.



INTRODUCTION

Cellular signaling has the bidirectional character of a switch.[1−3] However, unlike simple physical switches that use the same agent for modulation, cells often deploy two independent mechanisms for the on and off signals. Muscle contraction, protein phosphorylation, and nerve action potentials are important examples of such bidirectional biological signaling. Calcium concentration in muscle cells regulates contraction and is controlled by ion channels (on signal) and Ca ATPases (off signal).[4] Phosphorylation is initiated by kinases and terminated by phosphatases.[5] Membrane potentials are controlled by the selective flow of cations and anions through ion channels in the plasma membrane of the nerve.[6] Since the late 1970s photochemical uncaging of biological signaling molecules has been used as a powerful tool to interrogate such signaling cascades.[2,7] Typically this photochemical modulation uses protecting groups developed for organic chemistry in the 1960s[8] and is unidirectional in character. Such compounds are photolyzed with near-UV light (Figure 1a, violet), and some are also reasonably sensitive to two-photon photolysis using pulsed, near-IR lasers (710−740 nm range[7]).

The first report of chromatically selective photochemical deprotection appeared in 2000,[9] and because this was in the context of organic synthesis, only UV or near-UV light was used for photolysis. Unfortunately UV light is not compatible with cellular physiology, and thus uncaging in living cells typically requires wavelengths greater than 330 nm.[7] Since near-IR light is more compatible with cellular viability, we used two-photon excitation to enable the first example of chromatically selective two-color uncaging on living neurons in 2010.[10] However, because of the absorption overlap of the caging chromophores (Figure 1a, violet and blue), chromatically orthogonal two-color uncaging was quite challenging. Recently we have developed a new 7-diethylaminocoumarin (DEAC) caging chromophore, called DEAC450, which has an absorption maximum for one-photon excitation at 450 nm, that undergoes very effective two-photon uncaging with a femtosecond-pulsed laser at double this wavelength. Importantly, irradiation of DEAC450-Glu at dendritic spine heads with a pulsed laser at short wavelengths (i.e., 720 nm) produced little or no postsynaptic current in neurons when compared to 900 nm.[11] The nonlinear nature of two-photon excitation enabled clean chromatically selective uncaging of glutamate and was based on the >60-fold difference in two-photon excitation at the two wavelengths. However, it was unclear if the more modest 11-fold difference in linear light absorption at 350 nm versus 450 nm (Figure 1a, red) would enable useful and effective chromatically selective uncaging with normal lasers. Here we describe the design logic in the evolution of the DEAC450 caging chromophore and demonstrate that it can be applied to photorelease of an intracellular second messenger, cyclic-AMP (cAMP), in living cells, with excellent optical selectively using linear excitation. We show that photolysis of DEAC450-cAMP with 473-nm light is functionally orthogonal to photolysis of CDNI-GABA with near-UV light at 355 nm, by

Received: August 13, 2013
Published: October 11, 2013

   dx.doi.org/10.1021/ja408225k | J. Am. Chem. Soc. 2013, 135, 15948−15954

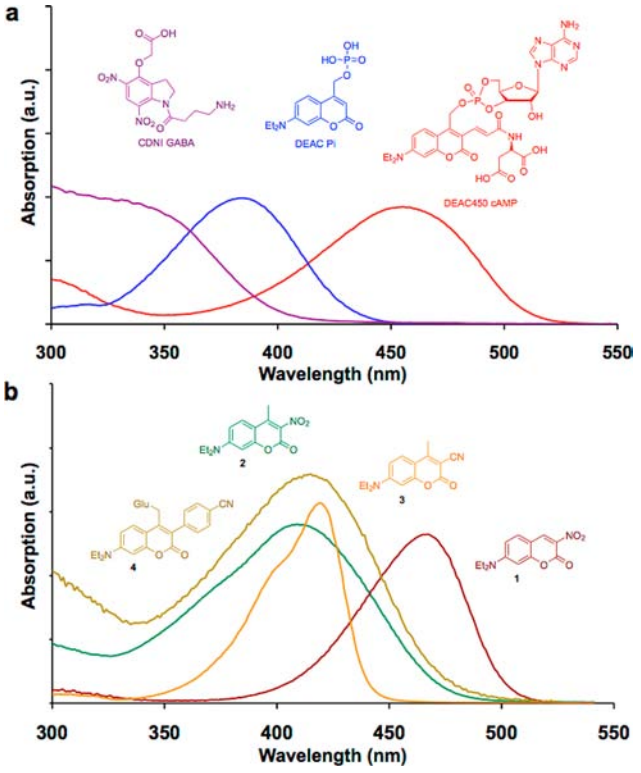


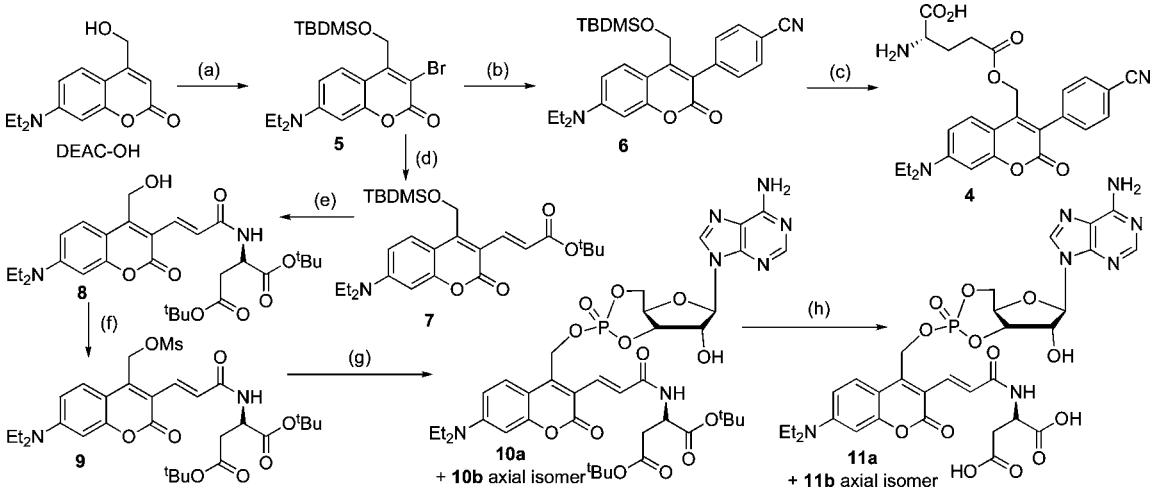
Figure 1. Comparison of the absorption spectra of nitroaromatic and aminocoumarin chromophores. (a) Spectra of CDNI-GABA (violet), DEAC (blue), and DEAC450-cAMP (red). (b) Spectra of 3-(*p*-cyanophenyl)-DEAC (**4**, khaki), 3-nitro-4-methyl-DEAC (**2**, green), 3-cyano-4-methyl-DEAC (**3**, orange), and 3-nitro-DEAC (**1**, dark red).

