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Toward the Development of New Photolabile Protecting Groups That Can Rapidly Release Bioactive Compounds upon Photolysis with Visible Light

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The synthesis and characterization of a new photolabile protecting group (caging group) for carboxylic acids, the 2-(dimethylamino)-5-nitrophenyl (DANP) group, is described. This compound has a major absorption band in the visible wavelength region with a maximum near 400 nm ($\epsilon_{400} = 9077\ M^{-1}\ cm^{-1}$ at pH 7.4 and 21 °C). The caging group is attached through an ester linkage to the carboxyl functionality of β-alanine, which activates the inhibitory glycine receptor in the mammalian central nervous system. Such caged compounds play an important role in transient kinetic investigations of fast cellular processes. Upon photolysis of DANP-caged β-alanine, the caging group is released within 5 μs. Quantum yields of 0.03 and 0.002 were obtained in the UV region (308 and 360 nm) and the visible region (450 nm), respectively. Laser-pulse photolysis experiments, using 337 or 360 nm light, were performed with the caged compound equilibrated with HEK 293 cells transiently transfected with cDNA encoding the α_1 homomeric, wild-type glycine receptor. The experiments demonstrated that neither DANP-caged β-alanine nor its byproducts inhibit or activate the glycine receptors on the cell surface. Under physiological conditions, the DANP-caged β-alanine is water-soluble and stable and can be used for transient kinetic measurements.

Introduction

First reported by Barltrop and Schofield in 1962,[1] photolabile protecting groups[2−5] have found numerous applications in biology in the past decade.[6,7] The protecting groups (also known as "caging" groups in biology) can render a bioactive compound inert until they are removed by photolysis, thus releasing the compound rapidly (within microseconds to milliseconds). These properties of caged compounds, the inertness of a precursor, and the rapid release of a bioactive compound after an equilibrium with another reactant has been achieved make the photolabile precursors particularly useful for transient kinetic investigations (reviewed in ref 8). High time resolution becomes important in the study of fast processes (e.g., the opening of ion channels).[9,10] In conventional kinetic investigations of cell surface proteins, ligand solutions flow over the cell. Equilibration of the ligands with the cell-surface proteins is determined by the velocity with which layers of solution covering the cell are displaced.[11,12] In kinetic investigations of neurotransmitter receptors on cell surfaces, the time resolution of these flow techniques is not adequate to resolve the elementary reaction steps in the response of the

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receptors to neurotransmitter.[5,13,14] The main advantage of photorelease of a bioactive compound from its caged form over conventional mixing of reactants is that caged compounds allow time resolutions of microseconds rather than milliseconds to be achieved. In addition, photolytic release of biologically active compounds can be performed with high spatial resolution, and this has been exploited for identifying specific receptors in neuronal circuits,[15−18] and in the synthesis of DNA chips.[19]

A caged compound suitable for the investigation of biological systems must satisfy the following criteria:

(1) The compound must be soluble and stable in water at neutral pH.

(2) The compound should be photolyzable at long wavelengths to avoid cell damage (visible region preferred, vide infra).

(3) The quantum yield of the photorelease should be sufficiently high that the bioactive compound is released in quantities adequate for the proposed studies.

(4) The release of the bioactive molecule should occur in microseconds so the photolysis is not rate limiting for the biological reaction to be investigated.

(5) The caged compound and the photolysis byproducts must be biologically inert.

The stability of the caged compound in water is important as any reaction or hydrolysis in the dark would reduce the effective concentration of the bioactive compound released by photolysis, and the released compound would activate the biological system before measurements begin. A second problem is that neurotransmitter receptors are transiently inactivated (desensitized) during prolonged (milliseconds) exposure to neurotransmitter. Dark hydrolysis would lead to liberation of the neurotransmitter and, therefore, to transient inactivation of the receptor before the reaction can be investigated. The wavelength of the radiation used to photolyze the caged compound is also an important parameter. Most of the photolabile caging groups developed so far are uncaged with high-energy UV light. Radiation at short wavelengths can damage living cells.[20] Moreover, expensive light sources, which are not simple to operate or maintain, are required. Recently, the substituted 2-nitroveratrole group,[21,22] the nitroindolines,[7] the phenacyl group,[23] and substituted coumarins[24,25] have been studied as caging groups that can be removed by visible light. However, they suffer from low solubility in an aqueous medium or, in the case of the 2-nitroveratrole group and coumarin derivatives, from a slow rate of release of the bioactive compound. Thus, a photolabile caging group that has all the desired characteristics of a compound suitable for investigating biological reaction mechanisms (vide supra) is still to be made.

