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Chemistry

Photolabile precursors of glutamate: Synthesis, photochemical properties, and activation of glutamate receptors on a microsecond time scale

(caged compounds/caged glutamate)

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ABSTRACT **Newly synthesized photolabile derivatives of glutamate, caged glutamate, that release free glutamate on a microsecond time scale after a pulse of UV laser light are described. 2-Nitrobenzyl derivatives were attached to the amino or carboxyl groups of glutamate. Substitution with a $-CO_2^-$ group at the benzylic carbon accelerates the photolysis reaction when compared to -H and $-CH_3$ substituents. γ-*O*-(α-Carboxy-2-nitrobenzyl)glutamate is stable at neutral pH. In 100 mM phosphate buffer at pH 7.0, the compound is photolyzed at 308 nm with a quantum product yield of 0.14. The half-life of the major component of the photolytic reaction, as judged by the transient absorbance change at 430 nm, is 21 μs (≈90%); the half-life of a minor component (≈10%) is 0.2 ms. The amine-linked derivatives have half-lives in the millisecond region and a 4-fold lower quantum yield. The potential of the newly synthesized compound for use in rapid chemical kinetic investigations of glutamate receptors is demonstrated. (*i*) The caged glutamate at 1 mM concentration does not desensitize glutamate receptors in rat hippocampal neurons. (*ii*) Caged glutamate (1 mM) does not inhibit activation of the receptors by 50 μM glutamate. (*iii*) Photolysis of the compound induces rapid onset of transmembrane currents in rat hippocampal neurons.**

The main excitatory receptors in the central nervous system are members of a family of membrane-bound proteins and are activated by glutamate (1, 2). The receptors are believed to be involved in learning and memory and pathological phenomena such as ischemia-related neuronal death (reviewed in ref. 2). Activation of signal transmission in the mammalian nervous system can occur on a submillisecond time scale. Rapid desensitization (transient inactivation) of glutamate receptors in the millisecond time region has been observed (3). For this reason, chemical kinetic investigations of such processes must employ techniques with an equivalent (or better) time resolution (4).

One strategy to overcome limitations in time resolution imposed by slow diffusion and mixing of reactants is photolytic release of an active substance from a precursor of the desired compound (a "caged" compound) (for reviews, see refs. 5–7). The first practical example of a photolabile precursor of a neurotransmitter was a 2-nitrobenzyl derivative of carbamoylcholine, a specific ligand for the acetylcholine receptor (8). Introduction of the α-carboxyl-2-nitrobenzyl (αCNB) group to protect the amino group of carbamoylcholine (9) led to a compound that could be photolyzed by a nanosecond pulse of UV light to carbamoylcholine with a quantum yield of 0.8 and a $t_{1/2}$ value of 45 μs. The compound has been used in kinetic investigations of the acetylcholine receptor in BC_3H1 muscle cells (10, 11) and in mapping the distribution of receptor sites on the surface of these cells (12).

Photolytic release of glutamate from the α-(4,5-dimethoxy-2-nitrobenzyl) ester of L-glutamate (13) has been used to map functional connections between neurons in tissue slice preparations from mammalian cortex (14). Another caged glutamate derivative, *N*-1-(2-nitrophenyl)ethoxycarbonyl-L-glutamate, was used to provide evidence for the role of glutamate as a neurotransmitter in the squid giant axon (15).

We report here the synthesis and photochemical characteristics of two additional series of caged glutamate derivatives. The first series links the photolabile protecting group to the α-amino position of glutamate, while the second series is protected at either the α- or γ-carboxyl group. The carboxyl-linked compound has a 4-fold higher product quantum yield. The experiments described indicate that the αCNB group has the most desirable properties of those so far discovered as a photolabile protecting group for the carboxyl group of neurotransmitters and amino acids.

