Synthesis and Photochemistry of Photolabile Derivatives of γ-Aminobutyric Acid for Chemical Kinetic Investigations of the γ-Aminobutyric Acid Receptor in the Millisecond Time Region†

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ABSTRACT: The γ-aminobutyric acid (GABA) receptor is an abundant neuronal receptor in the mammalian and invertebrate nervous systems and is associated with an inhibitory chloride ion channel. GABA is the endogenous neurotransmitter for the receptor and can trigger both fast activation and a reversible desensitization of the receptor. A series of photolabile amine-linked *o*-nitrobenzyl derivatives of GABA were synthesized that photolyze rapidly to release free GABA. The photochemical properties of the GABA precursors were determined; the compounds undergo rapid photolysis, initiated with UV irradiation at 308 nm, and release free GABA on a millisecond time scale. The pH of the photolysis medium affects both the quantum yield and the rate of photolysis. For example, the quantum yield observed for *N*-(α-carboxy-2-nitrobenzyl)-γ-aminobutyric acid increases from 0.06 at pH 5.0 to 0.1 at pH 10.5, and the half-life for the photolytic reaction decreases from 1.0 to 2.5 ms in the same pH range. Photolysis of the compounds induces rapid onset of transmembrane ion currents in mouse cortical neurons. The potential of the new compounds for use in rapid chemical kinetic investigations of the neuronal GABA receptor is demonstrated.

Rapid release of biologically active molecules from photolabile precursors is an important technique in the study of fast biological processes (Kaplan & Somlyo, 1989; Kaplan, 1990; Adams & Tsien, 1993; Corrie & Trentham, 1993). The use of these "caged compounds" provides control of spatial and temporal distribution of substrate concentration in systems where diffusional mixing delays are a barrier to observation of rapid kinetic events. Recently, photolabile derivatives of neurotransmitters that activate acetylcholine (Walker et al., 1986; Milburn et al., 1989) and glycine (Billington et al., 1992) receptors have been synthesized. In this study we report the synthesis and characterization of photolabile precursors of γ-aminobutyric acid (GABA) which can be used in kinetic studies of the GABA receptor in the millisecond time domain.

GABA is the most abundant inhibitory neurotransmitter of the mammalian central nervous system. The neuronal GABA receptor has been widely studied in both vertebrate (Olsen et al., 1991) and invertebrate (Atwood, 1982; Satelle et al., 1991) systems. GABA receptor function is complex and is currently the object of much active investigation (Sieghart et al., 1992; Stelzer, 1992). The GABA-A form of the receptor produces an anion-specific transmembrane ion channel. Activation of the channel is accompanied by a rapid change of transmembrane voltage which can counteract effects of cation channels such as the glutamate receptor. Thus, the GABA-A receptor–ionophore complex is thought to have a critical role in opposing excitatory modes of neuronal signal transmission and is involved in physiological functions such as nociception and anxiety, in convulsive states such as epilepsy, and in some psychiatric disorders (Vicini, 1991; Paredes & Agmo, 1992). The receptor is a target for centrally acting sedative and anticonvulsant drugs with extensive therapeutic utility (Bureau & Olsen, 1990). For example, the benzodiazepines specifically interact with the GABA receptor (Wieland et al., 1992) to produce anxiolytic effects without the general systemic depression that was seen with barbiturates.

Fast opening and closing processes of the GABA-A receptor chloride channel are important features of the receptor mechanism. Rapid kinetic techniques were shown to be necessary to distinguish the separate desensitization components of the GABA response of neuronal receptors in rat membrane vesicles (Cash & Subbarao, 1989). Recently, it has become possible to use a rapid chemical reaction technique, a cell-flow technique with a 10-ms time resolution (Udgaonkar & Hess, 1987), in investigations of the GABA-A receptor in single cells from the central nervous system (Geetha & Hess, 1992). Rate constants for the desensitization process of two receptor forms, the dissociation constants for the GABA activation site, and the concentration of the open receptor–channel as a function of GABA concentration were determined (Geetha & Hess, 1992). In experiments with the excitatory acetylcholine receptor it was found that the rate constants for channel opening and closing were too large to be determined by flow techniques (Matsubara et al., 1992) because those processes occur on a time scale that is faster than the diffusional limit of the flow method. For these reasons, an inactive photolabile precursor of carbamoylcholine was synthesized that could liberate carbamoylcholine on a microsecond time scale (Milburn et al., 1989). This compound was used to determine the rate constants for channel opening and closing (Matsubara et al., 1992) and the effect of inhibitors on these rate constants (Niu & Hess, 1993). Here we demonstrate that photolabile precursors of GABA, which we have synthesized and characterized, can be used in rapid chemical kinetic investigations of the GABA-A receptor.

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MATERIALS AND METHODS

N-(2-Nitrobenzyl)-γ-aminobutyric acid (**C-G-I**). A mixture of γ-aminobutyric acid (0.52 g, 5.0 mmol), 2-nitrobenzaldehyde (1.132 g, 7.5 mmol), sodium hydroxide (2.5 mL of 2 N solution, 5.0 mmol), and methanol (7.5 mL) was stirred at room temperature overnight. It was cooled to 0 °C and sodium borohydride (0.175 g, 4.6 mmol) was added in portions over a period of 4 h. After 12 h, 3 drops of acetic acid was added. The contents were concentrated under vacuum and chromatographed on Sephadex LH-20, using water as eluent, and the product was eluted with free γ-aminobutyric acid. The fractions were combined, acidified with hydrochloric acid (2 N) to pH 3, and lyophilized. The product was recrystallized from water twice. Yield = 0.78 g (65%), mp 148–150 °C. ^{1}H NMR (D_2O·K_2CO_3) 8.09 (d, J = 7.94 Hz, 1 H), 7.66 (t, J = 7.57 Hz, 1 H), 7.53 (m, 2 H), 4.33 (s, 2 H, Ar-CH_2-NH), 3.07 (t, J = 8.22 Hz, 2 H, NH-CH_2-), 2.15 (t, J = 7.07 Hz, 2 H, -CH_2-CO_2-), and 1.81 (p, J = 6.85 Hz, 2 H, -CH_2-CH_2-CH_2); ^{13}C NMR (D_2O·K_2CO_3) 184.26 (CO_2), 151.19 (C-NO_2), 138.18, 136.75, 134.43, and 129.02 (C_3, C_4, C_5, and C_6), 51.73 (Ar-CH_2), 50.85 (N-CH_2), 37.44 (CH_2-CO_2), and 25.11 (CH_2-CH_2-CH_2). Anal. Calcd for $C_{11}H_{14}N_2O_4$: C, 55.5; H, 5.9; N, 11.8. Found: C, 55.02; H, 5.97; N, 11.49.

