Caged Bioactive Carboxylates. Synthesis, Photolysis Studies, and Biological Characterization of a New Caged *N*-Methyl-D-aspartic Acid

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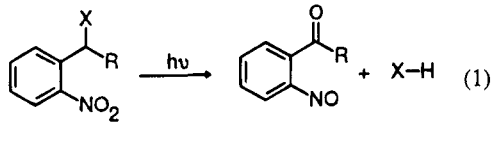
Photolytic release of bioactive compounds from "caged" precursors constitutes a useful method for reagent application with precise spatial and temporal control.[1] This method is particularly useful for the study of biological events that occur on the micro- to millisecond time scale. When cell-flow techniques using free reagents present inadequate time resolution, photolysis of inert caged precursors overcomes diffusional delay and has allowed for kinetic and mechanistic exploration of events that occur on the sub-millisecond time scale. For example, nanosecond UV photolysis of caged carbamoylcholine yields free carbamoycholine with a quantum yield of 0.8 and a $t_{1/2}$ value of 45 μs.[2a] These photolysis properties have allowed for the study of receptor activation and inhibition mechanisms of the nicotinic acetylcholine receptor.[2]

The most commonly used photoremovable, or caging, group is the *o*-nitrobenzyl group. The group is attached to moieties whose pK_a is low enough to allow for at least transient existence of the moiety in anionic form as the *o*-nitrobenzyl group is photolytically cleaved by near UV irradiation in an internal redox process (eq 1).[3]



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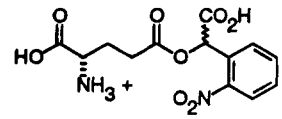
Moieties that have been caged include phosphates, amines, amides, carbamates, carbonates, carboxylates, phenols, and alcohols.[1a,2a] A wide range of uncaging rates and quantum yields has been observed, seemingly dependent upon both the leaving nucleofuge and the substitution pattern on the *o*-nitrobenzyl substituent.[1b] No model yet exists that allows one to predict with confidence that a particular caged compound will be rapidly photolyzed with sufficient quantum yield. In particular, photolysis of caged carboxylates has normally afforded free carboxylic acids only very slowly (on the order of 1–100 s^{-1}) and with low quantum yields.[1a,4] This dearth of useful caged carboxylates has been a hindrance to the use of these compounds in neurobiology, as many compounds active in the central nervous system are small carboxylate-containing materials, such as the ubiquitous amino acid neurotransmitters glutamate and γ-aminobutyric acid (GABA). Recently, we reported that the use of the α-carboxyl-2-nitrobenzyl (α-CNB) group to cage the γ-carboxyl group of glutamate (**1**)[5] and GABA (**2**)[6] yielded compounds that upon photolysis release free neurotransmitter in the microsecond time region with reasonable quantum yield (ca. 0.15). The caged carboxylates **1** and



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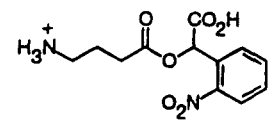

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2 have proven useful for the study of the rapid receptor activation, desensitization, and inhibition events of their respective receptors in neurons.[5,6] Building upon these results, we report here on the preparation, photolysis properties, and biological characterization of the caged β-(α-CNB) ester (**8**) of *N*-methyl-D-aspartic acid (NMDA), the prototypical agonist for its important glutamate receptor subtype.[7]

Results and Discussion

Synthesis. Esters of the carboxylate α to the electron-withdrawing amino group in NMDA (**3**) were expected to be more hydrolytically labile than esters of the side chain carboxylate.[5] Minimization of hydrolytic instability is an important goal, so as to prevent dark formation of the agonist in solution. Toward this end, *N-t*-BOC NMDA (**4**), prepared from **3** and *tert*-butyl pyrocarbonate in aqueous $NaHCO_3$/dioxane, was doubly esterified by alkylation of both carboxylates with an excess of the bromide **5**[5] in refluxing benzene in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (Scheme 1). Taking advantage of the differential reactivity toward hydrolysis of the two aspartic acid carbonyl groups,[8] the α-ester in tetraester **6** was selectively hydrolyzed using 1 equiv of hydroxide in water/dioxane. The remaining *tert*-butyl ester in **7** was selectively deprotected using trifluoroacetic acid (TFA), which concomitantly removed the *N-t*-BOC protecting group, giving the desired caged β-(α-CNB) ester of *N*-methyl-D-aspartic acid (**8**).

