Synthesis, Photochemistry, and Biological Activity of a Caged Photolabile Acetylcholine Receptor Ligand†

Tracy Milburn,‡,§ Norio Matsubara,‡,∥ Andrew P. Billington,‡ Jayant B. Udgaonkar,‡,⊥ Jeffery W. Walker,‡,# Barry K. Carpenter,○ Watt W. Webb,△ Jeffrey Marque,△,◇ Winfried Denk,& James A. McCray,+ and George P. Hess*,‡

Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University, Ithaca, New York 14853

Received May 16, 1988; Revised Manuscript Received August 11, 1988

ABSTRACT: A biologically inert photolabile precursor of carbamoylcholine has been synthesized; it is photolyzed to carbamoylcholine, a well-characterized acetycholine analogue, with a half-time of 40 μs at pH 7.0 and a quantum yield of 0.8. The compound, *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine, was synthesized from (2-nitrophenyl)glycine. The photolysis rates (of five compounds) and the biological activity (of two compounds) were determined, and both properties were found to depend on the nature of the substituents on the photolabile protecting group. Laser pulse photolysis at wavelengths between 308 and 355 nm was used to investigate the wavelength dependence, quantum yield, and rate of the photolysis reaction. Photolysis products were isolated by high-performance liquid chromatography and identified by chemical and spectroscopic analysis and by their ability to activate the nicotinic acetylcholine receptor. BC_3H1 muscle cells containing those receptors and a cell-flow method were used in the biological assays. The approach described may be useful in the preparation and characterization of other photolabile precursors of neurotransmitters that contain amino groups. The importance of these rapidly photolyzed, inert precursors of neurotransmitters is in chemical kinetic investigations of the reactions involving diverse neuronal receptors; such studies have been hampered because the available techniques have an insufficient time resolution.

The application of chemical kinetic investigations, using fast reaction techniques, to studies of neuronal receptor mediated reactions is recent (Hess et al., 1979, 1987). The use of such techniques with a time resolution of 5 ms has been restricted to studies of receptors in membrane vesicles (Hess et al., 1979) or to measurements with receptor-containing cells (Udgaonkar & Hess, 1987), which have been limited by a 20-ms time resolution. Here we describe an approach that utilizes photolysis, in the microsecond time region, of an inert precursor to carbamoylcholine itself, a well-characterized analogue of acetylcholine. Kinetic investigations of the nicotinic acetylcholine receptor, employing laser pulse photolysis to generate carbamoylcholine, are capable of a time resolution of at least 100 μs. In this paper we described the synthesis of the precursors, their photochemistry, and their biological activity.

The use of photolabile precursors for investigations of biological reactions is based on earlier studies (Kaplan et al., 1978; McCray et al., 1980; Gurney & Lester, 1987; Walker et al., 1987; Adams et al., 1988; Ellis-Davis & Kaplan, 1988; Karpen et al., 1988) that demonstrated the utility of photolabile precursors of biologically important phosphates and chelators for calcium and other divalent cations in such investigations. We previously synthesized *N*-[1-(2-nitrophenyl)ethyl]carbamoylcholine (Walker et al., 1986) (compound II—see Table I for structures of carbamoylcholine derivatives). Although compound II showed appropriate photolytic properties, it was not inert and both inhibited and inactivated receptor function (Walker et al., 1986). On the premise that the net positive charge of the compound was responsible for this phenomenon, we sought to produce a derivative that also carried a negative charge, in the hope that this derivative would not interact with the receptor.

Initially an aryl carboxyl substituent was introduced (compound IV). However, photolysis of compound IV was retarded compared to that of *N*-[1-(2-nitrophenyl)ethyl]carbamoylcholine. In contrast, α-carboxyl substitution (compound V) enhanced the rate of photolysis and, additionally, eliminated the inhibition and inactivation (desensitization) of the receptor that was observed with compound II.

Experimental Procedures

Apparatus

Visible and ultraviolet absorption spectra were obtained on a Cary 219 recording spectrophotometer (Varian Instruments). Proton NMR spectra were recorded on an XL-200 MHz Fourier transform machine (Varian Instruments). Chemical shifts are given in parts per million (ppm) relative to tetramethylsilane. Melting points were determined with a capillary

† This work was supported by grants awarded to G.P.H. by NIH (GM04842) and the Cornell Biotechnology Program, which is supported by the New York State Science and Technology Foundation, a consortium of industries, and the U.S. Army Research Office.

* Correspondence should be addressed to this author at 270 Clark Hall, Cornell University, Ithaca, NY 14853.

‡ Section of Biochemistry, Molecular and Cell Biology, Division of Biological Sciences, Cornell University.

§ Present address: Department of Physiology, Australian National University, Canberra, A.C.T. 2601, Australia.

∥ Supported by an award from the Cornell Biotechnology Program.

⊥ Present address: Department of Biochemistry, Stanford University Medical School, Stanford, CA 94305.

Present address: Department of Physiology, University of Wisconsin, Madison, WI 53706.

○ Department of Chemistry, Cornell University.

△ School of Applied and Engineering Physics, Cornell University.

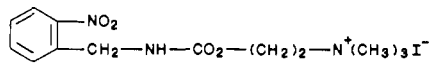
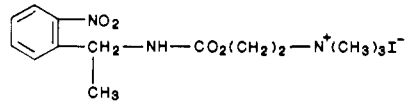
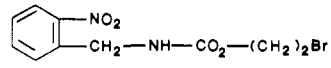
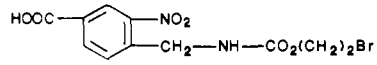
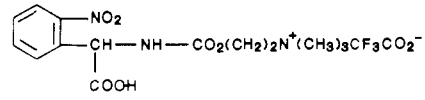
◇ Present address: Beckman Instruments, Inc., Spinco Division, 1050 Page Mill Rd, Palo Alto, CA 94303.

& Department of Physics, Cornell University.

+ Department of Physics, Drexel University.