The *o*-nitrobenzyl group is one of the most widely applied photolabile caging groups in use today. In 1966 Barltrop et al.[26] reported the release of benzoic acid from its nitrobenzyl ester upon photolysis. Since then, this caging group has found a variety of applications in biology, but it has a few shortcomings that limit its applicability. First, short-wavelength UV light is required for deprotection ("uncaging").[27] Second, after the initial photochemical excitation, the molecule goes through a series of "dark" steps before the bioactive molecule is released.[28] It is estimated that there is a lag of a few milliseconds after photolysis before the bioactive molecule is released.[28,29] This makes the caging group unsuitable for studying fast reactions, which frequently occur on the sub-millisecond time scale. Third, a reactive *o*-nitrosobenzaldehyde is formed as a byproduct of the photolysis reaction, and this can damage cells.[27] Introduction of the carboxyl group in the α-position of this group overcomes most of these problems.[5] Transient kinetic investigations with a microsecond time resolution of neurotransmitter-receptor-mediated reactions, which regulate signal transmission between $\sim 10^{12}$ nerve cells in the brain,[30] first became possible with the use of this caging group.[5,31] However, UV light is still required for uncaging.[5]

The desyl[32,33] and 2-methoxy-5-nitrophenyl (MNP)[34,35] groups are the two other photolabile caging groups that are in common use. The desyl group suffers from low solubility in aqueous medium, and both the desyl and MNP groups absorb in the UV region, thus requiring high-energy UV radiation for the uncaging step. Substituted coumarins were used by Furuta et al.[24,25,36] to cage phosphates and carboxylic acids. The quantum yield when the coumarin protecting group is used (photolysis wavelength 340 nm) was found to be dependent on the substitution at the C-7 position.[24] Although the quantum yield of the 7-methoxy-substituted coumarins is 0.12 at 340 nm, that of the 7-acetoxy, 7-propionyloxy, and 7-hydroxy derivatives is about 0.06 at the same wavelength. These caged compounds also have a low solubility

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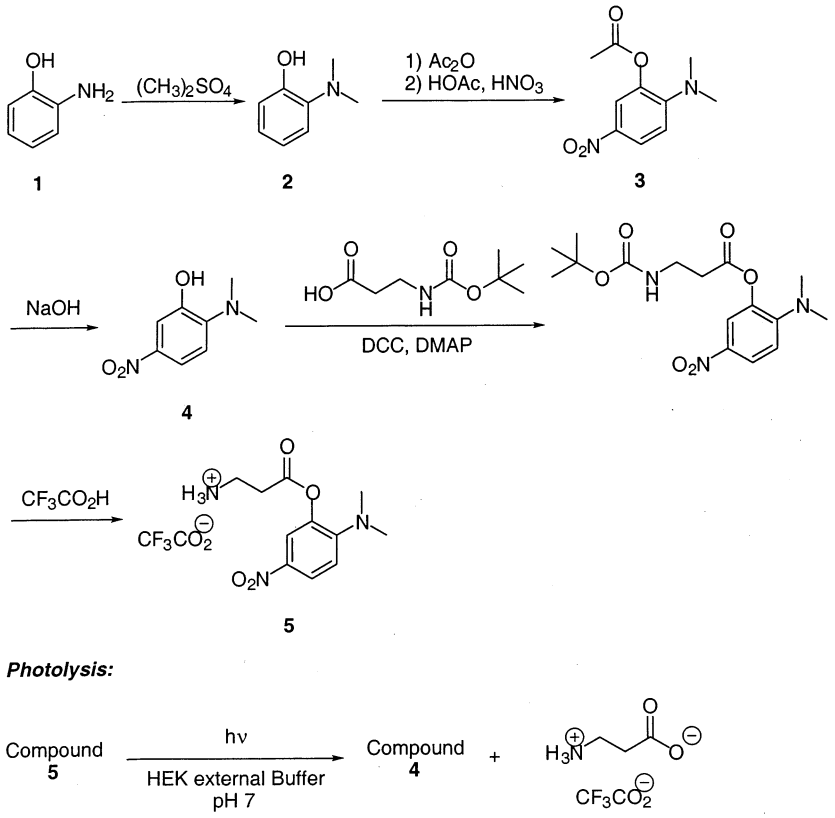
SCHEME 1[a]



[a] The overall yield of compound **4** was 23% of the starting material. The overall yield of compound **5**, starting from compound **4**, was about 77%.

in aqueous medium and must be dissolved in 1% DMSO before addition to an aqueous solution, and the rates of release of the phosphates and esters have not been reported. Recently, brominated 7-hydroxycoumarin-4-methyl was developed as a promising caging group for carboxylic acids that can be removed by longer wavelength UV light.[23] Although this caging group absorbs in the visible range ($\epsilon_{400} \approx 5-8000\ M^{-1}\ cm^{-1}$), the one-photon photolysis was carried out with 365 nm radiation, with a quantum yield of 0.019. Two-photon photolysis of coumarin derivatives with 800 nm light has been achieved, but the efficiency of this process is not known, and no data on the rate of release of the free compound are available. The brominated 7-hydroxycoumarin-4-methyl caging group is soluble in aqueous medium and can be used for mapping neurotransmitter-evoked currents.[23]

Carboxylic acids have been released from phenacyl esters upon photolysis with visible light (400 nm).[23] However, the deprotection requires the use of a sensitizer, 2-aminoanthracene. Moreover, the ester and the sensitizers used are insoluble in aqueous medium at physiological pH.