tert-Butyl 4-Bromobutyrate. A mixture of 4-bromobutyric acid (1.3 g, 7.8 mmol), isobutylene (9 mL), concentrated sulfuric acid (250 μL), methylene chloride (12 mL), and powdered molecular sieves (4 Å, 0.85 g) taken in a pressure bottle was shaken in a Parr hydrogenator–shaker for 2 days at room temperature. It was filtered through a Celite pad, poured into saturated sodium bicarbonate solution (10 mL) maintained at 0 °C, and extracted with methylene chloride (3 × 10 mL). The organic layer was washed with water (1 × 10 mL) and brine (1 × 10 mL) and dried over anhydrous sodium sulfate. The product was isolated as a yellow liquid on chromatography over silica gel using a hexane–ether mixture as eluant. Yield = 0.89 g (51%). ^{1}H NMR ($CDCl_3$) 3.44 (t, J = 6.62 Hz, 2 H, Br-CH_2-); 2.39 (t, J = 7.76 Hz, 2 H, -CH_2-CO_2-); 2.12 (p, J = 6.67 Hz, 2 H, -CH_2-CH_2-CH_2), and 1.44 (s, 9 H).

tert-Butyl N-(α-Methyl-2-nitrobenzyl)-γ-aminobutyrate. A mixture of (α-methyl-2-nitrobenzyl)amine hydrochloride (50 mg, 246 mmol), *tert*-butyl 4-bromobutyrate (68 mg, 305 mmol), anhydrous potassium carbonate (78 mg, 557 mmol), cetyltrimethylammonium bromide (18 mg, 49 mmol), *N,N*-dimethylformamide (750 μL), and acetonitrile (5 mL) was heated with stirring at 80 °C for 8 h. Ice (10 g) was added and the contents were extracted with ethyl acetate (3 × 10 mL). The combined ethyl acetate layer was washed with water (1 × 5 mL) and brine (1 × 5 mL) and dried over anhydrous sodium sulfate. The product was isolated as a yellow gum on chromatography over silica gel, using a hexane–ether mixture as the eluant. Yield = 40 mg (53%). ^{1}H NMR ($CDCl_3$) 7.77 (m, 2 H), 7.58 (m, 1 H), 7.34 (m, 1 H), 4.24 (quartet, J = 6.74 Hz, 1 H, CH-CH_3), 2.48 (m, 1 H, NCH_2-CH_2), 2.22 (m, 3 H, NCH_2-CH_2 and CH_2-CO_2-), 1.69 (p, J = 6.95 Hz, 2 H, CH_2-CH_2-CH_2), and 1.40 (s, 12 H, CH-CH_3 and CMe_3).

N-(α-Methyl-2-nitrobenzyl)-γ-aminobutyric Acid Hydrochloride (**C-G-II**). To a stirred solution of the *tert*-butyl ester (35 mg, 114 mmol) in ethyl acetate (200 μL) at 0 °C, a saturated solution of HCl(g) in ethyl acetate (500 μL) was added by syringe and the stirring continued overnight. The contents were concentrated under vacuum and the product was crystallized twice from ether–ethyl acetate. Yield = 20 mg (61%); mp 141 °C (decomp). ^{1}H NMR (D_2O) 7.93 (d, J = 8.02 Hz, 1 H), 7.88 (t, J = 7.81 Hz, 1 H), 7.61 (d, J = 7.32 Hz, 1 H), 7.51 (t, J = 7.63 Hz, 1 H), 4.81 (quartet, J = 6.64 Hz, 1 H, CH-CH_3), 3.03 (m, 1 H, NH-CH_2), 2.90 (m, 1 H, NH-CH_2), 2.27 (m, 2 H, CH_2-CO_2), 1.79 (m, 2 H, CH_2-CH_2-CH_2), and 1.55 (d, J = 6.78 Hz, 3 H, CH_3). Anal. Calcd for $C_{12}H_{16}N_2O_4$·HCl: C, 59.67; H, 5.25; N, 11.6. Found: C, 59.00; H, 5.37; N, 10.78.

Ethyl N-[α-(Methoxycarbonyl)-2-nitrobenzyl]-γ-aminobutyrate. A mixture of methyl 2-nitrophenyl-α-bromoacetate (137 mg, 0.5 mmol), ethyl γ-aminobutyrate hydrochloride (109 mg, 0.649 mmol), anhydrous potassium carbonate (102 mg, 0.75 mmol), and acetonitrile (5 mL) was stirred at room temperature for 2 days. The solvent was removed in a rotary evaporator, water (10 mL) was added, and the mixture was extracted with ether (3 × 8 mL). The ethereal layer was washed with sodium bicarbonate (3 × 5 mL, 5%), water (1 × 5 mL), and brine and then dried over anhydrous sodium sulfate. The product was isolated by silica gel column chromatography using hexane–ether (2:3) as the eluant. ^{1}H NMR (D_2O) 7.88 (d, J = 7.55 Hz, 1 H), 7.58 (m, 2 H, C_5-H), 7.45 (m, 1 H), 5.00 (s, 1 H, CH), 4.06 (quartet, J = 7.12 Hz, 2 H, $CO_2CH_2CH_2$), 3.69 (s, 3 H, CO_2CH_3), 2.72 (m, 1 H, NCH_2CH_2), 2.53 (m, 1 H, NCH_2CH_2), 2.30 (t, 2 H, J = 7.16 Hz, CH_2-CH_2-CO_2), 1.80 (m, 2 H, CH_2-CH_2-CH_2), and 1.2 (t, J = 7.15 Hz, 3 H, OCH_2CH_3).