Photolysis. Irradiation of **8** with a simple TLC illumination hand lamp cleanly produced free NMDA, as determined by coelution with an authentic sample on analytical TLC (Figure 1). Irradiation of aqueous solutions of **8** at 308 nm with an excimer laser (XeCl) produced a transient intermediate, spectroscopically characterized as the generally accepted *aci*-nitro inter-

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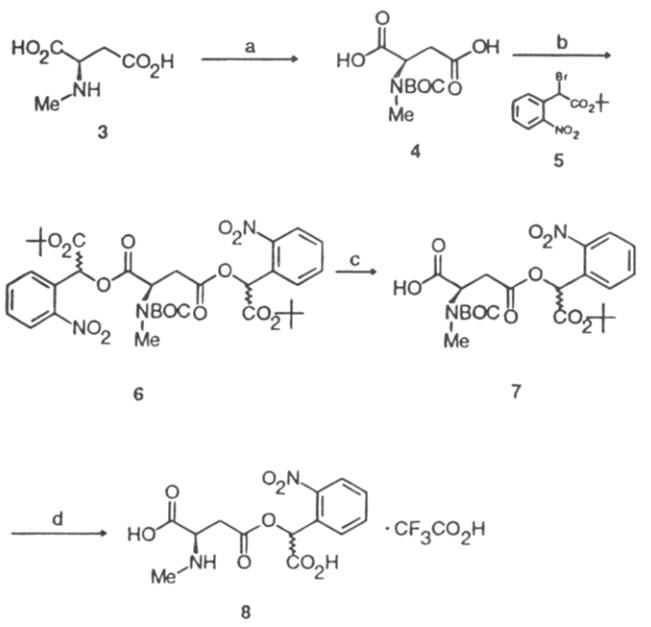
0022-3263/95/1960-4260$09.00/0

Scheme 1



[a] (a) *tert*-Butyl pyrocarbonate, $NaHCO_3$, H_2O/dioxane; (b) DBU, PhH/THF, reflux; (c) NaOH (1.0 equiv), H_2O/dioxane; (d) TFA/CH_2Cl_2.

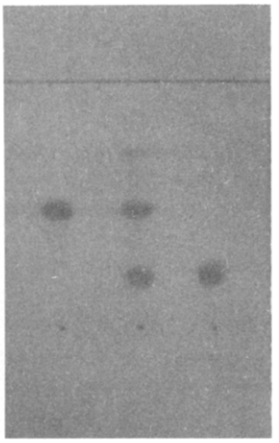


Figure 1. Analytical thin-layer chromatogram of caged NMDA (**8**) before and after photolysis. The components on the chromatogram were detected after treatment with ninhydrin. The solvent used was dioxane/2-propanol/water/ammonium hydroxide, 2:1:1:1. The upper horizontal line indicates the solvent front: (left lane) 5 μL of a 20 mM solution of **8** was applied at the base line; (center lane) 5 μL of a 20 mM solution of **8** was applied at the base line, followed by irradiation for 20 min at 254 nm with a TLC hand lamp at a distance of 1 cm, followed by elution; and (right lane) 5 μL of a 20 mM solution of NMDA (**3**) was applied at the base line.