0006-2960/89/0428-0049$01.50/0

Table I[a]

compound	λ_{max} (nm)	ϵ (M^{-1} cm^{-1})	$t_{1/2}$ (ms)	k (s^{-1})
 *N*-(2-nitrobenzyl)carbamoylcholine iodide (I)[b]	262	5200	1.7 ± 0.1	410
 N-[1-(2-nitrophenyl)ethyl]carbamoylcholine iodide (II)[b]	262	5200	0.067 ± 0.002	10000
 2-bromoethyl *N*-(2-nitrobenzyl)carbamate (III)			0.82[c]	850[c]
 2-bromoethyl *N*-(4-carboxy-2-nitrobenzyl)carbamate (IV)	260	5100	4.4	160
 *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine trifluoroacetate (V)	266	5200	0.04 ± 0.001	17000

[a] Measurements of $t_{1/2}$ and k were made in aqueous buffer at pH 7.0 and 23 °C. λ_{max} and ϵ_M were measured in aqueous solution and were independent of pH above 6. [b] Walker et al. (1986). [c] Solution contains 5% (v/v) methanol.

melting point apparatus (Thomas Hoover) and are uncorrected. Elemental analyses were done by Schwarzkopf Microanalytical Laboratories (Woodside, NY).

High-Performance Liquid Chromatography

A computer-controlled pump module (Perkin-Elmer Series 3B), rheodyne valve injector, spectrophotometric detector (Perkin-Elmer LC-75), and Omniscribe chart recorder (Houston Instruments) were used. The detector wavelength was set at 212 nm. An HPLC column (Dynamax modular macro C18) equipped with a guard column (Rainin Instruments) was used both to purify and to separate the photolysis products of compound V. The mobile phase was a linear gradient of acetonitrile in water containing 0.09% trifluoroacetic acid. The initial acetonitrile concentration was 10%, and this was increased linearly to 25% over a period of 30 min. A further increase to 30% in 5 min was made before returning to equilibrium conditions.

Synthesis

α-Bromo-2-nitro-4-toluic Acid (*a*). Nitration of α-bromo-4-toluic acid (Aldrich) was effected as described previously (Rich & Gurwara, 1975; Tjoeng et al., 1978) to yield 83% pure product a: mp 130.5–134.5 °C [lit. mp 120–126 °C (Rich & Gurwara, 1975) and 132–135 °C (Tjoeng et al., 1978)].

4-Carboxy-2-nitrobenzylamine (*b*). To a solution of product a (2 g, 7.7 mol) in a slight excess of 1 M sodium hydroxide was added aqueous hexamethylenetetraamine (1.29 g, 8.5 mol) with stirring at 0 °C. After 8-h stirring at room temperature the resulting betaine complex was filtered, washed with water, and dried in vacuo. The dry betaine (2.1 g) was refluxed for 2 h in a mixture of concentrated HCl (4 mL) and absolute ethanol (12 mL). The precipitated ammonium salt was filtered from the cooled solution. The filtrate was taken to dryness and then dissolved in a minimum amount of water; adjustment of the pH to 5.5 resulted in precipitation of the amine, which was filtered and air-dried to yield 42% pure product b: ^{1}H NMR (in D_2O containing a drop of HCl) δ 3.74 (s, 2 H, benzyl), 7.01 (d, 1 H, aromatic), 7.53 (dd, 1 H, aromatic), 7.89, (d, 1 H, aromatic).

2-Bromoethyl N-(4-Carboxy-2-nitrobenzyl)carbamate (*IV*). To a solution of product b (0.32 g, 1.6 mmol) in aqueous sodium carbonate (5 mL containing 0.18 g, 1.7 mmol) was added an ethereal solution of bromoethyl chloroformate (0.31 g, 1.7 mmol, in 3 mL; Aldrich). After 30-min stirring at room temperature, unreacted bromoethyl chloroformate was removed by ether extraction. The pH of the aqueous phase was adjusted to 3 with 2 M HCl, and the product was filtered and further extracted into dichloromethane from the aqueous layer. After being dried over anhydrous sodium sulfate, the organic phase was taken to dryness in vacuo, and the combined product was recrystallized from chloroform (0.36 g, 63%): ^{1}H NMR (acetone-d_6) δ 3.63 (t, 2 H, methylene), 4.36 (t, 2 H, methylene), 4.75 (d, 2 H, benzyl), 7.13 (br t, 1 H, amino), 7.83 (d, 1 H, aromatic), 8.31 (dd, 1 H, aromatic), 8.60 (d, 1 H, aromatic); mp 160–162 °C. Anal. Calcd for $C_{11}H_{11}BrN_2O_6$: C, 38.0; H, 3.2; N, 8.1. Found: C, 38.0, H, 3.0; N, 8.2.

2-Bromoethyl N-(2-Nitrobenzyl)carbamate (*III*). Compound III was prepared from 2-nitrobenzylamine hydrochloride (Overlook Industries) as described previously for 2-chloroethyl *N*-(2-nitrobenzyl)carbamate (Walker et al., 1986) to yield 60% of product: mp 73–74 °C. Anal. Calcd for $C_{10}H_{11}BrN_2O_4$: C, 39.6; H, 3.6; N, 9.2. Found: C, 39.9; H, 3.5; N, 9.4.

N-(α-Carboxy-2-nitrobenzyl)carbamoylcholine was synthesized from (2-nitrophenyl)glycine hydrochloride (Davis et al., 1973) as described in Figure 1.

