# Coumarin-Caged Glycine that Can Be Photolyzed within 3 $\mu$s by Visible Light†

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ABSTRACT: The synthesis and characterization of a new photolabile precursor of glycine (coumarin-caged glycine) are reported. The new compound is suitable for rapid chemical kinetic investigations of the membrane-bound neurotransmitter receptor activated by glycine. Unlike previously used caging groups for glycine, this precursor can be photolyzed rapidly and efficiently in the visible wavelength region. This allows the use of a relatively inexpensive light source. The α-carboxyl group of glycine was covalently coupled to the 7-(diethylamino)coumarin (DECM) caging group. The caged compound has a major absorption band with a maximum at 390 nm ($\epsilon_{390} = 13\,900$ $M^{-1}$ $cm^{-1}$). Photolysis was performed at wavelengths of ≥400 nm ($\epsilon_{400} = 12\,400$ $M^{-1}$ $cm^{-1}$). Under physiological conditions, DECM-caged glycine is water soluble and stable. In the visible wavelength region, it photolyzes rapidly to release glycine with a half-life of ∼2.5 $\mu$s and a quantum yield of 0.12 ± 0.01. The experimental results demonstrated that neither DECM-caged glycine nor its byproduct inhibits or activates human α1 glycine receptors expressed on the surface of HEK 293 cells.

The photorelease of bioactive molecules from biologically inert precursors (caged compounds) is a useful tool for studying, in cell or tissue culture, biochemical responses in which a target is not readily accessible, temporally or spatially, to a molecule that initiates the reaction (*1*−*8*). Many excellent techniques for investigating fast reactions of biomolecules in solution exist (reviewed in refs *9*−*12*), but they cannot be applied to membrane-bound proteins (*13*, *14*). For investigating reactions of membrane-bound proteins, the equilibration of inert caged compounds with their target protein followed by photolytic release of the active molecule in the microsecond time region has been especially useful (*7*, *8*). To be suitable for such investigations, caged compounds must be photolyzed rapidly (in microseconds) with sufficient quantum yield (*7*) and be soluble and stable in aqueous solution. The caged compound and its byproducts, other than the desired ligand, must be biologically inert. Most existing caged neurotransmitters that have these properties (reviewed in refs *7* and *8*) require relatively high energy UV light for their photolysis, and this can lead to undesirable damage to the biological material (*7*). This problem can be avoided by using a photolabile protecting group that has all the desirable properties listed above and that can be photolyzed in the visible wavelength region. Many molecules suitable as caging groups absorb in the visible wavelength region, including (coumarin-4-yl) methyl derivatives, which have been used to protect the biological activity of phosphates (*15*−*20*), carboxylates (*21*), sulfates (*20*), diols (*22*), alcohols (*23*), and carbonyl compounds (*24*). Our previous research showed that 7-(diethylamino)coumarin-caged glutamic acid (*21*) can be photolyzed by visible light and has the required properties for a caged neurotransmitter. However, when the DECM[1] group was used to cage the neurotransmitter γ-aminobutyric acid (GABA) (V. R. Shembekar *et al.*, unpublished observations), we found that the caged compound inhibits the $GABA_A$ receptor expressed in HEK 293 cells.

Here, we report the synthesis and characterization of DECM-caged glycine and demonstrate that the compound has the required properties for chemical kinetic investigations of the glycine receptor (reviewed in ref *25*). The receptor is a potential target for general anesthetics and alcohol (*26*−*28*). Mutations of the receptor are involved in malfunctions of the nervous system, such as in murine spastic disease (*29*) and human hyperekplexia (*30*).

## MATERIALS AND METHODS

*Synthesis*. See the reactions in Scheme 1. All the starting materials for synthesis were purchased from Aldrich.

