Brominated 7-hydroxycoumarin-4-ylmethyls: Photolabile protecting groups with biologically useful cross-sections for two photon photolysis


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**ABSTRACT** Photochemical release (uncaging) of bioactive messengers with three-dimensional spatial resolution in light-scattering media would be greatly facilitated if the photolysis could be powered by pairs of IR photons rather than the customary single UV photons. The quadratic dependence on light intensity would confine the photolysis to the focus point of the laser, and the longer wavelengths would be much less affected by scattering. However, previous caged messengers have had very small cross sections for two-photon excitation in the IR region. We now show that brominated 7-hydroxycoumarin-4-ylmethyl esters and carbamates efficiently release carboxylates and amines on photolysis, with one- and two-photon cross sections up to one or two orders of magnitude better than previously available. These advantages are demonstrated on neurons in brain slices from rat cortex and hippocampus excited by glutamate uncaged from N-(6-bromo-7-hydroxycoumarin-4-ylmethoxycarbonyl)-l-glutamate (Bhc-glu). Conventional UV photolysis of Bhc-glu requires less than one-fifth the intensities needed by the mate (Bhc-glu). Conventional UV photolysis of Bhc-glu requires less than one-fifth the intensities needed by the other (CNB-glu). Two-photon photolysis with raster-scanned femtosecond IR pulses gives the first three-dimensionally resolved maps of the glutamate sensitivity of neurons in intact slices. Bhc-glu and analogs should allow more efficient and three dimensionally localized uncaging and photocleavage, not only in cell biology and neurobiology but also in many technological applications.

Photolabile protecting groups have become a mainstay of organic synthesis (1), biotechnology, and cell biology (2, 3), because cleavage by light is a very mild deprotection step that usually is orthogonal to other experimental manipulations. The outstanding spatial and temporal precision with which light can be controlled enables diverse applications such as photolithographic construction of complex peptide and oligonucleotide arrays (4) or physiological release (uncaging) of bioactive substances in cells and tissues (2, 3). The ultimate in three-dimensional spatial precision would be obtained if the photochemistry were initiated not by the usual absorbance of a single UV photon but by excitation with two or more coincident IR photons of equivalent total energy (5–8). Such multiphoton excitation requires extremely high local intensities, typically obtained by focusing a femtosecond pulsed IR laser with a high-numerical-aperture lens and becomes insignificant away from the point of focus (Fig. 1). This nonlinear optical phenomenon is uniquely capable of noninvasively localizing the photochemistry to any given spot in three dimensions and will therefore be especially valuable in mapping biochemical sensitivities in complex tissues such as the brain (9, 10). Unfortunately, none of the commonly used photolabile protecting groups such as substituted 2-nitrobenzyls, 3-nitrophenyls, benzoins, and phenacyls (2, 11) have been reported to have sufficient photosensitivity for two-photon excitation without tissue damage. Photosensitivity is quantified as the uncaging action cross section $\delta_a$, which is the product of the two-photon absorbance cross section $\delta_d$ and the uncaging quantum yield $Q_{u2}$ (12). Ideally, $\delta_a$ should exceed 0.1 Goeppert–Mayer (GM), whose 1 GM is defined as $10^{-50}$ cm$^2$ s$^{-1}$ photon$^{-1}$, in the most popular groups, such as 3,4-dimethoxy-6-nitrobenzyl, fall considerably short of this criterion. For example, DM-nitrophen, a Ca$^{2+}$ chelator based on 3,4-dimethoxy-6-nitrobenzyl, has a maximum $\delta_a$ of 0.01 GM at 720 nm (12). The only existing caged biological messenger with adequate two-photon sensitivity is a Ca$^{2+}$ complex of an azide-substituted chelator ($\delta_a = 1.4$ GM at 700 nm), whose specific photochrome (12, 13) would not be readily generalizable to release any other important messengers. A partial substitute for a true two-photon-excited photolysis is to use conventional UV with molecules bearing two separate caging groups, both of which have to be photolyzed to release biological activity (2, 10). However, this approach requires that the target molecule be blockable at either of two positions and that the extent of photolysis remains low. Also, the feasible depth of uncaging inside tissue will be limited by the much stronger absorbance and scattering of UV compared with IR wavelengths. We now report that carboxylate, carbamate, and phosphate esters of brominated 6-hydroxycoumarin-4-ylmethanol have the requisite action cross sections ($\delta_a \approx 1$ GM), photolysis kinetics, synthetic accessibility, water solubility, and stability in the dark to be a reasonably general solution to the problem of true two-photon uncaging of biologically important acids and amines. In addition, their cross sections for one-photon uncaging with UV at 365 nm or longer wavelengths are about an order of magnitude better than that of most previous caged compounds.

