Synthesis and Photochemical Properties of a Kainate Precursor and Activation of Kainate and AMPA Receptor Channels on a Microsecond Time Scale†

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ABSTRACT: Kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolylpropionate (AMPA) receptors are transmembrane proteins that can form ion channels upon binding a specific ligand. The receptors are located at major excitatory synapses in the mammalian central nervous system. Kainate and AMPA receptors participate in many physiological activities of the brain, including learning and memory, and are involved in many neurological disorders. Elucidation of the mechanisms of receptor transmembrane channel formation, inhibition, and regulation is important in understanding fundamental central nervous system function and in designing potential therapeutic agents. Kainate can activate both kainate and AMPA receptors, leading to channel opening in the microsecond to millisecond time region. A newly developed laser pulse photolysis technique, with a microsecond time resolution, has been successfully used to study the chemical reactions of receptor proteins in the microsecond to millisecond time region. To apply the technique to kainate and AMPA receptors, a photolabile kainate precursor in which a caging group, the α-carboxy-2-nitrobenzyl group, is attached to the γ-carboxyl group of kainic acid has been synthesized. The photolytic release of free kainate from the caged kainate on the microsecond time scale, initiated by a pulse of laser light at 308 nm, was measured. The quantum yield is 0.34 at pH 6.8 and room temperature. The half-life of the major component (∼86%) of the photolytic reaction is 45 μs, while that of the minor component (∼14%) is 0.7 ms. The effects of the caged kainate on kainate and AMPA receptors endogenously expressed in rat hippocampal neurons were also evaluated. Caged kainate (750 μM) did not activate the receptor channels, nor did it potentiate or inhibit the kainate response. Photolysis of the caged kainate by a pulse of 333-nm laser light resulted in a rapid rise (with a $t_{1/2}$ of 0.4 ms) in the whole-cell current due to the opening of kainate-activated receptor channels. The results presented demonstrate that this kainate precursor is suitable for rapid chemical kinetic investigations of the kainate and AMPA receptors in the microsecond to millisecond time region.

Glutamate-activated receptors are the most abundant excitatory neurotransmitter receptors in the mammalian central nervous system (Mayer & Westbrook, 1987; Hollmann & Heinemann, 1994; Jonas & Spruston, 1994). On the basis of pharmacological (Collingridge & Lester, 1989) and molecular cloning and expression (Hollmann & Heinemann, 1994) studies, three glutamate receptor subtypes have been classified as *N*-methyl-D-aspartate (NMDA),[1] kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolylpropionate (AMPA) receptors. These glutamate receptors are cation-permeable channel proteins and are responsible for chemical synaptic transmission at the majority of excitatory synapses in the mammalian brain (Mayer & Westbrook, 1987; Hollmann & Heinemann, 1994; Jonas & Spruston, 1994). They are believed to be involved in learning and memory (Collingridge & Lester, 1989; Bashir et al., 1993) and are also involved in many neurological disorders, including strokes and Parkinson's disease (Hollmann & Heinemann, 1994).

Kainate can activate both kainate and AMPA receptors. Various EC_{50} values (defined as the activating ligand concentration at half-maximum current response) for kainate have been reported and in the case of hippocampal neurons are in the range 22−474 μM (Jonas & Sakmann, 1992; Lerma et al., 1993; Patneau et al., 1993). Kainate-activated channels were also reported to have wide ranges of conductance levels, from ≤5 to 49 pS, and of lifetimes, from 0.5 to 3 ms (Cull-Candy & Usowicz, 1987; Jahr & Stevens, 1987; Vyklicky et al., 1991). The time constant of kainate and AMPA receptor desensitization is as short as a few milliseconds, as was observed in measurements with outside-out-patches (patches of membrane a few micrometers in diameter pulled from the cell) (Raman & Trussell, 1992; Patneau et al., 1993; Trussel et al., 1993). The kainate and AMPA ion channels were found to be activated in the microsecond to millisecond time scale in measurements of miniature excitatory postsynaptic currents (Tang et al., 1991; Jonas & Spruston, 1994). The techniques used do not have the time resolution needed to measure elementary steps (for instance, the opening of the transmembrane channels) of the

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[1] Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolylpropionate; αCNB, α-carboxy-2-nitrobenzyl; BBNA, *tert*-butyl α-bromo-α-(2-nitrophenyl)acetate; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; GABA, γ-aminobutyric acid; NMDA, *N*-methyl-D-aspartate; TFA, trifluoroacetic acid.

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receptor-mediated reaction. Consequently, kinetic constants pertaining to individual reaction steps in channel activation have not yet been determined (Ambros-Ingerson & Lynch, 1993; Jonas & Spruston, 1994). Therefore, the relationship between neurotransmitter concentration and open receptor channels that determines the receptor-controlled change in transmembrane voltage (Hess et al., 1984), which triggers signal transmission between cells, is not known. Similarly, the chemical mechanism by which the receptor-mediated reaction is inhibited by abused drugs and therapeutic agents also is not well-understood.