N-(α-Carboxy-2-nitrobenzyl)-2-pyrrolidone. A mixture of ethyl *N*-[α-(methoxycarbonyl)-2-nitrobenzyl]-γ-aminobutyrate (80 mg, 0.247 mmol) and sodium hydroxide (0.3 mL of 6 N solution, 1.6 mmol) was stirred at room temperature in the dark for 3 days and the pH was adjusted to 3 with concentrated hydrochloric acid and cooled to 0 °C. The precipitated solid was filtered, washed with cold water, and dried. Yield = 12 mg (18%); mp 152–155 °C. ^{1}H NMR (D_2O·K_2CO_3) 7.93 (d, J = 8.68 Hz, 1 H), 7.43 (d, J = 8.84 Hz, 1 H), 7.33 (t, J = 6.70 Hz, 1 H), 4.78 (t, J = 7.08 Hz, 2 H, NCH_2CH_2), 2.04 (t, J = 7.33 Hz, 2 H, $COCH_2CH_2$), 1.93 (m, 2 H, $CH_2CH_2CH_2$). Anal. Calcd for $C_{12}H_{12}N_2O_5$: C, 54.54; H, 4.54; N, 10.61. Found: C, 54.84; H, 4.44; N, 10.44.

N-(α-Carboxy-2-nitrobenzyl)-γ-aminobutyric acid (**C-G-III**). A mixture of γ-aminobutyric acid (20 mg, 194 mmol), sodium α-oxo-(2-nitrophenyl)acetate (60 mg, 276 mmol), and sodium hydroxide (12 mg, 300 μmol) was stirred at room temperature for 8 h, cooled to 0 °C; sodium borohydride (16 mg, 421 mmol) was added in portions over a period of 4 h and left stirred for 12 h. Acetic acid (excess) was added and the contents were chromatographed over Sephadex LH-20, using water as eluant. The product eluted with free γ-aminobutyric acid and was chromatographed over silica gel using ethanol–water–17% NH_4OH to remove γ-aminobutyric acid and triturated with methylene chloride. Yield = 16 mg (28%); mp 142 °C (decomp). ^{1}H NMR (D_2O) 7.85 (d, J = 8.25 Hz, 1 H), 7.56 (m, 1 H0, 7.41 (m, 2 H), 4.49 (s, 1 H, CH), 2.45 (m, 2 H, N-CH_2), 2.02 (t, J = 7.43 Hz, 2 H, CH_2-CO_2), and 1.58 (quintet, J = 7.62 Hz, 2 H, CH_2-CH_2-CH_2); ^{13}C NMR (D_2O) 183.65 (CO_2), 173.26 (CO_2), 150.15 (Ar *C*-NO_2), 137.1, 134.71, 133.24, and 128.06 (C_3, C_4, C_5, and C_6), 129.93 (Ar C-C), 64.76 (*C*-CO_2H), 49.87 (N-*C*H_2), 36.74 (CH_2CO_2), and 24.64 (CH_2-*C*H_2-CH_2). Anal. Calcd for $C_{12}H_{13}N_2O_6$-Na: C, 47.38; H, 4.28; N, 9.21. Found: C, 46.54; H, 4.54; N, 9.15.

Laser Flash Photolysis. The 308-nm radiation used to photolyze the caged compounds was produced by a Lumonics TE861M XeCl excimer laser in pulses of 10–20-ns duration with energies between 10 and 70 mJ. The transient absorbance

of the photolytic intermediate was observed at right angles to the laser irradiation, by passing a collimated beam of light from a stabilized quartz tungsten halogen source (Newport Model 780) through a Corning WGS360 cutoff filter and then through the cuvette oriented with faces perpendicular to the irradiation and monitoring beams. The *aci*-nitro intermediate was detected by magnifying the image of a 0.1-mm section of the irradiated cuvette volume closest to the laser onto the 500-μm entrance slit of a 0.3-m McPherson 275 single-pass monochromator. Light from the monochromator was detected by a photomultiplier (Thorn EMI 9635QB), and the photocurrent was converted to voltage, filtered, and amplified with a Thorn EMI Model A1 preamp. Signal transients were digitized on a Phillips PM3305 digital storage oscilloscope at rates up to 2 MHz and then transferred to an AST Premium 286 personal computer for analysis. The data collection time was determined by the decay rate of the signal for each compound; it was a minimum of 6 times the value for the time constant of the slowest component observed in the signal to ensure adequate representation of the decay for subsequent fitting operations.

Chromatography. Analytical HPLC was performed on a Waters 600E instrument using a Waters μBondapak C-18 300- × 3.9-mm reversed-phase column. Phthaldialdehyde (OPA) derivatization was used to produce fluorescent analogs of free amino acids. Equal volumes of the buffered photolysis solutions (0.5–2 mM in 100 mM buffer) and of OPA reagent (Sigma) were allowed to react for 60 s. The pH and type of buffer (acetate, pH 5.0; phosphate, pH 7.5; bicarbonate, pH 10.5) in the photolysis solution did not affect the derivatization reaction in control samples. Twenty microliters of the mixture was loop-injected on the column, running with isocratic elution using 1.5 mL/min of 40% methanol–60% 50 mM phosphate buffer at pH 4.2. The peaks were recorded with an ISCO FL-2 fluorescence detector (350-nm excitation band and 430-nm detection band filters), and peak areas were measured with a chromatographic integrator (HP 3392).