**Abbreviations:** Bhc-glu, N-(6-bromo-7-hydroxycoumarin-4-ylmethoxycarbonyl)-l-glutamate; CNB-glu, l-glutamic acid γ-(a-carboxy-2-nitrobenzyl) ester; GM, Goeppert–Mayer or $10^{-50}$ cm$^2$ s$^{-1}$ photon$^{-1}$ per photon. **Present address:** Department of Molecular Science, Toho University, Miyama 2-2-1, Funabashi, 278-8510 Japan. **To whom reprint requests should be addressed:** Howard Hughes Medical Institute 0647, 310 CMM-W, University of California, San Diego, La Jolla, CA 92039-0647. e-mail: rtsien@ucsd.edu.
MATERIALS AND METHODS

Organic Synthesis. General. Chemicals and solvents were used directly as received unless otherwise noted. Acetonitrile, chloroform, and dichloromethane were dried over 4A molecular sieves. Proton magnetic resonance spectra (1H NMR) were recorded on a Gemini 200 MHz spectrometer (Varian) and reported in ppm (δ) downfield from tetramethylsilane with residual solvent peaks as the internal standards. UV spectra were recorded on a Cary 3E UV-Visible spectrophotometer (Varian). Fluorescence spectra were recorded on a Fluorolog spectrofluorometer (Spex Industries, Edison, NJ). Except where otherwise noted, KMops buffer consisted of 100 mM KCl/10 mM Mops titrated to pH 7.2 with KOH. Mass spectra were recorded on an electrospray mass spectrometer (5989B, Hewlett Packard). Thin layer and column chromatography were performed on precoated silica gel 60F-254 plates and 230–400 mesh silica gel 60, respectively (EM Separations Technology, Gibbstown, NJ). All manipulations of compounds sensitive to near-UV light were performed under an orange safety lamp.

Syntheses of the coumarins (Fig. 2) relied on condensation of ethyl 4-chloroacetoacetate with halogenated resorcinols (10b, 10c) in analogy to the known reaction with the unsubstituted parent resorcinol (10a) (14). The alternative of bromination after coumarin formation was hard to control short of tribromination, which yielded 11d. Acetate and glutamate esters (4–8) were prepared by direct displacement of chloride from 11a–11d. The preferred glutamate carbamate (9) was prepared by hydrosylation of the 4-chloromethyl side chain to hydroxymethyl in 13 (14), coupling to 4-nitrophenyl chloroformate to give 14, subsequent reaction with tert-butyldiphenylmethylglutamate hydrochloric acid salt, rt, 23 h; (b) CF3CO2H, CH2Cl2, r t, 2 d.

FIG. 2. Synthetic scheme for the new compounds prepared in this study. (a) Ethyl 4-chloroacetoacetate, H2SO4, rt, 6 d; (b) Br2, AcOH, rt, 1 h; (c) DBU, AcOH, benzene, reflux, 1.5 h; (d) DBU, N-(tert-butoxycarbonyl)-α-tert-butyloxycarbonyl glutamate, benzene, reflux, 1 h; (e) CF3CO2H, CH2Cl2, rt, 1 d; (f) H2O, reflux, 14 h; (g) i. DMAP, 4-nitrophenyl chloroformate, CH3CN, rt, 7 h; ii. DMAP, di-tert-butyloxycarbonyl glutamate hydrochloric acid salt, rt, 23 h; (h) CF3CO2H, CH2Cl2, rt, 2 d.

6-Bromo-4-chloromethyl-7-hydroxycoumarin (11c). A solution of 936.1 mg (4.953 mmol) of 4-bromoresorcinol (10c), concentrated sulfuric acid (5 ml), and ethyl 4-chloroacetoacetate (1 ml, 7.4 mmol) was stirred for 6 days at room temperature. The reaction mixture was poured into ice water and stirred for 2 h to give a fine precipitate. The precipitate was collected by filtration, washed with cold water, and dried under vacuum over P2O5 to yield 847.6 mg (2.928 mmol, 59.1% yield). The alternative of bromination of ethyl 4-chloroacetoacetate with halogenated resorcinols (10b, 10c) in analogy to the known reaction with the unsubstituted parent resorcinol (10a) (14). The alternative of bromination after coumarin formation was hard to control short
(CDCl3 + 5% CD3OD) δ 7.43 (1H, s), 6.85 (1H, s), 6.25 (1H, s), 5.16 (2H, s), 3.30 (1H, s), 2.14 (3H, s); MS (negative ion) 267.2 and 269.1; UV (K-Mops) λmax (29) 370 nm (16,000 M⁻¹·cm⁻¹).

