Synthesis and Characterization of a Caged Receptor Ligand Suitable for Chemical Kinetic Investigations of the Glycine Receptor in the 3-μs Time Domain[†]

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ABSTRACT: Here we report the development and characterization of a new photolabile protecting group for the carboxyl group of neurotransmitters, 2-methoxy-5-nitrophenyl. The synthesis and characterization of a photolabile derivative of β-alanine, caged β-alanine, are described. β-Alanine can activate the glycine receptor, a major inhibitory receptor in the mammalian central nervous system; the 2-methoxy-5-nitrophenyl derivative of β-alanine combined with a laser-pulse photolysis method makes it possible to investigate the chemical kinetic mechanism of the receptor in the 3-μs time domain. The derivative is photolyzed by a laser pulse to release free β-alanine within 3 μs and with a product quantum yield of 0.2. In aqueous solution in the dark and at neutral pH, the compound is more stable, by a factor of ~25, than the analogous derivative of glycine [Ramesh, D., Wieboldt, R., Niu, L., Carpenter, B. K., & Hess, G. P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11074−11078]. 2-Methoxy-5-nitrophenyl-β-alanine hydrolyzes in aqueous solution at neutral pH with a $t_{1/2}$ of approximately 1.5 h. Neither the 2-methoxy-5-nitrophenyl-β-alanine nor the 2-methoxy-5-nitrophenol photolysis side product activates, inhibits, or potentiates the response of glycine receptors in rat hippocampal neurons to glycine. Photolysis of 2-methoxy-5-nitrophenyl-β-alanine by irradiation with a 600-ns laser pulse at 333 nm releases β-alanine, which then activates glycine receptor-channels on neurons equilibrated with the caged compound, as detected by whole-cell current recording. Compared with the analogous derivative of glycine, in terms of quantum yield, photolysis rate, and stability, this new compound is not only a better candidate for use in chemical kinetic investigations of the glycine receptor, but can also be used in determining the location of glycine receptors in neuronal cells.

Signal transmission at chemical synapses in the central nervous system utilizes neurotransmitter-gated ion-channel receptor proteins (Kandel et al., 1995). Upon binding neurotransmitters secreted from a presynaptic terminal, receptors in the postsynaptic membrane can form open channels to allow ions to flow across the postsynaptic membrane. It is the ionic current flowing through the receptor-channels that generates a change in the membrane potential of the postsynaptic cell, thus triggering signal transmission to another cell. Activation of the excitatory cation-permeable receptor-channels, such as the glutamate and acetylcholine receptors, causes changes of the membrane potential to more positive values. Opening of anion-permeable channels causes a change in the membrane potential to more negative values and inhibits signal transmission. The glycine and γ-aminobutyric acid ($GABA_A$) receptors are examples of inhibitory receptors, which together with the excitatory receptors belong to a superfamily of receptor proteins (Betz, 1990; Stroud et al., 1990; Wisden & Seeburg, 1992; DeLorey & Olsen, 1992). Glycine and $GABA_A$ receptors are involved in physiologically based functions such as nociception and anxiety and also in many neurological disorders such as epilepsy (Meldrum, 1975; Lasley, 1991; Norris et al., 1994) and human hereditary hyperexplexia (Langosch et al., 1994). Pharmacological intervention at the glycine receptor may provide a means of controlling some kinds of seizures, and a better understanding of the glycine receptor may lead to new anticonvulsant drugs (Freed, 1985).

Both glycine and $GABA_A$ receptors desensitize (become transiently inactive) on the milliseconds-to-seconds time scale (Krishtal et al., 1988; Cash & Subbarao, 1987; Geetha & Hess, 1992; Walstrom & Hess, 1994). Glycine and $GABA_A$ receptors upon binding the corresponding neurotransmitters form open transmembrane channels on the sub-millisecond time scale (Twyman & MacDonald, 1991; Maconochie et al., 1994). Knowledge of the conductance of the open receptor-channel and of the mechanism of the receptor-mediated reaction, including the rate and equilibrium constants of the elementary steps, is required to relate the effect of neurotransmitter concentration and time to the receptor-controlled transmembrane voltage changes (Hess, 1993; Hess et al., 1995), which determine whether a cell will transmit a signal or not (Kandel, 1991). The single-channel current-recording technique enables one to measure the conductance and lifetime of receptor-formed transmembrane channels (Neher & Sakmann, 1976; Sakmann & Neher, 1995). The laser-pulse photolysis technique, which utilizes caged neurotransmitters, provides information about the reaction mechanism and the rate and equilibrium constants for channel opening and receptor desensitization (Matsubara et al., 1992; Niu & Hess, 1993; Niu et al., 1995).