the bidirectional modulation of membrane potential of neurons in acutely isolated brain slices. Since the DEAC450 chromophore could be used to cage many other molecules, this new technology may enable two-color uncaging with visible light to become a useful and widely used method.

RESULTS

Chromophore Design and Evolution. We sought to design a new caging chromophore that could be selectively photolyzed at long wavelengths relative to the well established nitroaromatic caged compounds.[7] It is well-known that addition of electron-withdrawing substitutents at the 3-position of 7-aminocoumarins produces large bathochromic shifts in the absorption maximum.[12] For example, 3-nitro-DEAC (**1**) has not only a large absorption maximum at 470 nm but also an extremely low minimum in the 340−360-nm range (Figure 1b, dark red) and thus was an attractive starting point for us to design a new, longer wavelength caging chromophore. However, addition of the crucial 4-methyl substituent to this chromophore (**2**), to allow attachment of biomolecules, dramatically perturbed these "near ideal" absorption features (Figure 1b, green). This is probably because the steric clash between the nitro and methyl groups in **2** twists the former out of planarity with the coumarin chromophore. This idea seemed to be confirmed when we found that 3-cyano derivative **3** had a similar absorption maximum compared to that of **2** but had a much more attractive absorption minimum in the 340−360 nm range (Figure 1b, orange). However, functionalization of the 4-methyl group of **3** was problematic, as the standard means of derivatization (oxidation with SeO_2[13]) requires hydrogen at the 3-position. Therefore, we revised our synthetic route by starting with the known DEAC-OH[13] (Scheme 1) to create a 3-(*p*-cyanophenyl) substituted DEAC-Glu,[14] **4**. We made a glutamate derivative first, as photolysis-evoked neuronal currents offer a potentially facile bioassay of uncaging efficacy.[15] The quantum yield of photolysis of this new caged glutamate was 0.05, but the absorption spectrum of **4** (Figure 1b, khaki) was similar to that of **2**. These data showed that DEAC uncaging was not greatly perturbed by substituents at the 3-position but confirmed that less bulky electron-withdrawing groups were needed to enhance the "absorption dip" in the 340−360 nm range. 3-Carboxy derivatives of aminocoumarins are known to have absorption spectra with bathochromic shifts similar to those of **1**−**4**,[12] but we found the synthesis of such simple derivatives in a form suitable for elaboration into caged

Scheme 1. Synthesis of DEAC450-cAMP[a]



[a]Reagents and conditions: (a) (i) TBDMSCl, Et_3N (83%); (ii) *N*-bromosuccinimide, NaOAc (59%). (b) 4-Cyanophenylboronic acid, CsF, $Pd(PPh_3)_4$ (69%). (c) (i) TBAF (85%); (ii) EDC, Boc-Glu-OtBu (76%); (iii) TFA, then HPLC purification (71%). (d) Reference 8. (e) TBAF (85%). (f) Methanesulfonyl chloride, TEA (59%). (g) cAMP, tri-*n*-butylamine (65%). (h) TFA, then HPLC purification (**11a**, 35%; **11b**, 44%).

dx.doi.org/10.1021/ja408225k | J. Am. Chem. Soc. 2013, 135, 15948−15954

Table 1. Summary of the Chemical and Photochemical Properties of Many Nitroaromatic and Coumarin-Caged cAMP Optical Probes[a]

caged cAMP[b]	ε, M^{-1} cm^{-1} (λ, nm)	QY	solubility in PB, mM	stability at pH 7.2	ε·QY
NB[17]	500 (350)	0.42	NR	NR	210
dcNPE[21]	500 (350)	0.2	>55	stable frozen	100
DMNB[20]	5,000 (350)	0.05	NR[c]	>100 h RT[d]	250
MEOC[39]	13,300 (325)	0.12	NR	stable frozen	1,600
DEAC[18]	18,600 (402)	0.21	0.15 (1% DMSO)	NR	3,900
DCAC[22]	17,000 (384)	0.26	2	>300 h RT[d]	4,420
Bhc[19]	14,600 (375)	0.081	0.5 (1% DMSO)	>750 h RT[d]	1,183
DEAC450	43,000 (456)	0.78	1.3	stable frozen	33,500

[a]Symbols, abbreviations, and notes. PB, physiological buffer without (or with) organic co-solvents; QY, quantum yield; ε, molar extinction coefficient; NR, not reported. [b]NB, *o*-nitrobenzyl; dcNPE, dicarboxy-(*o*-nitrophenyl)ethyl; DM, dimethoxy; MEOC, 7-methoxycoumarin; DCAC, dicarboxy-7-aminocoumarin; Bhc, 6-bromo-7-hydroxycoumarin. [c]Intracellular solution required 1% DMSO. [d]Approximate time for hydrolysis.

compounds surprisingly problematic (data not shown). In contrast, a functionalized DEAC-based caging chromophore could be created in a facile manner by Heck coupling of methylvinylacrylate[16] or *tert*-butylacrylate[11] to **5** to give the core DEAC450 chromophore (Supplemental Movie). Mesylation of alcohol **8**[11] gave **9**, and this derivative was used to alkylate the tri-*n*-butylamine salt of cAMP to give **10** in 35% overall yield. Treatment of **10** with TFA followed by HPLC purification gave equatorial and axial derivatives **11a** and **11b**, in yields of 35% and 44%, respectively (Scheme 1).