The synthesis and photolysis of DECM-caged glycine are outlined in Scheme 1. 7-(Diethylamino)-4-(hydroxymethyl)-coumarin (**I**) was synthesized as previously described (*31*) and was used here to cage *N*-*tert*-Boc-glycine. *N*-*tert*-Boc-glycine (998.5 mg, 5.7 mmol), 4-(dimethylamino)pyridine

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[1] Abbreviations: HEK 293, human embryonic kidney; DE4MCM, 7-(*N*,*N*-diethylamino)-4-methylcoumarin; DE4HMCM, 7-(*N*,*N*-diethylamino)-4-(hydroxymethyl)coumarin; *N*-*tert*-Boc-DECM, *N*-(butyloxycarbonyl)-7-(*N*,*N*-diethylamino)coumarin; DECM, 7-(*N*,*N*-diethylamino)-coumarin; DCC, 1,3-dicyclohexylcarbodiimide; DMAP, 4-(dimethylamino) pyridine; α-CNB, α-carboxyl-2-nitrobenzyl; TEACl, tetraethylammonium chloride; DNAP, 2-(dimethylamino)-5-nitrophenyl.

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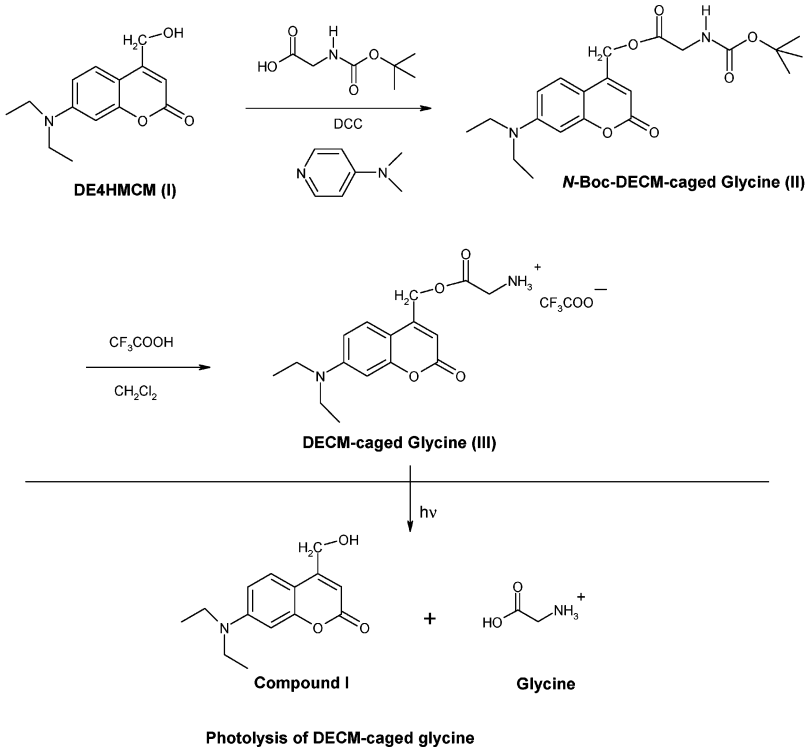
Scheme 1: Photolysis of DECM-Caged Glycine




(58.64 mg, 0.48 mmol), and 1,3-dicyclohexylcarbodiimide (DCC) (1.285 g, 6.23 mmol) in 100 mL of dichloromethane ($CH_2Cl_2$) were stirred at room temperature for ~10 min; 440 mg (1.78 mmol) of compound **I** was added to the reaction mixture, and the resulting mixture was stirred at room temperature in the dark for ~30 min. The reaction mixture was filtered and the solvent evaporated to give a yellow solid. The crude product was purified using a silica gel column and flash chromatography (10% acetone/$CH_2Cl_2$ mixture), yielding 0.647 g (1.6 mmol, 89%) of caged compound **II**: $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 7.28 (d, $J$ = 9.0 Hz, 1H), 6.58 (dd, $J$ = 9.0, 2.6 Hz, 1H), 6.49 (d, $J$ = 2.6 Hz, 1H), 6.15 (s, 1H), 5.2 (s, 2H), 3.35 (q, $J$ = 7.1 Hz, 4H), 2.4−2.6 (m, 2H), 1.45 (s, 9H), 1.17 (t, $J$ = 7.1 Hz, 6H).

