Synthesis and Photochemistry of Photolabile *N*-Glycine Derivatives and Effects of One on the Glycine Receptor†

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ABSTRACT: Three photolabile precursors of glycine containing a photosensitive 2-nitrobenzyl moiety attached to the amino group have been synthesized. When exposed to ultraviolet radiation between 308 and 350 nm, the compounds photolyze to release glycine, an important inhibitory neurotransmitter in the central nervous system. The identification of glycine as a photolysis product was determined by two different methods: separation of the photolyzed sample by thin-layer chromatography followed by a reaction with ninhydrin, and recognition of derivatized glycine using the Waters pico-tag method in conjunction with high-performance liquid chromatography. The photolysis of these compounds at 22 °C has been investigated, and the rate of decay of a transient intermediate in the reaction, which is assumed to reflect product release, has been measured. For *N*-(α-carboxy-2-nitrobenzyl)glycine this decay rate was found to be 940 s^{-1} at pH 6.8 and 600 s^{-1} at pH 7.5. Additionally, this compound was found to exhibit biological activity upon photolysis; cultured mouse spinal cord cells containing neuronal glycine receptors were used to detect the glycine liberation. The approach adopted here is useful in demonstrating the utility of photolabile precursors of neurotransmitters that have the protecting group linked to the neurotransmitter through the amino group. The rapid photolysis of such compounds to release free neurotransmitter is valuable in gaining access to chemical kinetic studies of neurotransmitter receptors. Previously, such studies have been limited because the available methods for neurotransmitter delivery did not give a sufficiently high time resolution.

Central to the transmission of signals in the nervous system are chemical reactions in which membrane-bound proteins form transmembrane channels upon interaction with specific chemical signals (neurotransmitters). The proteins include receptors activated by a series of amino-group-containing compounds, for instance, glycine, glutamic acid, *N*-methyl-D-aspartic acid, γ-aminobutyric acid, and serotonin. Because the reactions that govern the channel-opening processes of neurotransmitter receptors lie in the millisecond time region, fast chemical reaction techniques were employed to study these processes initially in the nicotinic acetylcholine receptor (Hess et al., 1979, 1983; Udgaonkar & Hess, 1987b). The most recent technique involves the use of photolabile precursors of carbamoylcholine (Walker et al., 1986; Milburn et al., 1989), a stable amino-group-containing analogue of the neurotransmitter acetylcholine. *N*-(α-Carboxy-2-nitrobenzyl)carbamoylcholine is photolyzed with a half-time in the region of 100 μs (Milburn et al., 1989; Matsubara et al., 1992). We are developing this approach further so that chemical kinetic studies of amino acid neurotransmitter receptors expressed on mammalian neurons in primary culture can also be undertaken. In contrast to chemical kinetic investigations of the nicotinic acetylcholine receptor in the microsecond to millisecond time region and the resulting minimum reaction scheme [Hess et al., 1979; Matsubara & Hess, 1992; Matsubara et al., 1992; reviewed by Hess et al. (1987), Ochoa et al. (1989), and Changeux (1990)], comparable measurements with the amino acid neurotransmitter receptors have not been reported.

The 2-nitrobenzyl moiety has been used as a photosensitive protector in organic synthesis [reviewed in Morrison (1969), Pillai (1980, 1987), and Zehavi (1988)]. Furthermore, this group has also been used to protect biologically important phosphates, calcium chelators, and neurotransmitters [Kaplan et al., 1978; McCray et al., 1980; Ellis-Davies & Kaplan, 1988; Karpen et al., 1988; reviewed in McCray and Trentham (1989)], which have been used to study biological processes in the millisecond time region. The functionalization of amino acids as photolabile carbamates has been demonstrated (Barltrop et al., 1966; Patchornik et al., 1970; Robertson et al., 1991). An alternative approach was to block the carboxyl group of the neurotransmitter with the photolabile 3,4-dimethoxy-2-nitrobenzyl moiety (Wilcox et al., 1990); but these studies indicated that the release of neurotransmitter is slow and occurs in the second time region and thus would be inapplicable for fast reaction techniques. The 2-nitrobenzyl moiety has also been used in preparative organic synthesis as a photolabile protecting group for the imidazole side chain of histidine in peptide synthesis (Kalbag et al., 1975) and for the amino group of 1-pyrazolidine-3-one in the synthesis of β-lactams (Perry et al., 1990). The quantum yield or rate of photolysis of these derivatives was not reported in these studies. In an extension of our previous investigations of photolabile precursors of amino-group-containing neurotransmitters, we synthesized three photolabile derivatives of glycine (Scheme I) utilizing the nitrobenzyl moiety as the photolabile protecting

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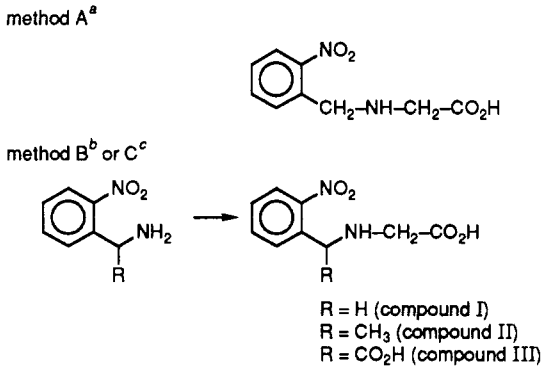
Scheme I: Structures of the Three *N*-Protected Photolabile Glycine Precursors: Compounds I, II, and III



[a] (i) glycine, aqueous NaOH, CH_3OH; (ii) $NaBH_4$, H^+. [b] (i) CHO CO_2H, aqueous NaOH, CH_3OH; (ii) $NaBH_4$; H^+ used for R = H, CH_3. [c] CH_2BrCO_2H, aqueous Na_2CO_3, used for R = CO_2H.

group of the amino group on glycine. Three compounds were made because the photolytic properties, specifically photolysis rate and product quantum yield, appear to depend upon the substituents on the α carbon of the protecting group (Walker et al., 1986; Zhu et al., 1987; Milburn et al., 1989). The three different photolabile protecting groups used here were also used previously in studies with carbamoylcholine, which is a carbamate (Walker et al., 1986; Milburn et al., 1989). This allows us to compare the effect of the leaving group on the photolytic reaction. Although compounds II and III exhibit similar photolytic properties, investigations with the carbamoylcholine derivative that contained the same photolyzable group as compound II indicated that it inhibits the nicotinic acetylcholine receptor (Walker et al., 1986). Therefore, we have more extensively studied compound III, *N*-(α-carboxy-2-nitrobenzyl)glycine. This compound, when applied to glycine receptors on cultured mouse spinal cord cells, did not activate or inhibit the receptor prior to photolysis and was found to activate channel opening upon photolysis.