Recently, Bochet has explored nitroveratrole derivatives as photolabile protecting groups.[21,22] Some of these groups can be removed in the visible wavelength region, but the compounds are insoluble in aqueous medium and are photolyzed in the minute time region.[21,22]

Thus, none of the caging groups that photolyze in the visible region reported so far meet all the criteria mentioned above. Current efforts in this laboratory are directed toward the development and study of photolabile caging groups for amino acid neurotransmitters that can be removed by visible light, and that have all the other desirable qualities of the caged neurotransmitters tested previously[37,38] (reviewed in ref 8). Here we report the synthesis of 2-(dimethylamino)-5-nitrophenol and the use of the 2-(dimethylamino)-5-nitrophenyl (DANP) group as a caging group for the carboxyl functionality. This caging group is analogous to the MNP caging group,[34] but it absorbs at wavelengths above 400 nm. Previous experiments in which the MNP group was used to cage β-alanine suggested that the analogous DANP group would also be suitable for transient kinetic investigations of neurotransmitter receptors on cell surfaces.[39]

Results and Discussion

Synthesis of the Caged Compound. The synthesis of DANP-caged β-alanine is described in Scheme 1. 2-(Dimethylamino)-5-nitrophenol (**4**) was synthesized as previously described.[40] Methylation of 2-aminophenol with dimethyl sulfate generated the methylated compound **2**. Acylation of the hydroxyl group in **2** reduces its ring-activating effect, and the nitro group was introduced in the desired 5 position. The acyl group was then removed with NaOH to form **4** with an overall yield of

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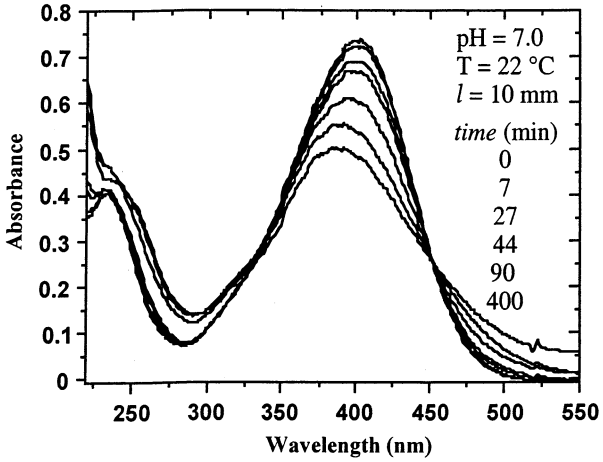

FIGURE 1. Thermal hydrolysis of DANP-caged β-alanine: UV−vis spectrum of DANP-caged β-alanine (100 μM in 50 mM phosphate buffer, pH 7.0, 22 °C) at times t = 0 min (top line at 400 nm) and t = 7, 27, 44, 90, and 400 min after the compound was dissolved. The path length of the cuvette was 10 mm. From the time dependence of the absorption at 400 nm, the rate constant for thermal hydrolysis was calculated to be $k = 0.007 \pm 0.001$ min^{-1} ($t_{1/2}$ = 99 min).

23% (from 2-aminophenol). The BOC (*N*-*tert*-butoxycarbonyl group) protected β-alanine was introduced in the presence of the coupling reagent DCC (1,3-dicyclohexylcarbodiimide)[41] and DMAP (4-(dimethylamino)pyridine). The BOC group was then removed by treatment with trifluoroacetic acid in dichloromethane to yield the caged compound **5**.[35]

Thermal Stability. The absorption spectrum and the thermal stability of the caged compound **5** were measured in a buffered solution at pH 7 (Figure 1). The caged compound decomposed in the dark by hydrolysis ($t_{1/2}$ = 99 min at 22 °C) to form **4**. On the basis of previous studies with 2-methoxy-5-nitrophenyl glycine (MNP-glycine) ester, the rates of thermal hydrolysis for DANP-caged α-amino acids are expected to be much faster.[35] The thermal hydrolysis rate of MAP-glycine is 6.1 min at pH 7.1 and room temperature. Hence, MAP-glycine was not considered for further study.

Laser-Flash Photolysis. Laser-flash photolysis experiments were carried out with 250 μM DANP-β-alanine (**5**) in 50 mM phosphate buffer (pH 7) using a 308 nm (Figure 2A) or 450 nm (Figure 2B) laser pulse (5−10 ns pulse duration). The transient absorbance changes were monitored at wavelengths where the absorption coefficient of the phenol **4** (Scheme 1) was significant while that of the caged compound was negligible (ca. 500 nm; see Figure 1). The absorbance was averaged over 20 pulses to achieve a better signal-to-noise ratio. At 22 °C, the phenol **4** is detected immediately after the laser pulse at both the wavelengths, within the time resolution of the method (see Figure 2). These experiments demonstrate that the photolysis products are formed within a few microseconds after the laser pulse. The experiments did not, however, reveal any reaction intermediates that would provide clues about the mechanism of the photolysis reaction, nor are the experiments capable of