Chemical Characterization of DEAC450-cAMP. *Aqueous Stability and Solubility.* Chemical stability and aqueous solubility are important properties for the practical application of any caged compound for neurophysiology.[15] The simple nitrobenzyl ester of cAMP was made in the late 1970s,[17] and subsequently several other caged cAMP probes have been made.[18−20] However, addition of uncharged aromatic chromophores decreases the solubility of the caged cyclic nucleotides (Table 1). To mitigate this problem, a dicarboxylate moiety has been added to both the nitrobenzyl and DEAC caged-cAMP probes.[21,22] We adopted the same strategy by conjugation of additional charge to the core DEAC450 chromophore (Supplemental Movie and Scheme 1). Thus, the solubility of DEAC450-caged cAMP was such that a solution of a concentration of at least 1 mM could be made in physiological buffer, without the addition of any organic cosolvent (see Supporting Information). In general, when the caged compound uses an ester linkage to attach the chromophore to the biomolecule, the stability of the linkage is defined by its electrophilic character.[21] For example, dimethoxy-*o*-nitrobenzyl-cAMP is much more unstable than its simple nitrobenzyl counterpart,[20] and an electron-deficient analogue is more stable.[21] Similarly, we have found that DEAC-Glu is quite unstable at pH 7.4 (half-life of 17 h; Momotake and Ellis-Davies, unpublished), whereas its DEAC450-Glu counterpart is highly stable.[11] DEAC450-cAMP also proved to be highly stable, as solutions made in buffer at pH 7.2 and frozen at −40 °C for 2 months showed no sign of hydrolysis.

Optical Properties. DEAC450 has a molar extinction coefficient of 43,000 M^{-1} cm^{-1} at 450 nm,[11] a value that is significantly higher than DEAC derivatives (range 15,000−25,000 M^{-1} cm^{-1}). The absorption maximum of DEAC450-cAMP has the same relatively large absorptivity, albeit slightly red-shifted to 456 nm. Another important photochemical property of any caged compound is the quantum yield of photolysis. Comparative irradiation of DEAC450-cAMP and DEAC450-Glu revealed that the former was photolyzed 2 times faster than the latter,[11] corresponding to a quantum yield of uncaging of 0.78 (both isomers were equally photosensitive). HPLC analysis of the reaction mixture showed that DEAC450-alcohol and cAMP were cleanly released as the sole products after complete photolysis (Supporting Information). These properties taken together make DEAC450-cAMP an extremely effective caged compound from a photochemical point of view (Table 1). Finally, in comparison to other recently developed longer wavelength caging chromophores such as "RuBi"[23] and amino-nitro-biphenyl,[24] DEAC450 is distinctive in having a pronounced absorption minimum in the wavelength region that has been used traditionally for photolysis of many nitroaromatic caged compounds such as NV-IP_3,[25,26] DM-nitrophen,[27] and MNI-Glu[28] (such compounds have absorption spectra similar to the violet trace in Figure 1a). We chose to examine if this feature of DEAC450-cAMP would allow selective photolysis at long wavelengths of visible light (i.e., 473 nm), while using shorter wavelengths to photorelease a caged neurotransmitter, CDNI-GABA[29] (Figure 1a, violet). We used two wavelengths of visible light for these experiments for the following reasons: first, if successful, such experiments would nicely complement two-color, two-photon uncaging,[24] and second, visible lasers, unlike two-photon lasers, allow facile irradiation of the entire cell body of large neurons.[30,31]

Intracellular Uncaging in Living Neurons. Striatal cholinergic interneurons are tonically active in vitro,[32] and this action potential firing rate is known to be upregulated by stimuli that lead to the production of cAMP,[32,33] while it is blocked by the inhibitory amino acid GABA.[34] We tested if DEAC450-cAMP and CDNI-GABA could be uncaged in an optically orthogonal manner using one-photon excitation, so as to modulate the firing rate of striatal interneurons in a bidirectional way. First, DEAC450-cAMP was loaded via the whole-cell patch clamp technique into single neurons in acutely isolated brain slices. After allowing several minutes for dialysis, irradiation with 473-nm light caused a prominent enhancement of the firing rate (Figure 2a, left), which lasted for tens of seconds after uncaging. Less intense 473-nm stimuli produced more transient responses (Supplemental Movie). When DEAC450-cAMP was omitted from the internal solution but CDNI-GABA was added to the bath, 355-nm light inhibited action potential firing (Figure 2b, right). However, the same dosage of 473-nm light used for DEAC450-cAMP above did not alter the firing rate (Figure 2b, left). Likewise, 355-nm light irradiation of DEAC450-cAMP inside cells did not increase the firing rate (Figure 2a, right). A 3-fold increase in energy at 355 nm also had no effect. Similar chromatic selectivity was