Experimental Procedures

Apparatus. Visible and ultraviolet absorption spectra were obtained using a Beckman model 25 spectrophotometer. NMR spectra were recorded using a Varian XL-400 (400 MHz, ^1H reference signal at 4.64 δ; 100 MHz ^{13}C); chemical shifts are expressed in parts per million, δ, downfield of tetramethylsilane. Melting points were determined on a Thomas–Hoover apparatus and are uncorrected. Elemental combustion analyses were performed by Schwarzkopf Microanalytical Laboratory, Inc. (Woodside, NY) and Desert Analytics, Inc. (Tucson, AZ). Glycine concentration determinations were carried out by the Cornell University Biotechnology Program Analytical and Synthetic Facility (Ithaca, NY). Fast atom bombardment (FAB) mass spectrometry was done by the Mass Spectrometry Facility, Department of Chemistry, Cornell University.

High-Performance Thin Layer Chromatography (HPTLC). High-performance thin layer chromatography was performed using a *n*-butanol/acetic acid/water (3:1:1) solvent system. Samples were spotted onto silica gel 60 F-254 precoated plates (no. 5635; layer thickness, 0.2 mm; Merck). The plates were developed and observed under ultraviolet light before being sprayed with ninhydrin reagent to detect free glycine (Moffat & Lytle, 1959).

Synthesis. N-(2-Nitrobenzyl)glycine (I). Method A. Compound I was prepared as reported by Kessler et al. (1983) from 2-nitrobenzaldehyde (0.15 g, 1 mmol), glycine (0.113 g, 1.507 mmol), sodium hydroxide (0.06 g, 1.5 mmol, 0.75 mL of 2 N), methanol (1.5 mL), and sodium borohydride (0.03 g, 0.79 mmol) and purified by vacuum liquid chromatography (Coll et al., 1977) on silica gel absorbent (10–40 μm, type H, Sigma), using 0.1% acetic acid in ethanol as the eluent whose polarity was gradually increased by addition of water: mp 189–191 °C; yield, 15%. ^1H NMR (D_2O–K_2CO_3): δ 8.13 (m, 1 H, C_3–H), 7.72 (m, 1 H, C_6–H), 7.59 (m, 2 H, C_4 and C_5–H), 4.42 (s, 2 H, benzylic), and 3.61 (s, 2 H, methylene). ^{13}C NMR: δ 173.84 (CO_2K); 151.47 (C_2); 138.2, 137.08, 134.54, and 129.06 (C_3, C_4, C_5, and C_6); 128.73 (C_1); 52.1 (benzylic); and 51.91 (methylene). Anal. Calcd. for $C_9H_{10}N_2O_4$: C, 51.4; H, 4.8; N, 13.3. Found: C, 51.24; H, 4.81; N, 12.96.

Method B. Compound I was also synthesized from 2-nitrobenzylamine hydrochloride (0.19 g, 1.005 mmol), glyoxylic acid hydrate (0.14 g, 1.52 mmol), sodium hydroxide (0.1 g, 2.5 mmol, 1.25 mL of 2 N), methanol (2.5 mL), and sodium borohydride (0.028 mg, 0.737 mmol) and purified as described above: yield, 42%.

N-(α-Methyl-2-nitrobenzyl)glycine (II). Method B. Compound II was made from 2-nitro-α-phenethylamine (0.3 g, 1.8 mmol), which was prepared as reported by Walker et al. (1986), glyoxylic acid hydrate (0.25 g, 2.7 mmol), sodium hydroxide (0.11 g, 2.75 mmol, 1.4 mL of 2 N), methanol (2.8 mL), and sodium borohydride (0.05 g, 1.3 mmol). Compound II was purified by vacuum liquid chromatography as described for compound I, isolated as its hydrochloride salt, and crystallized from methylene chloride–ether: mp 165–170 °C; yield, 50%. ^1H NMR (D_2O): δ 7.91 (d, 1 H, J = 8.25 Hz, C_3–H), 7.66 (m, 2 H, C_4 and C_5–H), 7.51 (t, 1 H, J = 8 Hz, C_6–H), 4.91 (q, 1 H, J = 6.8 Hz, CH), 3.45 (m, 2 H, CH_2), and 1.57 (d, J = 6.8 Hz, CH_3). ^{13}C NMR: δ 173.84 (CO_2H); 137.75, 133.78, 131.55, and 128.71 (C_3, C_4, C_5, and C_6); 133.18 (C_1); 55.72 (CH); 50.18 (CH_2): and 20.02 (CH_3). Anal. Calcd. for $C_{10}H_{12}N_2O_4$·HCl: C, 45.97; H, 4.98; N, 10.7. Found: C, 46.27; H, 5.08; N, 10.44.