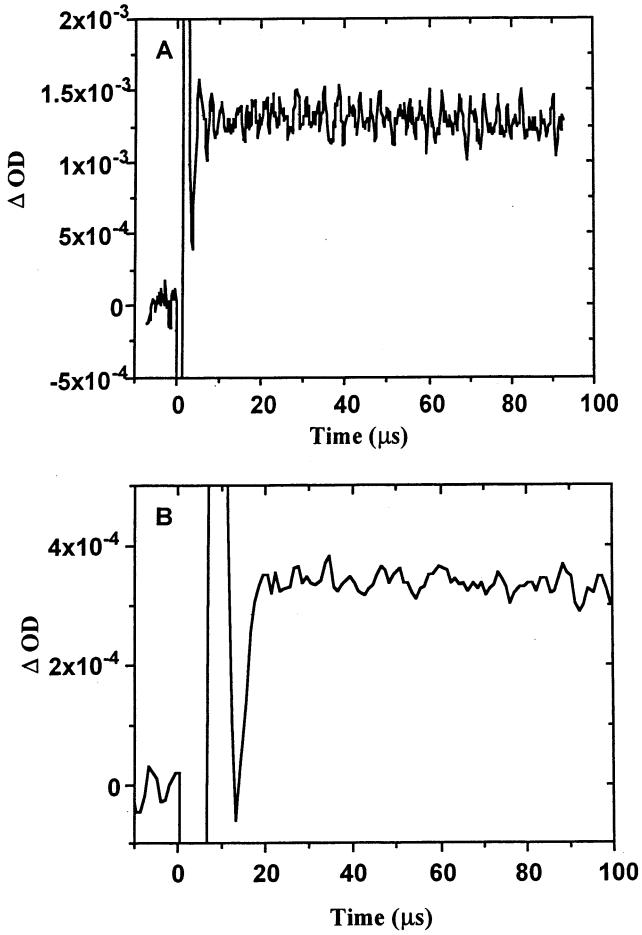

FIGURE 2. Absorbance change [Δ(OD)] measured as a function of time, produced by photolysis of DANP-caged β-alanine with a single laser pulse (A, λ = 308 nm, 75 mJ/cm^2; B, λ = 450 nm, 250 mJ/cm^2) and averaged over 20 laser pulses at 22 °C. Conversion to the product is complete within 5 μs. The wavelength of the analyzing light was 495 nm (A) and 480 nm (B). The concentration of the caged compound was 250 μM in 50 mM phosphate buffer (pH 7.0). The path length of the excitation beam was 2 mm, while that of the analyzing beam was 10 mm. The appearance of the phenol on photolysis is indicated by the wavy lines. The spikes immediately after the laser pulse are instrumental artifacts due to scattered light that saturates the detector.

detecting the free β-alanine as it is released from its caged precursor. As in the case of the similar MNP-caged compound,[35] the mechanism of the photolysis probably involves photohydrolysis of the ester linkage from the excited triplet state, as demonstrated by Kuzmic et al.[34] in the case of MNP-caged acetate. It is assumed that β-alanine is released stoichiometrically with the phenol (**4** in Scheme 1) and that the free β-alanine is released on the same time scale (within 5 μs) as the phenol (**4** in Scheme 1). Therefore, it appears that the caged compound is suitable for kinetic measurements of fast cellular processes in the microsecond time domain.

Quantum Yield. For a photolabile caging group to be useful for biological applications, the quantum yield of the photochemical step should be high (criterion 3, vide supra) so that the neurotransmitter is released in sufficient quantity at the desired site at a light intensity that is not harmful to the cell. The quantum yields of

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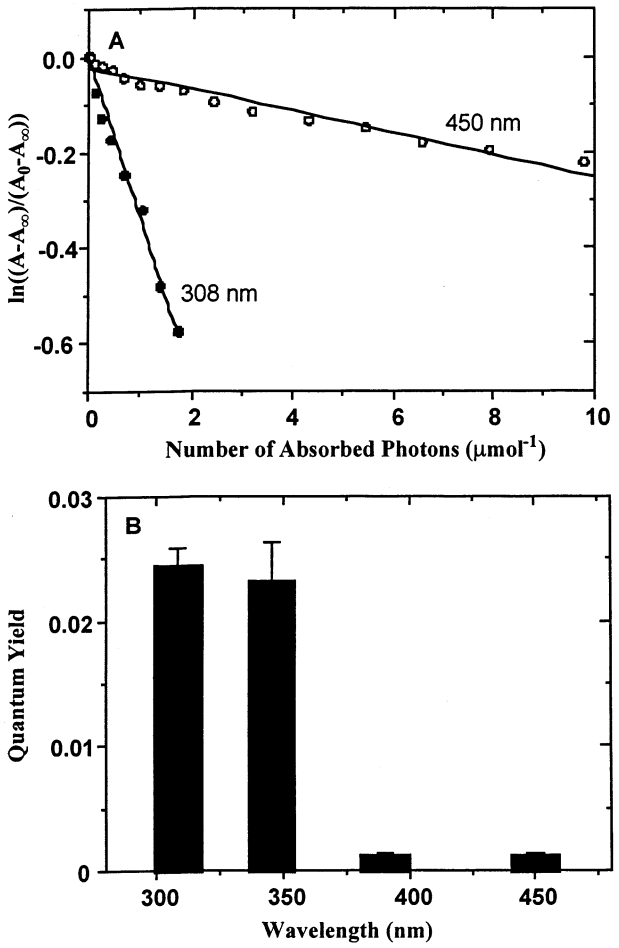