mediate **9** (eq 2).[3,9] Decay of **9** fit a double exponential decay, with a fast time constant of 31 μs (73%), a slow time constant of 590 μs (23%) at pH 6.8 (Figure 2), and a photolysis quantum yield of 0.43 ± 0.09. The decay of *aci*-nitro intermediates such as **9** is thought to lead directly to uncaged reagent in a concerted fashion,[10] likely via a six-electron electrocyclic ring closure of **9a** to **9b**, followed by elimination of ROH. The α-carboxy substituent of the caging group usually imparts an accelerating effect upon the photorelease of uncaged reagent, relative to substituents such as hydrogen or methyl.[1a,b,2a] This effect may be due to relief of steric and/or electronic repulsion between the exomethylenic substituents and the nitro group in **9a**, which is likely to be more significant in the case of the α-carboxy substituent. The dark decomposition, rather than the photolytic formation, of the *aci*-nitro intermediate is known to be the rate-determining process.[1,3] The rate of decay of **9** is pH dependent, with a maximum rate at ca. pH 5.5. The rate drops off at pH < 5.5 and pH > 9 (Figure 3); similar rate dependence on pH has been observed previously with caged phosphates.[10a] Although **8** was prepared as a mixture of diastereomers, which resulted from generation of a chiral center at the benzylic position of the caging group, it is expected that the difference in photolysis behavior of the two diastereomers is not significant. This expectation is based on previous studies in which caged diastereomers of optically active nucleotides were separated and photolyzed separately.[11]

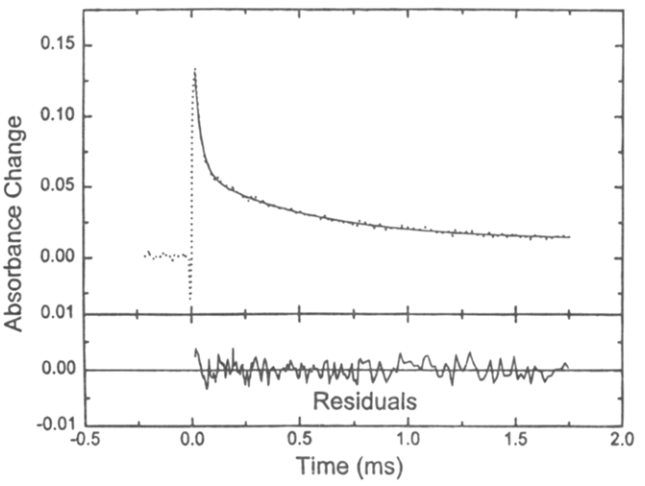
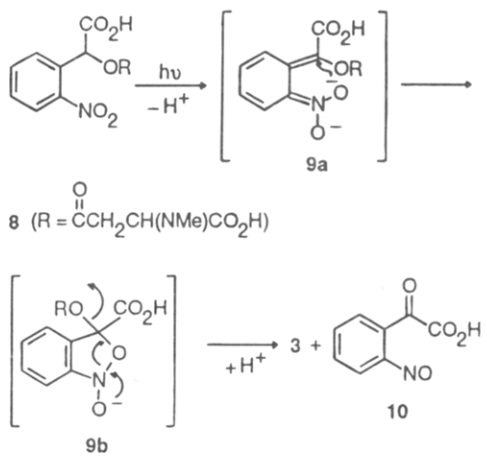


Figure 2. Absorbance transient of **9** at 430 nm produced by a 308 nm (XeCl) excimer laser flash in a 0.5 mM solution of **8** at pH 6.8 and 22 °C. A double exponential approximation to the data yields best fit decay time constants of 31 μs (73%) and 590 μs (23%). The dotted line represents the measured data and the solid line the fitted curve. The lower panel gives the residuals of the fitting.