7-(Diethylamino)-4-(hydroxymethyl)coumarin methyl-*N*-*tert*-Boc-glycine (0.2 g, 0.49 mmol) was dissolved in 100 mL of dichloromethane, and the reaction mixture was cooled to 0 °C. Trifluoroacetic acid (5 mL) was added slowly, and the resulting solution was stirred in the dark for ~24 h, bringing it to room temperature. The solvent was removed under reduced pressure. The residue [DECM-caged glycine (**III**)] was purified using a Sephadex LH-20 column with water as the eluent. The deprotection yield was 54%; 0.2 g (0.55 mmol) of compound **II** gave 0.11 g (0.27 mmol) of compound **III**: $^1$H NMR (300 MHz, $D_2O$) $\delta$ 7.63 (d, $J$ = 9.0 Hz, 1H), 7.20 (d, $J$ = 2.1 Hz, 1H), 7.15 (dd, $J$ = 9.0, 2.6 Hz, 1H), 6.38 (s, 1H), 5.42 (s, 2H), 3.96 (s, 2H), 3.48 (q, $J$ = 7.1 Hz, 4H), 0.98 (t, $J$ = 7.1 Hz, 6H). Anal. Calcd for $C_{18}H_{21}N_2O_6F_3$: C, 51.68; H, 5.03; N, 6.7. Found: C, 51.42; H, 5.29; N, 6.49.

*Hydrolysis in the Dark.* The DECM-caged glycine (1 mM) was rapidly dissolved in the extracellular buffer solution (140 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES; the pH was adjusted to 7.4 using 5 N NaOH), and the solution was immediately transferred to a cuvette (volume of 4 mL and path length of 10 mm), which was placed in an absorption spectrometer (OLIS-14C) at 22 °C. Whole spectra were recorded between 1 and 240 min after the compound was dissolved to determine the thermal stability of the caged compound (Figure 1A).

*Laser-Flash Photolysis (Transient Spectral Measurements).* Laser-flash photolysis experiments were conducted with 250 $\mu$M DECM-caged glycine **III** (Scheme 1) in extracellular buffer (see above for the composition), using a $XeCl_2$ excimer laser (Compex 102 Lambda Physik) and a single 10 ns pulse of 4.8 mJ. The energy of the beam was measured using a Molectron joule meter. Light (≥400 nm) produced by fluorescence from a $4.9 \times 10^{-4}$ M solution of Exalite 404 dye (Exciton Inc.) placed in a cuvette with a path length of 10 mm was used to initiate photolysis. The decay of the transient intermediate absorbance was recorded by digitizing the photomultiplier output at rates of up to 2 MHz. The transient absorption changes were monitored at a wavelength where there was a significant change in the absorption upon irradiation of the caged compound (*ca.* 460 nm). All photolysis rates and quantum yields were measured at room temperature. Nonlinear least-squares fitting of the single-exponential decays was used to determine the photolysis rate constants (Figure 1B).

*Quantum Yield.* The quantum yield of the caged compound was determined by an actinometric method (*32*) as described previously (*3*).

*Expression of cDNA and Cell Culture.* The cDNA encoding the human α1-subunit of the glycine receptor in a pCis construct for mammalian expression was kindly provided by H. Betz (Max-Planck Institute for Brain Research, Frankfurt, Germany) (*33*). The vector was amplified using the MaxiPrep kit from Qiagen (Valencia, CA). HEK 293 cells (American Type Cell Culture, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium supplemented with