The use of "caged compounds" and the light-induced release of active molecules (e.g., the laser-pulse photolysis technique) provide control of spatial and temporal resolution

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of ligand concentration in systems where a time delay associated with diffusional mixing prevents measurement of fast reactions [reviewed by Kaplan (1990), Adams and Tsien (1993), Corrie and Trentham (1993), and Hess (1993)]. Here we report the development and use of a new protecting group, 2-methoxy-5-nitrophenyl (MNP),[1] for the carboxyl group of neurotransmitters. We have used it to protect β-alanine, which can activate the glycine receptor. The laser-pulse photolysis technique, which employs biologically inactive photolabile precursors of neurotransmitters (caged neurotransmitters), was developed for measuring elementary steps in receptor-mediated reactions in the sub-millisecond time domain on a single living cell (Milburn et al., 1989; Matsubara et al., 1992; Billington et al., 1992; Niu & Hess, 1993; Ramesh et al., 1993; Gee et al., 1994; Wieboldt et al., 1994a,b; Niu et al., 1995). In this technique the cell is first equilibrated with the caged compound, the neurotransmitter is released by a laser pulse in the microsecond time region, and the resulting current, a measure of the concentration of open receptor-channels, is determined by a whole-cell current-recording technique (Hamill et al., 1981).

A report on the photolysis of 2-methoxy-5-nitrophenyl acetate (MNP-acetate) (Kuzmic et al., 1986) led to the synthesis of MNP-glycine (Ramesh et al., 1993). The half-life of photohydrolysis of MNP-glycine is less than 3 μs with a quantum yield of 0.2, but the compound is not stable at neutral pH and decomposes with a $t_{1/2}$ value of 4.2 min. In this report, we describe the use of MNP as a caging group to protect β-alanine, which can activate the glycine receptor. MNP-β-alanine is thermally stable at neutral pH but has the same desirable photochemical characteristics as MNP-glycine. MNP-β-Alanine can be equilibrated with a cell preparation at physiological pH for convenient time periods without appearance of a detectable background level of free β-alanine from thermal hydrolysis. We show that MNP-β-alanine can be used to investigate the kinetics of activation of glycine receptor-channels in rat hippocampal neurons using the laser-pulse photolysis technique.

MATERIALS AND METHODS

Synthesis. The procedure for the preparation of MNP-β-alanine follows the method presented previously (Ramesh et al., 1993). Reagents were obtained from Aldrich (Milwaukee, WI) unless stated otherwise. A Varian XL-200 was used to obtain NMR spectra in the indicated deuterated solvents, high resolution mass spectral analysis was performed at the Mass Spectroscopy Laboratory in the School of Chemical Sciences at the University of Illinois, and elemental analysis was performed at Galbraith Laboratories, Knoxville, TN.

N-BOC-(2-Methoxy-5-nitrophenyl) 3-Aminopropionate. A 7 mL dichloromethane solution of *N*-BOC β-alanine (220 mg, 1.2 mmol, Sigma, St. Louis, MO), 4-(dimethylamino)-pyridine (10 mg, 0.08 mmol), 1-hydroxybenzotriazole (10 mg, 0.075 mmol), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide·HCl (230 mg, 1.2 mmol) was stirred at −78 °C. 2-Methoxy-5-nitrophenol (170 mg, 1 mmol) was added rapidly in one portion, and the reaction was continued overnight and allowed to equilibrate to ambient temperature (22 °C). The methylene chloride solution was washed with water (3 × 5 mL), aqueous $NaHCO_3$ (5%, 1 × 5 mL), water (2 × 5 mL), aqueous citric acid (10%, 3 × 5 mL), and saturated NaCl (1 × 3 mL) and was dried over anhydrous Na_2SO_4. Chromatography on a silica gel column using hexane and ether eluent gave the protected adduct as a white powder. Yield: 80%. ^{1}H-NMR ($CDCl_3$): 8.16 (dd, J = 9.2 and 2.7, 1H, C_4-H); 7.98 (d, J = 2.7, 1H, C_6-H); 7.02 (d, J = 9.14, 1H, C_3-H); 5.12 (bs, 1H, -$NHCO_2$); 3.94 (s, 3H, OCH_3) 3.52 (dt, J = 6.03, 2H, CH_2-NH); 2.81 (t, J = 6.03, CH_2CO) and 1.64 (s, 9H, $CO_2C(CH_3)_3$).

(2-Methoxy-5-nitrophenyl) 3-Aminopropionate·HCl (MNP-β-Alanine). A solution of *N*-BOC-(2-methoxy-5-nitrophenyl) 3-aminopropionate (65 mg, 0.19 mmol) in ethyl acetate (saturated with HCl gas) initially at −78 °C was stirred overnight and allowed to reach room temperature. The precipitated solid was filtered, washed with ethyl acetate, and dried. Yield, 50 mg (95%); mp, 168−175 °C (dec). ^{1}H-NMR (D_2O; HOD = 4.6 ppm): 8.08 (d, J = 8.8, 1H, C_4-H); 7.9 (s, 1H, C_6-H); 7.1 (d, 1H, C_3-H); 4.4 (s, 3H, OCH_3); 3.2 (t, J = 5.8, 2H, CH_2-N); 2.97 (t, J = 5.8, 2H, CH_2CO). High-resolution mass spectroscopic analysis: calcd. for MH^+ as $C_{10}H_{13}N_2O_5$, 241.0824; found, 241.0827.