Choline Chloride Chloroformate (*B*). A suspension of choline chloride (A) (0.7 g 0.022 mol; Aldrich) in dry chloroform was stirred under dry nitrogen at room temperature. Trichloromethyl chloroformate (TCF) (1.2 mL, 0.01 mol; Alfa) was added through a rubber septum from a gas-tight syringe. ^{1}H NMR was used to monitor the reaction. At completion (~10 h) integration showed the resulting yellow oil to be a 3:1 mixture of product B and chloroform. Since no impurities and no starting materials were detected by NMR at the end of the reaction, 100% yield was assumed. Due to

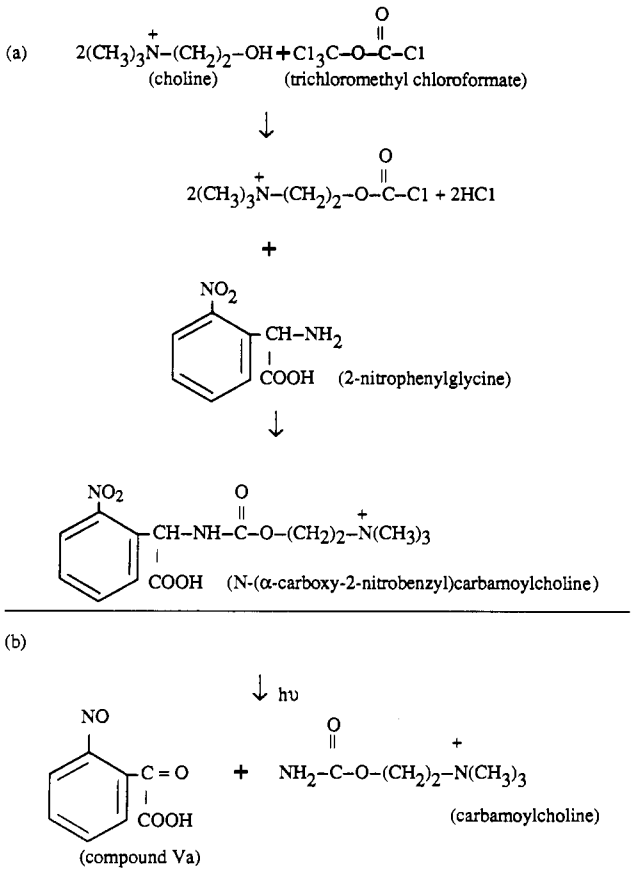


FIGURE 1: (a) Outline of the synthesis of *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine and (b) the proposed mechanism of the photolysis of *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine.

its high reactivity, product B was prepared as needed and used as the 3:1 mixture of product B and chloroform. ^{1}H NMR (acetone-d_6) showed peaks at δ 5.03 (m, 2 H, methylene), 4.20 (m, 2 H, methylene), and 3.52 (m, 9 H, trimethyl).

N-(α-Carboxy-2-nitrobenzyl)carbamoylcholine (D). Diisopropylamine (0.77 mL, 0.0055 mol; Aldrich) was added to a solution of (2-nitrophenyl)glycine (98 mg, 0.005 mol) in 2 mL of dry acetonitrile with stirring at 0 °C under a stream of dry nitrogen. A solution of the 3:1 mixture of choline chloride chloroformate and chloroform in dry acetonitrile (1 mL) was added dropwise from a gas-tight syringe. After being warmed to room temperature, the resulting slurry was filtered and was then evaporated to dryness in vacuo. Final purification of V as its trifluoroacetyl salt was effected by reversed-phase HPLC using a gradient of acetonitrile in water as described above: yield after HPLC 53 mg (23.3%); ^{1}H NMR (acetone-d_6) δ 8.00 (d, 1 H, aromatic), 7.71 (2 H, aromatic), 7.57 (m, 1 H, aromatic), 6.06 (d, 1 H, benzyl), 4.61 (m, 2 H, methylene), 3.91 (m, 2 H, methylene), 3.40 (m, 9 H, trimethyl). Anal. Calcd for $C_{16}H_{20}N_3O_8F_3$: C, 43.7; H, 4.6; N, 9.6. Found: C, 43.82; H, 4.28; N, 9.83.

Photolysis

Three different laser photolysis systems were used in these experiments. The first system used was a water-cooled Holobeam ruby laser with an output energy of typically 3 J. The laser was Q-switched with a Pockels cell, resulting in a pulse of 50-ns duration. The output was frequency doubled with an angle-tuned KDP (potassium dihydrogen phosphate) crystal. The 347-nm secondary beam with output energies up to 50 mJ was separated from the primary beam with a glass filter (U-350, Hoya Optics). The output of the primary beam was measured with a ballistic thermopile and a nanovoltmeter (Keithley) and that of the secondary beam with a disk energy meter (Scientec). For absorption measurements the secondary beam was allowed to fall on a 2-mm quartz cell set at 45° so that the path length was 2.83 mm.

Detecting light from a tungsten iodide lamp was directed through a monochromator (Bausch & Lomb) and was orthogonal to the laser beam and at 45° to the reaction cell, also resulting in the same path length. The transmitted light was monitored by use of a photomultiplier (EMI 9824B). Three blue glass filters (8 mm thick each) each with a transmission of 50% at 426 nm and a full-width at half-maximum of 84 nm were placed between the quartz cell containing the sample and the photomultiplier. In order to obtain the spectrum of an *aci*-nitro intermediate, measurements were made of the absorption after photolysis for different monochromator settings. To account for fluctuations in the pulse energy, a small fraction of the laser light was scattered into a photodiode, and the absorption signals were then normalized by the integrated photodiode output.

The second photolysis arrangement used was a "frequency-tripled" neodymium:YAG laser (15–25 mJ per 8–9-ns pulse at 355 nm) or an excimer laser (Lambda Physik 101) (10 mJ per 10-ns pulse at 308 nm). A quartz cuvette with a 2-mm light path length was used for absorption measurements. The wavelength of the detecting light (406 nm for compound III, 409 nm for compound IV, and 435 nm for compound V) was selected by a monochromator (McPherson Model 275). Absorption resulting from the *aci*-nitro intermediate was monitored with a photomultiplier (EMI Model 9635QB) after filtration through a Corion P10-405-S bandpass filter (for detection between 400 and 409 nm) or a Corning CS 375 edge filter (for detection at wavelengths longer than 410 nm).

The third system was a Candela SLL500 dye laser. Oxazine 720 perchlorate (Exciton) was used as the dye. The laser output at a wavelength of 704 nm was frequency doubled with an angle-tuned, temperature-stabilized ammonium dihydrogen phosphate crystal (Quantum Technology). In the experiments with BC_3H1 muscle cells described below, the 352-nm secondary laser beam was focused with a quartz lens (f = 12.7 mm) into a fiber optic cable of 200-μm core diameter (Newport FC 2 uv). By simultaneous projection of visible light through the same fiber, the cells could be illuminated sufficiently for experimental work, and hence, the location of the photolyzing beam was known. Throughput energies of up to 0.6 mJ, with a pulse length of 600 ns, were obtained by this technique.