MATERIALS AND METHODS

Synthesis. *1-(2-Nitrophenyl)ethyl bromide (1).* A yellow solution of 1-(2-nitrophenyl)ethanol (1.00 g; 5.98 mmol) (16) in 33% HBr/HOAc (20 ml) was heated at 75°C for 1 hr and then diluted with water. The resulting mixture was extracted with EtOAc (2 × 30 ml). The extract was washed with saturated sodium bicarbonate (1 × 20 ml), dried (sodium sulfate), and concentrated to give a yellow liquid. This liquid was purified by flash chromatography (10–20% EtOAc/hexanes) to give **1** as 1.12 g of a yellow liquid (81%): R_f 0.78 (5% EtOAc/$CHCl_3$); ^1H NMR (C^2HCl_3) 7.89 (d, J = 8.1 Hz, 1H, C_3 H), 7.83 (d, J = 8.1 Hz, 1H, C_6 H), 7.64 (t, J = 8.2 Hz, 1H, C_5 H), 7.43 (t, J = 8.2 Hz, 1H, C_4 H), 5.81 (q, J = 6.9 Hz, 1H, CHBr), 2.08 (d, J = 7.1 Hz, 3H, CH_3).

t-Butyl-(2-bromo-2-nitrophenyl) acetate (2). *t*-Butyl-(2-nitrophenyl) acetate was prepared by reaction of 2-nitrophenylacetic acid with oxalyl chloride to produce the acid chloride and then esterified with *t*-butyl alcohol. To a solution of *t*-butyl-(2-nitrophenyl) acetate (1.81 g; 7.63 mmol) and benzoyl peroxide (40 mg) in CCl_4 (75 ml) was added *N*-bromosuccinimide (NBS) (1.42 g; 8.00 mmol). The resulting mixture was refluxed for 90 hr and then cooled and filtered. The filtrate was charged with more benzoyl peroxide (40 mg) and NBS (1.44 g; 8.1 mmol) and refluxing was resumed for a further 48 hr. The filtrate was again recharged with benzoyl

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Abbreviations: αCNB, α-carboxy-2-nitrobenzyl; RT, room temperature; TFA, trifluoroacetic acid.

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peroxide (30 mg) and NBS (1.3 g; 7.3 mmol) and refluxed for 48 hr. After cooling and filtration, the filtrate was concentrated *in vacuo* to give a red-yellow oil that contained **2** and unreacted starting ester. Compound **2** was obtained as 1.72 g (71%) of a pale yellow solid via flash chromatography (100 g of silica gel; dichloromethane/hexanes, 1:1); m.p. 29–31°C; R_f 0.36 (dichloromethane/hexanes, 1:1; two elutions); ^{1}H NMR (C^2HCl_3) 8.0 (dd, J = 8.1, 1.3 Hz, 2H, C_3H and C_5 H), 7.68 (dt, J = 8.0, 1.2 Hz, 1H, C_4 H), 7.51 (dt, J = 7.7, 1.3 Hz, 1H, C_6H), 5.96 (s, 1H, CHBr), 1.47 [s, 9H, $C(CH_3)_3$]. Analysis calculated for $C_{12}H_{14}NO_4Br$: C, 45.59; H, 4.46; N 4.43. Found: C, 45.06; H, 4.44; N, 4.31.

γ-(α-Carboxy-2-nitrobenzyl)-L-glutamic acid ester (3). A solution of *N*-t-Boc-L-glutamic acid, α-*t*-butyl ester (240 mg; 0.79 mmol; Sigma), *t*-butyl-(2-bromo-2-nitrophenyl) acetate (**2**; 250 mg; 0.79 mmol), and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (125 mg; 0.82 mmol) in benzene (10 ml) was refluxed for 5 hr. After cooling, the reaction mixture was partitioned between ethyl acetate (20 ml) and water (20 ml). The organic layer was dried (sodium sulfate) and concentrated to give a pale brown oil, which was purified by flash chromatography (0–5% EtOAc/$CHCl_3$) to give the alkylated and still protected product as 0.34 g (80%) of a clear colorless oil: R_f 0.35 (5% EtOAc/$CHCl_3$). Deprotection was accomplished at room temperature (RT) under argon by addition of 30% trifluoroacetic acid (TFA)/$CHCl_3$ (10 ml) to the product of the previous reaction and letting the mixture stand for 3 hr. The volatile compounds were removed *in vacuo*, and toluene (2 × 15 ml) was evaporated from the residual pale brown oil. This oil was purified by chromatography on Sephadex LH-20, using water as eluant, giving **3** as 135 mg (50%) of a fluffy white powder after lyophilization; m.p. 122–125°C (dec.); ^{1}H NMR (^2H_2O) 8.13 (d, J = 7.8 Hz, 1H, C_3 H), 7.80 (m, 1H, C_4 H), 7.7 (m, 2H, C_4 H and C_6 H), 6.68 (s, 1H, ArCH), 4.08 (t, J = 6.4 Hz, 1H, CO_2CHN), 2.7 (m, 2H, $CO_2CH_2CH_2$), 2.25 (m, 2H, $CO_2CH_2CH_2$). Analysis calculated for $C_{15}H_{15}N_2O_{10}F_3$: C, 40.92; H, 3.43; N, 6.36. Found: C, 42.27; H, 3.81; N, 6.74.