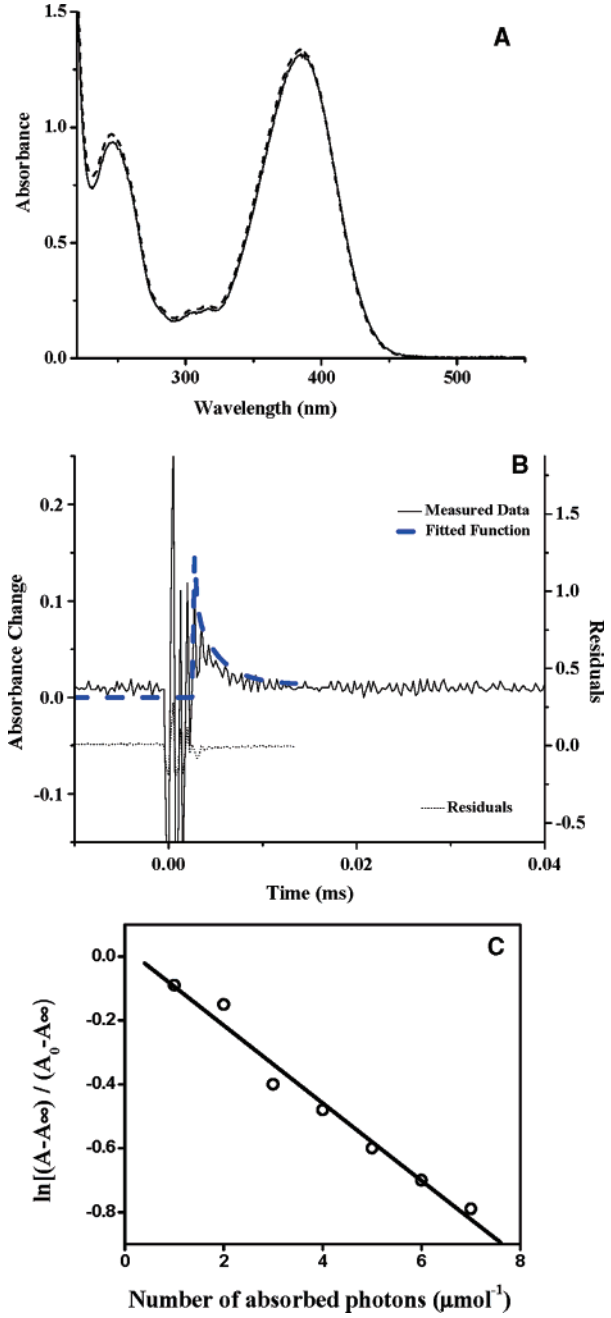



FIGURE 1: (A) Thermal hydrolysis of DECM-caged-glycine. UV−vis spectrum of DECM-caged glycine (250 $\mu$M in extracellular buffer at pH 7.4 and 22 °C) 0 (—) and 240 min (- - -) after the compound was dissolved. The data at 5, 10, 20, 30, 60, and 120 min are not shown. The path length of the cuvette was 10 mm. (B) Absorption transient at 460 nm observed in the photolysis of 0.8 mM DECM-caged glycine (compound **III** in Scheme 1) in extracellular buffer induced by a light pulse at ≥400 nm, pH 7.4, and 22 °C. The single-exponential absorbance decays with a half-time of ~2.5 $\mu$s. The solid line represents the measured data and the dotted line the fitted curve. The absorbance change observed at time zero is produced by discharge of the laser power supply and is also observed in the absence of DECM-caged glycine. (C) Absorbance (*A*) of a 250 $\mu$M DECM-caged glycine solution in extracellular buffer at pH 7.4 and 22 °C, measured as a function of the number of laser flashes at the excitation wavelength of 400 nm. $A_0$ is the absorbance before photolysis, and $A_\infty$ is the absorbance after ~500 flashes. The solution (3 mL) was irradiated in a 10 mm × 10 mm cuvette. The solution was stirred after every 20th laser flash. The line corresponds to the results of a linear regression representing a slope of 0.12 ± 0.01, which gives the quantum yield of the photolysis of the DECM-caged glycine (eq 1) (*3*).