Cell-Flow Studies

BC_3H1 cells were obtained from Dr. Gregory Weiland, Cornell University, and maintained in culture as described elsewhere (Sine & Taylor, 1979). For cell-flow measurements (Udgaonkar & Hess, 1987), cells were plated on 35-mm plastic dishes and differentiated in serum-poor medium (Olson et al., 1983). Measurements with cells were made in solutions of 140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.7 mM $MgCl_2$, and 25 mM HEPES (pH 7.4). Experiments were done at 22–23 °C. A cell-flow technique with a 20-ms time resolution was used that allowed the channel-forming process to be measured prior to receptor desensitization (Udgaonkar & Hess, 1987; Hess et al., 1987). Rapid application of ligand was achieved as previously described (Krishtal & Pidoplichko, 1980; Fenwick et al., 1982; Clapham & Neher, 1984; Udgaonkar & Hess, 1987). In brief, a U-shaped stainless steel capillary tube (250-μm i.d.) with a circular porthole of approximately 150-μm diameter at the base of the U was con-

nected to a peristaltic pump (Gilson) at both ends. This porthole was kept within 50–150 μm of the cell, which had been placed under a whole-cell voltage clamp (Hamill et al., 1981). The electrode contained a solution of 145 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, and 25 mM HEPES (pH 7.4). Ligand solution was applied to the cell surface by closing the solenoid valve at the exit of the U-tube and removed by opening the valve. Solution emerged from the porthole at a rate of ~20 μL min^{-1} when the valve was closed and entered the porthole when the valve was open at a rate of ~40 μL min^{-1}. Data were low-pass filtered (Krohn-Hite 3322) with a 2-kHz cutoff frequency (−3dB point), digitized at 100-Hz sampling frequency with a PDP 11/23 minicomputer, and stored on a hard disc. The data were then transferred to a Prime 750 digital computer (Material Science Center, Cornell University). The observed current was corrected for receptor desensitization (Udgaonkar & Hess, 1987).

Results

A number of photosensitive carbamoylcholine derivatives were synthesized as described and obtained in purified form by either HPLC or recrystallization. The proposed structures for these compounds (see Table I) were verified by proton NMR, UV absorption, and elemental analysis.

Photolysis and Identification of Products. Compounds I and II have been synthesized previously (Walker et al., 1986) and are shown for comparison with the newly synthesized compounds III–V in Table I. Compound V, *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine trifluoroacetate (Table I), appears to be the most promising compound for kinetic investigations of the acetylcholine receptor in the millisecond time region, and this paper deals mainly with its chemical and biological properties.

UV Spectra and Identification of Photolysis Products. The UV spectrum of compound V is shown in Figure 2a. The HPLC profile of a photolyzed solution of compound V is shown in Figure 2b. Peak A (Figure 2b) represents a new compound, which absorbs in the UV; the size of the peak varied with the degree of photolysis. The present hypothesis regarding the mechanism of the photolysis reaction (McCray et al., 1980) suggests that this compound is compound Va (see Figure 1).

Peak B (Figure 2b) had an elution volume typical of compound V, and peak C had an elution volume characteristic of authentic samples of carbamoylcholine. The material in peak C was detected by a chemical assay specific for amino nitrogen after acid hydrolysis of the sample (Schiffman et al., 1964).

Flash Photolysis. The cell-flow method in combination with flash photolysis was used to show that caged carbamoylcholine (compound V) does not induce the formation of transmembrane channels until it is photolyzed. In the experiment illustrated in Figure 2c, a BC_3H1 muscle cell was prepared for whole-cell current recording. The cell-flow method (Udgaonkar & Hess, 1987) was used to flow a solution of 100 μM caged compound over the cell. Under the flow conditions used, equilibration of the ligand with receptors on the cell surface is complete within 100 ms (Udgaonkar & Hess, 1987). After an initial period of solution flow of approximately 2 s, recording of the current was started at $t = 0$ in Figure 2c. No current was observed until the caged compound was photolyzed at t = 265 ms (Figure 2c) by a 600-ns, 352-nm laser flash. Three distinct phases of the whole-cell current can be seen. When the rate of photolysis of a caged neurotransmitter is rapid compared to the rates of receptor desensitization and channel formation, the rate constants of the elementary steps can be determined (Hess et al., 1985). (1) The rising phase of the current reflects the rate constants for the ligand-binding