α-(α-Carboxy-2-nitrobenzyl)-L-glutamic acid ester (4). A solution of *N*-t-Boc-L-glutamic acid, γ-*t*-butyl ester (221 mg; 0.73 mmol), bromide **2** (0.21 g, 0.66 mmol), and DBU (116 mg; 0.76 mmol) in benzene (10 ml) was refluxed overnight. After cooling, water (30 ml) was added and the layers separated. The aqueous layer was extracted with ethyl acetate (2 × 15 ml). The combined organic portions were washed with brine once, dried over sodium sulfate, and concentrated *in vacuo* to give 0.37 g of a brown oil, which was purified by flash chromatography (0–5% EtOAc/$CHCl_3$) to give the protected product as 0.30 g (84%) of a clear colorless oil as a 1:1 mixture of diastereomers. A solution of the alkylated product (0.29 g; 0.54 mmol) in 40% TFA/$CHCl_3$ (10 ml) was allowed to stand at RT for 24 hr and then concentrated *in vacuo*. Benzene (2 × 10 ml) was evaporated from the residue, leaving a pale brown oil. This oil was dissolved in water (20 ml) and lyophilized, giving the title compound as 22 mg (94%) of a pale brown crystalline solid that was a mixture of diastereomers: ^{1}H NMR (^2H_2O) 8.17 (d, J = 8.1 Hz, 1H, C_3 H), 7.84 (t, J = 7.6 Hz, 1H, C_5 H), 7.70 (m, 2H, C_4 H and C_6 H), 6.8 (two s, 1H, $ArCHCO_2$), 4.4 (two t, J = 6.6 Hz, 1H, CO_2CHN), 2.76 (dt, J = 8.0, 1.9 Hz, 1H, $CO_2CH_2CH_2$), 2.6–2.2 (m, 3H, $CO_2CH_2CH_2$). An analytical sample was prepared by chromatography on Sephadex LH-20, giving a fluffy white powder; m.p. 130–134°C (dec.). Analysis calculated for $C_{15}H_{15}N_2O_{10}F_3$: C, 40.92; H, 3.43; N, 6.36. Found: C, 41.45; H, 3.82; N, 6.62.

γ-(α-Methyl-2-nitrobenzyl)glutamic acid ester (5). A mixture of 2-nitroacetophenone hydrazone (180 mg; 1.0 mmol), MnO_2 (700 mg; 8.05 mmol), and $CHCl_3$ (8 ml) was stirred for 10 min in the dark at RT. The solution was filtered through a pad of Celite, washed with more $CHCl_3$, and cooled to 0°C. *N*-t-Boc-α-*t*-butyl-L-glutamic acid (150 mg; 0.5 mmol) was added to the diazo solution and was stirred overnight. The solution was filtered, dried over sodium sulfate, and then slurried with silica gel. The product was eluted twice with 5 ml of hexane/diethyl ether (1:1). The solvent was removed *in vacuo* to yield 130 mg (60%) of yellow solid. Deprotection was accomplished by mixing the entire product with 2 ml of ethyl acetate saturated with dry HCl cooled with a dry ice/ethanol bath. The mixture was allowed to warm to RT overnight. The solid product was recovered by filtration and washing with ethyl acetate. ^{1}H NMR (C^2HCl_3): 7.95 (d, J = 8.15, 1H), 7.63 (m, 2H), 7.42 (m, 1H), 6.32 (q, J = 7.35, 1H, benzylic CH), 4.19 (m, 1H, α-CH), 2.4 (m, 2H, $CO_2CH_2CH_2$), 1.8 (m, 2H, $CO_2CH_2CH_2$). Analysis calculated for $C_{13}H_{17}O_6N_2Cl$: C, 46.93, H, 5.15, N, 8.42; Found: C, 46.72, H, 5.15, N, 8.22.