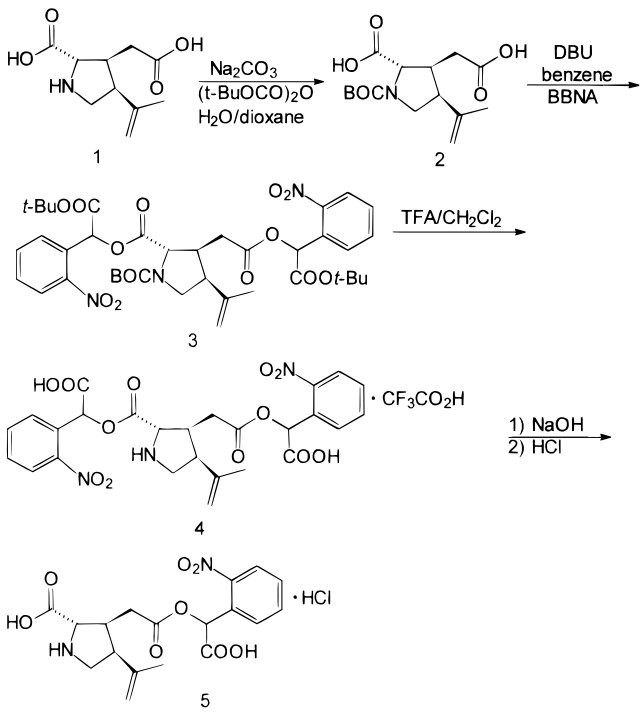
A recently developed laser pulse photolysis technique is suitable for measuring the kinetic constants of these receptor-mediated reactions because it provides adequate time resolution (Milburn et al., 1989; Matsubara et al., 1992; Billington et al., 1992; Niu & Hess, 1993; Ramesh et al., 1993; Hess, 1993; Wieboldt et al., 1994a,b; Gee et al., 1994; Hess et al., 1995; Niu et al., 1995). In this approach, photolytic release of an active substance from a precursor of the compound, a so-called caged compound, is employed to overcome slow diffusion and mixing of reactants [see reviews in Kaplan (1990), Adams and Tsien (1993), Corrie and Trentham (1993), and Hess (1993)]. To obtain caged compounds suitable for chemical kinetic investigations of neurotransmitter receptors, we modified the *o*-nitrobenzyl photolabile protecting group. The α-carboxy-2-nitrobenzyl (αCNB) caging group was introduced and attached to the amino group of carbamoylcholine (Milburn et al., 1989). The resulting compound is biologically inert, is readily photolyzed by a nanosecond pulse of laser light at 308 nm to carbamoylcholine with a quantum yield of 0.8 and $t_{1/2}$ of 45 μs, and has been used successfully in investigations of the nicotinic acetylcholine receptor in BC_3H1 muscle cells (Milburn et al., 1989; Matsubara et al., 1992; Niu & Hess, 1993; Hess, 1993; Hess et al., 1995; Niu et al., 1995). The same caging group was also used to protect the amino group of γ-aminobutyric acid (Wieboldt et al., 1994a) and glycine (Billington et al., 1992), which are ligands for the GABA and glycine receptors respectively. Recently, αCNB was also used to protect the γ-carboxyl group of glutamate (Wieboldt et al., 1994b), a ligand for glutamate receptors, and the carboxyl group of GABA (Gee et al., 1994). These caged compounds were found to be effective precursors of neurotransmitters in rapid chemical kinetic investigations of the corresponding neurotransmitter receptors.

In this report, we describe the synthesis of γ-*O*-(α-carboxy-2-nitrobenzyl)-(−)-kainate (caged kainate) and its photochemical properties. The potential of this caged kainate for use in rapid kinetic investigations of kainate and AMPA receptors was explored by using rat hippocampal neurons.

MATERIALS AND METHODS

N-(*tert-Butoxycarbonyl*)-(−)-*kainic Acid* (**2**). Scheme 1 outlines the chemical synthesis of γ-*O*-(α-carboxy-2-nitrobenzyl)-(−)-kainate (**5**). (−)-Kainic acid (**1**, Research Biochemicals International, 0.25 g, 1.2 mmol) was suspended in dioxane/water (1:1, 8 mL). Sodium bicarbonate (0.21 g, 2.5 mmol) was added. To the resulting solution was added a solution of *tert*-butyl pyrocarbonate (0.44 g, 2.0 mmol) in 1 mL of dioxane. The mixture was stirred at room temperature for 16 h, diluted with water (5 mL), and then extracted with EtOAc (3 × 5 mL) to remove excess

Scheme 1



pyrocarbonate. The pH of the aqueous portion was adjusted to 2.7 by the dropwise addition of 5% HCl, followed by extraction with EtOAc (3 × 5 mL). The extract was dried (sodium sulfate) and concentrated to give **2** as 0.37 g (100%) of a colorless foam: R_f (MeCN H_2O/AcOH, 8:1:1) 0.72; mp 145−148 °C (dec); ^{1}H NMR ($CDCl_3$) 4.96 (br s, 1H C═CH_2), 4.72 (br s, 1H, C═CH_2), 3.71 (s, 1H, $NCHCO_2$), 3.5 (m, 2H, NCH_2), 3.0 (m, 2H, CH_2CO_2), 2.35 (m, 2H, CO_2-CH_2CH, NCH_2CH), 1.73 (two s, 3H, CH_3), 1.43 (two s, 9H, $C(CH_3)_3$). Anal. Calcd for $C_{15}H_{29}NO_6$: C, 57.50; H, 7.90; N, 4.47. Found: C, 57.30; H, 7.56; N, 4.30.