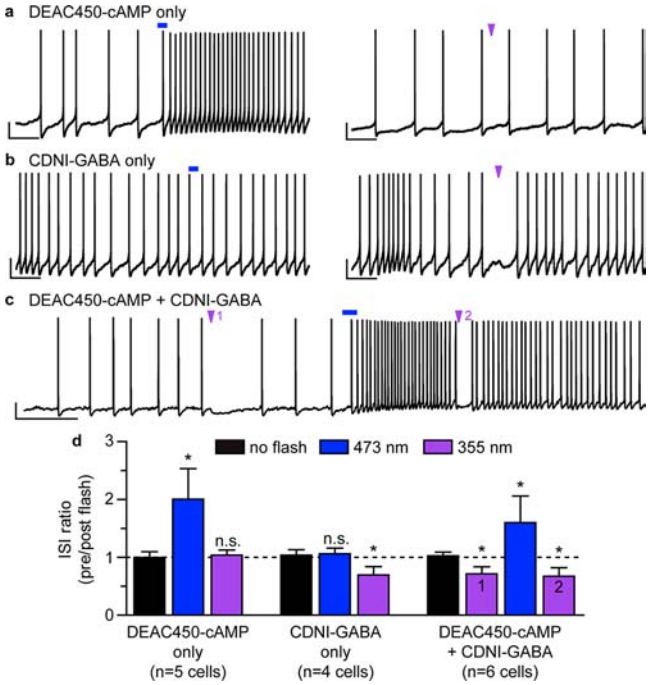


Figure 2. Bidirectional modulation of neuronal firing rates by optically orthogonal uncaging of cAMP and GABA. Whole-cell current clamp recordings from acute mouse brain slices were used to monitor spontaneous action potential firing in striatal cholinergic interneurons. DEAC450-cAMP (25−75 μM) was included in the intracellular solution, and CDNI-GABA (1 mM) was bath-applied. Cell bodies were irradiated at 473 nm (~20 μm spot and 410 mW/cm^2 for 100−200 ms) and/or 355 nm (50 μm spot and 16 mW/cm^2 for 2 ms). Scale bars: y = 10 mV, x = 1 s. (a) 473-nm light causes an increase in the spontaneous firing rate of neurons loaded with DEAC450-cAMP (left trace), but 355-nm light does not (right trace). (b) 473-nm light does not alter the spontaneous firing of neurons bathed in CDNI-GABA (left trace), but 355-nm light transiently inhibits spontaneous firing (right trace). (c) In the presence of both DEAC450-cAMP and CDNI-GABA, sequential illumination with 355-nm and 473-nm light produces bidirectional changes in action potential firing. (d) Bar graphs show summaries of light-induced changes in action potential firing by quantifying the ratiometric change in interspike interval (ISI) before (pre) and after (post) each light flash for many cells. The "no flash" condition corresponds to ISI ratios measured from each cell before and after an arbitrary time point. Data are presented as geometric means + the standard deviation upper limit. Asterisks denote a significant difference from the "no flash" condition ($p < 0.05$) according to paired sample t test analysis.

observed in multiple experiments (Figure 2d) and suggested that DEAC450-cAMP and CDNI-GABA could be photolyzed on the same cell in an optically independent manner using 473-nm and 355-nm light. Such chromatic orthogonality was demonstrated by varying the order of the applied wavelengths as shown in Figure 2c. When DEAC450-cAMP was loaded into cells and CDNI-GABA was included in the bath solution, firing was paused transiently when 355-nm light irradiation was applied first. After recovery, irradiation at 473 nm produced an enhanced neuronal firing rate, which was briefly blocked again by irradiation at 355 nm (Figure 2c).

To quantify the effect of illumination on action potential firing rate, the time between action potentials, or interspike interval (ISI), was measured before (pre) and after the light flash (post) and the pre/post flash ISI ratio was used as a metric. The pre/post flash ISI ratio accurately reveals relative changes in action potential firing rates across different cells, even if individual cells have vastly different absolute firing rates. To control for any inherent drift in basal firing rate and provide a control condition for statistical analysis, ISI ratios were also measured from each cell before and after an arbitrary time point during a time window that contained no light flash (no flash). Figure 2d summarizes the effects of light on action potential firing under each condition. In the presence of DEAC450-cAMP only, when compared to the control condition (no flash: *mean* 1.00, SD *range* 0.91−1.10), blue light produced a large increase in the ISI ratio (473 nm: *mean* 2.01, SD *range* 1.59−2.53, $p < 0.05$), whereas violet light did not significantly affect the firing rate (355 nm: *mean* 1.04, SD *range* 0.95−1.13, $p > 0.05$). In the presence of CDNI-GABA only, in comparison to the control condition (no flash: *mean* 1.04, SD *range* 0.95−1.13), blue light did not significantly affect firing (473 nm: *mean* 1.06, SD *range* 0.97−1.16, $p > 0.05$), but violet light reduced the ISI ratio (355 nm: *mean* 0.70, SD *range* 0.58−0.84, $p < 0.05$). In the presence of both compounds, all three light flashes significantly altered spike rates (355 nm (1): *mean* 0.72, SD *range* 0.61−0.84, $p < 0.05$; 473 nm: *mean* 1.60, SD *range* 1.24−2.06, $p < 0.05$; 355 nm (2): *mean* 0.67, SD *range* 0.55−0.82, $p < 0.05$) in comparison to control (no flash: *mean* 1.03, SD *range* 0.97−1.09).

DISCUSSION

Photochemical protecting groups were introduced in 1966 by Barltrop and co-workers, when they showed that simple *o*-nitrobenzyl carboxylic esters could be photolyzed so as to liberate the free carboxylate.[8] The generality of this protecting group was established subsequently by the work of several laboratories.[8,35,36] In 1970, Woodward and Patchornik extended the wavelength range of the *o*-nitrobenzyl protecting group by substitution of electron -donating methoxy substituents onto the aromatic ring.[37] These two photochemical protecting groups have been extensively used by biologists for caging a very wide range of important biological signaling molecules.[2,7] However, extending the absorption range of the *o*-nitrobenzyl protecting group significantly beyond 400 nm has proved very challenging.[38] In contrast, coumarins offer a wide spectral range as they are extensively used as laser dyes, but being fluorescent, such molecules would not seem to be prime candidates for useful photochemical protecting groups. However, it was discovered in 1995 that 7-methoxycoumarin derivatives could be used as such for acids,[39] photorelease coming through a photosolvolysis mechanism (reviewed in ref 40). The DEAC chromophore, first used for uncaging in 2001,[18] extends the absorption spectrum of coumarin cages well beyond 400 nm. However, there is considerable overlap of the DEAC and nitroaromatic caging chromphores spectra (Figure 1a), making bimodal two-color uncaging experiments challenging. However, the flexibility of coumarin substitution and the extensive fluorophore chemistry developed around aminocoumarins[12] made it very attractive for chromphore evolution into a new useful long wavelength caging chromphore. We used Heck coupling of methylvinylacrylate[16] or *tert*-butylacrylate to **5** to give the core DEAC450 chromophore (Supplemental Movie). A simple but key feature of the product (**7**) from the latter acrylate is the side chain carboxylate, which enables further flexible elaboration of the chromophore with molecules such as D-Asp in order to aid solubility. Thus, **7** was converted into mesylate **9**, which was used for direct caging of cAMP, to give, after deprotection, pure