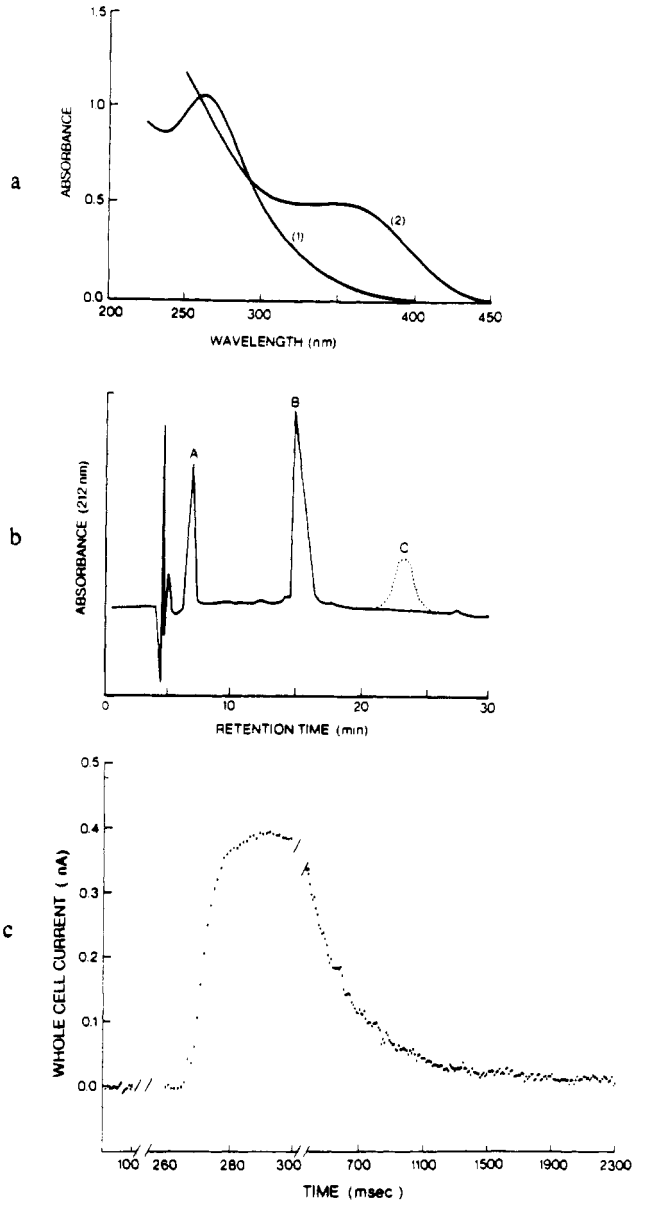


FIGURE 2: (a) Effect of laser photolysis upon the visible and ultraviolet spectrum of compound V. An aqueous solution of 0.2 mM compound V (0.6 cm^3) in 0.2 M 4-morpholinepropanesulfonic acid (MOPS) at pH 8.0 was exposed to an excimer laser (Lambda Physik Eng 101) at 308 nm for 100 shots rated at 5.8 mJ/pulse. Spectra were recorded before (1) and after (2) photolysis; measurements were made with a Cary 219 spectrophotometer. (b) Elution profile. A 1-mL sample of 2 mM caged carbamoylcholine (compound V) was exposed to five laser flashes at 355 nm (15–25 mJ per pulse) in 0.1 M Tris, pH 8.0, room temperature, and a 100-μL aliquot was placed on the column. The flow rate was 16 mL/min. Peak A represents a new compound, presumably the photolyzed protecting group of caged carbamoylcholine; peak B represents caged carbamoylcholine and peak C carbamoylcholine (see the text). (c) A cell-flow laser flash experiment with 100 μM caged carbamoylcholine (compound V), with a BC_3H1 cell, pH 7.4, 23 °C. The whole-cell current was measured; at 265 ms, a 600-ns 352-nm laser flash was delivered (see Experimental Procedures and Results for further detail).

process at low ligand concentrations. At high ligand concentration, when the opening of the channel becomes rate limiting, the channel-opening and -closing rate constants can be determined from the current rise time (Hess et al., 1985). (2) The peak amplitude is a measure of the maximum number of receptor molecules that are in the open-channel form at a given concentration of neurotransmitter. From the effect of ligand concentration on the peak current amplitude one can determine the dissociation constant of the receptor–ligand