3,6,8-Tribromo-4-chloromethyl-7-hydroxycoumarin. (11d). To a suspension of 212.6 mg (1.01 mmol) of 4-chloromethyl-7-hydroxycoumarin (11a) in acetic acid (2 ml) was added 0.2 ml (3.9 mmol) of bromine. The mixture first became a brown solution and then a yellow-orange slurry. After stirring for 9 h at room temperature, another intermediate carbonate product. Purification with column chromatography (8 g of SiO2, 25% methanol/chloroform containing 0.4% acetic acid) gave 12.7 mg (0.0286 mmol, 94.1% yield) of 9 as a yellow solid.

N-(6-bromo-7-hydroxycoumarin-4-yl)methoxycarbonyl-L-glutamic acid (9). (Bhc-glu). A solution of 16.9 mg (0.0304 mmol) of 15 in dry CH2Cl2 (3 ml) and CF3COOH (1 ml) was stirred for 2 days at room temperature and evaporated to dryness. Purification with column chromatography (8 g of SiO2, 25% methanol/chloroform containing 0.4% acetic acid) gave 12.7 mg (0.0286 mmol, 94.1% yield) of 9 as a yellow solid.

Quantum Efficiencies for One-Photon Excitation and Rates of Hydrolysis in the Dark. The photolysis quantum efficiencies for one-photon excitation were determined by irradiating a KMops-buffered solution of the substrate with 365-nm UV light from a mercury lamp (B-100; Spectronics, Westbury, NY). The duration of each irradiation period was controlled by an electronic shutter. Between each duration, a small aliquot (10 µl) of the solution was removed for analysis by reversed-phase HPLC with a BetaBasic-18 column (Keystone Scientific, Bellefonte, PA) eluted with an isocratic mixture of 40% acetonitrile and 60% water containing 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min, using absorbance detection at 325 nm. Optical densities at 365 nm were kept around 0.1 so that inner-filtering of the irradiation and spatial gradients of concentrations could be neglected, and the progress curves were simple decaying exponentials. Quantum efficiencies were calculated as (1/Φt)⁻¹, where 1/Φt is the irradiation intensity in einsteins·cm⁻²·s⁻¹, and σ is the decadic extinction coefficient in cm²·mol⁻¹·10⁻³ times ε, the usual extinction coefficient in M⁻¹·cm⁻¹, and τ050% is the irradiation time in seconds for 90% conversion to product (15). The total UV intensity 1 was measured by using chemical actinometry with 6 mM potassium ferrioxalate in the same setup (16). Dark hydrolysis rates were measured similarly except without illumination.

Quantitative Analysis of Photolysis Product from Bhc-Glu (9). Glutamate released from Bhc-Glu (9) on photolysis was quantified as its fluorescamine derivative by HPLC (17). For example, the production of glutamate from 100 µM 9 exposed to 365 nm irradiation was as follows: 30 s, 27.6% yield; 60 s, 52.7%; 90 s, 74.5%; 120 s, 98.7%. Therefore, glutamate was quantitatively released with the appropriate stoichiometry.

In Vitro Measurements of Uncaging Cross Sections. Measurements of cross sections for two-photon uncaging were made in microcuvets with 10 x 1 x 1 mm illuminated dimensions and about 15 µl effective filling volume (26.10F-O; Starna Cells, Atascadero, CA). Femtosecond IR pulses pumped by a Millenium; both from Spectra-Physics) were aimed along the long axis of the sample chamber and focused in its center with a 25 mm focal length lens (06LXP003; Starna Cells, Atascadero, CA). Femtosecond IR pulses were estimated by replacing the caged compound by a solution of fluorescein, a reference compound of known fluorescence quantum yield Qf(0.9) and two-photon absorbance crosssection δα (30 and 38 GM at 740 and 800 nm respectively) (18). The time-averaged fluorescence (F(t)) collected by the detector is given by the equation (18)
where φ is the collection efficiency of the detector, Cφ is the concentration of fluorescein, \( I_F(t) \) is the mean squared light intensity, \( S(r) \) is a unitless spatial distribution function, and the integral is over the volume of the microcuvet. The fluorescence emitted at right angles to the laser beam was measured through a 535 ± 22.5 nm bandpass filter in front of a silicon photodiode radiometer (II1700; International Light, Newburyport, MA). The collection efficiency \( \phi \) was estimated as \( A/y/(4\pi s^2) \), where \( A \) is the area of the detector (0.38 cm²), \( y \) is the fraction of the integrated emission spectrum transmitted by the interference filter (0.377, measured separately in a spectrofluorometer), \( R \) is the distance from the center of the cuvet to the detector (3.83 cm), and \( n \) is the refractive index of water (1.333). For photoysis of a caged compound or substrate, the number \( N_p \) of molecules of product formed per unit time is