Cell Culture and Whole-Cell Current Recording. The experimental details of the current measurements have been described previously (Geetha & Hess, 1992). In brief, neurons derived from embryonic mouse cortical tissue at the 16th day of gestation were cultured on 35-mm Falcon plates treated with collagen to provide a substrate for cell attachment. The cells were supplied with minimal Eagle's medium (MEM) supplemented with 10% fetal calf serum and 10% horse serum (all supplied by Gibco) and held in culture for up to 3 weeks. Bipolar neurons were chosen for whole-cell current recording on the fourth or fifth day of culture. This facilitated the lifting of intact cells from the substrate by the recording electrode and the flow application of control ligand solutions. The cell-flow method used to equilibrate GABA receptors on the cell surface rapidly with ligands (Krishtal & Pidoplichko, 1980; Udgaonkar & Hess, 1987) and the recording of the whole-cell current at constant transmembrane voltage (Hamill et al., 1981) have been described (Geetha & Hess, 1992). The extracellular recording buffer consisted of 145 mM NaCl, 18 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES (pH 7.4). The intracellular electrode buffer was 140 mM CaCl, 1 mM $CaCl_2$, 10 mM EGTA, and 10 mM HEPES (pH 7.2).

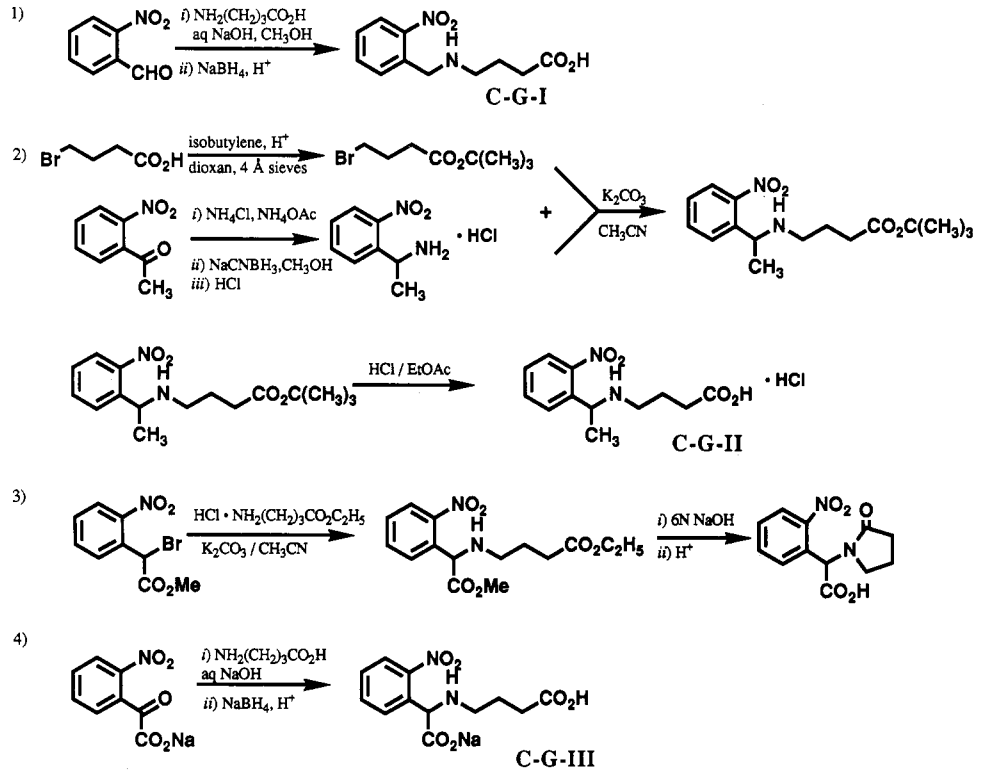
Laser Pulse Photolysis. Solutions of caged compounds in extracellular buffer, as well as control solutions of GABA, were delivered to cells suspended by a current-recording electrode with a rapid-flow device (Krishtal & Pidoplichko, 1980). Single laser pulses of 308-nm light and energies of 0.2–1.0 mJ, generated with the excimer laser, were directed to the cell via a UV-grade 200-μm quartz fiber (Fiberguide Industries). Current transients were recorded either with a Labmaster DMA digitizer driven by the Pclamp (Axon) program running on an AST personal computer or on a digital oscilloscope with subsequent transfer to the PC. Laser and data collection synchronization was triggered with the timing capabilities of the Labmaster DMA hardware.

Spectra of the Photolysis Products. Solutions of the GABA derivatives at concentrations of 1–5 mM in 100 mM buffer (acetate, pH 5.0; phosphate, pH 7.5; carbonate, pH 10.5) were photolyzed by single pulses of UV light at 308 nm from the excimer laser; energy absorbed per pulse was 5 mJ. The photolysis was performed on 40 μL of solution in a 2- × 2-mm quartz cuvette with full exposure of the front face of the solution volume to the laser light. The cuvette was transferred immediately after irradiation to a Hewlett-Packard Model 8452A diode array spectrophotometer for measurement of the absorption spectrum. The absorbance differences produced by photolysis were permanent and fast. The overlay spectra shown in Figure 2 were produced by adjusting the average baseline absorbance of each trace to zero at 650 nm.

RESULTS

The synthesis strategy for the *N*-protected photolabile GABA derivatives **C-G-I**, **-II**, and **-III** is given in Scheme 1. Reductive amination of 2-nitrobenzaldehyde with GABA in the presence of sodium borohydride yields *N*-(2-nitrobenzyl)-γ-aminobutyric acid in 65% yield. This procedure is an extension of the method reported by Kessler et al. (1983) for the synthesis of *N*-(2-nitrobenzyl)glycine. *tert*-Butyl 4-bromobutyrate was prepared from 4-bromobutyric acid, isobutylene, and catalytic amounts of sulfuric acid in the presence of 4-Å molecular sieves with dioxan as the solvent, in a pressure bottle (Anderson & Callahan, 1960). The addition of molecular sieves to the reaction mixture was found to increase the yield of conversion of the acid to the *tert*-butyl ester. (α-Methyl-2-nitrobenzyl)amine was prepared by the reductive amination of 2-nitroacetophenone with ammonium acetate, using sodium cyanoborohydride in 18% yield (Borch et al., 1971; Walker et al., 1986). The amine was then reacted with *tert*-butyl 4-bromobutyrate in the presence of anhydrous potassium carbonate in acetonitrile to yield *tert*-butyl *N*-(α-methyl-2-nitrobenzyl)-γ-aminobutyrate in 53% yield. Deblocking of the above *tert*-butyl ester to *N*-(α-methyl-2-nitrobenzyl)-γ-aminobutyric acid was achieved in 61% yield with anhydrous hydrogen chloride in ethyl acetate. 2-Nitrophenylacetic acid was esterified with anhydrous methanol in the presence of sulfuric acid to yield methyl 2-nitrophenylacetate in 93% yield. Treatment of the methyl ester with *N*-bromosuccinimide, in the presence of benzoyl peroxide as the radical initiator, in carbon tetrachloride gives methyl 2-nitrobenzyl-α-bromoacetate in 23% yield. Reacting the bromo ester with ethyl γ-aminobutyrate in the presence of anhydrous potassium carbonate in acetonitrile furnishes ethyl *N*-[α-(methylcarbonyl)-2-nitrobenzyl]-γ-aminobutyrate in 68% yield. Saponification of the diester, followed by acidification, resulted in *N*-(α-carboxy-2-nitrobenzyl)-2-pyrrolidone in 13% yield and not the anticipated *N*-(α-carboxy-2-nitrobenzyl)-γ-aminobutyric acid. Hence, 2-nitrophenyl-α-oxoacetate, prepared from selenium dioxide oxidation (Hatanaka & Ishimaru, 1973) of 2-nitroacetophenone in pyridine (21% yield), was reductively aminated with γ-aminobutyric acid in the presence of sodium borohydride to yield *N*-(α-carboxy-2-nitrobenzyl)-γ-aminobutyric acid with 28% yield.