N-BOC-(2-Methoxy-5-nitrophenyl) 4-Aminobutyrate. A procedure identical to the method used to prepare MNP-β-alanine above was used starting with the protected amino acid *N*-BOC-γ-aminobutyrate. The protected product was isolated in 88% yield with a melting point of 105−106 °C. ^{1}H-NMR ($CDCl_3$): 8.17 (dd, J = 9.1 and 2.7, 1H, C_4-H); 7.98 (d, J = 2.7, 1H, C_6-H); 7.03 (d, J = 9.1, 1H, C_3-H); 4.7 (bs, 1H, -$NHCO_2$); 3.94 (s, 3H, OCH_3) 3.27 (dt, J = 6.35 and 6.67, 2H, CH_2-NH); 2.67 (t, J = 7.28, CH_2CO); 1.96 (m, 2H, $CH_2CH_2CH_2$) and 1.46 (s, 9H, $CO_2C(CH_3)_3$). Anal. Calcd. for $C_{16}H_{22}N_2O_7$: C, 54.24; H, 6.22; N, 7.91. Found: C, 54.24; H, 6.03; N, 7.84.

(2-Methoxy-5-nitrophenyl) 4-Aminobutyrate·HCl (MNP-GABA). Removal of the BOC protecting group was performed as above with a yield of 85% and mp 178−180 °C. To minimize hydrolysis, the sample was dissolved in dry solvent immediately before recording the spectra. ^{1}H-NMR (d_6-DMSO) 8.23 (dd, J = 9.15, 2.7, 1H, C_4-H); 8.18 (bs, 2H, -NH_2); 8.12 (*d*, J = 2.7, 1H, C_6-H); 7.40 (d, J = 9.7, 1H, C_3-H); 3.95 (s, 3H, OCH_3) 2.93 (t, J = 7.5, 2H, CH_2-NH); 2.78 (t, J = 7.30, CH_2CO) and 1.96 (m, 2H, CH_2CH_2-CH_2). Anal. Calcd for $C_{11}H_{15}N_2O_5Cl$: C, 45.36; H, 5.15; N, 9.62. Found: C, 45.39; H, 5.08; N, 9.59.

Laser-Flash Photolysis. Instrumentation used for determination of the transient spectroscopic characteristics of MNP-β-alanine has been described (Milburn et al., 1989; Ramesh et al., 1993). An XeCl excimer laser (TE861M, Lumonics, Ottowa, Ont.) produced 10-ns, 30-mJ pulses of 308-nm light to photolyze the 10−25-μL caged compound solutions in a 1 × 10-mm cuvette. The absorbance due to liberation of 2-methoxy-5-nitrophenol produced by the induced photolysis was monitored with a beam from a broad-spectrum tungsten halogen source (New Port 780) through a section of the cuvette perpendicular to the laser beam. Absorbance changes were observed at wavelengths between 350 and 450 nm with a 0.2-m McPherson 275 single-pass monochromator (Newport) and detected by a photomultiplier (9635QB, Thorn EMI, Fairfield, NJ). The photocurrent was converted to voltage with a preamplifier (model A1, Thorn EMI) that effectively limited the bandwidth of the signal to 500 kHz. Signals were digitized at rates up to 1 MHz with a digital oscilloscope (ScopeStation 140, LeCroy, Chestnut

[1] Abbreviations: MNP, 2-methoxy-5-nitrophenyl; *N*-BOC, *N*-*tert*-butoxycarbonyl; αCNB, α-carboxy-2-nitrobenzyl.

Ridge, NY) and stored for subsequent offline display and plotting.

Cell Culture and Whole-Cell Current Recording. Hippocampal neurons prepared from newborn Sprague-Dawley rats were cultured as previously described (Niu et al., 1996a). The preparation of the cells was also similar to the one described by Patneau et al. (1993). The whole-cell current was recorded at constant voltage (−60 mV) (Hamill et al., 1981) to measure the ionic currents activated by β-alanine using neurons which had been in culture for 5 days to 3 weeks unless otherwise specified. The extracellular buffer was 145-mM NaCl, 3-mM KCl, 1-mM $CaCl_2$, 2-mM $MgCl_2$, 10-mM glucose, and 10-mM HEPES (pH 7.1); the electrode solution consisted of 140-mM CsCl, 2-mM $MgCl_2$, 1-mM $CaCl_2$, 10-mM EGTA, 2-mM Na_2ATP, and 10-mM HEPES (pH 7.1). A neuron was attached to a patch electrode and suspended in the buffer to facilitate rapid exchange of solutions using a cell-flow device (Krishtal & Pidoplichko, 1980; Udgaonkar & Hess, 1987). Currents induced by rapid flow of free β-alanine, glycine, or GABA over the cell surface were amplified, low-pass filtered at 3 kHz, and digitized and stored for later display and analysis. The concentration of the released β-alanine in laser-pulse photolysis experiments (see below) was determined by comparing the whole-cell currents produced by known concentrations of β-alanine in cell-flow experiments with those produced by photolysis of MNP-β-alanine.

Laser-Pulse Photolysis. A Candela UV-500 dye laser with sulforhodamine 640 (Exciton) as the laser dye produced single 500-μJ laser pulses of 600-ns duration at 333 nm after a secondary harmonic generator assembly. The light was delivered *via* an optical fiber (200-μm core diameter, SFS200/220N, FiberGuide Industries) to the neuron attached to a patch electrode. Data collection was synchronized to triggering of the laser by the acquisition system, which consisted of a Labmaster DMA digitizer (Scientific Solutions) driven by the pClamp program (Axon). Electronic filtering of the signal from the current amplifier was set at 10 kHz (low pass) with digitization rates up to 50 kHz to accommodate the rapid current rise produced by receptor activation following photolysis of MNP-β-alanine.