\[
N_p = 0.5Q_{2s}\delta_sC_S(J_0(t))/F(\tau)dV,
\]

where \( Q_{2s} \), \( \delta_s \), and \( C_S \) are the quantum yield for two-photon uncaging, absorbance cross section, and concentration of the substrate, respectively. \( N_p \) was measured by HPLC sampling of the microcuvet as for one-photon photolysis. The uncaging action cross section \( \delta_s = Q_{2s}\delta_s/C_s \) can therefore be estimated by combining the results of the fluorescence calibration and HPLC measurements of uncaging in the same apparatus:

\[
\delta_s = \frac{N_p\delta_0 Q_{2s}D_0 C_r}{(F(\tau))/C_S}.
\]

Similarly, when the substrate is also fluorescent, its two-photon fluorescence action cross section \( \delta_{ys} = (Q_{2s}\delta_0/C_s) \) can be expressed as

\[
\delta_{ys} = (F(\tau))/\phi_1C_r (F(\tau))/\phi_2 C_s \delta_{yp}.
\]

Here the subscripts \( S \) and \( F \) distinguish values for the substrate and for the fluorescein reference. If one assumes that the one- and two-photon quantum efficiencies are the same, then \( \delta_0/Q_{2s} \) and \( \delta_{ys}/Q_{2s} \) should both equal \( \delta_s \). In fact, \( \delta_0/Q_{2s} \) is typically about twice \( \delta_s/Q_{2s} \). At present we cannot say which values for \( \delta_s \) are more reliable because the estimate of the geometric collection efficiency \( A/y/(4\pi s^2) \) is quite crude and because Parma & Omenetto (19) have claimed that \( Q_{2s} \) is only 0.32Q₂S for the closely related 7-hydroxyccoumarin. Therefore, the absolute magnitudes of the cross sections here are probably reliable to no better than a factor of two, although their relative magnitudes may be more accurate. Also, all cross sections are for the closely related 7-hydroxyccoumarin. Therefore, the absolute magnitudes of the cross sections here are probably reliable to no better than a factor of two, although their relative magnitudes may be more accurate. Also, all cross sections are directly proportional to the assumed value of \( Q_{2s}\delta_s \) for fluorescein.

One-Photon UV Uncaging on Brain Slices. Coronal visual cortex slices from Long-Evans rats at postnatal days 26–28 were cut to 400 µm thick by using an egg slicer-like device (20). Animals were anesthetized with a lethal dose of pentobarbital (100 mg/kg) and decapitated, and brains were rapidly placed in ice-cold artificial cerebrospinal fluid containing (mM) NaCl (120), KCl (3.5), CaCl₂ (2.5), MgCl₂ (1.3), NaH₂PO₄ (1.25), NaHCO₃ (25), CaCl₂ (0.5), MgSO₄ (4), Hepes (10), NaATP (3), and GTP (0.3), pH adjusted to 7.30 with KOH. For some experiments, this solution also contained 2 mM MgCl₂ (0.3), Na₂ATP (3), Na₂GTP (0.3), and K₂EGTA (0.5), pH adjusted to 7.30 with KOH. For some experiments, this solution also contained 0.1 mM K₃-fura-2 (Molecular Probes). Whole-cell patch clamp recordings were made under visual control by using video contrast enhancement and under oblique illumination. Recordings were made in voltage clamp mode with \( V_m \) between −60 and −90 mV and accepted only when the holding current was less than 100 pA.