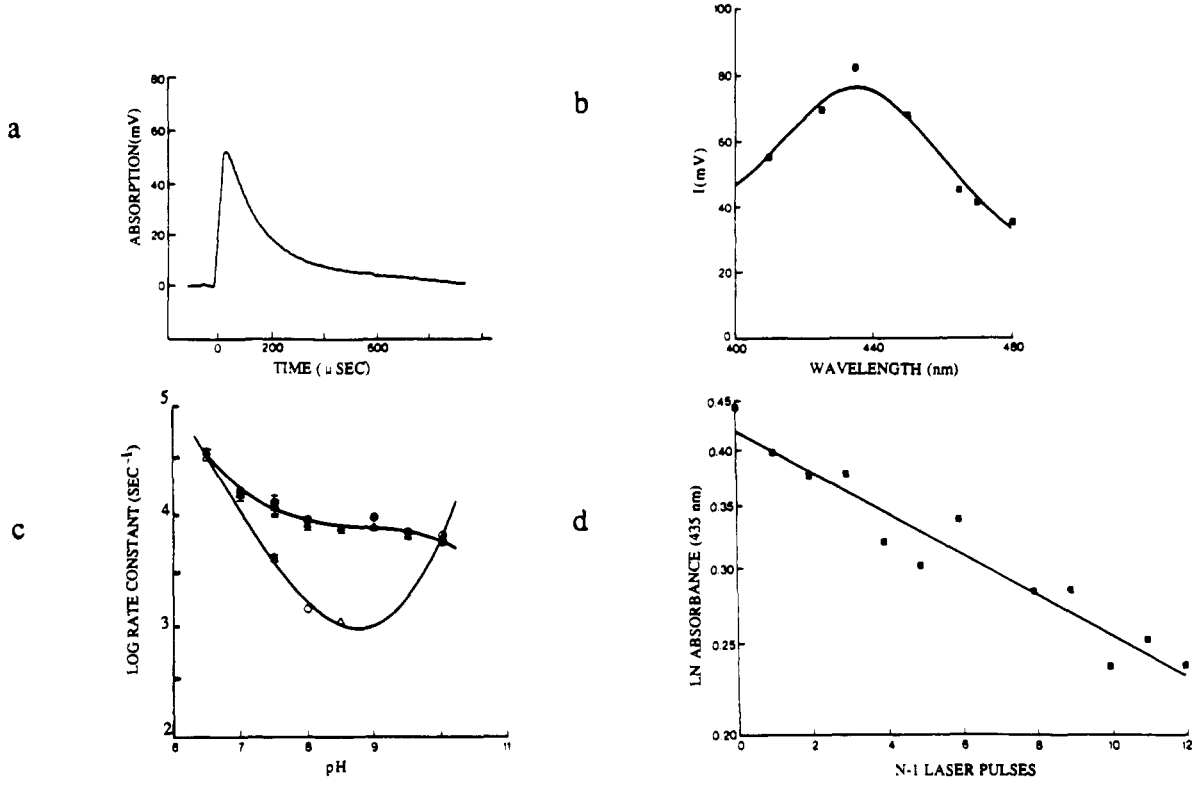


FIGURE 3: (a) Flash photolysis of 2 mM compound V, 0.1 M Tris buffer, pH 10.0, room temperature. A Holobeam ruby laser (see Experimental Procedures) was used. The transient intermediate in the reaction was observed at 429 nm. (b) Maximum observed absorbance of the transient intermediate in the photolysis of 5 mM compound V (■), pH 7.0, 0.16 M MOPS, room temperature. (c) Rate constants for the decay of the transient intermediate in the photolysis of 2 mM compound V as a function of pH measured at both 429 (▲) and 400 nm (●). The data for compound II measured at 429 (△) and 406 nm (○) are shown for comparison. The error bars represent standard deviations of two to three measurements. The following buffers were used: 0.1 M 4-morpholineethanesulfonic acid (MES), pH 6.5; 0.1 M MOPS, pH 7.0 and 7.5; 0.1 M 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES), pH 7.0 and 7.5; 0.1 M Tris, pH 8.0, 8.5, and 9.0; 0.1 M borate, pH 9.0, 9.5, and 10.0. (d) A sample cell containing 4.4 mM compound V in 0.1 M phosphate buffer at pH 7.5 (0.048 cm^3) was irradiated repeatedly, mixing the sample between laser pulses, with 4.5-mJ pulses at 308 nm. Absorption measurements indicated that over 97% of the energy was absorbed. The peak absorption of the *aci*-nitro intermediate was measured at 435 nm as a function of the number of laser pulses, n. The absorption is an indirect measure of the *aci*-nitro concentration; a semilogarithmic plot of absorption against $n - 1$ was found to give a straight line, from which the quantum yield, ϕ, and molar absorption coefficient, ϵ, were calculated (see text). The experimental parameters were as follows: path length = 2 mm, C_0 = 4.1 mM, and fraction of volume illuminated, F = 0.25. Ratio of absorbed photons to target molecules, K_E = 0.24. A linear least-squares computer program was used to calculate the parameters of the line in the figure. The error in intercept and slope is calculated to be ±20%.

complexes and the channel-opening equilibrium constant (Udgaonkar & Hess, 1987). (3) The falling phase of the current allows evaluation of the rate coefficients for receptor desensitization.

Analysis of the caged carbamoylcholines (see Table I) during flash photolysis revealed a spectral transient, characterized by an instantaneous increase in absorbance (<10 μs) followed by a first-order decay (of more than 90% of the initial absorbance change) (Figure 3a). For example, the rate constant for the decay in the experiment shown in Figure 3a has a value of 6×10^3 s^{-1} at pH 10 and 23 °C. The absorbance maximum of the transient intermediate was found to be near 435 nm (Figure 3b). In comparable experiments with compound II, the absorbance maximum of the transient intermediate was 406 nm (Walker et al., 1986) similar to that observed in experiments with a nitrobenzyl derivative of ATP (McCray et al., 1980). The magnitude of the absorbance increase was proportional to the concentration of the solution in the cuvette and to the energy output of the laser. The decay rate was dependent upon pH (Figure 3c) and the structure of the protecting group (Table I). The effects of pH on the rates of decay of compounds II and V are shown in Figure 3c. The decay rate of the transient intermediate of compound V is the same whether it is observed at 400 nm or at 429 nm. For caged compound V there was a dependence on pH, with a plateau in the region pH 8–9; the rates were faster at lower pH. These properties of the transient species are characteristic of the *aci*-nitro intermediate. A considerable amount of evidence (McCray et al., 1980; Walker et al., 1986, 1988) indicates that the rate of decay of the *aci*-nitro intermediate is a measure of the rate of product formation, in this case carbamoylcholine. The fastest photolysis rate observed was 3.8×10^4 s^{-1} at pH 6.5 for compound V. A comparison of the photolysis rates at pH 7.0 of the different compounds synthesized is given in Table I. The determination of the quantum yield of compound V was based on the assumption that the concentration of the transient intermediate, with an absorption maximum at 406 nm in the photolysis of nitrobenzyl phosphates, is directly proportional to the concentration of the compound (in this case carbamoylcholine) liberated in the photolysis reaction. The absorbance of the intermediate, A, after a single laser pulse is given by

$$A = \epsilon_M l C_0 \phi K_E \quad (1a)$$

C_0 is the initial concentration of compound V, ϕ the quantum yield, ϵ_M the extinction coefficient of the transient intermediate at 435 nm, l the length of the light path, and K_E the ratio of the number of absorbed photons to the number of target molecules in the laser beam. Mixing the solution after each laser pulse and subjecting it to a subsequent pulse give an

absorbance, A_n, determined at the nth laser pulse. When K_E remains constant

$$A_n = \epsilon_M l C_0 \phi K_E \exp[-\phi K_E F(n-1)] \quad (1b)$$

where F is the fraction of the solution containing the cage compound through which the laser beam passed. The monitoring beam, 2 mm × 2 mm × 1 mm in dimension, passed at right angles through the center of the laser beam, which had a dimension of 2 mm × 2 mm × 3 mm. A semilogarithmic plot of A_n versus $n - 1$ is shown in Figure 3d. The quantum yield, obtained from the slope of the line, was 0.8. From the intercept of the graph, the value for ϵ_M was determined and found to be 2600 M^{-1} cm^{-1} at 435 nm. A number of errors can affect the measurements: (1) In the series of laser pulses one of the products of the photolysis reaction (the nitrosophenyl derivative, compound Va, Figure 1) absorbs energy at the expense of the starting material. Therefore, a systematic change in the slope of the line in Figure 3d is predicted. This was not observed because the experiment was performed near the isosbestic point of the absorption spectra of the starting material and the product. (2) There is an exponential decay of the energy of the laser beam as it passes through the solution. This is not a major cause of error because the monitoring beam measures an average of all the absorbance changes that occur along the path of the laser beam propagation through the solution. (3) The energy of the laser beam fluctuates. We have found measurements of the energy of the laser beam between laser pulses in determination of quantum yields to be experimentally difficult at the moment. Therefore, we have determined the average energy of the pulse as 4.5 mJ. We consider the energy flucturation of the laser to be the dominant error in our measurements. We calculated the average error in the constants to be ±20%.