Bis-α,γ-*[O-[*α-*(tert-butoxycarbonyl)-2-nitrobenzyl]]-N-(tert-butoxycarbonyl)-*(−)-*kainate* (**3**). To a colorless solution of **2** (0.73 g, 2.3 mmol) and *tert*-butyl α-bromo-α-(2-nitrophenyl)acetate (Wieboldt et al., 1994b) (1.48 g, 4.68 mmol) in benzene (55 mL) was added diazabicyclo[5.4.0]-undec-7-ene (0.72 mL, 4.8 mmol). The resulting blue solution was heated at 70 °C for 3 h and then cooled and partitioned between EtOAc (30 mL) and water (30 mL). The organic portion was dried (sodium sulfate) and concentrated to give a brown gum, which was purified by flash chromatography (Still et al., 1978) to give **3** as 1.12 g (61%) of a clear, very pale brown immobile oil as a mixture of diastereomers: R_f (5% EtOAc/$CHCl_3$) 0.47; ^{1}H NMR ($CDCl_3$) 8.00 (m, 2H, Ar-3-H), 7.7 (m, 4H, Ar-4,5-H), 7.5 (m, 2H, Ar-6-H), 6.8 (m, 2H, ArCH), 5.0, 4.9 (two br s, 1H, C═CH_2), 4.72 (two br s, 1H, C═CH_2), 4.4 (two m, 1H, $NCHCO_2$), 3.7 (m, 1H, NCH_2), 3.5 (m, 1H, NCH_2), 3.1 (m, 2H, CO_2CH_2), 2.5 (m, 2H, CO_2CH_2CH, NCH_2CH), 1.8 (four s, 3H, CH_3), 1.35 (m, 27H, $C(CH_3)_3$). Anal. Calcd for $C_{39}H_{49}N_3O_{14}$ H_2O: C, 58.42; H, 6.41; N, 5.24. Found: C, 58.04; H, 6.43; N, 5.29.

Bis-α,γ-*[O-(*α-*carboxy-2-nitrobenzyl)]-*(−)-*kainate Trifluoroacetate Salt* (**4**). To a solution of **3** (1.04 g, 1.33 mmol) in dichloromethane (15 mL, anhydrous) under argon was added trifluoroacetic acid (6.0 mL, 78 mmol). The resulting solution was incubated at room temperature overnight and

evaporated to dryness. Toluene (1 × 5 mL) was evaporated from the residue, which was then purified by chromatography on Sephadex LH-20, using water as eluant. The product fractions were pooled and lyophilized, giving **4** as 0.72 g (79%) of a white powder as a mixture of diastereomers: R_f (MeCN/H_2O/AcOH, 8:1:1) 0.55; ^{1}H NMR (D_2O) 8.1 (m, 2H, Ar-3-H), 7.9−7.5 (m, 6H, Ar-4,5,6-H), 6.6 (m, 2H, ArCH), 5.1−4.9 (m, 2H, C═CH_2), 4.2 (dd, J = 24, 3.3 Hz, 1H, NCHCO_2), 3.7−3.4 (m, 2H, NCH_2), 3.2−3.0 (m, 2H, CO_2-CH_2), 2.7 (m, 2H, CO_2CH_2CH, NCH_2CH), 1.7 (four s, 3H, CH_3). Anal. Calcd for $C_{28}H_{26}N_3O_{14}F_3$: C, 49.07; H, 3.82; N, 6.13. Found: C, 49.82; H, 4.07; N, 6.25.

*γ-[O-(α-Carboxy-2-nitrobenzyl)]-(−)-kainic Acid Hydrochloride Salt (**5**).* The pH of a suspension of **4** (0.71 g, 1.0 mmol) in water (23 mL) was raised from 2 to 8.5 by the careful addition of aqueous NaOH with stirring. The colorless solution was incubated at room temperature for 12 h, and the pH was adjusted to 1.7 by dropwise addition of aqueous HCl. The resulting mixture was lyophilized, followed by purification on Sephadex LH-20 (2 × 10 cm); elution with water gave **5**, after pooling of product fractions and lyophilization, as a white powder of 0.31 g (70%) as a mixture of diastereomers: R_f (MeOH/$CHCl_3$/H_2O/AcOH, 12.5:10:3.5:0.2) 0.65; mp 159−163 °C (dec); UV−vis (H_2O) λ = 263 nm (ϵ = 4800); ^{1}H NMR (D_2O) 8.12 (dd, J = 8.1, 2.7 Hz, 1H, Ar-3-H), 7.81 (t, J = 8.0 Hz, 1H, Ar-5-H), 7.70 (m, 2H, Ar-4,6-H), 6.62 (s, 1H, ArH), 5.09, 4.74, 4.72, 4.66 (four s, 2H, C═CH_2), 4.23 (dd, J = 16.8, 3.3 Hz, 1H, NCHCO_2), 3.68 (m, 1H, NCH_2), 3.48 (m, 1H, NCH_2), 3.02 (m, 1H, NCH_2CH), 3.05 (m, 1H, NCH(CO_2H)CH), 2.65 (m, 2H, CO_2CH_2), 1.68, 1.59 (two s, 3H, CH_3); FAB HRMS for $C_{18}H_{21}N_2O_3$ 393.1298 (M^+ + H), found 393.1305. Mass spectroscopy was done at the Mass Spectroscopy Laboratory of University of Illinois, Champaign, IL. Elemental combustion analysis was performed by Galbraith Laboratories Inc., Knoxville, TN.