N-(α-Carboxy-2-nitrobenzyl)glutamic acid (6). To a solution of L-glutamic di(*t*-butyl) ester hydrochloride (300 mg; 1.01 mmol) and bromide **2** (304 mg; 0.96 mmol) in acetonitrile (7 ml) was added potassium carbonate (304 mg; 2.2 mmol). The resulting mixture was stirred at 50°C for 48 hr; more glutamate diester (44 mg; 0.15 mmol) was added, and heating was continued for another 24 hr. Water was added to dissolve the solids, and the resulting mixture was extracted with ethyl acetate (2 × 20 ml). The extract was dried over sodium sulfate and concentrated *in vacuo* to give 0.51 g of a pale yellow oil. The diastereomeric products were separated by flash chromatography, using $CHCl_3$/hexanes (3:1–1:0) as eluant. The diastereomers were treated separately with 40% TFA/dichloromethane (10 ml). After 20 hr at RT, the volatile compounds were removed *in vacuo* to give pale brown immobile oils. The crude product from the faster eluting diastereomer was purified by chromatography on Sephadex LH-20, using water as eluant, giving 44 mg of a fluffy white powder (36%); m.p. 112–116°C (dec.); ^{1}H NMR (^2H_2O) 8.30 (d, J = 8.1 Hz, 1H, C_3 H), 7.90 (t, J = 7.4 Hz, 1H, C_5 H), 7.82 (t, J = 7.7 Hz, 1H, C_4 H), 7.70 (d, J = 7.4 Hz, 1H, C_6 H), 5.60 (s, 1H, $ArCHCO_2$), 3.98 (t, J = 6.0 Hz, 1H CO_2CHN), 2.6 (m, 2H, $CO_2CH_2CH_2$), 2.25 (m, 2H, $CO_2CH_2CH_2$). Analysis calculated for $C_{15}H_{15}N_2O_{10}F_3$: C, 40.92; H, 3.43; N, 6.36. Found: C, 41.61; H, 3.45; N, 6.39. The crude product from the slower-eluting diastereomer was purified similarly to give 90 mg of a fluffy, pale yellow powder (60%); m.p. 126–129°C (dec.). Preparation of **6** has been reported in a review (6) but synthesis and characterization of the compound have not been published.

N-(α-Methyl-2-nitrophenyl)-L-glutamic acid (7). To a pale yellow solution of γ-*t*-butyl glutamic acid ester (900 mg; 3.04 mmol) and 1-(2-nitrophenyl)ethyl bromide (**1**; 0.64 g; 2.8 mmol) in anhydrous acetonitrile (20 ml) at RT were added potassium carbonate (0.90 g; 6.5 mmol) and catalytic sodium iodide (50 mg). The resulting mixture was stirred at 60°C for 5 days and then filtered. The filtrate was concentrated *in vacuo* to give a pale brown residue, which was purified by flash chromatography using EtOAc/$CHCl_3$ (0–5%) as eluant. The product diastereomers were obtained together as 0.60 g of a clear, pale brown oil (53%): R_f 0.54, 0.48 (5% EtOAc/$CHCl_3$). A solution of the protected products (0.17 g; 0.42 mmol) in 40% TFA/$CHCl_3$ (5 ml) was allowed to stand at RT overnight. The volatile compounds were removed *in vacuo*, and benzene (1 × 10 ml) was evaporated from the residue, leaving a brown oil. This oil was purified by chromatography on Sephadex LH-20 using water as eluant, giving the title product as a mixture of diastereomers as 0.16 g of a pale brown hygroscopic foam (94%): ^{1}H NMR (^2H_2O) 8.06 (d, J = 8.1 Hz, 2H, C_3 H), 7.80 (m, 4H, C_4 H and C_5 H), 7.69 (m, 2H, C_6 H), 5.19 (q, J = 6.7 Hz, 1H, ArCH), 5.14 (q, J = 6.8 Hz, 1H, ArCH), 4.06 (dd, J = 8.4, 5.0 Hz, 1H, CO_2CHN), 3.81 (t, J = 6.6 Hz, 1H, CO_2CHN), 2.5 (m, 4H, $CO_2CH_2CH_2$), 2.3–2.1 (m, 4H, $CO_2CH_2CH_2$), 1.75 (t, J = 6.5 Hz, 6H,