For delivery of caged glutamate, a 50-µm wide Kaption-coated fused-silica capillary tube (Polymicro Technologies, Phoenix, AZ) was positioned at the surface of the slice before recording, and its back end was immersed in a reservoir of local perfusion solution. Basic local perfusion solution contained (mM) NaCl (119), KCl (3), NaH₂PO₄ (1), CaCl₂ (0.5), Mg₂SO₄ (4), Hepes (25), and d-glucose (10). The patch pipette solution contained (mM) potassium methanesulfonate (135), Hepes (10), MgCl₂ (0.3), Na₂ATP (3), Na₂GTP (0.3), and K₂EGTA (0.5), pH adjusted to 7.30 with KOH. For some experiments, this solution also contained 0.1 mM K₃-fura-2 (Molecular Probes). Whole-cell patch clamp recordings were made under visual control by using video contrast enhancement and under oblique illumination. Recordings were made in voltage clamp mode with \( V_m \) between −60 and −90 mV and accepted only when the holding current was less than 100 pA.

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experiments slow scan speeds (2 s per line) were used to allow low-resolution images to be acquired (32 × 32 pixels; Fig. 7b).

In tests of the power dependence and for the involvement of α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA)-type glutamate receptors (Fig. 5), currents were recorded during scans with fast line rates (5 ms per line, fast scan direction is vertical in Fig. 5) that did not permit temporal and hence spatial resolution along the scan lines. To verify cell responsiveness, glutamate was uncaged also by conventional, one-photon absorption by using a pulsed mercury arc lamp (23).

RESULTS

In Vitro Measurements. Large two-photon cross sections are found in molecules with extended conjugation and high extinction coefficients for π–π* transitions. Of the caging groups commonly used in biology, nitrobenzyl groups without alkoxy substituents have so far shown unmeasurably low cross sections (19), so we did not attempt to measure an uncaging cross section δ0 for CNB-glu (1). The 3,4-dimethoxy-6-nitrobenzyl chromophore has the longest wavelengths and highest extinction coefficients for one-photon absorption and therefore would seem to have more promise. However, we were unable to detect any two-photon photosensitivity for the commercially available L-glutamic acid-(3,4-dimethoxy-6-nitrobenzyl) ester (2), because its reaction kinetics (Fig. 3) were entirely dominated by rapid spontaneous dark hydrolysis with a time constant of 19.8 h, presumably because of the electron-withdrawing -NH3+ on the α-carbon of the glutamate. A simpler ester without this background problem, 3,4-dimethoxy-6-nitrobenzyl acetate (3), showed δ0 = 0.03 and 0.01 GM at 740 and 800 nm, respectively (Table 1). These values are comparable to that measured for DM-nitrophen (0.01 GM at 720 nm) by a very different analytical procedure. We therefore turned to 7-hydroxycoumarin-4-ylmethyl as the most promising member of a newer generation of caging groups (24, 25), because the unattached 7-hydroxycoumarin chromophore has an acceptable cross section for two-photon excitation of fluorescence (19). 7-Hydroxycoumarin-4-ylmethyl acetate (4) proved to have much higher δ values (1.07 and 0.13 GM at 740 and 800 nm) for hydrolysis to acetic acid and 7-hydroxycoumarin-4-ylmethanol. We hypothesized that halogenation of the coumarin would further increase the two-photon cross sections by lowering the pKa of the chromophore below physiological pH and promoting formation of the more strongly absorbing anion. Also, heavy atoms would promote intersystem crossing to the triplet, which is believed to be the photochemically reactive state (T.F., M. Kanehara, and M. Iwamura, unpublished data). Compounds 5–7 were indeed mostly anionic at pH 7.2. Table 1 shows that their maximum δs increased in the order 6-chloro < 6-bromo < 3,6,8-tribromo substitution as predicted. Although 7 had the highest cross section at 800 nm, its photolysis was complicated by multiple secondary photoproducts, and useful derivatives proved relatively difficult to synthesize, whereas analogs of the monobromo compound 6 were much more tractable. Compounds 4–7 had half-lives for dark hydrolysis of 202, 228, 183, and 218 h, respectively. To prepare physiologically useful caged compounds, the 6-bromo-7-hydroxycoumarin-4-ylmethyl (Bhc) group was linked to L-glutamate at either the γ-carboxyl or the amino group via a carbamate linker (Fig. 2). The γ-ester (8) and the carbamate (9) had about the same δ0, 0.9–1.0 and ~0.4 GM at 740 and 800 nm, respectively (Table 1). The superior sensitivity to two-photon photolysis of 9 compared with prior caged glutamates such as 2 is qualitatively obvious (Fig. 3). The main disadvantage of 8 was its spontaneous hydrolysis. At pH 7.2, 24°C, the percentages of 8 remaining after 17, 40, 65, 91, and 119 h in the dark were 84, 63, 41, 21, and 8, respectively, whereas there was no detectable loss of 9 (>99% remaining) over 35 h. This superiority of glutamate carbamates over γ-esters and especially α-esters with respect to dark hydrolysis is similar to that already reported for α-carboxy-2-nitrobenzyl protecting groups (26). Background hydrolysis is often a serious drawback because free glutamate readily desensitizes glutamate receptors, so carbamate (9) is preferred.