FIGURE 3. (A) Determination of the photolysis quantum yield. The absorbance A of a 500 μM caged β-alanine solution in 50 mM phosphate buffer at pH 7.0 and 22 °C was measured as a function of the number of laser flashes. For comparison between two experiments, on the x-axis the number of laser flashes was converted to the number of absorbed photons (μmol^{-1}). The excitation wavelengths were 308 nm (●) and 450 nm (○). A 40 μL portion of a total volume of 50 μL of the solution was irradiated in a 3 × 3 mm cuvette. The solution was stirred after each laser flash (308 nm) or after every 10th laser flash (450 nm). The lines represent the results of a linear regression analysis with slopes of $(3.4 \pm 0.1) \times 10^5$ and $(2.1 \pm 0.1) \times 10^4$ μmol^{-1}, respectively. (B) Quantum yield plotted as a function of wavelength. The experiments were performed in 50 mM phosphate buffer at pH 7.0 and 22 °C. The data were fitted to the equation[5] $A_n = \epsilon_M l C_0 \phi K_E \exp[-\phi K_E F(n-1)]$. A_n is the absorbance after the nth pulse, ϵ_M the extinction coefficient of the product, l the path length, C_0 the initial concentration of the caged compound, ϕ the quantum yield, K_E the ratio of the absorbed photons to the number of target molecules (constant), and F the fraction of the solution containing the caged compound through which the laser beam passes.

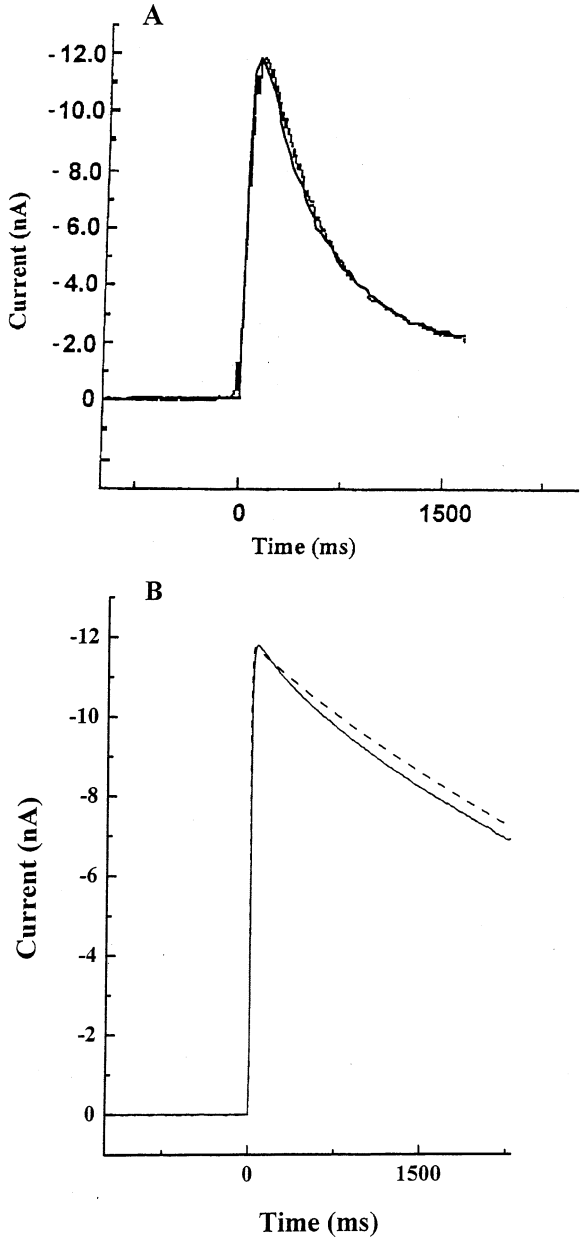

FIGURE 4. (A) The 2-(dimethylamino)-5-nitrophenyl ester of β-alanine (DANP-caged β-alanine) does not inhibit the α_1-glycine receptor. 100 μM β-alanine flowed over the surface of an HEK 293 cell transfected with α_1-glycine receptor cDNA ($V = -60$ mV, pH 7.4, $T = 22$ °C) in the absence (solid line) or presence (dotted line) of 1 mM DANP-caged β-alanine, using the cell-flow technique. The induced current was recorded in the whole-cell configuration.[44] The experiments were carried out three times on different cells. (B) The 2-(dimethylamino)-5-nitrophenol does not inhibit the α_1-glycine receptor. 100 μM β-alanine flowed over the surface of an HEK 293 cell transiently transfected with α_1-glycine receptor cDNA ($V = -60$ mV, pH 7.4, $T = 22$ °C) in the presence (solid line) and absence (dashed line) of 1 mM 2-(dimethylamino)-5-nitrophenol using the cell-flow technique, and the β-alanine-induced current was recorded in the whole-cell configuration. The experiments were carried out twice with the same cell.