Biological Characterization. The response of rat hippocampal neurons to free NMDA is shown in Figure

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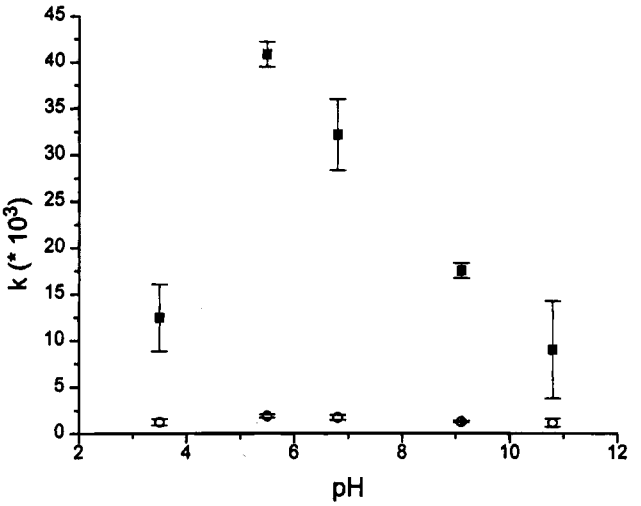

Figure 3. Dependence of the rate of decay of intermediate **9** on pH. For all experiments, 0.5 mM **8** in different buffer solutions was photolyzed using an excimer laser (308 nm, XeCl) at room temperature. The intermediate spectra were recorded at 430 nm. Each data point represents the average of four measurements; the error bars describe the standard deviations. The following buffers were used: pH 3.5, 100 mM citrate; pH 5.5, 100 mM acetate; pH 6.8, 100 mM phosphate; pH 9.1, 100 mM borate; and pH 10.8, 100 mM glycine. Filled squares represent the fast rate constants and the open circles the slow rate constants.

4a, as measured by whole-cell current recording.[12] Receptor activation by free neurotransmitter results in a transmembrane ion current. Figure 4b shows that when the cell is preincubated for 5 s with 300 μM **8** at pH 7.4 the response to 300 μM free NMDA is significantly diminished. This inhibition appears to be a function of incubation time, as shown in Figure 4c,d, where experiments on another cell show essentially no inhibition when free NMDA and **8** are applied to the neuron together, i.e. without preincubation with the caged precursor **8**.

The excellent photolysis properties of **8**, i.e. rapid uncaging with high quantum yield, continue to demonstrate that carboxylates caged as α-carboxy-2-nitrobenzyl esters are promising candidates for use in the study of rapid biological events such as receptor activation and desensitization. The inhibitory nature of **8** toward receptor activation by free NMDA likely makes the probe less useful than we had hoped. Thus, the synthesis of **8** constitutes a step toward an ideal version of caged NMDA. The synthetic method described herein also should prove useful in the synthesis of amino acids selectively esterified on their side chain carboxylates with complex alcohols.

Experimental Section

General. All reagents were used as received. NMDA was purchased from Research Biochemicals, Inc. Flash chromatography was performed according to the method of Still et al.[13] Fast atom bombardment mass spectra (FAB^+) were measured at the Oregon State University Mass Spectrometry facility. Melting points are uncorrected.

(*S*)-Bis-α,β-[α-(*tert*-butoxycarbonyl)-2-nitrobenzyl] *N*-(*tert*-Butoxycarbonyl)-*N*-methylaspartate (6). NMDA (1.00 g, 6.80 mmol) was suspended in water/dioxane (1:1, 10 mL) at rt.