One-Photon UV Uncaging in Brain Slices. To compare the physiological effects of invoking currents in neurons from uncaging Bhc-glu (9) or CNB-glu (1) at the same concentration (50 μM), eight cells from layer II/III of rat visual cortex were directly stimulated by focusing 10-ms pulses of UV laser light (351–364 nm) below the cell body. Fig. 4a shows typical current traces from stimulating cells at increasing light intensities. The three upper traces are from a cell bathed in Bhc-glu. The responses were evoked by stimulation at UV intensities of 2, 3, and 4 mW at the slice, whereas similar sized current responses from a different cell bathed in CNB-glu (lower three traces) required intensities of 10, 16, and 21 mW, about five times higher intensity. The times from onset of shutter opening to the peak inward current were longer for the Bhc-glu responses compared with CNB-glu. The mean peak times for all responses from the 4 cells sampled with Bhc-glu ranged from 29.8 to 45.7 ms (mean ± SEM = 38.9 ± 3.6 ms, n = 4 cells) whereas the values for cells sampled with CNB-glu ranged from 14.1 to 25.8 ms (mean ± SEM = 18.3 ± 5.2 ms, n = 4). Carbamates are known to release glutamate more slowly than do γ-esters (26), probably because of the time required for loss of CO2 from the carbamic acid of glutamate rather than any intrinsic slowness of coumarin-4-ylmethoxymethyl hydrolysis. The mean normalized current for all of the cells as a function of pulse energy, plotted on a log scale, is shown in Fig. 4b. Normalization of each cell to its own average maximum response was required because the neurons can vary in their absolute sensitivity to glutamate, partly because the neurons were patched blindly, so that the exact position of the cell body
The form of the dose-response curve is expected to be complex because of the cooperativity of the gluamate receptor and the competition between its activation and desensitization (27). Furthermore, saturating activation of the receptor was not achievable with CNB-glu because the maximal laser power was limited. Nevertheless, the data in Fig. 4B confirm that Bhc-glu is physiologically about five-fold more UV-sensitive than CNB-glu. The superiority of Bhc-glu is not as marked as implied by the thirty-fold ratio of $\epsilon_{Q_{1}(365)}$ values in Table 1, probably because the 351–364 nm wavelengths from the laser are better for CNB-glu and worse for Bhc-glu and because the slightly slower kinetics of the carbamate allow more receptor desensitization to limit the peak amplitude.

Two-Photon IR Uncaging in Brain Slices. Bhc-glu was applied to slices locally by pressure-ejection from a capillary tube. The compound’s presence could be monitored directly by its fluorescence at low levels of two-photon excitation, which allowed cells to be seen in “negative” stain (Fig. 5a). During whole-cell recordings, application of Bhc-glu did not cause detectable changes in holding current (15 cells), showing that this compound lacks intrinsic agonist activity.

The stream of pulses from the Ti:sapphire laser was raster scanned across cell bodies and dendrites. In the absence of caged compound, scans caused no changes in holding current (15 cells). After application of Bhc-glu, scans evoked inward currents (Fig. 5b) whose amplitude was strongly dependent on beam power (Fig. 5c). The peak current amplitude increased as a supralinear function of beam power, mostly caused by the nonlinear power dependence expected for a two-photon process relative to the focus point of the objective was not determined (see Materials and Methods). The form of the dose-response curve is expected to be complex because of the cooperativity of the gluamate receptor and the competition between its activation and desensitization (27). Furthermore, saturating activation of the receptor was not achievable with CNB-glu because the maximal laser power was limited. Nevertheless, the data in Fig. 4B confirm that Bhc-glu is physiologically about five-fold more UV-sensitive than CNB-glu. The superiority of Bhc-glu is not as marked as implied by the thirty-fold ratio of $\epsilon_{Q_{1}(365)}$ values in Table 1, probably because