Scheme 1



The application of caged compounds for the investigation of rapid kinetic processes depends critically on the ability of the photolysis reaction to give the desired products rapidly and with good yield (Kaplan & Somlyo, 1989). It is therefore necessary to determine the rate of the photodeprotection reaction and confirm the release of the products. The rate-limiting step of the internal redox reaction of *o*-nitrobenzyl photodeprotection is identified with the disappearance of an *aci*-nitro intermediate (Schupp et al., 1987). Figure 1a shows the transient absorbance of the intermediate, which is produced by photolysis of **C-G-III** in pH 7.5 buffer with a single 10-ns pulse from an excimer laser at 308 nm. The half-life of the transient part of the signal is 1.5 ms at this pH. The half-life increases with the pH of the photolysis buffer from 0.5 ms at pH 4.5 to 2.5 ms at pH 11.5. The effect of pH on the rate of disappearance of the transient intermediate is much smaller than was observed for analogous photolabile precursors of biologically active compounds with leaving groups attached to the nitrobenzyl photoprotecting group by carbamate, phosphate, and amide linkages (Milburn et al., 1989; McCray & Trentham, 1989; Ramesh et al., 1993). The half-lives ($\sim$1.5 ms) of intermediate absorbance of **C-G-I** and **-II** at pH 7.5 were identical to the half-life of the **C-G-III** signal within experimental error. Figure 1a shows that a baseline absorbance shift at 440 nm, which persists after the transient absorbance has disappeared, has a magnitude that is larger than the transient absorbance. The magnitude of both the transient and baseline shift absorbance signals increases in buffer at higher pH.

The spectral distribution of the transient absorption has been shown to be characteristic of the *aci*-nitro intermediate (Wettermark, 1962a,b; Schupp et al., 1987). In Figure 1b, the *aci*-nitro intermediate spectrum is shown; construction of the spectrum required deconvolution of the contribution of the transient signal absorbance from the persistent shift of the baseline absorbance. The average baseline shift, determined 20 ms after initiation of the photolysis, was subtracted from the transient signal absorbance maximum. Spectral data were collected at 10-nm intervals over the range 380–500 nm to give the absorbance of the transient intermediate. The resulting spectral distribution of the photolysis intermediate of **C-G-III** resembles the spectra of the carboxyl derivative of amine-linked glycine (Billington et al., 1992), analogous carboxyl derivatives of caged carbamoylcholine (Milburn et al., 1989), and amino acid amides (Ramesh et al., 1993). It is important to note that these characteristic *aci*-nitro spectra are only produced after subtraction of the shift of the baseline absorbance that remains constant during a 200-ms time period of observation. Since the maximum transient absorbance is observed within microseconds after the laser pulse, we assume that the photolysis product responsible for the nondecaying absorbance change is produced in a similar time region. The absorbance profile of the baseline shift remaining after subtraction of the decay of the transient signal (data not shown) is similar to the absorbance change produced by the photoproducts of the reaction over the range 350–500 nm (see Figure 2). The 350-nm end point represents the lower wavelength limit of the transient spectrophotometer so a full comparison of the spectra was not possible. Also, the absorbance of the *aci*-nitro transient is greater at higher pH; the measured transient absorbance increases 3-fold between pH 5.0 and 10.5. There appears to be a correlation between the magnitude of the *aci*-nitro intermediate absorbance and the quantum yield (see below). The *aci*-nitro transient absorbance observed with the amine-linked caged compounds is less intense than the absorbance transient observed with the 2-nitrobenzyl-α-carboxy derivatives of carbamoylcholine (Milburn et al., 1989) and amino acid amides (Ramesh et al., 1993) observed under similar photolysis conditions.

The formation of products and disappearance of the caged derivative was followed by measuring the UV–vis spectrum of the reaction mixture during photolysis. Figure 2 illustrates the changes in the UV–vis absorption spectra of **C-G-III** solutions buffered at pH 5.0 (Figure 2a) and 10.5 (Figure 2b)