ArCHCH$_3$). Analysis calculated for $C_{15}H_{17}N_2O_8F_3 \cdot H_2O$: C, 42.06; H, 4.47; N, 6.54. Found: C, 41.95; H, 4.00; N, 6.95.

Laser-Pulse Photolysis and Transient Spectral Measurements. The equipment used for transient absorption spectroscopy has been described (17). Briefly, pulses of 308-nm light from an excimer laser initiated photolysis, and transient absorbance signals were digitized at rates up to 2 MHz. A nonlinear least-squares analysis program, GENPLOT (Computer Graphics, Ithaca, NY), was used to fit one- or two-component exponential functions to the transient decay signals measured between 380 and 500 nm.

Quantum Product Yield. A 15-μl aliquot of caged glutamate solution [1–5 mM in 100 mM phosphate buffer (pH 7.4)] was photolyzed in a 0.2-mm quartz cuvette with 308-nm pulsed UV light from the excimer laser; 15–25 mJ was absorbed by the solution in each trial as measured by an energy meter (Gentec, Palo Alto, CA). Analytical HPLC was performed with a Waters Novapak C_{18} reversed-phase column (300 × 4.5 mm) to determine the amount of free glutamate released in the photolysis. *o*-Phthaldialdehye labeling (OPA reagent; Sigma) was used to derivatize the free amino acid, which elutes at $k' = 3.4$ using fluorescence detection and isocratic elution at 1 ml/min with 27% methanol/70% 50 mM phosphate buffer, pH 6.4.

Cell Culture and Whole-Cell Current Recording. Neurons from hippocampi of 1-day-old rats were enzymatically and mechanically separated and cultured on plates treated with collagen (18). Isolated neurons were chosen for whole-cell current recording (19) and lifted intact with attached neurites from the substrate or were caused to form a vesicle by pulling a large membrane patch from the soma of neurons strongly adherent to the substrate (20, 21). The cell or vesicle suspended by the recording electrode was positioned in front of a U-tube flow device (22) used for rapid equilibration of ligands with receptors on the cell or vesicle surface. An optical fiber was used to deliver UV light at 343 nm (300–600 μJ per pulse) from a dye laser to the cell (11). The extracellular recording buffer consisted of 145 mM NaCl, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM Hepes (pH 7.4); no Mg^{2+} or glycine was added. The intracellular electrode buffer was 140 mM CsCl, 1 mM $CaCl_2$, 10 mM EGTA, and 10 mM Hepes (pH 7.2).