dx.doi.org/10.1021/ja408225k | J. Am. Chem. Soc. 2013, 135, 15948−15954

Table 2. Summary of a range one- and two-photon actuation modalities using caged compounds or genetically encoded proteins[a]

λ (nm)	CDNI-Glu/GABA MNI-Glu	DEAC450-Glu	NV-IP$_3$	NPE-ATP	DEAC450-cAMP	ChR2	Halo	C1V1	optoXR	eArch3.0
355	✔	✘	✔	✔	✘	?	✘	?	?	✘
473	✘	✔	✘	✘	✔	✔	✘	✔	✔	✔
560-580	✘	✘	✘	✘	✘	✘	✔	✔	✘	✔
720	✔	✘	✔	✘	✘	✘	✘	✘	✘	✘
830	✘	✔	✘	✘	✔ (b)	✘ (c)	✘	✘	?	✘
900	✘	✔	✘	✘	✔ (b)	✘ (c)	✘	✔	?	✔
1040	✘	✘	✘	✘	✘	✘	✘	✔	✘	✔

[a]The table is divided into quadrants: upper left, one-photon photolysis of caged compounds; lower left, two-photon photolysis of caged compounds; upper right, one-photon actuation of genetically encoded proteins; lower right, two-photon activation of genetically encoded proteins. Green ticks and red crosses show known activation of each probe in living cells at the indicated wavelength, ? = not reported but absorption spectra suggest activation possible. Caged compounds and genetically encoded proteins have been chosen merely to illustrate a range possible optical actuators, a comprehensive list can found in recent reviews.[40,43,44,56,73] [b]By analogy with DEAC450-Glu. [c]Specialized illumination enables two-photon excitation of ChR2 in the 830−900nm-range.[75]. Abbreviations: MNI, 4-methoxy-7-nitroindolinyl; CDNI, 4-carboxymethoxy-5,7-dinitroindolinyl; NPE, 1-(*o*-nitrophenyl)ethyl; NV, *o*-nitroveratryl.

equatorial (**11a**) and axial (**11b**) DEAC5450-caged cAMP compounds (Scheme 1 and Supplemental Movie).

DEAC450-cAMP was loaded via whole-cell patch clamp into neurons in brain slices acutely isolated from transgenic mice with tdTomato selectively expressed in striatal cholinergic neurons. This method has two essential properties for such experiments and has been widely used by many neurophysiologists.[41,42] It enables the delivery of a precise concentration of probe to the intracellular space and facile monitoring of the optically targeted neuron's electrical function. We chose to probe striatal cholinergic neurons as these cells are tonically active because the resting membrane potential is typically around threshold for firing action potentials.[34] Irradiation of the cell body with 473-nm light produced the expected rapid and robust increase in the firing rate of the targeted neurons (Figure 2a, left). In separate experiments we bath applied a nitroaromatic-caged neurotransmitter, CDNI-GABA, to brain slices. Irradiation of the cell with 355-nm light uncaged the inhibitory neurotransmitter on the patch-clamped neurons and caused a transient pause in the tonic action potential firing (Figure 2b, right). When the same amount of violet and blue light was used to irradiate cells with DEAC450-cAMP or CDNI-GABA, no effects from uncaging were detected (Figure 2a, right, and b, left; respectively). In these experiments each caged compound was applied to cells individually, and we were able to determine the level of optical cross talk for each probe. In our final set of experiments we co-applied the probes to striatal cholinergic neurons and varied the order of photolysis with the 355-nm and 473-nm lasers. As before, irradiation with violet light caused a transient pause in the firing (Figure 2c), and after full recovery, irradiation with 473-nm light increased tonic firing. This increase could then be blocked with another flash of 355-nm light. This sequence of light flashes demonstrated that our two caged compounds could be photolyzed with sufficient orthogonality so as to allow arbitrary application of two opposing stimuli in order to control cell signaling in a bidirectional manner.

Two-color uncaging has been studied since 2000, when Bochet pioneered this field for photochemical synthesis[9] (reviews, refs 43 and 44). Of course, optically selective uncaging of one of two optical probes at long wavelengths is trivial, as excitation of one chromophore is facile as a result of non-overlap in the redder part of the spectrum. The real challenge with optical orthogonality lies in the absorption overlap of the long wavelength probe in the region where the short wavelength probe is photolyzed.[10] The best orthogonality ratio reported for chemical synthesis by irradiation of two compounds in equal concentration is about 10 (ref 45; note, under different conditions this ratio has been improved recently[46]). In all cases the wavelengths of light used for such synthetic organic chemistry (254 and 420 nm) are not compatible with standard microscopes, as normal optical glass only transmits visible light (i.e., >330 nm). Visible light has been used for high-resolution, two-color photolithography with an excellent orthogonality ratio of >50.[47] However in cells such orthogonality has been difficult to match, and this situation has been illustrated by several biological studies using several different caged compounds. Such work took advantage of the fact that simple nitrobenzyl caged compounds are photolyzed at short wavelengths (near-UV light in the 340−360-nm range) but are optically transparent at longer wavelengths (e.g., >420 nm), whereas DEAC-caged compounds are photolyzed selectively at such long wavelengths.[48,49] However, *complete photolysis* of the DEAC probe was required before the nitroaromatic probe was uncaged because short wavelengths of light photolyzed both probes due to significant overlap at ca. 350 nm (see also refs 50−53). We found that the power threshold for 355-nm light uncaging of DEAC450-cAMP was at least 3-fold higher than that used for CDNI-GABA uncaging before effects of cAMP released could be detected, but with ever increasing laser power at the shorter wavelength we could eventually elicit a neuronal response from DEAC450-cAMP photolysis. This is because there is an absorbance ratio of only 7.5 for DEAC450 between 473 and 355 nm, and this finite difference has inevitable consequences for the degree of orthogonality that is possible in such two-color experiments when one is using linear[54] and not nonlinear excitation[10,55] for uncaging. Nevertheless, our data taken together show that our