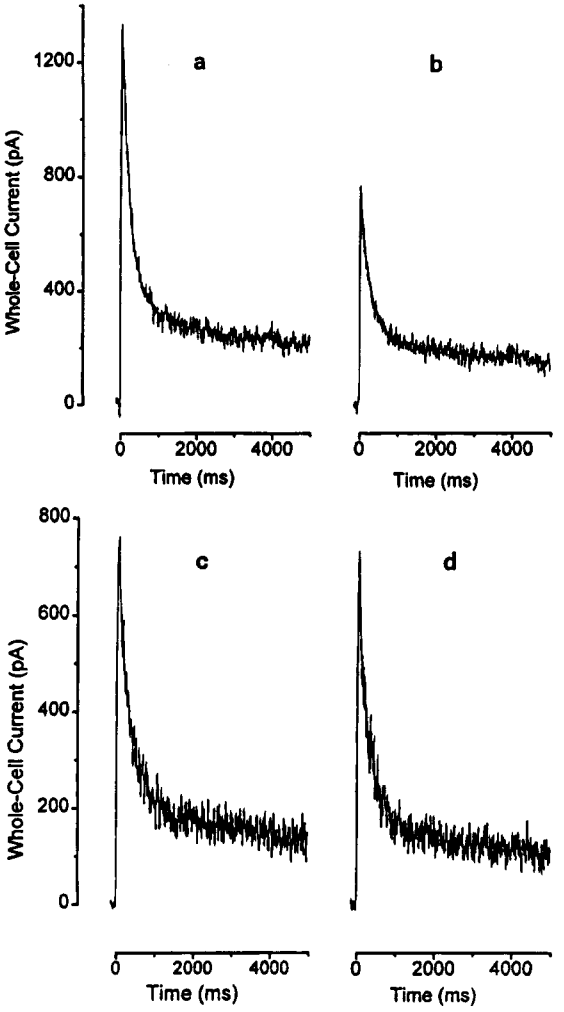

Figure 4. Whole-cell current induced by NMDA and recorded from individual neonatal rat hippocampal neurons at, −60 mV, pH 7.4, and 22 °C. Transmembrane ion current generated by (a) flow[15,16] of 300 μM free NMDA (**3**) over a cell; (b) simultaneous flow of **3** (300 μM) and **8** (300 μM) over the same cell, after 5 s preincubation with 300 μM **8**; (c) the same process as in (a) but with a different cell; and (d) simultaneous flow of **3** (300 μM) and **8** (300 μM) over the same cell as in (c) but without preincubation with **8**.

Sodium bicarbonate (1.77 g, 21.1 mmol) was added, followed by a solution of *tert*-butyl pyrocarbonate (1.75 g, 8.00 mmol) in 4 mL of dioxane. The resulting colorless mixture was stirred for 48 h and then treated with another portion of pyrocarbonate (1.00 g, 4.58 mmol) in 2 mL of dioxane and sodium bicarbonate (0.47 g, 5.6 mmol). After another 24 h, the reaction mixture was diluted with water (10 mL) and extracted with EtOAc (3 × 10 mL). The pH (8.5) of the aqueous mixture was adjusted to 3.0 with 5% HCl, followed by extraction of the mixture with EtOAc (3 × 10 mL). The extract was dried (sodium sulfate) and concentrated to give **4** as 1.30 g (77%) of a clear colorless viscous oil: R_f (MeCN/H_2O/AcOH, 8:1:1) 0.73; ^1H NMR ($CDCl_3$) δ 4.76, 4.55 (two br s, 1H), 3.15 (dd, J = 17.0, 7.0 Hz, 1H), 2.94 (br d, J = 19.9 Hz, 3H), 2.83 (td, J = 17.0, 7.3 Hz, 1H), 1.45 (s, 9H). Anal. Calcd for $C_{10}H_{17}NO_6$: C, 48.58; H, 6.93; N, 5.67. Found: C, 48.81; H, 7.20; N, 5.29.

To a colorless solution of **4** (0.42 g, 1.7 mmol) and **5**[5] (1.04 g, 3.40 mmol) in benzene/THF (20 mL/5 mL) at rt was added DBU (0.52 mL, 3.50 mmol). The resulting deep blue mixture was heated at reflux for 6 h and then cooled and filtered (Whatman no. 1). The filtrate was concentrated, and the residue was purified by flash chromatography (EtOAc/$CHCl_3$, 0−5%) to give tetraester **6** (0.57 g, 50%), as an approximately 2:2:1:1 mixture of diastereomers; the ratio is based on *N*-methyl ^1H NMR integration ratios. The tetraester **6** was a clear, pale brown photosensitive viscous oil that readily entrained moisture and

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solvents, slowly decomposing upon attempts to completely remove residual volatiles: R_f (5% EtOAc/$CHCl_3$) 0.42; ^1H NMR ($CDCl_3$) δ 8.0 (m, 2H), 7.7–7.4 (m, 6H), 6.76 (m, 2H), 5.2–4.8 (m, 1H), 3.5–3.0 (m, 2H), 2.96 (m, 3H), 1.5–1.3 (m, 27H). Anal. Calcd for $C_{34}H_{43}N_3O_4 \cdot 0.5H_2O$: C, 56.19; H, 6.10; N, 5.78. Found: C, 56.22; H, 6.08; N, 5.52.