N-(α-Carboxy-2-nitrobenzyl)glycine (III). Method C. To a solution of 2-nitrophenylglycine hydrochloride (500 mg, 2.15 mmol) in 50 mL of water, sodium carbonate (456 mg, 4.30 mmol) and bromoacetic acid (299 mg, 2.15 mmol) were added. The reaction mixture was protected from light and was stirred at room temperature for 11 days. The pH was adjusted to 2.0 with 1 N hydrochloric acid, and the resulting acid solution was washed three times with chloroform. The pH of the aqueous portion was adjusted to 7.0 with 1 N sodium hydroxide and then lyophilized. The residue was subjected to Sephadex LH-20 chromatography (4 × 50 cm) with water elution to yield three bands. The bands, in order of elution, consisted of an *N,N*-dialkylated side product, the title compound, and unreacted 2-nitrophenylglycine. Lyophilization of the product band yielded 220 mg (34%) of a light yellow powder: mp 123–125 °C (dec). ^1H NMR (D_2O): δ 8.05 (d, 1 H, J = 8.2 Hz, C_3–H), 7.65 (t, 1 H, J = 7.7 Hz, C_5–H), 7.56 (t, 1 H, J = 7.5 Hz, C_4–H), 7.47 (d, 1 H, J = 7.6 Hz, C_6–H), 4.99 (s, 1 H, CH), 3.52 (d, 1 H, J = 16.2 Hz, CH_AH_B), and 3.46 (d, 1 H, J = 16.2 Hz, CH_AH_B). ^{13}C NMR: δ 170.7 and 170 (2 × CO_2); 147.87 (C_2); 135.05, 131.48, and 126.14 (C_3, C_5, and C_4); 134.35 and 126.41 (C_1 and C_6); 63.31 (CH); and 48.35 (CH_2). Anal. Calcd. for $C_{10}H_9N_2O_6Na$ H_2O: C, 40.83; H, 3.77; N, 9.52. Found: C, 40.56; H, 3.65; N, 9.48. Mass spectrum (8 keV xenon, glycerol/1% TFA matrix), normalized to the molecular ion: m/z 255 (corresponding to $[C_{10}H_{11}N_2O_6]^+$).

Product Release and Quantum Yield Studies. So that we might observe glycine as a photolysis product, and also estimate the degree of photolysis under known irradiation conditions (product quantum yield), we employed several different techniques. To begin with, we wished to test the photolability of the compounds. Aqueous solutions of 15 mM compounds I (potassium salt), II (hydrochloride), or III (sodium salt) were irradiated at λ = 350 nm in a Pyrex tube using a Rayonet photochemical reactor (Srinivasan–Griffin model) (16 × 15 W) for 20 min at room temperature. TLC analysis [glass plate/silica gel 60F-254 (no. 5714-3, Merck); solvent, *n*-butanol/acetic acid/water, 3:1:1] performed every 5 min showed the presence of both the starting material and the product glycine.

Product release from compound III was also examined, by using an Excimer laser (Lumonics TE-861M) as the excitation source. An aqueous 3 mM solution (0.03 mL) of compound III was placed in a quartz cuvette (Spectrocell, Inc.) with a path length of 2 mm. The area of sample exposed to the laser pulse was 6 mm^2 (40% of the available surface), and the energy/pulse delivered was 12 ± 0.5 mJ. Each solution was irradiated 60 times, and thorough mixing was ensured every 10 laser pulses. High-performance thin layer chromatography was used to monitor the liberation of glycine. After irradiation, 0.02 mL of the sample was spotted on to an HPTLC plate, along with 0.02 mL of the following in adjacent lanes: an unphotolyzed reference sample and two glycine standards, 0.5 and 1 mM in water.

The final procedure was designed to estimate the extent of photolysis that results from known irradiation conditions. Samples of 0.2 mM compound III (0.2 mL), in 0.1 M ammonium acetate buffer, pH 5.8, were placed into a quartz cuvette (Spectrocell, Inc.) with a path length of 1 cm. The samples were exposed to 5–8 excimer laser pulses, with thorough stirring of the solution between each irradiation. The beam incident upon the surface of the cuvette had a cross-sectional area of 6 mm^2 and so covered 30% of the available surface. The energy absorbed by the sample was measured by subtracting that which was transmitted from the amount transmitted by the buffer alone (10 mJ); all the energy measurements were made by using a Gentec ED200 Joule meter. Samples were then analyzed by precolumn derivitization of the free amino acid with phenylisothiocyanate, followed by high-performance liquid chromatography (HPLC) according to the Waters pico-tag method (Bidlingmayer et al., 1984).

Laser Pulse Photolysis. Two different experimental arrangements were used to obtain the results reported here. For investigating the kinetics of photolysis, an excimer laser (Lumonics TE-861M) was used to provide short pulses (10–20 ns) of 308-nm radiation, rated at 35 mJ/pulse. So that we could detect and measure photochemically induced optical changes in the sample, a crossed beam apparatus was employed. For this work, the excited sample was viewed by a monitor beam which propagated through the sample at 90° to the direction of the excimer beam. This monitor beam was produced by a stabilized light source (Newport 780) and was passed through the 0.16-mL quartz photolysis cuvette; the path length for both monitor and excitation beams was 2 mm. The intensity of the monitor beam was measured by using a photomultiplier (Thorn EMI 9635QB), which was fitted with a monochromator (McPherson 275, 2-nm bandwidth). The photomultiplier output was treated by a current-to-voltage converter (Thorn EMI Gencom A1), and the resultant output was fed to a storage oscilloscope (Tektronix type 549), where the results were displayed and recorded for further analysis. The experimental traces were then digitized by a Convex computer (Material Science Center, Cornell University). More recently, data were recorded by using an AST minicomputer equipped with an A/D convertor (Metrabyte DAS100). These data were subsequently analyzed using a Northgate minicomputer with the GENPLOT software package (Computer Graphics Services).

The second system was a flashlamp-pumped dye laser (Candela SLL-UV500), and this was used to investigate the photolysis-induced biological activity of compound III. This laser system was capable of producing 5–10 mJ pulses of ultraviolet radiation (600 ns/pulse) after passing the primary output through a thermally stabilized "frequency-doubling" A.D.P. crystal (Quantum Technologies) and then removing the residual primary beam with an optical filter (Corning CS-754). For the work described here, 0.5–3 mJ laser pulses were used, and the final output was 320 ± 5 nm; the dye used was 40 μM Rhodamine 640 Perchlorate (Exciton) with methyl alcohol as the solvent. The laser output was delivered to the experiment by coupling it to a single optical fiber of 200-μm core diameter (Fiberguide Industries, SFS-200N).