the DANP-caged β-alanine were determined at different wavelengths as described previously.[5] A 40 μL sample of **5** (Scheme 1) was photolyzed with repetitive laser flashes at appropriate wavelengths, and the concentration of **4** (Scheme 1) was determined spectrophotometrically. Figure 3A shows the change in the absorbance of the caged compound as a function of the number of photons absorbed from the laser flashes at 308 and 450 nm. The quantum yield at a number of different excitation wavelengths is shown in Figure 3B; it is approximately 0.03 at 308 and 350 nm. This value is higher than that of the brominated coumarins,[25] but it drops more than 10-fold to 0.002 at 400 nm and above. Thus, even though the

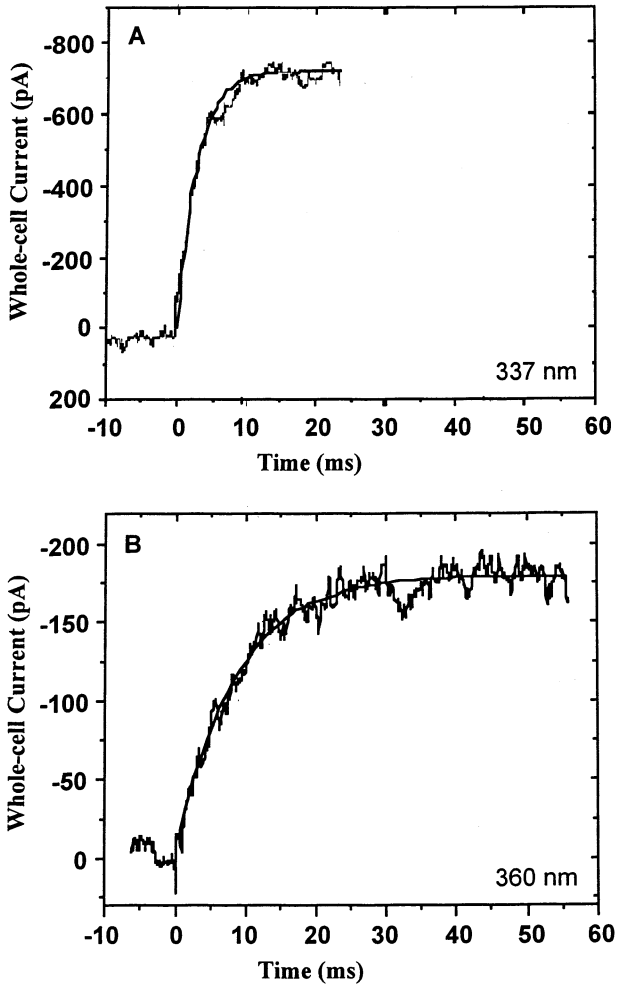

FIGURE 5. Whole-cell current recorded[44] from an HEK 293 cell transfected with α_1-glycine receptor at $V = -60$ mV, pH 7.4, and $T = 22$ °C. The current was induced by the photolytic release of β-alanine. (The concentration of liberated β-alanine was estimated as described in the Experimental Section.) (A) From 0.94 mM DANP-caged β-alanine, with a 337 nm pulse used for the photolysis (500 μJ). The concentration of liberated β-alanine was estimated to be 50 μM. The smooth line represents the best fit according to the equation $I(t) = I_\infty[1 - \exp(-k_{obs}t)]$ with $k_{obs} = 368 \pm 3$ s^{-1} and $I_\infty = 720$ pA. $I(t)$ is the current at time t, I_∞ is the current at $t = \infty$ (in the absence of desensitization), and k_{obs} is the apparent pseudo-first-order rate constant of the current rise.[31] (B) From 2 mM DANP-caged β-alanine, with a 360 nm laser pulse (500 μJ). The concentration of liberated β-alanine was estimated to be 30 μM. The smooth line represents the best fit with $k_{obs} = 115 \pm 5$ s^{-1} and $I_\infty = 180 \pm 2$ pA.

caged compound absorbs well into the visible range, efficient release of the bioactive compound does not occur at 400 nm and above. In contrast, quantum yields of different caged neurotransmitters in the UV wavelength region were found to be in the range of 0.8−0.14.[8]