<table>
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<th>Compd</th>
<th>$\lambda_{\text{abs}}$ (nm)</th>
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<th>$\epsilon(400)$</th>
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<td>5,900*</td>
<td>4,900</td>
<td>1,050</td>
<td>0.006</td>
<td>29</td>
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<td>4</td>
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<td>5,000</td>
<td>1,000</td>
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<td>103</td>
<td>0.20</td>
<td>3.85 ± 0.31</td>
<td>1.26 ± 0.04</td>
<td>1.07 ± 0.05</td>
<td>0.13 ± 0.01</td>
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<tr>
<td>5</td>
<td>370</td>
<td>16,000</td>
<td>5,000</td>
<td>0.01</td>
<td>302</td>
<td>374</td>
<td>0.37</td>
<td>9.68 ± 0.32</td>
<td>3.22 ± 0.09</td>
<td>1.07 ± 0.07</td>
<td>0.34 ± 0.02</td>
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<tr>
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<td>15,000</td>
<td>5,000</td>
<td>0.037</td>
<td>548</td>
<td>224</td>
<td>0.22</td>
<td>4.24 ± 0.12</td>
<td>1.41 ± 0.05</td>
<td>1.99 ± 0.09</td>
<td>0.42 ± 0.01</td>
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<td>3.1 ± 0.2</td>
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<td>8</td>
<td>369</td>
<td>19,550</td>
<td>7,560</td>
<td>0.019</td>
<td>364</td>
<td>89</td>
<td>0.24</td>
<td>0.89 ± 0.11</td>
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<td>0.37 ± 0.06</td>
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<td>5,400</td>
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<td>329</td>
<td>0.38</td>
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<td>2.23</td>
<td></td>
<td>0.95 ± 0.21</td>
<td>0.37 ± 0.06</td>
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$\lambda_{\text{abs}}$, wavelength in nm of absorbance maximum; $\epsilon$, extinction coefficient in M$^{-1}$ cm$^{-1}$ at wavelength indicated; $Q_{11}$, quantum efficiency for uncaging with one-photon excitation at 365 nm; $\lambda_{\text{em}}$, wavelength in nm for fluorescence emission peak; $\delta$, fluorescence action cross section in 10$^{-50}$ cm$^4$ s/photon for two-photon excitation at wavelength indicated ($\delta_{740}Q_{740}$, where $\delta_{740}$ is the absorbance cross section and $Q_{740}$ is the quantum efficiency for fluorescence, both with two-photon excitation); $\delta_{em}$, uncaging action cross section in GM (10$^{-50}$ cm$^4$ s/photon) for two-photon excitation at wavelength indicated ($\delta_{em} = \delta_{740}Q_{740}$, where $Q_{740}$ is the quantum efficiency for uncaging with two-photon excitation). Measurements were made in K-Mops at 24–25°C. Error estimates are standard deviations.

*Values taken from the literature (30, 31); For compound 1, the entries listed under $\epsilon(365)$ and $Q_{11}(365)$ actually refer to 360 nm.
†$\lambda_{\text{em}}$ could not be measured because breakdown was dominated by dark hydrolysis with a 19.8-h exponential time constant.
‡Value was measured at 780 nm on an independent apparatus.

Two photon uncaging of Bhc-glu. (a) Two-photon fluorescence image of a hippocampal neuron bathed in 0.5 mM Bhc-glu. (b) Whole-cell currents recorded in response to full-field raster scans like the one used for the fluorescence image (2 ms per line, 256 lines) with the beam power between 0 to 75 mW. (c) Dependence of peak current on beam power. The curves indicate fits by eye to second- and third-power functions. (d) Block of uncaging-evoked currents by a glutamate receptor antagonist (laser power 80 mW). Traces show responses at the same location in the absence and presence of 50 $\mu$M CNQX (6-cyano-7-nitroquinoxaline-2, 3-dione), an antagonist of non-N-methyl-D-aspartate receptors.
DISCUSSION