new coumarin cage (in the form of DEAC450-cAMP) and a nitroaromatic cage (in the form of CDNI-GABA) can be photolyzed with excellent optical orthogonality at long and short wavelengths of visible light, so as to induce bidirectional signaling with signal cell precision.

Genetically encoded actuation methods offer an alternative to caged compounds for bidirectional control of neuronal signaling, and these have been the subject of intensely active development since 2004.[56−59] For example, cis−trans isomerization of an azobenzene probe tethered to a mutant cysteine residue near a potassium channel was used for reversible control of action potential firing with two wavelengths of light (on with 500 nm, off with 380 nm).[60] Similar on−off gating was achieved with two rhodopsin-based probes (on with channelrhodopsin2[61,62] (ChR2) at 473 nm, off with halorhodopsin (Halo or NpHR) at 600 nm).[60,63,64] Recently, some mutant rhodopsins (e.g., C1V1 and eArch3.0) have been shown to be very active with two-photon excitation, thus extending the wavelength range available for two-color photolysis.[65] Importantly, the rhodopsin-based approach can easily be used in living animals[66−68] because only a single component is required for probe delivery. The rapid adoption of these genetically encoded methods by the neuroscience community[69] poses the question, "Are caged compounds still useful?" We believe a careful examination of the chemical and spectral properties of caged probes and genetically encoded photoactuators shows that the two methods are *highly complementary*, with one report even showing that the two methods can be combined in vitro.[70] Table 2 summarizes a selection of the basic chemico- and geneto-optical actuation modalities currently available to neuroscientists and illustrates the wavelength and biochemical diversity of these two methods.

CONCLUSION

In this report we describe the design logic behind the spectral evolution of the DEAC cage that lead to the development of a new photochemical protecting group, which we call "DEAC450". This new caging chromophore has a large absorptivity in the 440−480-nm range, a wavelength region that is chromatically isolated from the widely used nitroaromatic caging chromophores, such as nitrobenzyl,[17,37] nitroindolinyl,[71] and their derivatives.[7,72] Furthermore, DEAC450 has a striking absorption minimum in the wavelength region where such nitroaromatic protecting groups are photolyzed. We made DEAC450-caged cAMP as the first example of an intracellular second messenger that is highly sensitive to photolysis with blue light. Our work shows how logical chromophore evolution can be used to refine a caging chromophore so as to "fill in the gap" in the optical arsenal available for photochemically orthogonal modes of actuation available to neurophysiologists who study cells using caged compounds, with chromatically selective, dual 1P uncaging joining dual 2P photolysis[10] and 2P/1P excitation.[70] Of course caged compounds have been used to control the concentration of a very wide variety of molecules[40,43,44,73,74] that extends well beyond those involved in neuronal membrane potential. Further, this generality of actuation is in stark contrast to genetically encoded methods. Thus, when paired with other traditional caging chromophores that are highly active at short wavelengths, DEAC450-caged biomolecules may enable further examples of arbitrary two-color uncaging of two signaling molecules with living cells using visible light beyond those in our current work.

EXPERIMENTAL SECTION

Chemical Synthesis. CDNI-GABA was made as described previously.[29] Full details of the synthesis of DEAC450-cAMP and compound **4** are described in the Supporting Information.

Photochemical Methods. The quantum yield of uncaging was measured by comparative photolysis as described previously for other caged compounds.[10,11,27,29] Concentrations of DEAC450-Glu and DEAC450-cAMP were set to give an OD = 0.2 at 473 nm in a 1-mm cuvette. A defocused 473-nm laser was used for irradiation. Compound **4** was compared to *N*-DCAC-GABA[10] by matching the absorbance at 410 nm. The solutions were photolyzed with a defocused 410-nm laser. Solutions were made in HEPES (40 mM) and KCl (100 mM) at pH 7.4.

Physiology Methods. All animal handling was performed in accordance with guidelines approved by the Harvard Institutional Animal Care and Use Committees and federal guidelines. Coronal forebrain slices 300 μm in thickness were acutely isolated as described previously[31] from P30-P45 mice expressing the fluorescent protein TdTomato in cholinergic neurons, used to facilitate in their identification. Full details are described are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

Chemical and physiological experimental details, analytical data for chemical synthesis, and a video flowchart illustrating the design logic in the development of DEAC450 and its application to caging cAMP. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

graham.davies@mssm.edu

Author Contributions

[§]These authors contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the NIH (GM053395 and NS069720 to G.E.-D., MH085498 to B.L.S.), the HFSP (RG0089/2009C to G.E.-D.), postdoctoral fellowships from the Helen Hay Whitney Foundation and the Charles A. King Trust, N.A., Bank of America, Co-Trustee (M.R.B.), and the Howard Hughes Medical Institute (B.L.S.).