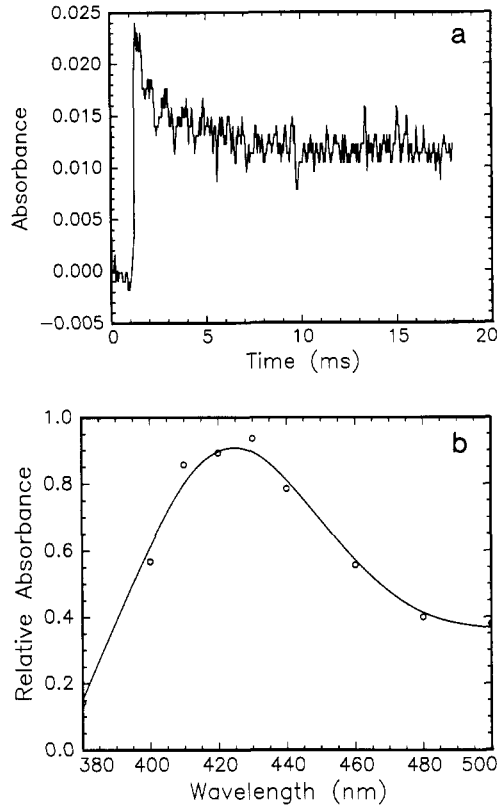


FIGURE 1: (a) *aci*-Nitro intermediate absorbance decay of a 2 mM solution of **C-G-III** in 100 mM phosphate buffer at pH 7.5 and 22 °C. The transient absorbance was monitored at 440 nm over a 2-mm path length in a thin section of solution exposed to the UV light as it entered the cuvette in order to obtain the best signal-to-noise ratio. A single excitation pulse was produced by an excimer laser at 308 nm with an energy at the cuvette face of 65 mJ. (b) Spectral distribution of the transient *aci*-nitro absorbance. The conditions of photolysis are the same as in panel a. The permanent baseline shift appearing after photolysis has been subtracted from the transient part to generate the *aci*-nitro spectrum. The spectrum of the permanent shift (not shown) reflects the spectrum of the photolysis products over the wavelength range 350–500 nm.

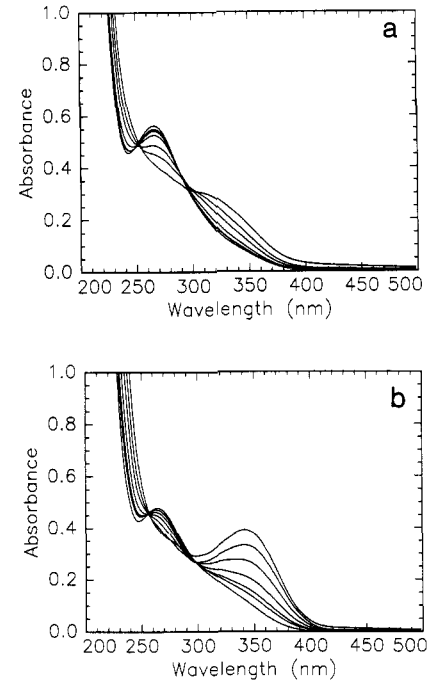


FIGURE 2: Photolysis product spectra. Solutions of **C-G-III** at concentrations of 0.7 mM in 100 mM buffer (panel a, acetate, pH 5.0; panel b, carbonate, pH 10.5) were photolyzed by single flashes of UV light at 308 nm from the excimer laser. The lower trace at 330 nm in each series corresponds to the spectrum of unphotolyzed starting material and the next spectra in succession were produced by photolysis with respectively 1, 2, 4, 10, 20, and 50 laser shots.

Table 1: Estimation of Photorelease of GABA and Quantum Yield (Φ) Determination for **C-G-I**, **C-G-II**, and **C-G-III** by HPLC[a]

pH	no. of shots	C-G-I		C-G-II		C-G-III	
		% conv	Φ	% conv	Φ	% conv	Φ
5.5	1	0.04	0.004	0.29	0.036	0.42	0.052
5.5	2	0.08	0.004	0.62	0.040	0.95	0.060
7.5	1	0.2	0.024	0.33	0.040	0.26	0.032
7.5	2	0.4	0.024	0.62	0.040	0.66	0.048
10.5	1	0.55	0.068	1.31	0.160	0.82	0.10
10.5	2	1.1	0.068	2.32	0.144	1.5	0.092

[a] Excimer laser incident pulse energy per shot was 28–30 mJ at 308 nm; absorbed laser energy was 4–5 mJ. The total volume of 35 μL in a 2- × 2-mm cuvette was irradiated. Solutions were 4.1 mM in 10 mM buffer (pH 5.5, acetate; pH 7.5, phosphate; pH 10.5, bicarbonate). The half-life values of the *aci*-nitro decay at 440 nm, pH 7.5, and 22 °C for compounds **C-G-I**, **C-G-II**, and **C-G-III** are 1.5 ms.

that occur following photolysis with 5-mJ pulses of 308-nm laser light. The spectra were obtained by irradiating the solution in a 2- × 2-mm cuvette and then immediately transferring the cuvette to a diode array spectrophotometer to acquire the UV–vis spectrum from 200 to 800 nm. No absorbance changes were found above 500 nm. Photolysis with 308-nm light was repeated until no further changes in the spectra were produced; that limit was 50 laser pulses with these irradiation conditions. The absorbance traces began to deviate from the clearly defined isosbestic points of earlier spectra after 100 or more laser pulses, indicating the possibility of secondary photolysis of the product mixture. The increase in absorbance at 330 nm reflects the conversion to products, which presumably include the nitroso keto acid side product of the reaction (Chow, 1982). At wavelengths above 400 nm there is a shoulder in the absorbance of the products. The baseline shift seen in Figure 1 reflects the appearance of products. Therefore, the spectral changes that occur during the first 10 ms after initiation of photolysis may be interpreted as a convolution of the *aci*-nitro intermediate absorbance decay with an absorbance signal arising from the formation of the photolysis products.