(*S*)-β-[α-(*tert*-Butoxycarbonyl)-2-nitrobenzyl]-*N*-(*tert*-Butoxycarbonyl)-*N*-methylaspartic Acid (7). To a solution of tetraester **6** (0.54 g, 0.75 mmol) in dioxane (5 mL) at rt was added a solution of NaOH (30 mg, 0.75 mmol) in water (1 mL). The resulting pale brown mixture was stirred for 48 h. The volatiles were removed in vacuo, and the residue was dissolved in 25 mL of saturated sodium bicarbonate solution. The resulting mixture was extracted with EtOAc (2 × 15 mL), and the aqueous layer was acidified to pH 2.7 by the addition of 5% HCl. The resulting mixture was extracted with EtOAc (3 × 15 mL). The combined extracts were treated with activated charcoal, filtered (Celite), and concentrated to a pale brown oil. This oil was purified by flash chromatography ($CHCl_3$/MeOH/AcOH, 1:0:0 to 50:5:1) to give bis-ester **7** as 0.17 g (47%) of a ca. 1:1 mixture of diastereomers; the ratio is based on ^1H NMR integrations of NCH signals. The bis-ester **7** was a pale, clear brown photosensitive viscous oil that readily entrained moisture and solvents, slowly decomposing upon attempts to completely remove residual volatiles. Unreacted starting material (**6**) was recovered as 0.12 g (22%) of a colorless oil. For **7**: R_f ($CHCl_3$/MeOH/AcOH, 15:3:1) 0.64; ^1H NMR ($CDCl_3$) δ 8.03 (d, J = 8.1 Hz, 1H), 7.65 (m, 2H), 7.5 (m, 1H), 6.8 (two s, 1H), 4.61 (two m, 1H), 3.25 (dd, J = 17.0, 5.5 Hz, 1H), 2.92 (br s, 3H), 2.09 (s, 1H), 1.4 (two s, 18H). Anal. Calcd for $C_{22}H_{30}N_2O_{10} \cdot CH_3CO_2H$: C, 53.13; H, 6.32; N, 5.16. Found: C, 53.01; H, 6.49; N, 4.90.

(*S*)-β-(α-Carboxy-2-nitrobenzyl) *N*-Methylaspartic Acid, Trifluoroacetate Salt (8). To a pale brown solution of **7** (0.13 g, 0.27 mmol) in dichloromethane (2.0 mL, anhydrous) under argon at rt was added TFA (1.0 mL, 13 mmol). The resulting solution was incubated at rt for 16 h and then concentrated in vacuo. Toluene (3 mL) was evaporated from the residue, leaving an immobile pale brown oil. This oil was purified by chromatography on Sephadex LH-20, with water as eluant. After the product fractions were pooled, lyophilization gave the title compound as 90 mg (75%) of a fluffy white powder, as a ca. 1:1 mixture of diastereomers (ratio based on integration of ^1H NMR ArCH signals): mp 116–119 °C dec; UV max (pH 7.0 phosphate buffer) 264 nm (ϵ 5100); ^1H NMR (D_2O) δ 8.15 (d, J = 7.9 Hz, 1H), 7.82 (t, J = 7.3 Hz, 1H), 7.7 (m, 2H), 6.73 (two s, 1H), 4.23 (br s, 1H), 3.3 (m, 2H), 2.84 (br s, 3H); FABMS *m/e* (relative intensity) 327 (M − CF_3CO_2, 100), 219 (8), 154 (21), 136 (16); HRMS calcd for $C_{13}H_{15}N_2O_8$ 327.0824, found 327.0828. Anal. Calcd for $C_{15}H_{15}N_2O_{10}F_3$: C, 40.92; H, 3.43; N, 6.36. Found: C, 41.24; H, 3.74; N, 6.76.