REFERENCES

(1) Sjulson, L.; Miesenbock, G. *Chem. Rev.* **2008**, *108*, 1588−1602.
(2) Mayer, G.; Heckel, A. *Angew. Chem., Int. Ed.* **2006**, *45*, 4900−4921.
(3) Gorostiza, P.; Isacoff, E. Y. *Science* **2008**, *322*, 395−9.
(4) Berridge, M. J.; Lipp, P.; Bootman, M. D. *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 11−21.
(5) Bauman, A. L.; Scott, J. D. *Nat. Cell Biol.* **2002**, *4*, E203−206.
(6) Neher, E. *Angew. Chem., Int. Ed.* **1992**, *31*, 824−829.
(7) Ellis-Davies, G. C. R. *Nat. Methods* **2007**, *4*, 619−628.
(8) Barltrop, J. A.; Plant, P. J.; Schofield, P. *Chem. Commun.* **1966**, 822−823.
(9) Bochet, C. *Tetrahedron Lett.* **2000**, *41*, 6341−6346.
(10) Kantevari, S.; Matsuzaki, M.; Kanemoto, Y.; Kasai, H.; Ellis-Davies, G. C. R. *Nat. Methods* **2010**, *7*, 123−125.
(11) Olson, J. P.; Kwon, H. B.; Takasaki, K. T.; Chiu, C. Q.; Higley, M. J.; Sabatini, B. L.; Ellis-Davies, G. C. R. *J. Am. Chem. Soc.* **2013**, *135*, 5954−5957.