Laser Flash Photolysis. The equipment used for transient spectroscopy has been described (Milburn et al., 1989). Briefly, the pulses of 308-nm laser light used to photolyze the caged compound were produced from a Lumonics TE861M XeCl excimer laser; the pulse length was 10 ns and the energy about 50 mJ from the primary output. The wavelength of 308 nm was chosen because it is near the isosbestic point of caged kainate and its photoproducts (∼300 nm). The transient absorbance of the *aci*-nitro intermediate (McCray et al., 1980; Schupp et al., 1987) was monitored at a right angle through the center of the laser beam, using a halogen lamp (Newport 780) with a Corning WGS360 cutoff filter and a 0.2-m McPherson 275 single-pass monochromator in the range 350−500 nm. Tbe illumination volume of the monitoring beam was about 10 × 1 × 2 mm^3 and that of the laser beam was 1 × 10 × 2 mm^3 (l × w × h). The signal from the monochromator was detected by a photomultiplier (Thorn EMI 9635QB), and the photocurrent was converted to voltage, filtered, preamplified (Thorn EMI Model A1), and stored using a digital storage oscilloscope (LeCroy Scope Station 140) with digitization rates of up to 1 MHz. The Origin program (MicroCal Software) was used for fitting (nonlinear least-squares method; Marquardt algorithm) and plotting the data. The buffers and their concentrations used in characterizing the transient intermediate were as follows: pH 2−5.5, 100 mM citrate; pH 6−8, 100 mM phosphate; pH 8.5−10, 100 mM borate; pH 10.5−12, 100 mM glycine.

Quantum Yield. Determination of the product quantum yield of caged kainate was based on the assumption (McCray et al., 1980; Schupp et al., 1987; Walker et al., 1988; Wootton & Trentham, 1989) that the concentration of the transient intermediate is directly proportional to the concentration of the compound liberated in the photolysis reaction. The absorbance of the transient intermediate, A_n, measured at the nth laser pulse with mixing of the solution after each pulse, is given by the following (Milburn et al., 1989):

$$A_n = \epsilon_M L C_0 \phi K_E \exp[-\phi K_E F(n-1)] \qquad (1)$$

ϵ_M is the molar extinction coefficient of the transient intermediate, L is the length of the light path, C_0 is the initial concentration of the compound that is photolyzed, ϕ is the quantum yield, K_E is the ratio of the number of absorbed photons to the number of target molecules in the laser beam, and F is the fraction of the solution containing the compound that is photolyzed through which the laser beam passes. We ascertained that the absorbance of the solution at 308 nm does not change, within experimental error, during these experiments. This ensures that energy absorption by the product at the expense of the starting material does not have to be considered. The quantum yield and the molar extinction coefficient of the *aci*-nitro intermediate can be obtained from a semilogarithmic plot of A_n versus (n − 1) (see Figure 2). The quantum yield evaluated is based on the experiments and assumptions given earlier that the concentration of the *aci*-nitro intermediate is directly proportional to the concentrations of the released protecting group and the desired product, in this case the neurotransmitter.

Cell Culture and Whole-Cell Current Recording. Neurons were obtained from the hippocampi of 2-day-old Sprague−Dawley rats and cultured on 35-mm Falcon dishes pretreated with 0.5 mg/mL collagen (rat tail type I from Sigma) and maintained in 5% CO_2. Minimal Eagle's medium was supplemented with 10 mM glucose, 2.7 mM glutamine, 5% fetal bovine serum, and 5% horse serum for the first day; on the second day fetal bovine serum was omitted (all from Gibco). On the fifth day, the dishes were treated with 41 μM 5-fluoro-2′-deoxyuridine and 102 μM uridine (final concentration; all from Sigma) for 24−48 h. The cells used in experiments were from 5 days to 3 weeks old. Some measurements were made with vesicles. The method of obtaining vesicles has been described by Walstrom and Hess (1994) and is similar to that described by Sather et al. (1992). In brief, a vesicle was obtained from a cell body by first making a whole-cell seal (Hamill et al., 1981) and then gently lifting the recording electrode (glass pipet) until the membrane pinched off from the cell body, thus forming a vesicle. The vesicles typically had a diameter of about 10 μm and a capacitance of about 1−3 pF. When an entire cell was used in the measurements, the cell was also lifted up from the bottom of a culture dish and suspended in the external solution. The cell body had a diameter of about 15 μm and a capacitance of about 7 pF: The glass electrodes were made with a List L/M-3P-A pipet puller and were fire-polished using a Narishige MF-83 polisher. The electrode resistance was about 3 MΩ. The extracellular solution consisted of 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10

mM glucose, and 10 mM HEPES (pH 7.4), and 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 7.4). A cell flow device (Krishtal & Pidoplichko, 1980; Udgaonkar & Hess, 1987) was used to equilibrate the receptors on the cell surface with ligands, including kainate (Sigma) and caged kainate **5**. Whole-cell currents (Hamill et al., 1981) were detected with a List L/M-EPC-7 amplifier, filtered through a low-pass RC filter (Krohn-Hite 3322) with a cutoff frequency of 2 kHz (−3-dB point), and recorded with a sampling frequency of 300−500 Hz by a Labmaster DMA digitizer (Scientific Solutions) driven by the PClamp program (Axon). The Origin program (MicroCal Software) was used for fitting (nonlinear least-squares method; Marquardt algorithm) and plotting data.