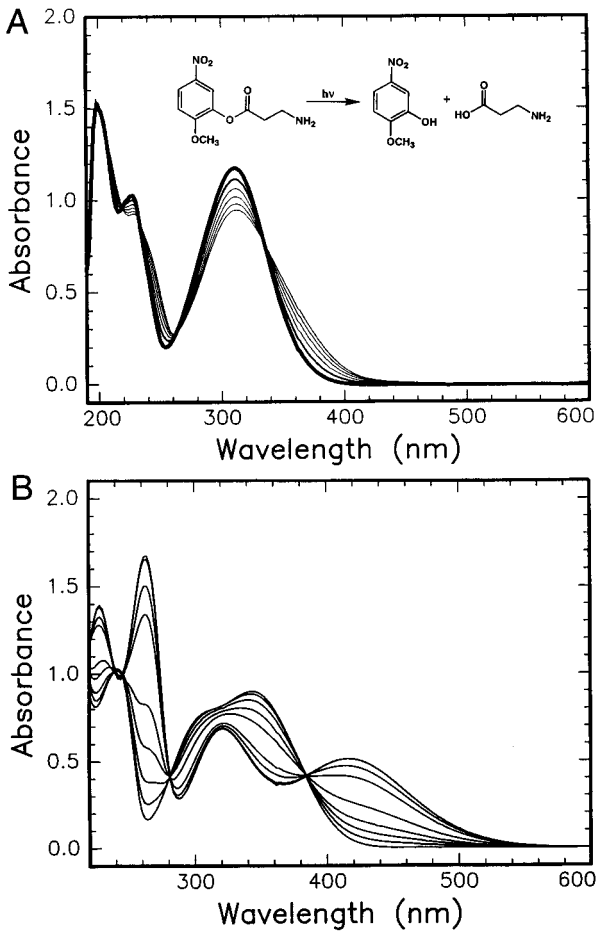

FIGURE 1: (A) Thermal hydrolysis of MNP-β-alanine at pH 7.1 and at room temperature. The concentration of the caged compound was 800 μM in 100-mM phosphate buffer. Spectra were recorded at 5-min intervals and are displayed at 10-min intervals. The heavy trace corresponds to the starting material and the isosbestic points are at 334, 262, 232, and 219 nm. (B) pH dependence of the spectra of 2-methoxy-5-nitrophenol. The concentration of the phenol solution was 0.75 mM in buffers of 100 mM; pH 3.5 (citrate), 6.9, 7.3, 7.6 (phosphate), 8.0, 8.5, 9.1 (Tris), 10.0 (borate), and 11.5 (borate). Lower trace at 400 nm corresponds to pH 3.5, and isosbestic points are at 385 and 280 nm. The next higher traces correspond to increasing pH values. The structures of MNP-β-alanine and its photodecomposition products are shown in the inset.

RESULTS

The chemical and biological properties of caged compounds determine the suitability of a new derivative for a particular application. In photolysis experiments, the maximum rate of receptor-channel activation that can be observed and the accessible range of released neurotransmitter concentrations are determined by the rate and quantum yield of the photochemical reaction of the caged compound used. It is also important to characterize biologically any activating or inhibitory effects of the caged compound and of the photolytic side products [Milburn et al., 1989; reviewed by Niu et al. (1996b)]. Finally, the stability and solubility of the caged compound in the pH range normally encountered in biological experiments must be determined.

Stability of MNP-β-Alanine in Aqueous Solutions. In solutions buffered at pH 7.1, MNP-β-alanine slowly hydrolyzes to produce free β-alanine and a side product, 2-methoxy-5-nitrophenol. Figure 1A shows spectra which reflect this conversion. A solution of the caged compound was placed in a cuvette, and spectra were taken at 5-min intervals. The absorbance decays exponentially, and the rate of hydrolysis was found by fitting absorbance *versus* time data to a single-exponential function. The rate constant of the thermal hydrolysis is 7.4×10^{-3} min^{-1} (i.e., $t_{1/2} = 93$ min) at pH 7.1 and 23 °C. In comparison, an analogous compound, MNP-glycine (Ramesh et al., 1993), decomposes about 20 times faster, with a rate constant of 0.16 min^{-1}, under identical conditions.

A series of spectra of 2-methoxy-5-nitrophenol at pH values between 3.5 and 11.5 (Figure 1B) serves as a reference for the thermal hydrolysis spectra and confirms the identity of this side product of the photohydrolysis of the MNP-β-alanine. The spectrum of the free phenol at pH 7.1 was identical to that of MNP-β-alanine when the hydrolysis of the caged compound was allowed to go to completion. Analysis of the absorbance change *versus* pH provides a value of 8.2 for the pK_a of 2-methoxy-5-nitrophenol.