Cell Experimentation. Cultured spinal cord neurons were prepared from 12–13-day mouse embryos and maintained as previously described (Ransom et al., 1977). Neurons were kept in culture for 10 days prior to the experiments. Whole-cell recordings (Hamill et al., 1981) were made, using a patch-clamp amplifier (List L/M-EPC7), on cells that were attached to the culture dish, in the presence of extracellular buffer of the following composition: 145 mM NaCl, 1.8 mM $MgCl_2$, 1.0 mM $CaCl_2$, 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) with additional KOH, pH 7.4. The electrodes were made from hard glass (World Precision Instruments), prepared on a puller (List L/M-3P-A), and fire polished. The electrodes contained the following solution: 140 mM $CsCl_2$, 1 mM $CaCl_2$, 10 mM EGTA, 2 mM Na_2-ATP, 4 mM $MgCl_2$, and 10 mM HEPES with NaOH, pH 7.4. The electrode resistance was typically 2–3 MΩ and the series resistance between 4 and 6 MΩ. The membrane potential was clamped at −40 mV and the series-resistance compensation adjusted so as to maintain the voltage change beneath the pipette to within 4 mV (Sigworth, 1983). An optical fiber, used to deliver the excitation pulse, was held approximately 2 mm away from the cell. As a control, a single laser pulse of 0.64 ± 0.1 mJ was delivered to the cell in the presence of buffer alone. Compound III was then dissolved in the extracellular buffer around the cell (620 μM final concentration), allowed to equilibrate with the receptors on the cell surface, and then irradiated.

The data were low-pass filtered at 2 kHz (Krohn-Hite 3322, −3 db cutoff point) and digitized between 100 and 200 Hz with an AST minicomputer in association with the pClamp 5.03 software package (Axon). These data were later analyzed using the same computer with the GENPLOT data analysis program (Computer Graphics Service).

RESULTS

Synthesis of Photolabile Glycine Derivatives. Three photolabile derivatives were synthesized as described in Scheme I and characterized by their ^1H and ^{13}C NMR spectra and by elemental analyses. Fast atom bombardment mass spectroscopy was also used to confirm the structure of compound III.

Photolysis and Identification of Products. The absorption spectra of compound III, before and after repeated illumination with excimer laser light at 308 nm, are shown in Figure 1.

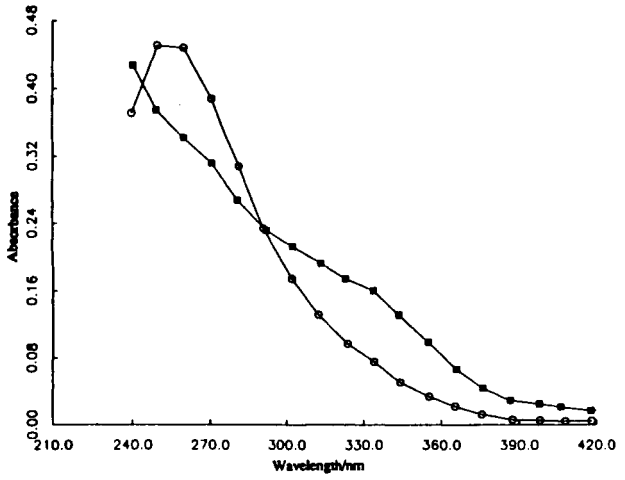


FIGURE 1: Effect of laser photolysis upon the visible and ultraviolet spectrum of compound III. An aqueous solution of 3 mM compound III (30 μL) was placed into a quartz cuvette (2 mm × 2 mm × 40 mm) and then exposed to pulses of ultraviolet radiation of 308 nm, delivered by an excimer laser (Lumonics TE-861M). A total of 60 pulses rated at 12 mJ/pulse was delivered to the sample; approximately 40% of the solution was illuminated by the laser, and the solution was throughly stirred every 10 shots. After diluting the samples by a factor of 30 with 0.1 M 4-morpholinepropanesulfonic acid (MOPS) at pH 7.5, spectra were recorded using a quartz cuvette with a 1-cm path length (Spectrocell, Inc.) both before (○) and after (■) photolysis. The spectra are corrected for the absorption of 0.1 M MOPS.

Prior to photolysis, the compound shows an absorbance maximum at approximately 262 nm, which is typical for the 2-nitrobenzyl moiety (Morrison, 1969). After photolysis, this peak flattens out, and the absorbance increases at wavelengths greater than 290 nm. The spectra obtained with compound II were similar to those of compound III, with the exception that photolysis produces only small changes in absorbance between 250 and 290 nm. Compound I, in which the 2-nitrobenzyl moiety was used as the protecting group when measured under similar conditions, also showed absorbance changes resulting from photolysis, but the effect was even less pronounced; the absorbance increase for compound I at 340 nm was only about 50% of that for compound III (which is shown in Figure 1). Additionally, when the photolabile protecting group used with compound I was used to protect the amino group of carbamoylcholine (an acetylcholine analogue), the resulting compound interacted significantly with the acetylcholine receptor (Walker et al., 1986). For these reasons, compound I was not further investigated.

To ascertain whether glycine was released as a photolysis product, compound III was run on an HPTLC plate, using both pre- and postphotolysis samples, together with glycine standards. Observation of the plate under UV light revealed the presence of the starting material. Compound III produced observable aromatic photoproducts, and a smear of photoproducts with higher mobilities was also seen. When sprayed with ninhydrin, glycine could clearly be identified in the lanes containing photolyzed solution. No photodecarboxylation of compound III to I was noticeable, unlike the observation of Margerum and Petrusis (1969) and as suggested by McCray and Trentham (1989).

Additional work with compound III was then conducted so that we could estimate the product quantum yield of glycine for this compound. After exposure of the samples to a series of laser pulses at 308 nm and precolumn derivitization of the sample (see Experimental Procedures) followed by HPLC, analysis clearly revealed the presence of photo-produced glycine. From the calibration, we were able to estimate that for compound III, at pH 5.8 and 22 °C, the product quantum yield for glycine was about 0.02.