Laser Pulse Photolysis. All compounds, including caged kainate and kainate, were dissolved in the extracellular buffer. A Candela UV-500 flash-lamp-pumped dye laser with sulforhodamine 640 (Exciton) as the laser dye was used for all of the laser pulse photolysis measurements. Single laser pulses at 333 nm, tuned by a secondary harmonic generator, were coupled into an optical fiber (core diameter = 200 μm, SFS200/220N, FiberGuide Industries) and directed to the cells, with an output energy of 500 μJ from the end of the fiber, as measured with a joulemeter (Gentec ED-200). Triggering of the laser discharge and data collection were synchronized by using the timing capabilities of Labmaster DMA hardware (Scientific Solutions) driven by the PClamp program (Axon). Kainate of known concentrations was used to calibrate the response by using the cell-flow method (Udgaonkar & Hess, 1987) before and after photolysis of the caged kainate. The whole-cell current generated by released kainate was detected and recorded as described previously (Hamill et al., 1981). The filter cutoff frequency was 10−20 kHz, and the sampling frequency was 10−50 kHz. The rising phase of the whole-cell current generated by photoreleased kainate can be described by a single-exponential rate equation (Matsubara et al., 1992; Niu & Hess, 1993):

$$I_t = I_{max}[1 - \exp(-k_{obs}t)] \quad (2)$$

I_t is the current observed at time t, I_{max} is the maximum amplitude of the observed current in the absence of desensitization, and k_{obs} is the observed first-order rate constant for the channel opening process.

RESULTS

Rate of Photoproduct Release and Quantum Yield. The photochemical reaction of the kainate precursor was characterized with transient absorbance spectroscopy after the reaction was initiated by a single 10-ns pulse of light at 308 nm, with an energy output of 50 mJ from a XeCl excimer laser. Transient absorbance spectra between 350 and 500 nm for the photolysis of the kainate precursor are shown in Figure 1A. The spectral distribution of the intermediate species with a maximum absorbance at 420 nm corresponds to that of the characteristic *aci*-nitro intermediate in the internal redox reaction of *o*-nitrobenzyl photodeprotection (McCray et al., 1980; Schupp et al., 1987). Figure 1B shows a representative trace of the formation and decay of this transient intermediate, which was followed by measuring the change in UV−vis absorbance of the reaction mixture during

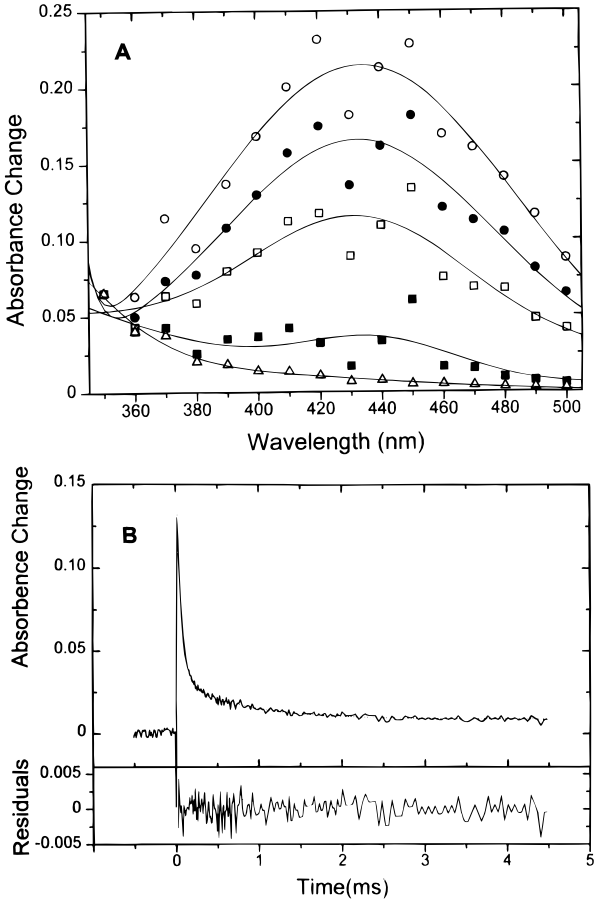


FIGURE 1: (A) Spectral distribution of the transient absorbance signal produced by photolysis of 0.5 mM caged kainate **5** in 100 mM phosphate at pH 6.8 and 22 °C. The spectra were constructed from the maxima of the transient absorbances observed at various wavelengths, indicated as the data points, and at various time periods: ○, 0 μs (time zero is defined as the maximum absorbance after photolysis was initiated by a 308-nm laser pulse); ●, 20 μs; □, 50 μs; ■ 200 μs; △, 10 000 μs. (B) *aci*-Nitro intermediate decay produced by 0.5 mM caged kainate **5** in 100 mM phosphate at pH 6.8 and 22 °C. A 10-ns pulse of 308-nm laser light (path length = 1 mm) was given at time zero, and the monitoring wavelength (path length = 10 mm) was set at 430 nm. The solid line represents the measured data, and the dotted line represents the double-exponential fit. The lower panel displays the residuals of the fitting. The fitting results are indicated in the text.