The absorbance values of the free phenol determined above also serve as end-point reference values for obtaining rate constants for the thermal hydrolysis of MNP-β-alanine over a range of pH values. Dilute (between 400 and 800 μM) solutions of the caged compound were prepared and

Table 1: Thermal Hydrolysis Rates of MNP Caged Compounds (min^{-1})

pH[a]	MNP-β-alanine	MNP-glycine[b]	MNP-GABA
2.5	nd[c]	3.7×10^{-3}	1.2×10^{-3}
3.6	nd	3.2×10^{-3}	1.4×10^{-2}
4.6	nd	3.6×10^{-3}	1.2×10^{-1}
4.9	nd	5.9×10^{-3}	3.0×10^{-1}
5.5	nd	4.3×10^{-3}	1.0
6.2	3.5×10^{-3}	7.3×10^{-2}	nd
6.5	8.1×10^{-4}	1.1×10^{-1}	nd
7.1	7.4×10^{-3}	1.9×10^{-1}	nd
8.0	5.6×10^{-2}	3.3×10^{-1}	nd
9.0	1.4×10^{-1}	nd	nd
10.0	2.9×10^{-1}	nd	nd

[a] The concentration of buffers used in the rate measurements was 100 mM; pH values were 2.5 (HCl/NaOH), 3.6 (citrate), 4.6, 4.92, 5.5 (acetate), 6.1, 6.5, 7.1 (Hepes), 8.0 (Tris), 9.0, and 10.0 (borate). [b] Ramesh et al. (1993). [c] nd, not determined (see text).

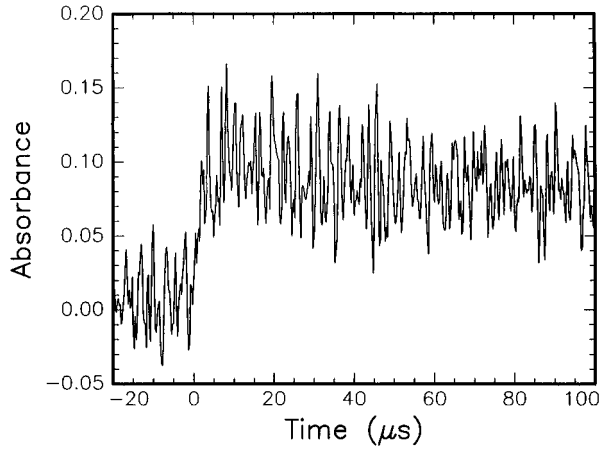

FIGURE 2: Rapid absorbance jump produced by photolysis of MNP-β-alanine. A 2-mM solution of the caged compound in 100-mM phosphate buffer, pH 7.1, at room temperature was photolyzed by a single pulse of 308-nm light from an XeCl excimer laser (approximately 50 mJ). The absorbance change was monitored at 405 nm with a single-beam transient spectrophotometer, the signal transfer parameters of which effectively produced 500-kHz filtering of the rapid absorbance jump. The rapid absorbance change reflects the release of free 2-methoxy-5-nitrophenol and presumably of β-alanine from the precursor.

immediately transferred to a cuvet thermostated at 23 °C in a spectrophotometer set up to collect absorbance values at timed intervals. The initial rates of the hydrolysis reaction were obtained from measurements at 310, 360, and 384 nm after subtraction of the absorbance of the completely hydrolyzed solution obtained from the experiments shown in Figure 1B, with scaling to compensate for concentration differences. Hydrolysis rates for three MNP-compounds, β-alanine, GABA, and glycine (Ramesh et al., 1993), are given in Table 1. Notably, at pH 7.1 the rate of hydrolysis of MNP-β-alanine is ∼25 times slower than that of MNP-glycine, indicating that the former can be used at physiological pH without the special precautions needed to prevent thermal hydrolysis that were necessary with MNP-glycine (Ramesh et al., 1993). The MNP-GABA, on the other hand, hydrolyzes too rapidly to be measured at pH 7.1. It is, therefore, of little use in investigations of the $GABA_A$ receptor.

Rate of Photoproduct Release and Quantum Yield. Figure 2 illustrates the fast conversion to products caused by photolysis of MNP-β-alanine. Photohydrolysis of the starting material leads to production of free 2-methoxy-5-nitrophenol. This is accompanied by the appearance of the characteristic phenol absorbance spectrum. The fast conversion was followed by monitoring at wavelengths where there are differences between the spectra of the starting material and of the phenol. A solution of MNP-β-alanine was photolyzed by a single flash of 308-nm light from an excimer laser. The response of the photomultiplier preamplifier imposes an effective time resolution of 2 μs on the sampled absorbance change, and the conversion appears as a step change broadened by the transfer function of the data collection instrumentation. However, it is apparent that the photodecomposition went to completion in less than 3 μs (Figure 2). The triplet-state lifetime of the MNP-acetate, considered to be the rate-limiting step in the photohydrolysis reaction, is 0.15 μs (Kuzmic et al., 1986).

The quantum yield of MNP-β-alanine was determined as follows. A 20-μL aliquot of the solution was photolyzed by a single 30-mJ light pulse at 308 nm from the excimer laser and the absorbed energy was measured with a ballistic thermopile; the concentration of 2-methoxy-5-nitrophenol, which is assumed to be produced stoichiometrically with the amino acid, was determined spectrophotometrically. The quantum yield of photolysis of MNP-β-alanine at pH 7.1 was measured as 0.2. This is the same as the quantum yield determined for MNP-glycine (Ramesh et al., 1993) and indicates that the leaving group does not significantly influence the efficiency of the photolysis reaction. A similar value, 0.13, for the quantum yield has been reported for MNP-acetate (Kuzmic et al., 1986). In comparison, a caged glycine derivative in which the α-amino group was protected with the αCNB group (Billington et al., 1992) had a quantum yield of only 0.02.