Effect of Caged Carbamoylcholine on the Nicotinic Receptor in BC_3H1 Muscle Cells. The results of a cell-flow experiment with BC_3H1 muscle cells are shown in Figure 4a. The solid line represents the whole-cell current observed as a function of time in the presence of 100 μM carbamoylcholine and 1 mM compound V. The same (within experimental error) result was obtained when compound V was omitted. The falling phase of the current allows evaluation of the rate coefficient for receptor desensitization. The dashed line gives the amplitude after correction for receptor desensitization and is proportional to the number of receptor channels open at a given concentration of ligand (Udgaonkar & Hess, 1987). The experiments show that when compound V is present in 10-fold excess over carbamoylcholine, caged compound V affects neither the number of receptor channels opened (as measured by the current amplitude) nor the rate of receptor desensitization (as measured by the rate of the falling phase of the current). In contrast, when 100 μM caged compound II is added to 100 μM carbamoylcholine in a cell-flow experiment, the current amplitude is reduced by 50%. The inhibitory effect of compound II on the ability of carbamoylcholine to open receptor channels in BC_3H1 cells is shown in Figure 4b. In this experiment the ratio of the current amplitude, I_A, in the absence of compound II to the current amplitude, $I_{A(C)}$, in the presence of compound II is plotted as a function of the concentration of compound II in the presence of a constant concentration of 100 μM carbamoylcholine, according to Udgaonkar and Hess (1987):

$$I_A/I_{A(C)} = 1 + [\text{caged II}]/K_I \quad (2)$$

where K_I is an apparent dissociation constant of compound II and has value of 103 μM. Similar results were obtained with the *Electrophorus electricus* electroplax acetylcholine receptor. They indicated that compound II both inhibits receptor-controlled ion translocation and desensitizes the receptor (Walker et al., 1986).

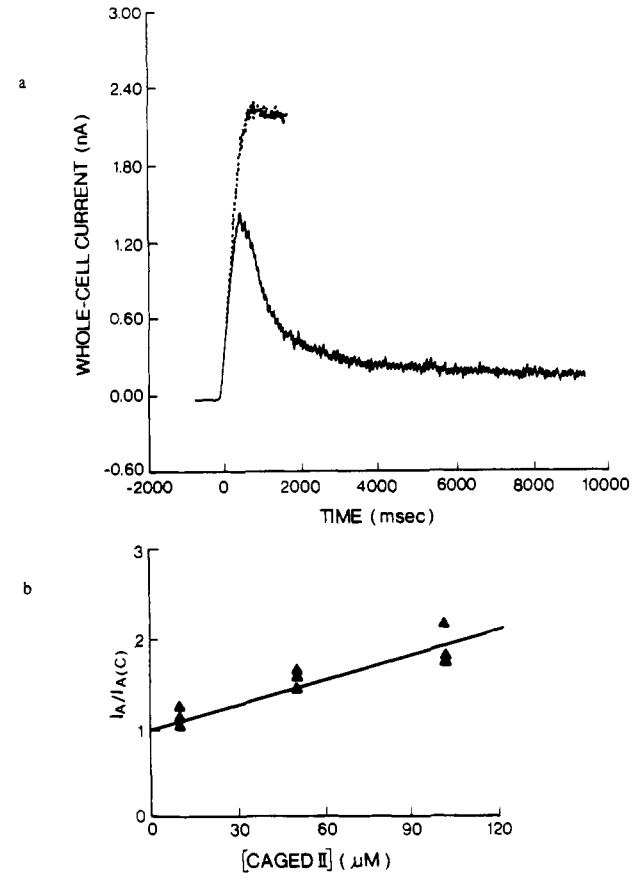


FIGURE 4: (a) Effect of cage compound V on carbamoylcholine-induced currents, 25 mM Hepes buffer, pH 7.4, room temperature. The whole-cell currents at 100 μM carbamoylcholine in a BC_3H1 cell were measured at −60 mV in the presence of 1 mM cage compound V with the cell-flow technique (Udgaonkar & Hess, 1987). The solid lines are the observed whole-cell current, and the dotted lines are the calculated corrected amplitude based on the rate of desensitization (Udgaonkar & Hess, 1987). (b) Inhibition of carbamoylcholine-induced currents by cage compound II. The whole-cell currents in a BC_3H1 cell were measured at −60 mV with the cell-flow method. The ratio of the current amplitude (I_A) in the absence of cage compound to the current amplitude [$I_{A(C)}$] in the presence of cage compound is plotted as a function of the concentration of the cage compound. The carbamoylcholine concentration (100 μM) was not varied. From the slope of the line the value of the apparent dissociation constant for the inhibitory site (Udgaonkar & Hess, 1986; Hess et al., 1987), K_I, was calculated to be 103 μM.

Discussion

Following the work of Walker et al. (1986), three more carbamate derivatives featuring the extensively studied photolabile 2-nitrobenzyl moiety were synthesized. The proposed mechanism for the photolysis of caged phosphates (McCray et al., 1980) suggested (Walker et al., 1986) that the photolytic cleavage of the 2-nitrobenzyl moiety involves an intramolecular oxidation–reduction reaction between the nitro group and the adjacent benzylic carbon, resulting in cleavage of the carbon–nitrogen bond to release free carbamate.

The rates of carbamate release and details of the photochemical mechanism were analyzed by flash photolysis. When spectral changes in the near UV were monitored, an intermediate was detected with absorption properties different from those of the parent compound. The intermediate was similar in its absorption spectrum to the *aci*-nitro intermediate ori-

ginally identified in the flash photolysis of 2-nitrotoluene (Wettermark, 1962), caged ATP (McCray et al., 1980), and more recently caged carbamoylcholine (compound II) (Walker et al., 1986). The presumed *aci*-nitro species showed a pH-dependent exponential decay to products with time constants in the microsecond to millisecond time region.

The approach we have introduced to measure quantum yield also allows one to measure the molar extinction coefficient of the transient intermediate in the photolysis reaction. It is of interest that the quantum yield for compound V, 0.8, is almost three times larger than that for compound II (Table I), 0.25, measured by an independent conventional method (Walker et al., 1986). For comparative purposes we have used the technique described here to determine the quantum yield for compound II and obtained a value of 0.29.