Biological Characterization. Neither the caged compound nor its photolysis product, 2-(dimethylamino)-5-nitrophenol (Scheme 1), should inhibit or activate the glycine receptors on the cell surface (criterion 5, vide supra) to be applicable for kinetic measurements of ion-channel opening. The HEK 293 cells transfected with α_1-glycine receptor cDNA were, therefore, exposed to 100 μM β-alanine in the presence and absence of 1 mM DANP-β-alanine (Figure 4A) or in the presence and absence of 1 mM 2-(dimethylamino)-5-nitrophenol (Figure 4B) at pH 7.4 and 22 °C. The whole-cell currents recorded as a response to β-alanine, in both the presence and the absence of the caged compound (Figure 4A) or the presence and absence of 2-(dimethylamino)-5-nitrophenol (Figure 4B) at concentrations as high as 1 mM, were the same. These control experiments demonstrate that neither the caged compound before photolysis nor its photolysis product, 2-(dimethylamino)-5-nitrophenol, activates or inhibits the α_1-glycine receptor.

Laser-Pulse Photolysis. Laser-pulse photolysis of the caged compound at excitation wavelengths of 337 nm (Figure 5A) and 360 nm (Figure 5B) was carried out with HEK 293 cells transiently transfected with cDNA encoding the α_1 homomeric glycine receptor. The whole-cell currents generated due to the opening of the ion channels induced by the released β-alanine were measured as a function of time (Figure 5). They demonstrated that at these wavelengths DANP-β-alanine is useful for kinetic investigations of the opening of the glycine receptor channel. Before and after each laser-pulse photolysis measurement, standard solutions of β-alanine flowed over the same cell and the maximum current amplitudes were determined[8,13] to ascertain that neither the cell membrane nor the receptors were damaged. These control experiments, together with those illustrated in Figure 4, demonstrated that neither the caged compound nor its photolysis byproducts affect the cells or the glycine receptor. (We routinely use such experiments to assess the effects of drugs and inhibitors on the kinetics of ion-channel openings.[5,8]) However, upon irradiation of the caged compound with laser light at wavelengths higher than 360 nm glycine-receptor-associated currents could hardly be detected. Thus, these results confirm that efficient photolysis takes place only at or below 360 nm and not at higher wavelengths.

Conclusions

Laser-pulse photolysis data for the DANP-caged β-alanine indicate that the DANP caging group can be used for biological applications requiring rapid release of carboxylic acid-containing molecules. As judged by experiments with the MNP caging group,[35,39] DANP-caged α-amino acids are likely to be less stable in aqueous media than are DANP-caged β-amino acids and are, therefore, likely to be more difficult to use in kinetic measurements. While the DANP-caged compound releases β-alanine a few microseconds after the laser flash, at visible wavelengths the quantum yield of the release step is low. Ultraviolet light of wavelengths 337 and 360 nm can be used to release the active compound with moderate efficiency. The DANP-caged compound can easily be synthesized from readily available starting material.

Experimental Section

Synthesis. 2-(Dimethylamino)-5-nitrophenol (4) (Scheme 1). The phenol **4** was synthesized by methods previously published[41] starting from commercially available 2-aminophenol: ^{1}H NMR ($CDCl_3$) δ 2.77 (s, 6H), 7.16 (d, J = 9 Hz, 1H), 7.77−7.81 (m, 2H).

2-(Dimethylamino)-5-nitrophenyl-*N*-*t*-BOC-*β*-alanine. *N*-*t*-BOC-*β*-alanine (0.172 g, 9.1×10^{-4} mol), DMAP (10 mg), and DCC (2 mL of a 1 M solution in CH_2Cl_2) were stirred at room temperature for about 10 min. **4** (0.124 g, 6.8×10^{-4} mol) was added to the reaction mixture, and the resulting reaction mixture was stirred at room temperature for about 2 h. The mixture was diluted with diethyl ether and washed several times with saturated $NaHCO_3$ solution. The organic layer was dried over anhydrous $MgSO_4$ and filtered over silica gel to give a yellow solid: yield 0.202 g (84%); ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9H), 2.84 (t, J = 6 Hz, 2H), 2.97 (s, 6H), 3.51 (q, J = 6 Hz, 2H), 5.08 (s (br), 1H), 6.86 (d, J = 9 Hz, 1H), 7.87 (d, J = 3 Hz, 1H), 8.03 (dd, J_1 = 9 Hz, J_2 = 3 Hz, 1H). Anal. Calcd for $C_{16}H_{23}N_3O_6$: C, 54.38; H, 6.56; N, 11.89. Found: C, 55.08; H, 6.56; N, 11.84.