β-Alanine Activates Glycine Receptors on Rat Hippocampal Neurons. What receptor does β-alanine activate? Although β-alanine is considered to be an activating ligand for glycine receptors (Choquet & Korn, 1988) one report suggests it also activates $GABA_A$ receptors (Horikoshi et al., 1988). The following experiments were performed to determine that the response to β-alanine observed in the rat hippocampal neurons used for this study arose from glycine receptors. Rat hippocampal neurons express both glycine and $GABA_A$ receptors. We took advantage of the fact that the $GABA_A$ response is detectable as early as 3 days in culture while the glycine response is not observed until a later stage. Figure 3A shows the response of a 3-day-old cell to 1-mM GABA (trace a, 900 pA). On the same cell, no response to either 1-mM β-alanine (trace b) or 1-mM glycine (trace c) was observed. In a 14-day-old cell the response to 300-μM glycine was 180 pA (Figure 3B, trace a) while the response to 300-μM β-alanine was 160 pA (Figure 3B, trace b). Krishtal et al. (1988) reported EC_{50} values of 100 and 400 μM for the response of rat hippocampal cells to glycine and β-alanine, respectively; the response to β-alanine was also shown to be blocked by strychnine, a specific inhibitor of glycine receptor-channels. It was thus concluded (Krishtal et al., 1988) that β-alanine activates the glycine receptor. The results shown in Figure 3 confirm that the β-alanine activates glycine receptors in rat hippocampal neurons.

Characterization of the MNP-β-Alanine. Cell-flow experiments (Udgaonkar & Hess, 1987) using rat hippocampal neurons were designed to address the question: Is MNP-β-alanine inactive (i.e., it does not activate, inhibit, or potentiate the β-alanine-induced response)? 300-μM β-alanine was