Laser Pulse Photolysis and Spectroscopy. All three compounds were found to give a spectral intermediate when a laser pulse of 308-nm wavelength was passed through the solution; a representative photolysis trace for compound III, monitored at 449 nm, is shown in Figure 2a. This clearly shows that photolysis induces a rapid change in the optical transmission of the sample (increased absorbance), which is accompanied by a decay that does not return to the original transmission level. The observed transient is presumably the aci-nitro intermediate for this compound (see Figure 2c and discussion below). From these data, one can see that in addition to the transient signal (Milburn et al., 1989), a permanent transmission change, a_2, is also observed (see Figure 2c and the figure legend). In experiments with the analogous photolabile carbamoylcholine derivative, Milburn et al. (1989) observed only a small a_2 component compared to the aci-nitro signal when monitoring between 410 and 500 nm. A plot of the maximum transmission change is shown as a function of wavelength in Figure 2b. The spectral distribution is not consistent with that characteristic of the aci-nitro intermediate (McCray et al., 1980; Walker et al., 1986, 1988) in product formation (McCray et al., 1980; Walker et al., 1988). However, by subtracting the permanent transmission change, a_2, from the spectra given in Figure 2b, one can find the wavelength dependence for the decaying component of the photolysis trace, a_1; a plot of the spectral distributions for a_1 and a_2 is given in Figure 2c. The time-dependent decaying component a_1 has a spectral distribution that is consistent with that of the aci-nitro intermediate. We, therefore, conclude that two compounds are formed within the time resolution of the apparatus: the concentration of one is time-dependent and exhibits spectral characteristics that would be expected for the aci-nitro intermediate, and the concentration of the other, an as yet unidentified species, is time-invariant during a period of observation of 5 s.

The rate of decay of the aci-nitro intermediate has also been studied. By regarding the trace as the sum of an exponentially-decaying and a step contribution, and by fitting it to the equation given in the legend to Figure 2c, we have been able to calculate the rate of decay of the aci-nitro intermediate as a function of the pH; the results for compound III are shown in Figure 2d. It can be seen that the decay rate is sensitive to the pH for pH values less than 7, in agreement with the results obtained for its analogous photolabile carbamoylcholine derivative (Milburn et al., 1989). At pH 6.8 the decay rate of the aci-nitro intermediate is 940 s^{-1}, and at pH 7.5 it is 600 s^{-1} at room temperature, 22 °C. This decay is considered to be a measure of the product release rate (McCray et al., 1980; Walker et al., 1988).

Compound II was found to give photolysis characteristics very similar to those for compound III. For compound II, the aci-nitro intermediate was found to have a spectral maximum at 420 nm and a decay constant of 824 s^{-1} in 0.2 MOPS, pH 7.0, at 22 °C. Although compound I clearly gave an intermediate on the millisecond time scale upon excitation, data interpretation was hampered by a complicated decay profile.

Effect of Compound III on Glycine Receptors on Cultured Mouse Spinal Cord Cells. Initially, to test the effect of compound III upon glycine receptors expressed on cultured mouse spinal cord cells, we made use of a cell-flow method (Udgaonkar & Hess, 1987). Depicted in Figure 3a are the responses of a cell to separate additions of 100 μM glycine (solid line, upper trace), 100 μM glycine and 1 mM compound

III (crosses, upper trace), and 1 mM compound III alone (lower trace). It can be seen in the upper traces that, upon additions of 100 μM glycine in the absence or presence of 1 mM compound III, first the current increases due to the formation of transmembrane channels and then decreases due to receptor desensitization induced by glycine (Nelson et al., 1977). The current amplitude is a measure of the number of receptors that have formed transmembrane channels. It can be seen that neither the current amplitude nor the decay rate of the current is measurably affected by the presence of compound III. Since 1 mM compound III alone (Figure 3a, lower trace) does not lead to the formation of observable transmembrane channels, it is concluded that compound III does not activate or inhibit the receptor. An experiment showing the photolysis of compound III in situ with a cell is given in Figure 3b. The lower trace represents the effect of illuminating the cell in extracellular buffer alone, and the upper one was obtained when 620 μM compound III was photolyzed. It can be seen that three processes of the receptor-mediated reaction have been separated along the time axis. The current versus time profile has been interpreted in similar experiments in which *N*-(α-carboxy-2-nitrobenzyl) was used as the photolyzable group to protect the amino group of carbamoylcholine (see also compound III), and the channel-opening rate of the nicotinic acetylcholine receptor in BC_3H1 muscle cells was investigated (Matsubara et al., 1992): (i) the rising phase of the whole-cell current is a measure of the ligand-binding and channel-opening step; (ii) the amplitude is a measure of the concentration of open receptor–channels; and (iii) the decaying phase is due to receptor desensitization. Receptor desensitization occurs in seconds and is shown on a different time scale. In a separate experiment, a solution containing 100 μM glycine was equilibrated with the receptors on the cell surface using a cell-flow technique, and the observed maximum current, corrected for receptor desensitization (Udgaonkar & Hess, 1987b), was determined. The same cell was then equilibrated with 410 μM compound III, and the maximum current obtained after photolysis (2.5 mJ, 313 nm) of compound III was measured. From measurements of the relationship between glycine concentration and the current amplitude obtained in cell-flow experiments with cultured spinal cord neurons (K. M. Walstrom, unpublished observation), we calculate that 5% compound III was photolyzed.

Discussion

Three photolabile glycine derivatives were synthesized to extend the use of photolabile neurotransmitters, following the work of Walker et al., (1986) and Milburn et al. (1989), which was concerned with photolabile carbamoylcholine derivatives. Photolysis of nitrobenzyl precursors occurs via the observable aci-nitro intermediate, as identified by its characteristic spectral distribution (McCray et al., 1980). By studying the time dependence for the disappearance of this intermediate, details concerning the photolytic reaction process, such as product formation, can be discerned.

Information concerning the rate of release of glycine was obtained in several ways. Primarily, the aci-nitro intermediate was studied. McCray et al. (1980), when using a photolabile ATP precursor, determined that the rate of decay of intermediate gave a measure of the rate of release of product. The intermediate observed for compound III was identified as the aci-nitro intermediate by its spectral distribution (Figure 2c). The glycine release rate was found to be 600 s^{-1} at pH 7.5 at room temperature. Further, the observed half-maximal rise time when glycine receptor–channels are opened by compound III upon photolysis is 50 ms at pH 7.4 and room temperature (Figure 3b); this response is consistent with a model in which glycine is rapidly released followed by a slower channel-opening process. Thus, the rate of glycine delivery to the cell surface from this compound is an order of magnitude faster than that which could be achieved by the cell-flow method (Udgaonkar & Hess, 1987b), which has a time resolution of 10–20 ms.