The pH of the photolysis medium also affected the yields of the photoproducts of each of the caged GABA compounds. The quantum yields of the **C-G-I**, **-II**, and **-III** derivatives at pH 5.0, 7.5, and 10.5 are given in Table 1. Solutions of the three derivatives were photolyzed in buffer by single 5-mJ shots of 308-nm light from an excimer laser. Conditions of irradiation and concentration were chosen such that photoproducts were produced at the level of less than 5% of the starting material. These conditions minimize the possibility of side reactions and secondary photolysis of products which can occur when multiple laser shots are used to photolyze the material. The amount of free GABA in the trial solutions was measured by derivatizing the amino acid with OPA to produce a fluorescent derivative that could be quantitated by standard HPLC methods (Jones et al., 1981). Measurements were made with the unphotolyzed solutions and after one and two shots. The values reported in Table 1 were produced by subtracting the background concentration found for the unphotolyzed solution from concentrations found of the photolyzed samples. Control measurements were done to ensure that the different pHs and buffer constituents used for the photolysis had no influence on the OPA derivatization or HPLC measurements. The differences in yield shown in Table

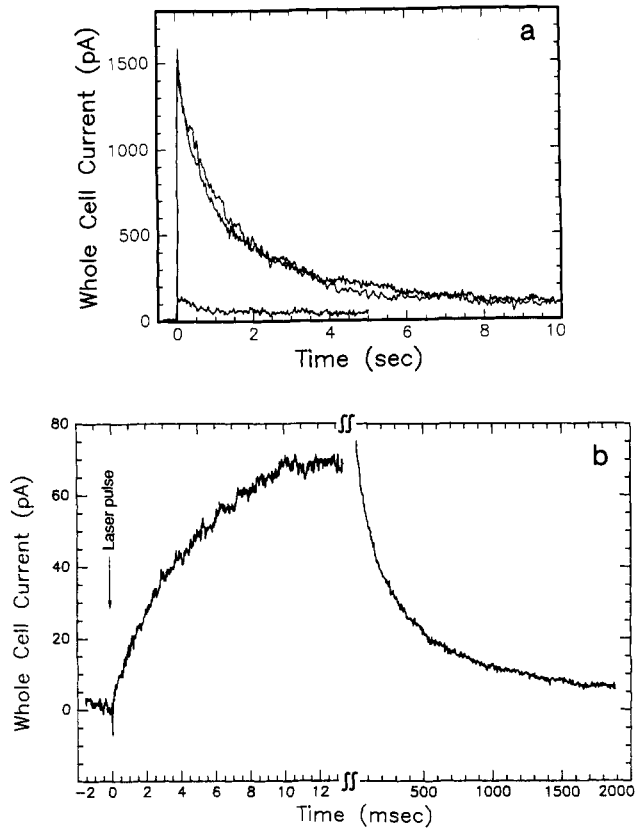


FIGURE 3: (a) Whole-cell current responses of embryonic mouse cerebral cortical cells, pH 7.3, 21–23 °C, at a transmembrane voltage V_m of −70 mV. (a) Cell-flow experiments. The upper two curves represent experiments in which the cell was equilibrated with 250 μM GABA in the absence and presence of 10 mM **C-G-III**. In the lower trace the concentration of **C-G-III** was 10 mM. (b) Laser-pulse photolysis experiment. The cell was exposed to a 500 μM solution of **C-G-III** in extracellular buffer for 3 s before being photolyzed by a laser pulse (308 nm, ~500 μJ). In a cell-flow experiment with the same cell, 250 μM GABA produced a maximum current amplitude of 2.2 nA.

I are directly attributable to the effect of pH on quantum yield. The maximum production of all three derivatives was seen at pH 10.5. The yield of photoreleased GABA depends on the pH of the irradiation medium. For **C-G-I**, the product yield of GABA increases 5-fold from pH 5.0 to 7.5; from pH 7.5 to 10.5 there is a 2.8-fold increase. The α-methyl derivative, **C-G-II**, shows no significant difference in yield at pH 5 and 7.5 but exhibits a 4-fold increase at pH 10.5. The α-carboxyl derivative, **C-G-III**, shows a small decrease in yield from pH 5 to 7.5 but the yield increases 2.7 times from pH 7.5 to 10.5.

Comparison of the series of spectra performed at pH 5.0 and 10.5 (Figure 2) indicates that the reaction proceeds with greater yield at pH 10.5 and thus supports the conclusions drawn from the HPLC data given in Table 1. It is important to note that a shift in pH from 5.0 to 10.5 produces changes in the spectra that partially mask the changes that occur with photolysis (see Figure 2; 330 nm). However, spectra measured at each of these pH values before and after photolysis confirm that the relative magnitude of the absorbance increase seen at 330 nm correlates with the amount of product released and that the differences seen are not merely effects of the pH change. Thus, overall photoproduct yield for these amine-linked caged compounds is determined by pH.

The three derivatives **C-G-I**, **-II**, and **-III** were used to activate whole-cell currents in fetal mouse cortical neurons in culture. The experiment shown in Figure 3a indicates that in cell-flow experiments with 250 μM GABA the whole-cell current response is the same in the absence and presence of 10 mM **C-G-III**. It was found that aqueous solutions of the caged material slowly decompose under room light to produce free GABA, so the purification, storage, and handling of the compounds must be performed under low-light conditions. When the caged compounds are photolyzed at 308 nm it is especially important to adjust the laser energy below the damage threshold for the cell (Rapp et al., 1989). The whole-cell current response shown in the lower trace in Figure 3a was produced by flow of a solution of 10 mM caged **C-G-III** over the voltage-clamped neuron. It does show a small activation current that is attributable to residual free GABA in the solution. A normalized dose–response calibration curve for GABA (Geetha & Hess, 1992), and the current produced by flow of the 250 μM control GABA solution over the cell, are used to determine the amount of free GABA in the unphotolyzed caged compound solution. In the example shown in Figure 3a, the lower trace indicates that the 10 mM solution of caged GABA contains approximately 18 μM free GABA. This concentration of free GABA was further confirmed by HPLC analysis of the OPA derivative of the same solution used for the flow experiment. Thus, the response seen in the lower trace of Figure 3a can be explained by contamination of the caged compound solution by free GABA. Typically, no detectable response was produced by freshly prepared caged compound solutions up to concentrations of 500 μM when carefully protected from room light.