Photolysis. The equipment used for transient absorption spectroscopy has been described in detail elsewhere.[14] Briefly, pulses of 308 nm (XeCl) light from an excimer laser (Lumonics TE 861 M) initiated photolysis of 0.5 mM solutions of **8** in various buffers. The transient intermediate (**9**) absorption was observed at 430 nm at right angles to the photolysis beam using a halogen lamp (Newport model 780) with a Corning WGS360 cutoff filter, a monochromator set to 380/500 nm (0.3 m McPherson 275 single pass), and a photomultiplier (Thorn EMI9635QB). The signal was amplified with a preamplifier (Thorn EMI model A1) and stored using a digital storage oscilloscope (LeCroy Cope Station 140). The signals were digitized at a rate of 0.5 MHz. A nonlinear least-squares analysis program, Origin 3.5, was used to fit one- or two-component exponential functions to the transient (**9**) decay signals measured between 380 and 500 nm.

The method used for the determination of the photolysis quantum yield of **8** has been described in detail elsewhere.[2a] Briefly, a 0.5 mM solution of **8** at pH 6.8 (phosphate buffer) in a quartz cuvette was irradiated using the 308 nm excimer laser, in which the laser beam was focused using a cylindrical lens on a spot of ca. 1 × 10 mm. The absorbed energy was measured with a joulemeter (ballistic thermopile) behind the cuvette and was found to average 3 mJ. The transient (**9**) was measured at right angles at 430 nm as described above. The solution was photolyzed with 10 consecutive laser shots. For each shot, the absorbed laser energy and the transient spectra were determined. With the presumption that the amount of **9** formed during photolysis is proportional to the amount of free NMDA generated, the amount of freed NMDA can be calculated using the relationship[2a]

$$A_n = \epsilon_m l c_0 \Phi K_E \exp[-\Phi K_E F(n-1)]$$

$$\ln A_n = \ln(\epsilon_m l c_0 \Phi K_E) - \Phi K_E F(n-1)$$

where A_n is the absorbance after the nth laser shot, ϵ_m is the molar extinction coefficient, l is the path length, c_0 is the initial concentration, Φ is the quantum yield, K_E is the ratio of the number of photons absorbed in each shot to the number of molecules in the target beam, F is the fraction of solution through which the laser beam passed, and n is the number of the current shot. The product K_EF is given by the ratio of the total number of photons absorbed in each shot to the total number of molecules in the cuvet. K_EF is constant becaue **8** and its photoproduct **10** have their isosbestic point at the irradiation wavelength (308 nm). A plot of $\ln A_n$ vs $n - 1$ thus gives a line, the slope of which provides the quantum yield Φ.

Whole-Cell Electrophysiology. The method used in these experiments for whole-cell current recording[12] has been described in detail elsewhere.[15] Briefly, hippocampal neurons from 2-day-old Sprague–Dawley rats were mechanically isolated[16] and cultured on dishes coated with collagen and in minimum essential medium with Earle's salts supplemented with 2.7 mM glutamine and 10 mM glucose. Cells used in the experiments were 10–18 days old. The cell suspended by the current-recording electrode was positioned in front of a U-tube flow device[17] used for rapid equilibration of ligands with receptors on the cell surface.[15] The extracellular recording buffer consisted of 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES (pH 7.4); the pipet solution was 140 mM CsCl, 1 mM $CaCl_2$, 10 mM EGTA, and 10 mM HEPES (pH 7.2). The response to NMDA was measured in the presence and absence of caged NMDA (**8**).

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