Compounds II and III were found to release glycine upon photolysis as does compound I. However, the photolysis characteristics for compound I appear to be more complex than for compound III, as the time dependence of the aci-nitro intermediate cannot be adequately described by a single exponential (data not shown). A smaller change in the absorption spectrum of compound I when compared to compound III could be seen after photolysis; it is, therefore, likely that compound I has a lower product quantum yield than compounds II and III. Compound II exhibits a baseline shift, aci-nitro spectra, and decay rate resulting from photolysis that are comparable to those of compound III (data not shown).

The α-carboxy-2-nitrobenzyl protecting group that Milburn et al. (1989) introduced may be generally useful in protecting a variety of amino acid neurotransmitters. This protecting group has several favorable characteristics, including a negative charge which decreases the membrane solubility of the compound. The photolysis release rate for both amines and carbamates is rapid, and the photolabile precursors do not appear to interact with either the glycine receptor or the nicotinic acetylcholine receptor (Milburn et al., 1989). When the *N*-(α-methyl-2-nitrobenzyl) amino-protecting group (Walker et al., 1986) was used with carbamoylcholine (see also compound II), it was found that the compound both inhibited and inactivated (desensitized) the nicotinic acetylcholine receptor (Walker et al., 1986). Since the photolytic properties of compound II, which has this protecting group, do not appear to be better than those of compound III, we have not yet investigated its use in kinetic measurements of receptor function.

The experiments with compound III, and the comparison with *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine, indicate that the leaving group has an important effect on the photolytic reaction. For compound III, the product quantum yield is estimated to be 0.02, from laser-pulse experiments at pH 5.8 followed by HPLC analysis. In an experiment in which a single 2.5-mJ laser pulse was used and glycine release was assayed by a whole-cell current-recording technique, we estimated that 5% of compound III was converted to glycine. The product quantum yield is considerably lower than that reported for the analogous photolabile carbamoylcholine. This may be due to the formation of the unidentified species that we report here and is currently under investigation in our laboratory. However, the dissociation constant of the site of the glycine receptor controlling channel opening (Akaike et al., 1989) is lower, by a factor of approximately 3, than the dissociation constant of the carbamoylcholine site that controls opening of the acetylcholine receptor–channel (Udgaonkar & Hess, 1987a). Therefore, much smaller quantities of released glycine are required in kinetic investigations of the glycine receptor than were required in similar kinetic investigations of the acetylcholine receptor using a carbamoylcholine derivative analogous to compound III (Milburn et al., 1989; Matsubara et al., 1992). The rate of photolysis of compound III is considerably lower than that of the analogous carbamoylcholine derivative. Under the conditions chosen for kinetic investigations of the acetylcholine receptor in BC_3H1 cells, the maximum rate of photolysis of the photolyzable carbamoylcholine derivative was 7300 s^{-1}. Under optimum con-