The two-photon uncaging cross sections ($\delta_u$) for Bhc and analogously halogenated 7-hydroxycoumarin-4-ylmethyl groups are on the order of 1 GM, about two orders of magnitude larger than those previously measured for any photolabile protecting groups. If one assumes equality of one- and two-photon quantum efficiencies, their absorbance cross sections ($\delta_s$) would be 20–50 GM, reasonably consistent with previous values for coumarin dyes (18, 19). Molecules with $\delta_s$ as high as several thousand GM have recently been reported, but they are very large, hydrophobic, and not yet harnessed to any useful photolytic reaction (28). The mechanism of Bhc photolysis has not yet been analyzed, although experiments on the related 7-methoxycoumarin-4-ylmethyl diethylphosphate indicate that the solvolysis occurs via the triplet state with a lifetime of about 1 s (T.F., M. Kanehara, and M. Iwamura, unpublished data). The intermediacy of a triplet state is supported by the increase in photolysis quantum yields from 6-chloro to 6-bromo to 3,6,8-tribromo substitution (Table 1). The kinetics of glutamate release from Bhc-glu after a photolysis flash remain to be fully characterized, although the above triplet-state kinetics suggest that the initial release of the carbamic acid is unlikely to be rate-limiting. Instead, the slow step is probably the final decarboxylation of the carbamic acid to regenerate glutamate, which takes a few ms at pH 7.2 and is faster yet at more acidic pH (26). All glutamates that are caged via a carbamate linkage have to go through this additional step. In the present trials, we preferred the better hydrolytic stability in the dark of Bhc-glu (9) over the potentially faster kinetics of the $\gamma$-ester (8). If one were confident that the tissue could handle background release of glutamate, e.g., by active uptake, and if maximal temporal and spatial resolution were required, the $\gamma$-ester (8) might prove superior. A modified $\gamma$-ester combining both fast kinetics and resistance to hydrolysis in the dark would be optimal.

Bhc-glu is the first caged glutamate to allow nondestructive three-dimensional mapping of the glutamate responsivity over the surface of a neuron. In various previous attempts to map glutamate sensitivities by using nitrobenzyl-based compounds (K. Svoboda and W.D., unpublished data) with two-photon excitation, no significant current signals were seen. The intrinsic fluorescence of Bhc-glu is helpful for visualizing the delivery of the caged compound but can hinder simultaneous use of UV- or violet-excited fluorescent indicators. Further modification of the chromophores to accelerate intersystem conversion (5, 10), but also probably including some contribution from positive cooperativity of the AMPA-type glutamate receptor (27). When the antagonist CNQX (50 $\mu$M) was included in the local perfusion solution, light-evoked currents were reversibly abolished (Fig. 5d) indicating that evoked currents arose from the activation of non-N-methyl-D-aspartate glutamate receptors.

A central property of two-photon excitation is the elimination of out-of-focus background (5). Two-photon uncaging should therefore allow the production of glutamate only in the plane of focus. This was tested by using two-photon scanning photochemical microscopy (8). Cells were filled with the indicator dye fura-2 to visualize their dendrites (Fig. 6a) and scanned with uncaging illumination with the focal planes set at different depths (Fig. 6b). Changes in holding current were recorded as a function of the beam position and displayed as pixel intensity, generating a map of the response amplitude. Responses varied as a function of the focal plane and corresponded with the dendritic elements that were in focus under fluorescence viewing (Fig. 6b). Scans over the cell body gave ring-like current maps characteristic of optical sectioning (Fig. 7b).

![Image](image_url)
crossing should reduce such fluorescence and increase the photolysis quantum yields. Although the present work focused on caged glutamates, we have also prepared the Bhc ester of cAMP, which is hydrolytically stable in the dark but efficiently photolyzed by two-photon excitation (T.F. and T.M.D., unpublished results). Therefore, the Bhc group can cage carboxylates, phosphates, and amines (via carbamates), the major acidic and basic functionalities of biological relevance.

The good performance of the above coumarin-based caging groups has important implications in both photochemistry and practical applications. Traditional caging groups are based on small chromophores absorbing only in the UV and often utilizing weak \( n-\pi^* \) transitions. The present coumarins are conceptually derived from well known fluorescent dyes by attaching the leaving group via a \(-\text{CH}_2-\) to a site that undergoes a large increase in electron density on excitation of the delocalized chromophore. Also, heavy atoms are added to promote intersystem crossing to the triplet state. We suspect that these design principles can be applied to other fluorescent dyes to create other caging groups of even longer wavelengths and larger 1- and 2-photon cross sections. In conventional 1-photon photolysis, longer wavelengths and greater photosensitivity should help minimize photodamage, decrease irradiation times, simplify apparatus, make semiconductor light sources feasible, and permit separately controllable photolyses of two or more protecting groups at different wavelengths.

Two-photon photolysis should facilitate higher-resolution three-dimensional optical memories (29), spatially arrayed combinatorial libraries (4), photodynamic therapy, and deeper and less-invasive mapping of the local responses of complex tissues to neurotransmitters and messengers.

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