10% heat-inactivated fetal bovine serum, in a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C. Exponentially growing HEK 293 cells were transiently transfected with 1.4 $\mu$g of cDNA encoding the α1-subunit of the human glycine receptor by using 15 $\mu$L of PolyFect transfection reagent (Qiagen). The cells were cotransfected with 0.6 $\mu$g of cDNA encoding the green fluorescent protein (pGreen Lantern plasmid, Life Technologies, Gaithersberg, MD) for detection of transfected cells (*34*). After 6−8 h, the cDNA and transfection reagent were removed and fresh medium was added. The cells were used for electrophysiological experiments between 24 and 72 h after each transfection.

*Whole-Cell Current Recording.* Whole-cell currents evoked by 100 $\mu$M glycine were recorded using the whole-cell configuration (*35*), at room temperature, −60 mV, and pH 7.4. The experiments were carried out in the absence or presence of 1 mM DECM-caged glycine (Figure 2). The solution in the recording pipet contained 120 mM CsCl, 2 mM $MgCl_2$, 10 mM TEACl, 10 mM EGTA, and 10 mM HEPES, adjusted to pH 7.4. The bath solution contained 140 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl, 1 mM $MgCl_2$, and 10 mM HEPES (pH 7.4). The resistance of the recording electrode filled with buffer solution was typically 3−5 MΩ, and the series resistance was 5−6 MΩ. The cells were held at a constant transmembrane voltage of −60 mV and room temperature (22 °C). Whole-cell currents were amplified by using an Axopatch 200B (Axon Instruments) amplifier and filtered at 1−2 kHz by using a 40-pole, low-pass, Bessel filter incorporated in the amplifier. The filtered signal was digitized by using a Labmaster DMA 100 kHz digitizing board (Scientific Solution) controlled by pCLAMP6 software (Axon Instruments).

*Cell-Flow Technique.* The use of a U-tube flow device for rapid solution exchange at the surface of a cell has been described in detail (*14*, *36*, *37*).

*Flash-Lamp Photolysis (Electrophysiological Measurements).* The light produced by a Rapp xenon flash lamp (SP-20) (Rapp OptoElectronic GmbH, Wedel, Germany) was coupled to an optical fiber (internal diameter of 600 $\mu$m), which delivered the light to the cell. A band-pass filter (385−450 nm) was used. Typical light energies were ~350 $\mu$J/pulse. DECM-caged glycine (1 mM) was used to obtain the results shown in Figure 2. The amount of glycine liberated was calibrated (*38*) by measuring the whole-cell currents before and after the light pulse and using a standard glycine solution (100 $\mu$M) and the known dose−response curve for glycine (*38*). Data were sampled at 20 kHz and low-pass filtered at 5 kHz. Data were analyzed with Origin 7 software (Microcal, Northampton, MA).

## RESULTS

The synthesis of 7-(*N*,*N*-diethylamino)-4-(hydroxymethyl)-coumarin-caged glycine (compound **III**) is shown in Scheme 1 and described in detail in Materials and Methods.

The absorption spectrum and the thermal stability of caged compound **III** (Scheme 1) were measured in extracellular buffer solution at pH 7.4. When protected from light, the caged compound exhibited very little hydrolysis at room temperature during 2 h of measurements (Figure 1A). When the caged glycine dissolved in the extracellular buffer (pH 7.4) was stored at −20 °C in the dark, no measurable hydrolysis was observed over the course of 20 h.