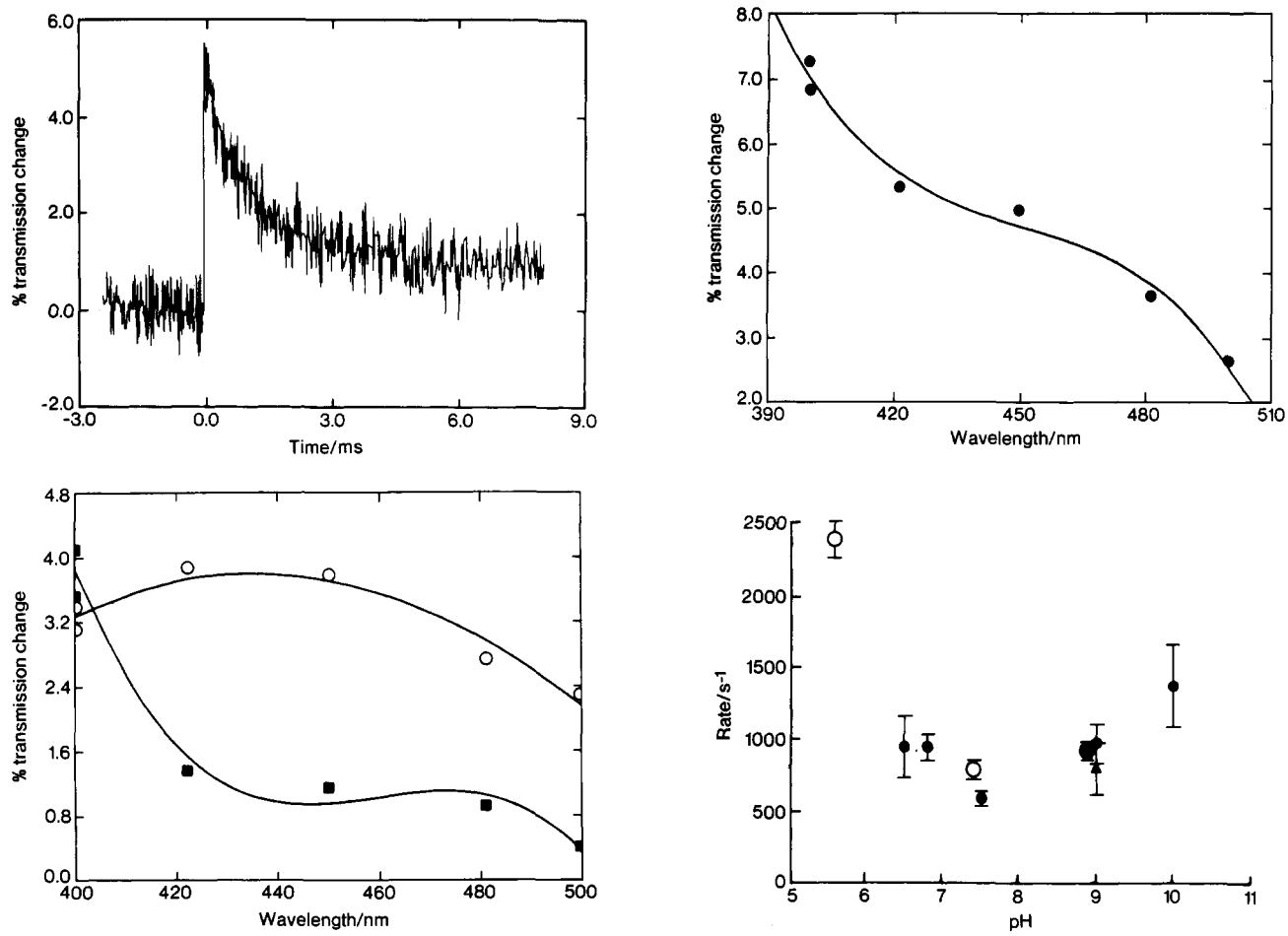


FIGURE 2: (a, top left) Transmission change observed upon photolysis of 3.7 mM compound III, 0.2 mM HEPES, pH 7.4, at 22 °C, and monitored at 449 nm. An excimer laser was used to deliver a pulse of 308 nm, 20-ns duration, and approximately 38 mJ, into a sample cuvette containing compound III; the path length used in both excitation and monitor directions was 2 mm. (b, top right) Maximal changes in transmission observed, as a function of wavelength, for the solution described in panel a. (c, bottom left) Characteristic spectra observed for the two components, one decaying exponentially (○), a_1, and the other a simple baseline shift (■), a_2. Each photolysis trace was digitized and then fitted to the equation, transmission change = $a_1 \exp(-\lambda t) + a_2$, to yield values of the amplitudes of a_1 and a_2 and of λ, the decay constant for species a_1. This figure illustrates the results obtained for one experimental data set, but the spectra were generated four times, with comparable results. (d, bottom right) Rate constants for the decay of the transient intermediate in the photolysis of compound III as a function of pH. Each measurement was recorded at 22 °C, and a monitoring wavelength between 400 and 500 nm was used. The decay constant, λ, was calculated as described in panel c and then plotted. The following buffers were used: 0.1 M 4-morpholineethanesulfonic acid (MES), pH 5.5; 0.1 M piperazine-*N*,*N*-bis(2-ethanesulfonic acid) (PIPES), pH 6.5; 0.1 M MOPS, pH 6.8; 0.1 M and 0.2 M HEPES, pH 7.4; 0.1 M HEPES, pH 7.5; 0.1 M tris(hydroxymethyl)aminomethane (TRIS), pH 8.8; 0.2 M TRIS (●), pH 9.0; 0.1 M borate, pH 9.0 (▲), and 0.1 M 3-(cyclohexylamino-1-propanesulfonic acid) (CAPS), pH 10.0. The error bars show the standard deviation for the measurements. Open symbols, $n = 2$; filled symbols, $n = 4$–10. The two different filled symbols indicate that different preparations of compound III were used for the experiments at pH 9.

ditions in experiments with cells, the photolysis rate of compound III is 600 s^{-1}. However, the observed rise time of the current in the kinetic experiment (Figure 3b), which we consider to reflect the channel-opening process, has a half-time, $t_{1/2}$, of 50 ms. In kinetic investigations of the acetylcholine receptor in BC_3H1 cells, at the lowest concentration of carbamoylcholine employed, the $t_{1/2}$ value for the current rise time was 50-fold smaller ($t_{1/2} \sim 1$ ms) (Matsubara et al., 1992). From the effect of ligand concentration on the three phases of the whole-cell current observed in laser-phase photolysis experiments with the acetylcholine receptor in BC_3H1 cells (Milburn et al., 1989; Matsubara et al., 1992), and with the glycine receptor in mouse spinal cord neurons (Figure 3b), information about elementary steps of the channel-opening process, the equilibrium constants determining channel opening, and the rate coefficient for receptor desensitization can be obtained (Udgaonkar & Hess, 1986). Although compound III is photolyzed considerably less than the analogous carbamoylcholine derivative, this is in part offset by the more favorable dissociation constant and slower rise time observed in experiments with the glycine receptor.

Three different rapid chemical kinetic techniques have been adopted for investigations of neurotransmitter receptors: flow techniques with a 5–20-ms time resolution suitable for kinetic measurements of receptors in membrane vesicles (Hess et al., 1979), flow techniques with a 10–20-ms time resolution suitable for kinetic measurements with single cells (Udgaonkar & Hess, 1978b; Matsubara & Hess, 1992; Geetha & Hess, 1992), and laser-pulse photolysis with a 100-μs time resolution in the case of the carbamoylcholine analogue of compound III (Milburn et al., 1989). The last technique has so far only been used in kinetic investigations of the channel-opening process of the mammalian nicotinic acetylcholine receptor in a muscle cell line, in which an acetylcholine analogue was used (Matsubara et al., 1992). The results presented here suggest that the laser-pulse photolysis technique, utilizing compound III and analogous derivatives of the other amino-group-containing neurotransmitters, may be useful not only in investigations of the mechanism governing the channel-opening process of the glycine receptor, and the other important inhibitory receptor in the central nervous system, the γ-aminobutyric acid receptor, but also in investigations of the ex-