(12) Li, H.; Cai, L.; Chen, Z. In *Advances in Chemical Sensors*; Wang, W., Ed.; InTech: Changhai, 2012.
(13) Ito, K.; Maruyama, J. *Chem. Pharm. Bull.* **1983**, *31*, 3014−3023.
(14) Shembekar, V. R.; Chen, Y.; Carpenter, B. K.; Hess, G. P. *Biochemistry* **2005**, *44*, 7107−7114.
(15) Ellis-Davies, G. C. R. *Beilstein J. Org. Chem.* **2013**, *9*, 64−73.
(16) Pottie, I. R.; Nandaluru, P. R.; Benoit, W. L.; Miller, D. O.; Dawe, L. N.; Bodwell, G. J. *J. Org. Chem.* **2011**, *76*, 9015−9030.
(17) Engels, J.; Schlaeger, E. J. *J. Med. Chem.* **1977**, *20*, 907−911.
(18) Hagen, V.; Bendig, J.; Frings, S.; Eckardt, T.; Helm, S.; Reuter, D.; Kaupp, U. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 1045−1048.
(19) Furuta, T.; Takeuchi, H.; Isozaki, M.; Takahashi, Y.; Kanehara, M.; Sugimoto, M.; Watanabe, T.; Noguchi, K.; Dore, T. M.; Kurahashi, T.; Iwamura, M.; Tsien, R. Y. *ChemBioChem* **2004**, *5*, 1119−1128.
(20) Nerbonne, J. M.; Richard, S.; Nargeot, J.; Lester, H. A. *Nature* **1984**, *310*, 74−76.
(21) Wang, L.; Corrie, J.; Wootton, J. *J. Org. Chem.* **2002**, *67*, 3474−3478.
(22) Hagen, V.; Dekowski, B.; Nache, V.; Schmidt, R.; Geissler, D.; Lorenz, D.; Eichhorst, J.; Keller, S.; Kaneko, H.; Benndorf, K.; Wiesner, B. *Angew. Chem., Int. Ed.* **2005**, *44*, 7887−7891.
(23) Fino, E.; Araya, R.; Peterka, D. S.; Salierno, M.; Etchenique, R.; Yuste, R. *Front. Neural Circuits* **2009**, *3*, 2.
(24) Donato, L.; Mourot, A.; Davenport, C. M.; Herbivo, C.; Warther, D.; Leonard, J.; Bolze, F.; Nicoud, J. F.; Kramer, R. H.; Goeldner, M.; Specht, A. *Angew. Chem., Int. Ed.* **2011**, *51*, 1840−1843.
(25) Li, W.; Llopis, J.; Whitney, M.; Zlokarnik, G.; Tsien, R. Y. *Nature* **1998**, *392*, 936−941.
(26) Kantevari, S.; Hoang, C. J.; Ogrodnik, J.; Egger, M.; Niggli, E.; Ellis-Davies, G. C. R. *ChemBioChem* **2006**, *7*, 174−180.
(27) Kaplan, J. H.; Ellis-Davies, G. C. R. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 6571−6575.
(28) Canepari, M.; Nelson, L.; Papageorgiou, G.; Corrie, J. E.; Ogden, D. *J. Neurosci. Methods* **2001**, *112*, 29−42.
(29) Matsuzaki, M.; Hayama, T.; Kasai, H.; Ellis-Davies, G. C. R. *Nat. Chem. Biol.* **2010**, *6*, 255−257.
(30) Schneggenburger, R.; Neher, E. *Nature* **2000**, *406*, 889−893.
(31) Banghart, M. R.; Sabatini, B. L. *Neuron* **2012**, *73*, 249−259.
(32) Bennett, B. D.; Wilson, C. J. *J. Neurosci.* **1999**, *19*, 5586−5596.
(33) Pisani, A.; Bonsi, P.; Centonze, D.; Martorana, A.; Fusco, F.; Sancesario, G.; De Persis, C.; Bernardi, G.; Calabresi, P. *J. Neurosci.* **2003**, *23*, 5272−5282.
(34) Pisani, A.; Bernardi, G.; Ding, J.; Surmeier, D. J. *Trends Neurosci.* **2007**, *30*, 545−553.
(35) Amit, B.; Zehavi, U.; Patchornik, A. *Israel J. Chem.* **1974**, *12*, 103−113.
(36) Binkley, R. W.; Flechtner, T. W. *Photoremovable Protecting Groups*; Plenum: London.
(37) Patchornik, A.; Amit, B.; Woodward, R. B. *J. Am. Chem. Soc.* **1970**, *92*, 6333−6335.
(38) Aujard, I.; Benbrahim, C.; Gouget, M.; Ruel, O.; Baudin, J. B.; Neveu, P.; Jullien, L. *Chem.—Eur. J.* **2006**, *12*, 6865−6879.
(39) Furuta, T.; Torigai, H.; Sugimoto, M.; Iwamura, M. *J. Org. Chem.* **1995**, *60*, 3953−3956.
(40) Givens, R. S.; Rubina, M.; Wirz, J. *Photochem. Photobiol. Sci.* **2012**, *11*, 472−488.
(41) Neher, E. *Neuron* **1998**, *20*, 389−399.
(42) Sakmann, B. *Pflugers Arch.* **2006**, *453*, 249−259.
(43) Lee, H.-M.; Larson, D. R.; Lawrence, D. S. *ACS Chem. Biol.* **2009**, *4*, 409−427.
(44) Klan, P.; Solomek, T.; Bochet, C. G.; Blanc, A.; Givens, R.; Rubina, M.; Popik, V.; Kostikov, A.; Wirz, J. *Chem. Rev.* **2013**, *113*, 119−191.
(45) Blanc, A.; Bochet, C. G. *J. Org. Chem.* **2002**, *67*, 5567−5577.
(46) San Miguel, V. n.; Bochet, C. G.; Del Campo, A. n. *J. Am. Chem. Soc.* **2011**, *133*, 5380−5388.
(47) Scott, T. F.; Kowalski, B. A.; Sullivan, A. C.; Bowman, C. N.; McLeod, R. R. *Science* **2009**, *324*, 913−917.
(48) Priestman, M. A.; Sun, L.; Lawrence, D. S. *ACS Chem. Biol.* **2011**, *6*, 377−384.
(49) Goguen, B. N.; Aemissegger, A.; Imperiali, B. *J. Am. Chem. Soc.* **2011**, *133*, 11038−11041.
(50) Priestman, M. A.; Shell, T. A.; Sun, L.; Lee, H. M.; Lawrence, D. S. *Angew. Chem., Int. Ed.* **2012**, *51*, 7684−7687.
(51) Kotzur, N.; Briand, B.; Beyermann, M.; Hagen, V. *J. Am. Chem. Soc.* **2009**, *131*, 16927−16931.
(52) Menge, C.; Heckel, A. *Org. Lett.* **2011**, *13*, 4620−4623.
(53) Schafer, F.; Joshi, K. B.; Fichte, M. A.; Mack, T.; Wachtveitl, J.; Heckel, A. *Org. Lett.* **2011**, *13*, 1450−1453.
(54) Callaway, E. M.; Katz, L. C. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7661−7665.
(55) Denk, W. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6629−6633.
(56) Yizhar, O.; Fenno, L. E.; Davidson, T. J.; Mogri, M.; Deisseroth, K. *Neuron* **2011**, *71*, 9−34.
(57) Miesenbock, G. *Science* **2009**, *326*, 395−399.
(58) Hegemann, P.; Nagel, G. *EMBO Mol. Med.* **2013**, *5*, 173−176.
(59) Nagel, G.; Szellas, T.; Kateriya, S.; Adeishvili, N.; Hegemann, P.; Bamberg, E. *Biochem. Soc. Trans.* **2005**, *33*, 863−866.
(60) Banghart, M.; Borges, K.; Isacoff, E.; Trauner, D.; Kramer, R. H. *Nat. Neurosci.* **2004**, *7*, 1381−1386.
(61) Boyden, E. S.; Zhang, F.; Bamberg, E.; Nagel, G.; Deisseroth, K. *Nat. Neurosci.* **2005**, *8*, 1263−1268.
(62) Nagel, G.; Szellas, T.; Huhn, W.; Kateriya, S.; Adeishvili, N.; Berthold, P.; Ollig, D.; Hegemann, P.; Bamberg, E. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 13940−13945.
(63) Han, X.; Boyden, E. S. *PLoS One* **2007**, *2*, e299.
(64) Zhang, F.; Wang, L. P.; Brauner, M.; Liewald, J. F.; Kay, K.; Watzke, N.; Wood, P. G.; Bamberg, E.; Nagel, G.; Gottschalk, A.; Deisseroth, K. *Nature* **2007**, *446*, 633−639.
(65) Prakash, R.; Yizhar, O.; Grewe, B.; Ramakrishnan, C.; Wang, N.; Goshen, I.; Packer, A. M.; Peterka, D. S.; Yuste, R.; Schnitzer, M. J.; Deisseroth, K. *Nat. Methods* **2013**, *9*, 1171−1179.
(66) Gradinaru, V.; Thompson, K. R.; Zhang, F.; Mogri, M.; Kay, K.; Schneider, M. B.; Deisseroth, K. *J. Neurosci.* **2007**, *27*, 14231−14238.
(67) Diester, I.; Kaufman, M. T.; Mogri, M.; Pashaie, R.; Goo, W.; Yizhar, O.; Ramakrishnan, C.; Deisseroth, K.; Shenoy, K. V. *Nat. Neurosci.* **2011**, *14*, 387−397.
(68) Nagel, G.; Brauner, M.; Liewald, J. F.; Adeishvili, N.; Bamberg, E.; Gottschalk, A. *Curr. Biol.* **2005**, *15*, 2279−2284.
(69) Deisseroth, K. *Nat. Methods* **2009**, *8*, 26−29.
(70) Zhang, Y. P.; Holbro, N.; Oertner, T. G. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 12039−12044.
(71) Amit, B.; Ben-Efraim, D. A.; Patchornik, A. *J. Am. Chem. Soc.* **1976**, *98*, 843−844.
(72) Pillai, V. N. R. *Synthesis* **1980**, 1−16.
(73) Yu, H.; Li, J.; Wu, D.; Qiu, Z.; Zhang, Y. *Chem. Soc. Rev.* **2010**, *39*, 464−473.
(74) Deiters, A. *ChemBioChem* **2010**, *11*, 47−53.
(75) Andrasfalvy, B. K.; Zemelman, B. V.; Tang, J.; Vaziri, A. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 11981−11986.