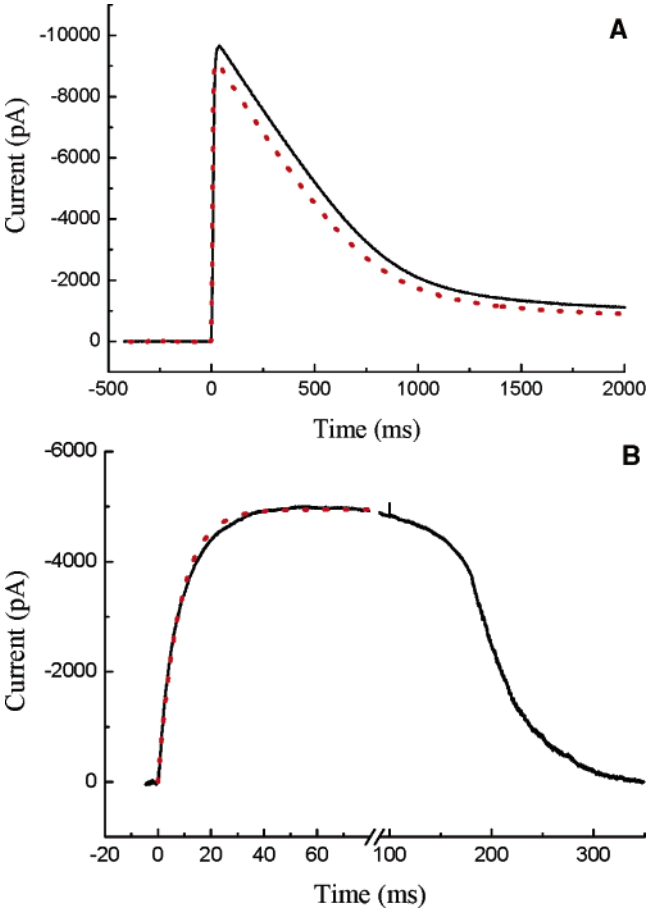



FIGURE 2: (A) DECM-caged glycine does not inhibit the whole-cell current evoked by glycine. Using the cell-flow technique (*14*, *36*, *37*), 100 $\mu$M glycine flowed over the surface of an HEK 293 cell transfected with cDNA encoding the glycine receptor (transmembrane voltage of −60 mV, extracellular buffer, pH 7.4, and 22 °C) in the absence (solid line) or presence (dotted line) of 1 mM DECM-caged glycine. Experiments were carried out at least three times on at least two different cells. (B) Whole-cell current recorded from an HEK 293 cell transfected with cDNA encoding the glycine receptor, at a transmembrane voltage of −60 mV, pH 7.4, and 22 °C. The current was induced by the photolytic release of glycine from 1 mM DECM-caged glycine, which was equilibrated with the receptors for 400 ms before being exposed to a 250 $\mu$s pulse of visible light (385 nm < $\lambda$ < 450 nm), delivering ~350 $\mu$J of energy. The concentration of liberated glycine was estimated to be ~60 $\mu$M (see the text). The dotted line represents the best fit according to the equation $I_{(t)} = I_\infty[1 - \exp(-k_{obs}t)]$ (*3*), where $k_{obs} = 125 \pm 16$ s$^{-1}$ and $I_\infty = -4950 \pm 57$ pA. $I_{(t)}$ is the current at time $t$, $I_\infty$ the current at infinite time (in the absence of desensitization), and $k_{obs}$ the apparent pseudo-first-order rate constant of the current increase.

The stability of DECM-caged glycine in aqueous solution was further tested by using HEK 293 cells transfected with cDNA encoding the α1-subunit of the human glycine receptor. The receptor is a highly sensitive glycine detector (*38*). The concentration of free glycine liberated as a result of hydrolysis from a 1 mM solution of DECM-caged glycine was measured as a function of time after solubilization at pH 7.4 and room temperature, by whole-cell current recordings. After 20 h in the dark at −80 °C and pH 7.4, no free glycine was detected (data not shown).

How rapidly is DECM-caged glycine photolyzed? 7-(*N*,*N*-Diethylamino)-4-(hydroxymethyl)coumarin **I** (*31*) (Scheme 1) is one of the products of the photolysis reaction. The rate constant for its formation was determined. A solution of caged glycine was excited with a 10 ns pulse of light at ≥400 nm (see Materials and Methods). The absorbance of **I** was measured at 460 nm as a function of time (Figure 1B). The transient absorbance decays with a single-exponential component and a half-life of ~2.5 $\mu$s. Thus, DECM-caged glycine is suitable for kinetic measurements of the membrane-bound glycine receptor in the microsecond time domain.