Figure 3b shows the response produced by photolysis of the caged GABA at the surface of a voltage-clamped neuron by a single flash of 308-nm UV light from an excimer laser. The cell response to a 250 μM GABA solution and the known dose response of the cortical neuron preparation to free GABA was again used to calibrate the concentration of free GABA released by photolysis. The concentration of free GABA produced by photolysis of 500 μM **C-G-III** was calculated in this example to be 25 μM. The maximum release of free GABA at the surface of a voltage-clamped neuron suspended in solution is influenced by the concentration of caged compound and the energy of UV laser light delivered from the fiber optic to the cell surface. The 308-nm light fluence was set just below the empirically determined damage threshold for the suspended cell. The second half of the trace in Figure 3b shows the falling phase of the signal, monitored after a second laser flash on the same cell using a longer time scale for the data acquisition. The observed rate of desensitization after photolysis activation of the current response is faster than the rate produced by flow of 25 μM GABA over the cell. The cell is equilibrated with caged compound using the cell-flow technique (Udgaonkar & Hess, 1987) and the laser pulse is applied while the solution containing the caged compound is still flowing over the cell. The decaying phase of the current amplitude is attributable, therefore, to two processes: receptor desensitization and the movement of the solution containing the GABA liberated by the laser pulse away from the cell.

DISCUSSION

The synthesis of the photolabile GABA precursors is accomplished from readily available starting materials with good yields. It is important to note that manipulations of these compounds in aqueous solution must be performed in such a way as to protect the solutions from light whenever possible. This is especially critical during the purification steps. A sensitive HPLC method, such as the one used here, is useful for monitoring the amount of free GABA in prepared solutions. The compounds are all readily soluble in aqueous buffers in the range pH 5–11. The new caged derivatives

provide a source for rapidly producing free GABA. The caged compound functions as an inert pool of sequestered amino acid until photolysis by a pulse of UV light. When a light source similar to the fiber optic employed here for photolysis on individual neurons is used, the production of free GABA may be precisely controlled in volumes below 1 μL. Therefore, these caged GABA compounds could be useful in studies where spatial and temporal control of free GABA is important.

At least two different receptors can be distinguished on the basis of rapid chemical kinetic measurements of transmembrane flux of anions in rat brain membrane vesicles (Cash & Subbarao, 1987, 1988, 1989) and in mouse cortical neurons (Geetha & Hess, 1992). Desensitization of both receptor forms is rapid with rates of 4.4 and 0.7 s^{-1}; a kinetic technique with time resolution longer than 100 ms will sample a population of receptors that is predominantly in a desensitized state. The rate of channel opening is faster than desensitization, and kinetic measurements of it require techniques for activating the channel with at least millisecond time resolution (Matsubara et al., 1992). The ability to rapidly produce free GABA at the cell membrane of a voltage-clamped neuron can be used to determine effects of activators and inhibitors on the initial rapid steps of the mechanism, which lead to channel opening, without interference by onset of desensitization. It was shown that single pulses of light released free GABA from the caged derivatives **C-G-I**, **-II**, and **-III** and that this can be used to activate ion currents on the receptors of cortical neurons in culture. We mainly used **C-G-III** in experiments with cells. This compound is photolyzed with the same rate and quantum yield as the other two caged GABA derivatives we synthesized. The caging group and the nitroso keto side product expected in the photolysis of **C-G-III** do not affect the acetylcholine receptor in BC_3H1 cells (Milburn et al., 1989; Matsubara et al., 1992; Niu & Hess, 1993). In contrast, the nitroso side product expected in the photolysis of **C-G-I** and **C-G-II** has been associated with adverse effects on biological preparations (Kaplan et al., 1978; Walker et al., 1988). The inherently rapid receptor activation provided by the laser-pulse photolysis technique using one of the GABA derivatives reported here can provide new information about opening and closing rates of the receptor–channel.

The assignment of the rapid absorption decay seen during photolysis to an *aci*-nitro intermediate relies on the similarity of the spectrum of the intermediate to known examples of *aci*-nitro spectra in other compounds (Wettermark, 1962a). The substituent on the benzylic carbon has been shown to produce characteristic absorbance maxima. For example, when the α-substituent is a hydrogen or carboxy group, the *aci*-nitro signal has a maximum around 435 nm, whereas a methyl group shifts the maximum to lower wavelengths, typically 420 nm. This was found for caged compounds with a variety of leaving groups: carbamate (Milburn et al., 1989), amide (Ramesh et al., 1993), caged glycine (Billington et al., 1992), and the α-carboxyl amine derivatives reported here. However, the photolytic intermediate observed for caged ATP with a phosphate ester leaving group and an α-methyl substituent produces an absorbance maximum at a lower wavelength [at 380 nm at pH 8.0 reported by McCray et al. (1980) and at 406 nm at pH 6.7 by Walker et al. (1988)]. The relatively large absorbance baseline shift produced by photolysis of **C-G-III** was subtracted to separate out the contribution of the transient part of the signal. This operation is valid as long as the contribution of the baseline shift has a rate of appearance as fast as the appearance of the *aci*-nitro intermediate.

The pH of the photolysis medium influences the rate of the *aci*-nitro intermediate decay and, presumably, the rate of product release as well. The carbamate (Walker et al., 1986) and amide linkages (Ramesh et al., 1993) showed strong dependence of the intermediate decay rates on pH when observed at the absorbance maximum of the intermediates. Faster rates were found at lower pH in all cases. In contrast to this, pH has much less influence on the relative rates for each of the amine-linked compounds that we report here.

The data on the photoproduct yields (Table 1) shows the effects of pH on the photolysis of the *o*-nitrobenzyl protecting group. Higher quantum yields are found at the upper pH for each of the GABA amine derivatives investigated here. The overall product yield of GABA is dependent on the energy of the radiation used for photolysis and on the inherent quantum yield of each compound. The maximum yield of free GABA is limited practically by the ability of the target preparation (in this case isolated cortical neurons) to withstand increasing levels of UV light fluence from the photolysis source. The *o*-nitrobenzyl group has been used with other linkages to produce photolabile compounds with distinctly different yield behavior in aqueous buffers than that observed here. Patchornik et al. (1970) reported a maximum yield of some amino acid derivatives when the carbamates were photolyzed below pH 1. In another study (Hayes et al., 1985), the yield of amine nucleotides and nucleic acids increased when the photodeprotection was carried out under low pH conditions (pH 3.5) rather than higher pHs. Schupp et al. (1987) have shown that the quantum yield is independent of pH for a series of 2-nitrobenzyl caged esters that they studied. Thus, there is evidence that the photolysis mechanism may differ for each linkage that has been studied.

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