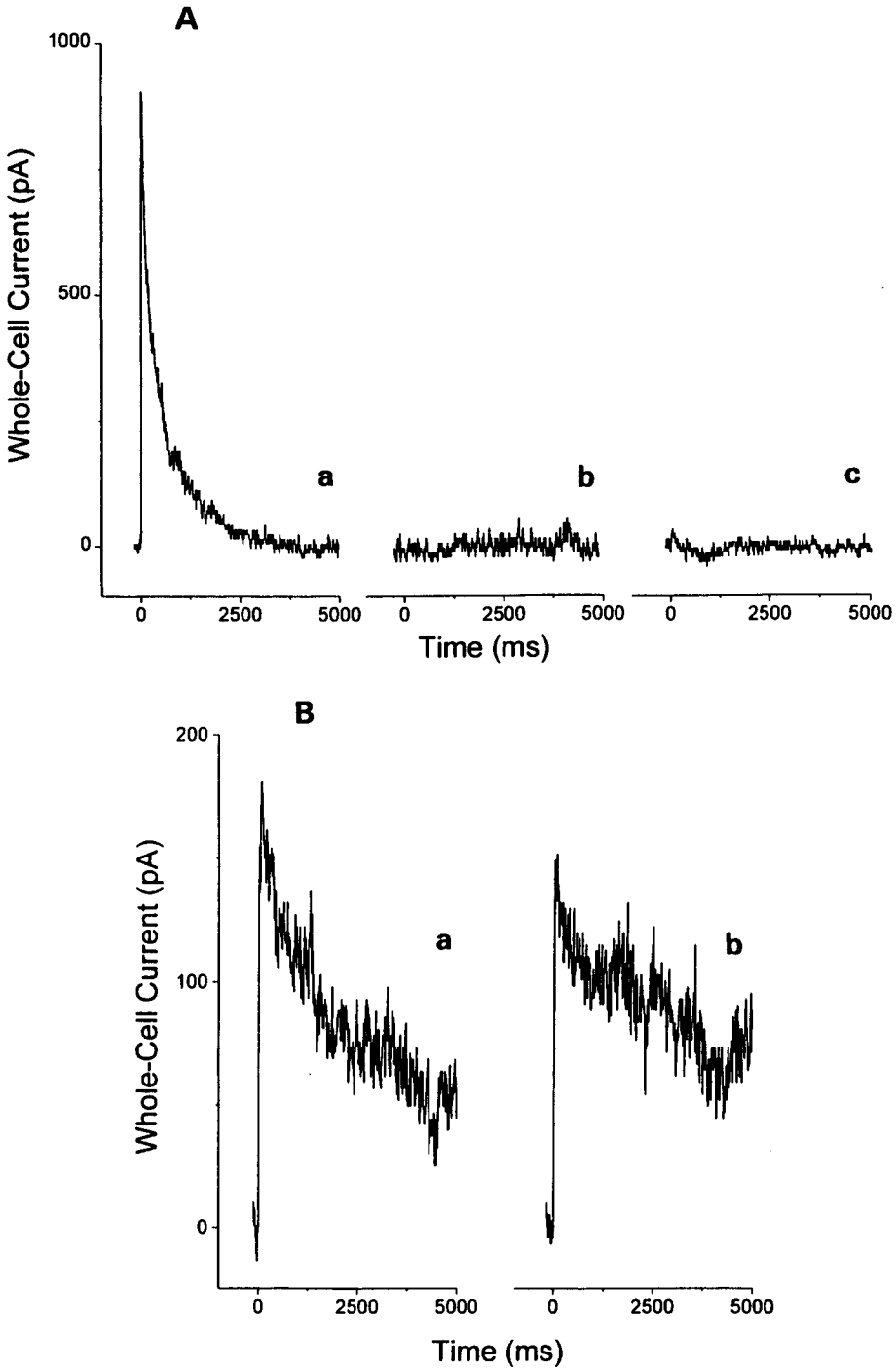

FIGURE 3: Whole-cell current responses of rat hippocampal neurons held in culture for 3 days (A) or 2 weeks (B). The cell in A showed a response to 1-mM GABA (a) but not to 1-mM β-alanine (b) or to 1-mM glycine (c). The cell in B showed responses to both 300-μM glycine (a) and 300-μM β-alanine (b). All measurements were made at -60 mV, pH 7.1, and 22 °C using a cell-flow technique (Udgaonkar & Hess, 1987). All current traces were collected for 10 s, but for clarity only the initial 5-s segment is shown for each of the traces.

chosen as the control concentration. Figure 4 shows the whole-cell current responses evoked by 300-μM β-alanine in the absence and presence of 750-μM MNP-β-alanine. When the two whole-cell current traces are superimposed (Figure 4), they are indistinguishable, indicating that the MNP-β-alanine is not an activator, inhibitor, or potentiator of the β-alanine response under the conditions of the experiment.

Laser-Pulse Photolysis of MNP-β-Alanine on a Single Rat Hippocampal Neuron. The photochemical properties of MNP-β-alanine were tested using the laser-pulse photolysis technique on a single neuron. Photolysis of 750-μM MNP-β-alanine induced a transmembrane ionic current as detected by whole-cell current recording (Hamill et al., 1981) (Figure 5). When the cell was preincubated with 750-μM MNP-β-alanine for 400 ms before photolysis activation of the glycine receptor was not observed (Figure 5), indicating both that no detectable level of free β-alanine was present in the solution, even 4 min after dissolving the compound, and that MNP-β-alanine is indeed inactive towards the receptor. The experiments confirmed that the compound is sufficiently stable in aqueous solution at neutral pH for at least 4 min to be used in experiments. Photolysis of MNP-β-alanine led to a 90-pA whole-cell current, which corresponds to the release of 20-μM β-alanine by photolysis (Figure 5). The current rise is fitted by a single exponential with a rate

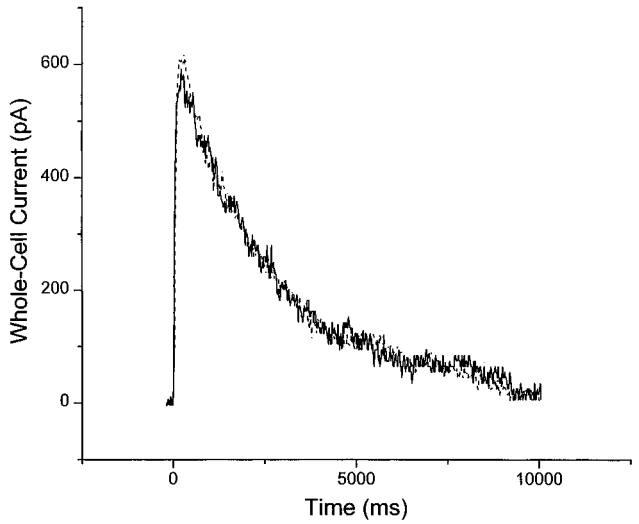

FIGURE 4: MNP-β-Alanine does not interfere with activation of whole-cell currents by β-alanine. The experiments were done using a cell-flow technique (Udgaonkar & Hess, 1987) with rat hippocampal neurons at −60 mV, pH 7.1, and 22 °C. The whole-cell currents induced by 300-μM β-alanine in the absence (solid line) and presence (dashed line) of 750-μM MNP-β-alanine are superimposed. The corrected current amplitudes of whole-cell responses induced by 300-μM β-alanine in the absence (solid line) and presence (dashed line) of 750-μM MNP-β-alanine are 630 and 670 pA, respectively. The desensitization proceeded in both cases in two phases. For the control (solid line), the rate of the fast component is 0.5 s^{-1} (62%) and that of the slow component is 0.2 s^{-1} (38%). In the presence of MNP-β-alanine (dashed line), the rate of the fast component is 0.6 s^{-1} (61%) and that of the slow component is 0.2 s^{-1} (39%).