2-(Dimethylamino)-5-nitrophenyl-*β*-alanine (5). 2-(Dimethylamino)-5-nitrophenyl-*N*-*t*-BOC-*β*-alanine (0.202 g, 5.7×10^{-4} mol) and 1 mL of trifluoroacetic acid were dissolved in 5 mL of dichloromethane, and the resulting solution was stirred for about 3 h at room temperature. The solvent was removed under reduced pressure. The residue was put under vacuum for about 24 h. The resulting solid was washed with small quantities of cold acetone and dried under vacuum: yield 0.194 g (92%); ^{1}H NMR ($CDCl_3$) δ 3.06 (s, 6H), 3.43 (t, J = 6 Hz, 2H), 4.28 (t, J = 6 Hz, 2H), 7.05 (d, J = 9 Hz, 1H), 7.89 (d, J = 3 Hz, 1H), 8.04 (dd, J_1 = 9 Hz, J_2 = 3 Hz, 1H), 8.75 (s (br), 3H). Anal. Calcd for $C_{13}H_{16}F_3N_3O_6$: C, 42.51; H, 4.39; N, 11.44. Found: C, 42.28; H, 4.42; N, 11.22.

Hydrolysis Experiments. The caged compound (100 μM) was rapidly dissolved in the buffer solution (50 mM phosphate, pH 7.0), immediately transferred to a cuvette (4.5 mL volume, 10 mm path length), and placed in an absorption spectrometer (Beckman) at 22 °C. Whole spectra were recorded at times between 1 and 400 min after the compound was dissolved.

Laser-Flash Photolysis. Transient absorption spectroscopy was performed as described.[5] The caged compound (250 μM in 50 mM phosphate buffer, pH 7.0) in a 2 × 10 mm sample cuvette was photolyzed with 308 nm light from an excimer laser, 10 ns pulse duration and 75 mJ/cm^2 energy per pulse, or with light from a dye laser pumped by the same excimer laser. The laser dyes used were *p*-terphenyl (λ_{max} = 343 nm), DMQ (λ_{max} = 360 nm), BiBuQ (λ_{max} = 388 nm), and coumarin 120 (λ_{max} = 450 nm). The dyes were available commercially. The absorption change after excitation of the caged compound was probed with the light output from a Xe lamp at a 90° angle to the laser beam and monitored with a photodiode (active area 1 mm^2). The output of the photodiode was amplified and measured with a digital oscilloscope. The wavelength of the analyzing light was selected with band-pass filters.

The quantum yield was determined as described earlier.[5] Briefly, a 40 μL sample of the caged compound solution (500 μM caged compound in phosphate buffer, pH 7.0) was photolyzed with repetitive laser pulses (λ = 308, 345, 390, and 450 nm), and the concentration of the liberated 2-(dimethylamino)-5-nitrophenol was determined spectrophotometrically (at wavelengths 400 and 492 nm). It is assumed that *β*-alanine is produced stoichiometrically with the 2-(dimethylamino)-5-nitrophenol. From these data the conversion to product after the first laser pulse was calculated, allowing the direct determination of the quantum yield together with the measured number of absorbed photons. This number was measured with an energy meter placed behind the cuvette.

Cell Experiments. HEK 293 cells were transiently transfected with cDNA encoding the α_1-glycine receptor as described previously.[38,42] Transient transfection of exponentially growing HEK 293 cells for control and photolysis experiments was performed using the Effectene reagent or calcium phosphate transfection method.[43] Cells were cotransfected with cDNA encoding the green fluorescent protein pGreenLantern to detect transfected cells. The cells were used for electrophysiological experiments between 24 and 72 h after transfection.

Whole-cell currents evoked by *β*-alanine (100 μM) were recorded under voltage-clamp conditions[44] at −60 mV and amplified with an amplifier or an integrating patch clamp amplifier. The experiments were carried out in the absence or presence of 1 mM DANP (Figure 4A) or 1 mM 2-(dimethylamino)-5-nitrophenol (Figure 5B). The recording pipet solution contained 120 mM CsCl, 2 mM $MgCl_2$, 10 mM TEACl, 10 mM EGTA, and 10 mM HEPES and was adjusted to pH 7.4. The bath buffer solution contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$ and 10 mM HEPES (adjusted to pH 7.4). Typical pipet resistances were 2.5−3.5 MΩ, and the series resistance was 4−6 MΩ. Series resistance compensation of 60−80% was used in the whole-cell recording experiments. Solutions were exchanged around the cell with a cell-flow device[13,45] with an effective time resolution of 10−20 ms.[13]

Laser-Pulse Photolysis (Figure 5). The laser light produced by a dye laser pumped by an excimer-pumped dye laser was coupled into an optical fiber (200 μm diameter), which delivered the laser light to the cell. The dyes used for the different wavelengths were the same as described above. In the case of photolysis with 337 nm light, the laser light was coupled into an optical fiber and delivered to the cell. The concentration of DANP-caged *β*-alanine used was 0.94 mM in Figure 5A and 2 mM in Figure 5B. Typical laser energies were 50−500 μJ/cm^2. The amount of *β*-alanine liberated was calibrated by cell-flow experiments before and after the laser pulse with a standard *β*-alanine solution (100 μM) and the known dose−response curve for *β*-alanine as described.[46] The pulse/flow system was computer-controlled with pClamp software. Data were sampled at 5−100 kHz and low-pass filtered at 2−10 kHz. Data were analyzed with Origin software.

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