RESULTS

Rate of Photoproduct Release. The characteristics of the photochemical reactions of the glutamate derivatives were evaluated with transient absorption spectroscopy after initiation of the reaction by a pulse of UV light. Fig. 1*a* shows the transient absorbance produced by a 308-nm pulse of light from a XeCl excimer laser in a solution of **3** in which the γ-carboxyl group of L-glutamate is protected by the αCNB group. Similar results are obtained for **4** when the α-carboxyl group of glutamate is protected by the αCNB group. The spectral distribution of the intermediate species absorbance, with a maximum at 430 nm (Fig. 1*b*), corresponds to the characteristic *aci*-nitro intermediate in the photolysis reaction (23, 24). Provided that decay of the putative *aci*-nitro intermediate leads to release of the desired compound, as is generally accepted (25, 26), the decay rates may be taken as reflecting the rate of photolytic release of free glutamate. The decay of **3** is a two-exponential process. About 90% of the reaction proceeds with a $t_{1/2}$ value of 21 μs and 10% proceeds with a $t_{1/2}$ value of 0.2 ms. The $t_{1/2}$ values of the decay of the faster component are given in Table 1. The three traces in Fig. 1*b* are constructed from absorbance values determined at different time points in the decay of **3** and show that the spectral distribution of the transient absorbance does not change during the first 50 μs. This suggests that the slower component has a spectral distribution similar to the fast component. The photolysis rates (Table 1) are influenced by the type of bond hydrolyzed (amine or ester) and by the substituents at the α position. For example, there is a large difference between the rates observed for the amine- and ester-linked derivatives. The rate of disappearance of the intermediate of **3** ($t_{1/2}$ = 21 μs) is ≈100 times faster than the corresponding decay of the amines. Photolysis of the amine compounds (**6**) produced an *aci*-nitro intermediate with an absorbance maximum at 430 nm and a transient lifetime of ≈2.6 ms at pH 7.4 in 100 mM phosphate buffer and with a quantum yield of 0.04. The photochemical properties of the two diastereomers of *N*-(α-carboxy-2-nitrobenzyl)-L-glutamic acid (**6a** and **6b**), however, were identical.

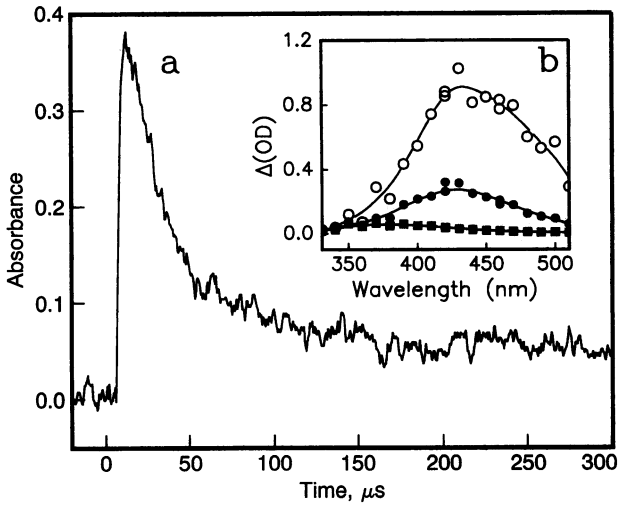

FIG. 1. (*a*) *aci*-Nitro intermediate decay produced by photolysis of 2.4 mM **3** (γ ester of glutamate) in 100 mM phosphate buffer (pH 7.4; 23°C). A 20-ns pulse of laser light at 308 nm was given at time 0 to initiate the reaction. The lifetime of the transient absorbance decay was the same when monitored at 380–500 nm. (*b*) Spectral distribution of the transient absorbance signal. Spectra of the intermediate species were constructed from absorbance transients observed at different wavelengths. ○, Absorbance values measured at the time of maximum absorbance after photolysis; ● and ■, values measured at 50 and 5000 μs, respectively.

Table 1. Photochemical properties of caged glutamate derivatives

Compound	Photolysis $t_{1/2}$*	λ_{max}, nm	Quantum yield
α-CO_2 amine (**6a**)	1.9 ms†	435	0.036 ± 0.011 (n = 6)
α-CO_2 amine (**6b**)	1.7 ms†	435	0.044 ± 0.019 (n = 3)
α-CH_3 amine (**7**)	310 μs‡	430	0.06 ± 0.02 (n = 3)
α-H amine§	3.3 ms¶	425	—
α-CO_2 γ ester (**3**)	21 μs¶	430	0.14 ± 0.05 (n = 4)
α-CO_2 α ester (**4**)	80 μs‡	430	0.16 ± 0.04 (n = 6)
α-CH_3 γ ester (**5**)	78 ms‖	430	—