photolysis at pH 6.8 and 430 nm. By assuming that the *aci*-nitro intermediate decays with concomitant release of the protected compound (Walker et al., 1988; Wootton & Trentham, 1989), the decay rate is taken as the rate of photolytic release of the protected compound, in this case the free kainate. The decay of **5** is a double-exponential rate process (Figure 1B). About 86% of the reaction occurs with a $t_{1/2}$ of 45 μs as the fast major component, while about 14% proceeds with a $t_{1/2}$ of 0.7 ms as the slow, minor component. The rate constants for both the slow and fast components have an optimum at pH 5 (data not shown). On a time base greater than 200 μs, the spectral distribution is similar to that observed during the first few microseconds and predominantly reflects the slow-component reaction (Figure 1A). This suggests that the same intermediate(s) give rise to both the fast and slow components of the photolytic reaction. These characteristics of the photolytic reaction have been reported previously for a glutamate precursor, which is also protected by the αCNB group (Wieboldt et al., 1994b).

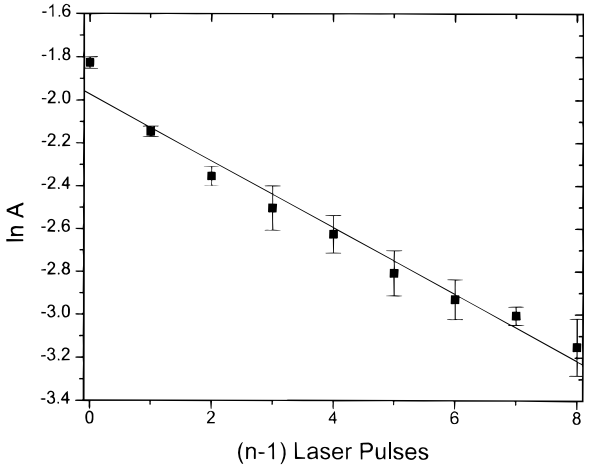


FIGURE 2: Determination of the product quantum yield for photolysis of caged kainate **5** in 100 mM phosphate at pH 6.8 and 22 °C. A sample cuvette containing 0.5 mM caged kainate was irradiated, with mixing of the solution between laser pulses of 308 nm with energy output of 50 mJ, and the absorbance, *A*, was monitored at 430 nm as a function of the number of laser shots, *n*. The ratio of absorbed photons to targeted molecules (K_E) was 0.87, the path length (*L*) was 10 mm, and the fraction of the volume irradiated (*F*) was 0.5. The product quantum yield and molar absorption coefficient were determined, by using eq 1, to be 0.34 ± 0.07 and 920 M^{-1} cm^{-1}, respectively. The measurements were made in duplicate, and the standard deviation from the mean is shown.

It is not known whether the same products are liberated as during the initial phases of the photolytic reaction.

The product quantum yield of caged kainate was determined. A semilogarithmic plot of A_n versus $(n - 1)$ according to eq 1 is shown in Figure 2. *A* represents the absorbance at 430 nm and *n* the number of laser shots. The product quantum yield, obtained from the slope of the line, was found to be 0.34 ± 0.07, and the molar extinction coefficient of the *aci*-nitro intermediate was evaluated to be 920 M^{-1} cm^{-1} at 430 nm and pH 6.8.

Activation of Kainate-Selective Receptors by Photolysis of Caged Kainate. The biological properties of the caged kainate were further tested by using rat hippocampal neurons, which express endogenous kainate and AMPA receptors (Patneau et al., 1993). To calibrate the kainate response in these cells, we used a cell-flow method with a 5-ms time resolution (Udgaonkar & Hess, 1987) and known concentrations of kainate. Figure 3A displays a typical trace of the kainate response under the whole-cell current-recording configuration (Hamill et al., 1981) in a cell-flow experiment. When 50 μM free kainate flowed over a vesicle of 6−10-μm diameter (Sather et al., 1992; Walstrom & Hess, 1994) obtained from a hippocampal cell, the amplitude of the response was about 30 pA. With the same vesicle, 30 μM kainate induced a whole-cell current of about 10 pA (data not shown). Also with the same vesicle, photolysis of the caged kainate induced a transmembrane ionic current; Figure 3B presents the whole-cell current response to about 100 μM free kainate generated by photolysis of 750 μM caged kainate. The observed first-order rate constant was determined by using eq 2 (Materials and Methods section) to be 1900 s^{-1}, and the amplitude of the response was about 70 pA.