One of the important criterion for a photolabile caging group to be useful for biological applications is that the efficiency of photolytic release of the biomolecule be high enough that a substantial amount is released at the desired site, at a light intensity that is not harmful to the cell. The quantum yield of DECM-caged glycine was determined at ≥400 nm, as described previously (*3*). The change in the absorbance of the caged compound as a function of the number of photons absorbed from the light pulses at ≥400 nm is shown in Figure 1C. The absorbance $A$, measured as a function of the number of consecutive light pulses, was plotted semilogarithmically (Figure 1C) according to eq 1 (*3*):

$$A_n = \epsilon_m l C_0 \phi K_E \, e^{-\phi K_E F(n-1)} \quad (1)$$

where $A_n$ represents the absorbance after the $n$th light pulse, $\epsilon_m$ the extinction coefficient of the product, $l$ the path length, $C_0$ the initial concentration of the caged compound, $\phi$ the quantum yield, $K_E$ the ratio of the absorbed photons to the number of target molecules (constant), and $F$ the fraction of solution containing the caged compound through which the laser beam passes. The quantum yield was determined from the slope of the plot in Figure 1C and was calculated by linear regression analysis to be 0.12 ± 0.01.

To be useful in investigations of neurotransmitter receptors, the caged compound and the photolysis byproducts must not inhibit or activate the receptors to be investigated. HEK 293 cells transfected with cDNA encoding the glycine receptor were exposed to 100 $\mu$M glycine in the presence and absence of 1 mM DECM-caged glycine (Figure 2A) at pH 7.4 and 22 °C. The whole-cell currents recorded in the presence and absence of the caged glycine were similar (Figure 2A). These control experiments indicate that in this system the DECM-caged glycine is biologically inert.

Flash-lamp photolysis in the 385−450 nm wavelength region of 1 mM DECM-caged glycine was also carried out with the transfected HEK 293 cells. The whole-cell current induced by the released glycine was recorded as a function of time (Figure 2B). To determine that neither the cell membrane nor the receptors were damaged, the whole-cell current induced by a standard solution (100 $\mu$M) of glycine was recorded before and after the flash-lamp photolysis measurement. These experiments were also used to estimate the concentration of glycine released from the caged compound (Figure 2B). Flash-lamp photolysis of 1 mM DECM-caged glycine released 60 $\mu$M glycine.

## DISCUSSION

Photolabile DECM-caged glycine is synthesized from readily available starting materials. The caged compound has to be protected from light, particularly while it is being handled in aqueous solutions. It is sufficiently soluble in water and the buffers used in transient kinetic experiments,

and it is stable under dark and cold storage conditions. Upon irradiation with a pulse of light in the visible wavelength region, glycine is released from the caged precursor rapidly ($t_{1/2} \sim 2.5$ $\mu$s) and with sufficient quantum yield ($\phi = 0.12 \pm 0.01$) to be used in transient chemical kinetic investigations (*7*, *39*, *40*).

We have previously reported a photolabile protecting group, 2-(dimethylamino)-5-nitrophenyl (DANP), for carboxylic acids that has a major absorption band in the visible wavelength region (*41*). However, DANP-caged $\beta$-alanine is photolyzed by visible light with a quantum yield of only 0.002 (*41*). An advantage of the coumarin-caged neurotransmitters glycine and glutamic acid (*21*) is their efficiency in the uncaging reactions upon irradiation. In biological applications with cells, the intensity of incident light for an uncaging reaction has to be limited to prevent significant cell damage. Previously, in transient kinetic investigations of neurotransmitter receptors, caged compounds had to be photolyzed in the UV region by using a laser (*8*). Now a relatively inexpensive, hazard-free, and simple-to-use light source, for instance a Rapp flash lamp, can be used to photolyze DECM-caged glycine and glutamate. This development is expected to facilitate investigations of the mechanisms of proteins that must be studied in a membrane-bound state.

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