*Expressed as half-life of the fast part of the intermediate decay, which is ≈90% of the transient signal in each case (see *Results*).
†Solution used to measure rate was 2.0 mM in 100 mM phosphate buffer (pH 7.4).
‡Solution used to measure rate was 2.0 mM in 100 mM phosphate buffer (pH 7.0).
§*N*-(2-Nitrobenzyl)-DL-glutamic acid was synthesized as described for the analogous γ-aminobutyric acid derivative (17) in 30% yield; m.p. 196–197°C. ^{1}H NMR (^2H_2O) 8.09 (d, J = 8.6 Hz, 1H, C_3H), 7.60 (m, 1H, C_4H), 7.55 (m, 2H, C_5 H and C_6H), 4.13 (s, 2H, $ArCH_2$), 3.34 (dd, J = 6.0, 3.2 Hz, 1H, CO_2CHN), 2.19 (m, 2H, $CO_2CH_2CH_2$), 1.8–1.9 (m, 2H, $CO_2CH_2CH_2$).
¶Solution used to measure rate was 2.4 mM in 100 mM phosphate buffer (pH 7.0).
‖Solution used to measure rate was 5.0 mM in 100 mM phosphate buffer (pH 7.0).

The α substituent affects both ester and amine derivatives. The rate of intermediate decay is slower when $-CO_2$ is replaced by $-CH_3$ in the ester-linked series but is slightly faster in the amines. Similar effects have been observed for analogous 2-nitrobenzyl derivatives of amides (27). Also, the γ ester of glutamate (3) photolyzes more rapidly than the α ester (4). Therefore, photolysis is sensitive to changes in configuration close to the bond that hydrolyzes and to substitution at the α position of the protecting group.

Quantum Yield and Hydrolysis. The product quantum yields measured for the derivatives are given in Table 1. Deprotection of the amine bond leads to an overall product yield for released glutamate that is ≈4-fold lower than the yield of corresponding esters. The α substituents do not exert a large influence on the yield for either the amine or ester linkage.

Buffered physiological saline solutions of **4** used for whole-cell studies were found to have a background level of free glutamate that increased with time. Solutions (1 mM) of **3** and **4** were prepared in 10 mM Hepes buffer (pH 7.4). The solutions were held at 27°C; aliquots were sampled at 1, 2, 4, 6, 24, and 48 hr, derivatized with *o*-phthaldialdehyde, and analyzed for free glutamate by HPLC with fluorescence detection. Under these conditions, the α-glutamate ester (**4**) hydrolyzes to produce free glutamate with a $t_{1/2}$ of ≈15 hr, whereas free glutamate in a solution of **3** increased <2% after 2 days.

Activation of Neuronal Glutamate Receptors by Photolysis of Caged Glutamate. Photolysis of the caged glutamate derivatives induced a transmembrane ion current in rat hippocampal neurons and membrane vesicles as monitored by whole-cell recording. Fig. 2 presents examples of currents initiated in neurons by photolysis of caged glutamate (Fig. 2*a*) or by free glutamate used with a cell-flow technique (29) (Fig. 2*b*). The $t_{1/2}$ value of the rising phase of the current (1.1 ms in Fig. 2*a*) represents the lifetime of an open glutamate channel(s). Values reported for the lifetime of open glutamate channels measured by single-channel current recordings are in the 1-ms time scale (e.g., ref. 20). The current decay, indicative of receptor desensitization, occurs in two time zones; only the rapid phase is shown. The maximum observed current, 3 nA, is 20 times larger than the current amplitude observed with the same cell when 300 μM glutamate was delivered to the cell by a flow device (22) (Fig. 2*b*). In addition, in the cell-flow experiment only a relatively slow falling phase of the current is observed. The explanation is that in the flow experiment, receptor desensitization occurs on a time scale comparable to equilibration of receptors on the cell surface with glutamate. Consequently, the receptor form associated with the rapid inactivation phase is not seen. In the experiment illustrated in Fig. 2*c*, a 600 μM solution of free glutamate in physiological saline was applied rapidly to an ≈7-μm round vesicle obtained from the membrane of the neuron. The total rise time for the response was 14 ms; the rapid desensitization (220 s^{-1}) that takes place during this equilibration period prevents a direct measurement of the true current amplitude. The slow phase associated with desensitization (shown in Fig. 2*b*) can no longer be detected because of the smaller signal when vesicles are used. The area of the cell membrane forming the vesicle is 10 times smaller than that of the intact neuron and the number of receptor sites is consequently expected to be smaller. In separate experiments, 1.0 mM **3** applied in the absence and presence of (50 μM) free glutamate neither potentiated nor inhibited the current response to free glutamate.