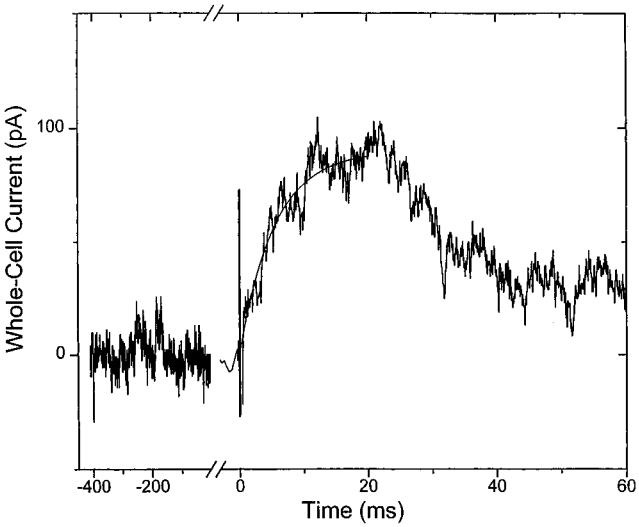

FIGURE 5: Whole-cell current induced by laser-pulse photolysis of MNP-β-alanine with a rat hippocampal neuron at −60 mV, pH 7.1, and 22 °C. The cell was preincubated with 750-μM MNP-β-alanine for an initial 400 ms. At time zero, a pulse of 333-nm laser light with an energy output of 500 μJ and pulse length of 600 ns was fired, as indicated by an electronic artefact which appears as a spike at the origin. The whole-cell current generated by free β-alanine released by the laser pulse was detected. The concentration of the photolyticaly released free β-alanine was estimated to be 20 μM as calibrated by a cell-flow technique (Udgaonkar & Hess, 1987). The whole-cell current trace is shown in two time domains. The maximum whole-current amplitude was 90 pA; at longer times (>25 ms) the current falls and reflects receptor desensitization. The solid line is calculated for a single-exponential process with a rate constant of 180 s^{-1}.

constant of about 180 s^{-1} over 85% of the rise. At the low concentrations of released β-alanine used, the value of 180 s^{-1} is assumed to reflect the channel-closing rate constant (Matsubara et al., 1992; Niu & Hess, 1993) and, therefore, a lifetime for the open channel of 5.6 ms. In spinal cord neurons the glycine receptor exhibits two conductance states, the main one having a lifetime in the range 3.9−5.9 ms (Twyman & MacDonald, 1991).

DISCUSSION

The choice of a suitable caging group is the first step in synthesizing a desired photolabile precursor of a neurotransmitter. Several criteria must be met in developing caged neurotransmitters for rapid kinetic investigations of neurotransmitter-mediated reactions. One is that the rate of neurotransmitter release from its precursor must be fast with respect to the rate of the reaction of interest. A successful caged compound will also be water soluble, biologically inert, and have a high quantum yield. In light of a report by Kuzmic et al. (1986), we have explored the feasibility of a new type of caging group, 2-methoxy-5-nitrophenyl, for the carboxyl group of neurotransmitters.

A distinctive feature of MNP-β-alanine is its stability in aqueous solution at neutral pH compared with MNP-glycine and MNP-GABA (Figure 1 and Table 1). The rate of thermal hydrolysis of MNP-β-alanine at neutral pH is ∼25 times slower than that of MNP-glycine (Table 1). MNP-GABA was found to hydrolyze instantly when dissolved at neutral pH (Table 1). We were, therefore, unable to use this compound to measure $GABA_A$ receptor activation with laser-pulse photolysis. The relative rates of hydrolysis of the three compounds are consistent with a mechanism in which the hydrolysis reaction is accelerated by intramolecular nucleophilic attack on the ester carbonyl by the amino group of the neurotransmitter (Page et al., 1986; Ramesh et al., 1993). Hydrolysis of MNP-glycine is favored by the spatial proximity of lone pair electrons on the primary amine to the ester carbon, brought about by formation of an intermediate with a three-membered ring. In the case of MNP-β-alanine, amino group participation involves formation of an intermediate with a four-membered ring whereas a five-membered intermediate is involved in the hydrolysis of MNP-GABA. The different hydrolytic stability exhibited by the three derivatives is an example of a well known neighboring group effect that involves intramolecular nucleophilic attack in cyclic intermediates (Carey & Sundberg, 1990). In general, compounds that can produce three-, five-, and six-membered ring intermediates during thermal hydrolysis will be less stable in aqueous solution at neutral pH, while compounds that involve four- and >6-membered ring intermediates are relatively more stable. The quantum yield and the rate of neurotransmitter release from MNP-β-alanine and MNP-glycine are similar. These results suggest that lactam formation, from intramolecular nucleophilic attack by the free amino group, may be an important pathway in the thermal hydrolysis of some MNP-caged amino acids, but that it plays little role in the photohydrolysis mechanism. Thus, measurement of the rate of formation of 2-methoxy-5-nitrophenol during photolysis provides information about the rate of liberation of the amino acid in its physiologically active, ring-opened form. Potentially the MNP group can be used to cage other compounds that have at least one carboxyl group and that cannot form three-, five-, or six-membered rings involving an amino group.

The rapid photolytic release of free β-alanine (<3 μs), a quantum yield of 0.2, and its relative stability at neutral pH

make this compound a suitable candidate for rapid kinetic investigations of the formation of glycine receptor-channels. The opening and closing rate constants for the glycine receptor-channel, and the intrinsic equilibrium dissociation constant(s) for ligand:receptor complexes, can now be determined by using the laser-pulse photolysis technique. Furthermore, the inhibition and activation of the receptor-channel by various compounds can now also be investigated in greater detail. Caged carbamoylcholine (Milburn et al., 1989) and caged glutamate (Wilcox et al., 1990) have been used for identifying and mapping the distribution of receptors on neurons (Denk et al., 1994; Denk, 1994) and brain tissue (Callaway & Katz, 1993; Katz & Dalva, 1994; Dalva & Katz, 1994). MNP-β-Alanine can be used in similar studies to identify and map the inhibitory glycine receptor because of its stability in aqueous solution at neutral pH.

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