The properties of the caged kainate have also been characterized in several other respects. Caged kainate (750

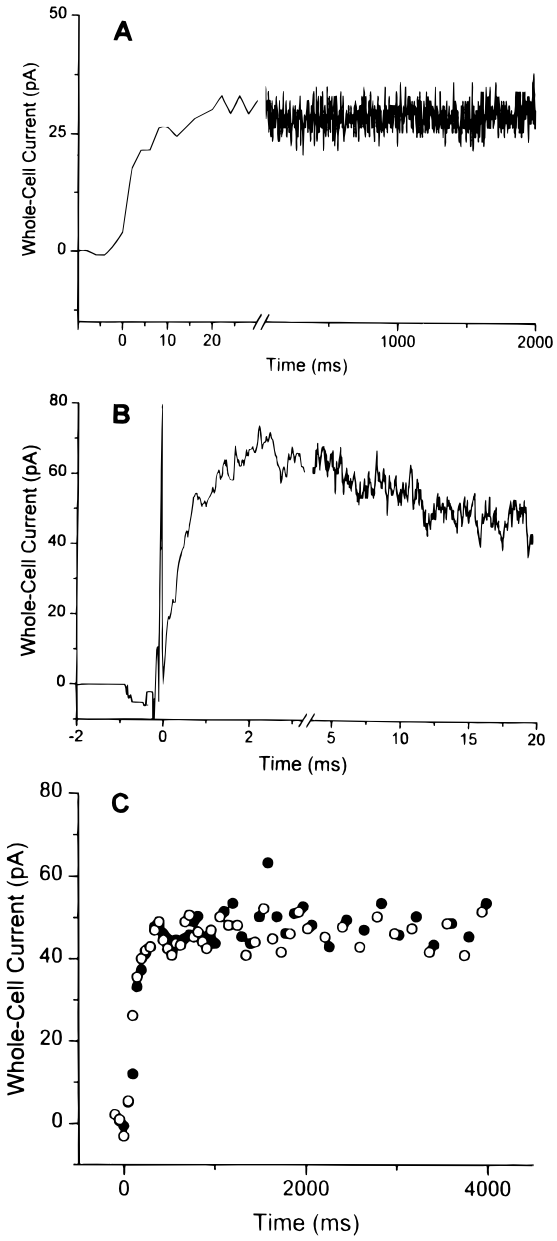


FIGURE 3: (A) Whole-cell response generated by 50 μM kainate in a cell-flow experiment using a vesicle from a hippocampal neuron at a transmembrane voltage of −60 mV, pH 7.4, and 22 °C. The current amplitude was about 30 pA. The current is shown on two different time scales indicated by a break in the *x*-axis. (B) Kainate-activated whole-cell response generated by about 100 μM free kainate released from 750 μM caged kainate by laser pulse photolysis, using the same vesicle as in (A) and under the same experimental conditions. The laser pulse length was 600 ns, and the wavelength was 333 nm. The spike at time zero is an instrumental artifact. The current amplitude and the observed rate constant for current rise fitted by one exponential were 70 pA and 1900 s^{-1}, respectively. The evoked whole-cell current is shown on two different time scales, indicated by a break in the *x*-axis. (C) Whole-cell response generated by 100 μM kainate in the absence (●) and presence (○) of 750 μM caged kainate after preincubating the cell with 750 μM caged kainate for 5 s. A whole cell, not a vesicle, was used in these experiments at −60 mV, pH 7.4, and 22 °C. For clarity, every eighth data point was used in plotting both traces.

μM) did not induce any detectable response (in experiments that were done with the same vesicle that was used for the experiments illustrated in Figure 3A,B). When 750 μM caged kainate was preincubated with receptors for up to 2 s, there was no decrease in the amplitude as assayed with 1

mM kainate, the concentration that gave the maximum response (data not shown). Furthermore, when 750 μM caged kainate was preincubated with the cell for up to 5 s, there was neither a decrease nor an increase in the amplitude of response as assayed with 100 μM kainate (Figure 3C). These results indicate that the caged kainate that we synthesized is biologically inert; it is not an inhibitor or a potentiator of the kainate response. In addition, spontaneous hydrolysis of the caged kainate, thus releasing free kainate, was not observed at ambient temperature in the time period of the experiments, ranging up to a few hours.

DISCUSSION

The αCNB caging group was introduced to protect the amino group of carbamoylcholine (Milburn et al., 1989). The resulting caged carbamoylcholine has been used to study the nicotinic acetylcholine receptor in BC_3H1 cells, including investigations of the mechanisms of activation (Matsubara et al., 1992) and inhibition by clinically important compounds (Niu & Hess, 1993; Niu et al., 1995) in the microsecond to millisecond time region. We have recently extended the use of the αCNB caging group to protect carboxyl groups in glutamate (Wieboldt et al., 1994b), GABA (Gee et al., 1994), and NMDA (Gee et al., 1995) derivatives, and we have now added a new compound, a kainate precursor. The results point to the potential of this caging group, which can also be used in the synthesis of precursors of other neurotransmitters and other biological compounds containing either amino or carboxyl groups.