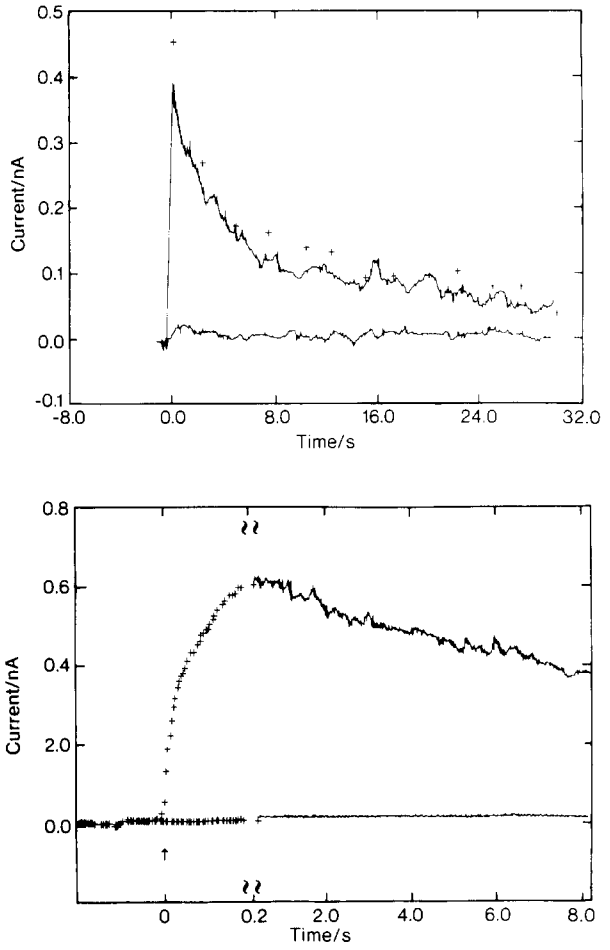


FIGURE 3: (a, top) Effect of 100 μM glycine (top trace) or 1 mM compound III (bottom trace) when applied to a cultured mouse spinal cord cell using the cell-flow method and the whole-cell recording technique (Hamill et al., 1981) at 22 °C. The experiment was conducted in extracellular buffer (see Experimental Procedures) containing physiological salts and 10 mM HEPES, pH 7.4. The current induced by 100 μM glycine plus 1 mM compound III, applied to the same cell and under the same conditions, is represented by the upper crosses. (b, bottom) Effect of photolysis of compound III on a cultured mouse spinal cord cell in the whole-cell current recording mode (Hamill et al., 1981) illustrating the response of glycine receptors to glycine release. The bottom trace is the response of the cell to laser light alone, and the upper current trace shows the response induced when 620 μM compound III, dissolved in extracellular buffer surrounding the cell at 22 °C, was exposed to the laser light. The 600-ns duration, 320-nm laser light (0.64 ± 0.1 mJ/pulse) was delivered by a Candela laser at the time indicated by the arrow.

citatory glutamate and *N*-methyl-D-aspartic acid receptors.

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Registry No. I, 42749-52-0; II·HCl, 139242-87-8; III·Na, 139242-88-9; 2-$NO_2C_6H_4CHO$, 552-89-6; 2-$NO_2C_6H_4CH_2NH_2$·HCl, 24835-08-3; 2-$NO_2C_6H_4CH(CH_3)_2$, 100311-54-4; 2-$NO_2C_6H_4CH_2NHCH_2CO_2H$·HCl, 24835-10-7; $NH_2CH_2CO_2H$, 56-40-6.

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How Fast Does an Acetylcholine Receptor Channel Open? Laser-Pulse Photolysis of an Inactive Precursor of Carbamoylcholine in the Microsecond Time Region with BC_3H1 Cells†

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ABSTRACT: The integrated function of the nervous system depends on specific and rapid transmission of signals between its constituent cells. The nicotinic acetylcholine receptor is the best known of a group of membrane-bound proteins responsible for such transmission; for this process to occur, a specific neurotransmitter, in this case acetylcholine, must bind to the receptor, which then forms transmembrane channels through which cations pass. The resulting change in transmembrane voltage determines whether or not a signal is transmitted. The question of how fast this process takes place in any neurotransmitter receptor has remained one of the interesting and most challenging in the field. To answer it, many attempts have been made to evaluate the rate constant for the opening of the acetylcholine receptor channel, but in almost all these studies the rate was measured after the receptor-mediated reaction, which involves the open channel and many intermediate states, had reached a quasi equilibrium. This resulted in a plethora of reported values for the rate constant that differ by a factor of up to 50-fold, even when the measurements were made with the same type of cell. The new approach described here involves the use of single cells of a mammalian cell line (BC_3H1), containing muscle-type acetylcholine receptors, and the rapid introduction of neurotransmitter to the cell surface. The rapid delivery was achieved by converting a previously synthesized photolabile precursor of carbamoylcholine to carbamoylcholine, a stable amino-group-containing analogue of acetylcholine, with a single laser pulse and an observed photolysis rate of 7300 s^{-1}. The resultant opening of the receptor channels creates a transmembrane current, which was measured in order to determine the rate constant for the formation of the open channel, k_{op}, the rate constant for channel closing, k_{cl}, and the dissociation constant of the receptor site controlling channel opening, K_1, the values of which were found to be 9400 s^{-1}, 580 s^{-1}, and 210 μM, respectively. Two of these constants, k_{cl} and K_1, were also measured by two independent methods, and good agreement was observed. The value of k_{op} is of interest because (i) it determines the rate at which signals can be transmitted between cells and (ii) signal transmission is determined by the concentration of receptors in the open-channel form. At any given concentration of neurotransmitter, the fraction of receptors in the open-channel form may be calculated from the values of K_1, k_{cl}, and k_{op}. These constants can now all be determined for the acetylcholine receptor, and for other receptors that are specific for neurotransmitters containing an amino group, using the laser-pulse photolysis technique described.

The nicotinic acetylcholine receptor is the best known member of a family of membrane-bound proteins responsible for transmission of signals at the junctions (synapses) between cells in the nervous system (Sakmann & Neher, 1984; Hess et al., 1987; Unwin et al., 1988; Claudio et al., 1989; Changeux, 1989; Stroud et al., 1990; Betz, 1990), ranging from about 10^{12} cells in humans (Kandel et al., 1985) to 302 cells in *Caenorhabditis elegans* (Chalfie et al., 1988). Here we describe a new approach to chemical kinetic investigations of receptor-mediated reactions, in which the reaction is initiated by a laser pulse, which converts an inactive precursor of carbamoylcholine (caged carbamoylcholine) (Walker et al., 1986; Milburn et al., 1989) to carbamoylcholine, a stable analogue of the neurotransmitter acetylcholine, with a $t_{1/2}$ value of 95 μs.

Rapid chemical reaction techniques, by virtue of their time resolution and the ability to vary the concentration of reactants, allow one to separate sequential steps of a complex reaction along the time axis. This has enabled kinetic studies to be made of individual steps of a reaction under conditions where simple rate laws are obeyed (Eigen, 1967; Hammes, 1982; Fersht, 1985). The approach has been used with considerable success in investigations of important biological reactions in solution (Eigen, 1967; Hammes, 1982; Fersht, 1985). A

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