Initially we were interested only in the rates of photolysis. A comparison of the photolysis rates of compounds I and III (Table I) indicates that the trimethylammonium group does not alter the rate significantly. Therefore, the trimethylammonium group was not included in compounds III and IV (Table I). When it was noted that the photolysis rate of compound IV is 65 times slower than that of compound II, its further investigation was abandoned.

The synthetic route to compound V is straightforward, and the complete carbamoylcholine derivative was made. Two properties of this compound make it useful: first, its photolysis rate is sufficiently fast, and second, it does not interact with the acetylcholine receptor prior to photolysis. Laser photolysis generates carbamoylcholine and provides a way of rapidly varying carbamoylcholine concentrations over a wide range. Used in conjunction with measurements of cell currents, photochemical release of carbamoylcholine allows a chemical kinetic approach to be applied to a wide variety of receptors on cell surfaces with a microsecond time resolution. A further extension of this work, now being undertaken, is the synthesis of caged neurotransmitters containing amino groups.

Acknowledgments

We are grateful to Professor Jack Kaplan, University of Pennsylvania, for helpful discussion regarding the synthesis of caged compound V and Professors Houston and Cool, Cornell University, for allowing us to use their excimer lasers.

Registry No. I, 100311-50-0; II, 100311-51-1; III, 117121-22-9; IV, 117121-23-0; V, 117121-25-2; $(CH_3)_3N^+(CH_2)_2$-OH, 62-49-7; $Cl_3COCOCl$, 503-38-8; $(CH_3)_3N^+(CH_2)_2OCOCl\cdot Cl^-$, 92442-84-7; 2-nitrophenylglycine, 50381-53-8; carbamoylcholine, 462-58-8; α-bromo-2-nitro-4-toluic acid, 55715-03-2; α-bromo-4-toluic acid, 6232-88-8; 4-carboxy-2-nitrobenzylamine, 2372-51-2; hexamethylenetetraamine, 100-97-0; α-bromo-2-nitro-4-toluic acid–hexamethylenetetraamine complex, 117121-26-3; 4-carboxy-2-nitrobenzylamine hydrochloride, 117121-27-4; bromoethyl chloroformate, 4801-27-8; (2-nitrophenyl)glycine, 108-18-9; *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine, 117121-28-5.

References

Adams, S. R., Kao, J. P. Y., Grynkiewicz, G., Minta, A., & Tsien, R. Y. (1988) *J. Am. Chem. Soc. 110*, 3212–3220.

Blicke, F. F., & Lilienfeld, W. M. (1943) *J. Am. Chem. Soc. 65*, 2281–2284.

Clapham, D. E., & Neher, E. (1984) *J. Physiol. (London) 347*, 255–277.

Davis, A. L., Smith, D. R., &McCord, T. J. (1973) *J. Med. Chem. 16*, 1043–1045.

Ellis-Davies, G. C. R., & Kaplan, J. H. (1988) *J. Org. Chem. 53*, 1966–1969.

Fenwick, E., Marty, A., & Neher, E. (1982) *J. Physiol. (London) 331*, 577–597.

Gurney, A. M., & Lester, H. A. (1987) *Physiol. Rev. 67(2)*, 583–617.

Hamill, O. P., Marty, E., Neher, E., Sakmann, B., & Sigworth, F. J. (1981) *Pfluegers Arch. 391*, 85–100.

Hess, G. P., Cash, D. J., & Aoshima, H. (1979) *Nature (London) 282*, 329–331.

Hess, G. P., Kolb, H.-A., Lauger, P., Schoffeniels, E., Schwarze, W., Udgaonkar, J. B., & Pasquale, E. B. (1985) in *Molecular Basis of Nerve Activity* (Changeux, J.-P., Hucho, F., Maelicke, A., & Neumann, E., Eds.) pp 317–334, de Gruyter, New York.

Hess, G. P., Udgaonkar, J. B., & Olbricht, W. L. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 507–533.

Kaplan, J. H., Forbush, B., & Hoffman, J. F. (1978) *Biochemistry 17*, 1929–1935.

Karpen, J. W., Zimmerman, A. L., Stryer, L., & Baylor, D. A. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1287–1291.

Krishtal, O. A., & Pidoplichko, V. I. (1980) *Neuroscience (Oxford) 5*, 2325–2327.

McCray, J. A., Herbette, L., Kihara, T., & Trentham, D. R. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7237–7241.

Nerbonne, J. M. (1986) *Opt. Methods Cell Physiol. 40*, 417–446.

Olson, E. N., Glaser, L., Merlie, J. P., Sebanne, R., & Lindstrom, J. (1983) *J. Biol. Chem. 258*, 13946–13953.

Rich, D. J., & Gurwara, S. K. (1975) *J. Am. Chem. Soc. 97*, 1575–1579.

Schiffman, G., Kabat, E. A., & Thompson, W. (1964) *Biochemistry 3*, 113–120.

Sine, S., & Taylor, P. (1979) *J. Biol. Chem. 254*, 3315–3325.

Tjoeng, F.-S., Tong, E. K., & Hodges, R. S. (1978) *J. Org. Chem. 43*, 4190–4194.

Udgaonkar, J. B. (1986) Ph.D. Thesis, Cornell University.

Udgaonkar, J. B., & Hess, G. P. (1986) *J. Membr. Biol. 93*, 93–109.

Udgaonkar, J. B., & Hess, G. P. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8758–8762.

Walker, J. W., McCray, J. A., & Hess, G. P. (1986) *Biochemistry 25*, 1799–1805.

Walker, J. W., Somlyo, A. V., Goldman, Y. E., Somlyo, A. P., & Trentham, D. R. (1987) *Nature (London) 327*, 249–252.

Walker, J. W., Reid, G. P., McCray, J. A., & Trentham, D. R. (1988) *J. Am. Chem. Soc. 110*, 7170–7177.

Wettermark, G. (1962) *J. Phys. Chem. 66*, 2560–2562.