Caged kainate was synthesized from readily available starting materials, and the photochemical characterization proved that this kainate precursor has several desirable properties, including a satisfactory quantum yield and a rapid photodecomposition rate constant for the release of free kainate. The usefulness of the compound was demonstrated with rat hippocampal neurons. The photolytically released free kainate induced whole-cell currents, and the caged kainate neither activated nor inhibited the kainate response in these neurons. The new kainate derivative is, therefore, suitable for rapid chemical kinetic investigations of kainate and AMPA receptors on the surface of a single neuron using the laser pulse photolysis technique (Matsubara et al., 1992; Niu & Hess, 1993; Niu et al., 1995).

Kainate and AMPA receptors coexist in hippocampal neurons (Lerma et al., 1993). In these neurons, both nondensitizing and desensitizing kainate responses have been observed (Trussel et al., 1988; Tang et al., 1989; Patneau & Mayer, 1990; Patneau et al., 1992, 1993; Lerma et al., 1993; Paternain et al., 1995; Spruston et al., 1995). In our preparation of hippocampal neurons, activation of receptors by kainate does not give rise to an observable fast desensitizing component in either the cell-flow experiments or the laser pulse photolysis measurements (Figure 3). The slowly decaying phase of the current observed in the laser pulse photolysis experiment (Figure 3B), but not in the cell-flow experiment (Figure 3A), presumably is due to diffusion of the photoreleased kainate away from the cell surface. For the nondesensitizing kainate response, it may be possible to measure the maximum whole-cell current by using conventional flow techniques with low time resolutions. The time constant for channel opening, however, which is in the 500-μs time region (Figure 3B), can be determined by laser pulse photolysis but not by rapid perfusion techniques (Figure 3). In laser pulse photolysis experiments, the time resolution is determined by the photolysis of caged kainate with a time constant of about 65 μs. In the cell-flow technique (Figure 3C), the time resolution is determined by the time required for neurotransmitter to equilibrate with receptors on the cell surface; the rise time of the current, associated with a time constant of ~20 ms, is considered to be a measure of this equilibration time (Udgaonkar & Hess, 1987). The equilibration time of receptors with ligands in flowing solutions can be considerably reduced by recording from a small membrane patch rather than from an entire cell, with a diameter usually about 10 times larger than that of the membrane patch; this will, however, reduce the number of receptors sampled by over 2 orders of magnitude. Reaction intermediates that exist in low concentrations will, therefore, not be observed. Examples where low concentrations of intermediates may not be observed include the slowly desensitizing acetylcholine receptor forms in BC_3H1 cells (Udgaonkar & Hess, 1987), γ-aminobutyric acid receptors in cortical cells (Geetha & Hess, 1992), and glycine receptors in spinal cord cells (Walstrom & Hess, 1994). The laser pulse photolysis technique has a microsecond time resolution and allows one to sample a high concentration of receptor molecules in a whole cell. The ability to observe low concentrations of reaction intermediates is also important in investigations of the mechanism of inhibition by neurotoxins, therapeutic agents, and abused drugs (Niu & Hess, 1993; Niu et al., 1995). These compounds can have different effects on the major and minor receptor components (Geetha & Hess, 1992).

What have we learned and what can we learn from measurements using the laser pulse photolysis technique and the caged kainate? Firstly, the current rise due to the opening of the kainate-selective receptor channels can be fitted by a single exponential for over 90% of the reaction. This indicated that a single rate process for channel opening was observed, although it is not certain whether this rate represents a single channel type or multiple channel types with the same rate constant. With the laser pulse photolysis technique and caged kainate, both kinetic and equilibrium constants for receptor channel formation can be measured. At low kainate concentrations, the rising phase of the current reflects the rate of formation of receptor−kainate complexes, while at high kainate concentrations, when the opening of the receptor channels becomes rate-limiting, the rate constants for channel opening and closing can be determined (Matsubara et al., 1992; Niu & Hess, 1993; Niu et al., 1995). Secondly, the observed maximum amplitude of the whole-cell current depends upon the concentration of kainate released and is a measure of the concentration of open channels. The amplitude can also be used to determine the value of the receptor−ligand dissociation constant and the equilibrium constant for channel opening (Hess, 1993). Lastly, once the chemical kinetic constants of channel formation are determined; the effects of an inhibitor on these constants can be measured (Niu & Hess, 1993; Niu et al., 1995). It has also been possible to determine the value of the inhibitor dissociation constants separately for the closed-channel forms and the open-channel form of the receptor (Niu & Hess, 1993). The kainate and AMPA receptors are inhibited by many compounds, including nootropic agents like aniracetam (Ito et al., 1990), antihypertensive drugs like

cyclothiazide (Patneau et al., 1993), lectin molecules like concanavalin A (Meyer & Vyklicky, 1989), and polyamine-containing toxins isolated from spider and wasp venoms (Eldefrawi et al., 1988; Jackson & Usherwood, 1988). The mechanism of action of these compounds, some of which are being explored clinically, is at present not well-understood at a molecular level. The availability of a photolabile inert precursor of kainate, reported in this paper, together with the laser pulse photolysis technique, may be a valuable tool in chemical kinetic investigations of these receptors and may lead to an increased understanding of how signal transmission in the central nervous system is affected by potential therapeutic agents for the treatment of neurological disorders.

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