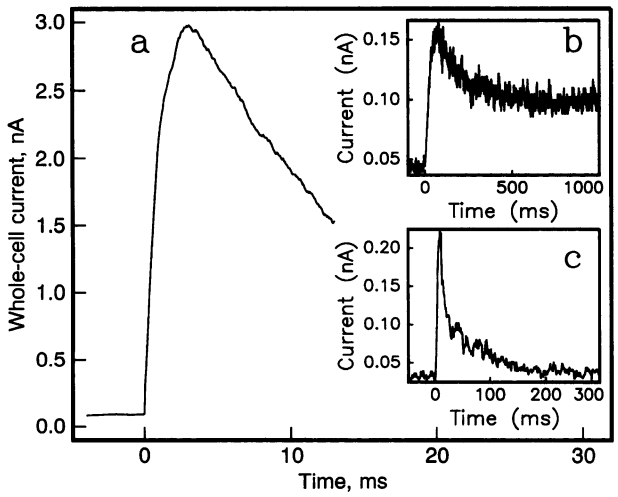

FIG. 2. Comparison of photolysis (10, 11, 28) and flow (22, 29) methods used to activate whole-cell current response of glutamate receptors in rat hippocampal neurons. Measurements were made in physiological saline at RT and the transmembrane voltage was clamped at −60 mV. (*a*) Current produced when 500 μM **3** was photolyzed over a neuron detached from the culture dish. No background response from the caged glutamate itself was observed. (*b*) Maximum signal produced when 300 μM free glutamate flowed rapidly over the same cell. (*c*) Response to rapid flow of 600 μM free glutamate over a membrane vesicle obtained from a hippocampal neuron.

DISCUSSION

The goal of the present investigation was to further develop photolabile derivatives of neurotransmitters (9, 13, 17, 27) suitable for rapid chemical kinetic investigations of receptors on the surface of single cells (10, 11) and for mapping the distribution of receptors on cell surfaces (12) and functional connections between neurons (14).

The results in Fig. 2 demonstrate the usefulness of the caged compound in rapid chemical kinetic investigations of glutamate receptors. The effect of glutamate concentration on the current rise time, the maximum current amplitude, and the falling phase of the current (Fig. 2*a*) can be determined in a single experiment that can give information about the rate and equilibrium constants for channel opening and receptor desensitization (10). Fig. 2 *b* and *c* shows that when techniques with inappropriate time resolution are used important information about the reaction is lost. The experiment in Fig. 2*b* gives information about only the receptor forms associated with the slow desensitization. By using a vesicle the time needed for the receptors to equilibrate with glutamate is considerably reduced (because the surface area of the membrane in contact with flowing solutions of glutamate is decreased), but the signal is also reduced and information about receptor forms that desensitize slowly (the minor component; Fig. 2*b*) is not obtained.

The αCNB group was introduced to protect the amino group of carbamoylcholine (9). The resulting caged compound was found to be suitable for rapid chemical kinetic investigations of the nicotinic acetylcholine receptor in the submillisecond time region (10, 11). The results described suggest that the αCNB group, successfully used in caging the amino group of carbamoylcholine, may be equally useful in caging the carboxyl group of neurotransmitters and other interesting biological compounds with carboxyl groups, including *N*-methyl-D-aspartic acid and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid, which are activating ligands for glutamate receptor subtypes.

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