Rapid Release of an α-Adrenergic Receptor Ligand from Photolabile Analogues†

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ABSTRACT: A series of 2-nitrobenzyl derivatives of the α_1-selective adrenergic agonist, L-phenylephrine [(*R*)-*N*-[2-(3-hydroxyphenyl)-2-hydroxyethyl]-*N*-methylammonium chloride], have been synthesized and characterized for the purpose of developing biologically inert compounds that can be rapidly converted to L-phenylephrine by near-UV irradiation. The compounds, derivatized on the phenolic oxygen, were *O*-(1-(2-nitrophenyl)ethyl)phenylephrine (I), *O*-(2-nitrobenzyl)phenylephrine (II), *O*-(4,5-dimethoxy-2-nitrobenzyl)phenylephrine (III), and *O*-(α-carboxyl-2-nitrobenzyl)phenylephrine (IV). All four compounds photolyzed to free phenylephrine following a brief exposure to 300–350-nm light or 347-nm laser light with steady-state quantum yields ranging from 0.05 to 0.28. The rates of phenylephrine formation on photolysis were estimated from the decay rates of *aci*-nitro intermediates detected by absorbance between 380 and 500 nm. Compound IV displayed the highest quantum yield (0.28) and most rapid photolysis rate (1980 s^{-1}) measured under near physiological conditions, pH 7.0, 22 °C. Biological properties of the compounds were examined in smooth muscle from rat caudal artery. Laser pulse photolysis of IV at 347 nm initiated a maximal contraction in Krebs buffer, pH 7.1, 25 °C, that mimicked the response to 50 μM phenylephrine but was faster in onset. Photoinitiated contractions were characterized by a delay of 0.93 ± 0.09 s followed by a rising phase with a 10–90% rise time of 3.56 ± 0.17 s (n = 7). Responses were fully blocked by the α_1-selective antagonist prazosin. Similar results were obtained using compounds I–III, but a slowly developing sustained phase of smooth muscle contraction was altered in the presence of compounds I–III. Compound IV represents a useful new biologically inactive 'caged' phenylephrine that should facilitate temporally and spatially resolved studies of α_1-adrenergic receptor mechanisms in cells and tissues.

In order to investigate functional properties of receptors in physiological preparations, it is necessary to have precise control over agonist and antagonist concentrations at the cell surface. Rapid and uniform administration of receptor ligands in isolated cells and multicellular tissues is hampered by diffusional delays and by unstirred layers. Surface receptors can also inactivate or desensitize in the presence of ligands (Katz & Thesleff, 1957; Hess et al., 1983), further complicating analyses that rely on bath application of agonists.

An emerging technique for resolution of the earliest events following receptor activation is photorelease of agonists from biologically inactive precursors (Walker et al., 1986; Milburn et al., 1989; Marque, 1989; Wilcox et al., 1990). The approach is based on earlier work that has demonstrated the utility of photorelease of phosphate compounds and divalent cations from photolabile 'caged' precursors (Gurney & Lester, 1987; McCray & Trentham, 1989; Adams & Tsien, 1992). In order to extend this photochemical strategy and to address new biological problems it is necessary to (1) develop new photolabile groups in order to improve the speed and photolysis efficiency of existing 'caged' substrates and (2) identify additional functional groups in biological molecules that are good sites for derivatization and photochemical deprotection.

We now report the development of photolabile derivatives of the α-adrenergic agonist, L-phenylephrine. The recently introduced α-carboxyl-2-nitrobenzyl protecting group (Milburn et al., 1989) appears to be ideal for use with phenylephrine. The photolysis half-time of *O*-(α-carboxyl-2-nitrobenzyl)phenylephrine (compound IV; Figure 1), determined by decay of an *aci*-nitro intermediate, was in the microsecond domain, and its quantum yield was greater than 0.25. *O*-(α-Carboxyl-2-nitrobenzyl)phenylephrine also displayed no agonist or antagonist effects toward α_1-adrenergic receptors in vascular smooth muscle prior to photolysis.

One compound described here (compound I) had been used previously to characterize the time course of tension development in vascular smooth muscle (Somlyo et al., 1988). While the photolysis kinetics were slow (2 s^{-1}), it was concluded that compound I at high concentrations produced free phenylephrine rapidly enough to establish the latency time and rise time of the onset of vascular smooth muscle contraction (Somlyo et al., 1988). We now confirm this conclusion with compound IV. Furthermore, the photochemical and biological properties of compound IV should permit α-adrenergic receptor-mediated events to be characterized over a wider range of agonist concentration and with higher time resolution than was previously possible with the topical addition of phenylephrine or with compound I. The derivatives described here also represent model compounds for 'caging' aromatic hydroxyl groups on other molecules of biological interest such as catecholamines, tyrosine, reduced quinones, and fluorescein-based fluorescent probes.

EXPERIMENTAL PROCEDURES

Chemicals for synthesis were obtained from Aldrich Chemical Co. (Milwaukee, WI) and used without further

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[1] Abbreviations: TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; TEAB, triethylammonium bicarbonate; DTT, dithiothreitol; TEAF, triethylammonium formate; ^{1}H-NMR, proton nuclear magnetic resonance; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; FAB, fast atom bombardment.

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purification. TLC was carried out on Silica Gel K60 F_{254} plates (Merck, Darmstadt, Germany). Merck silica gel (grade 60, 230–400 mesh, 60 Å) was used for flash chromatography. A Bruker 200 MHz instrument was used for NMR measurements. Fast atom bombardment (FAB) mass spectrometry was performed in the Department of Chemistry at the University of Wisconsin on a VG Analytical ZAB-2F double-focusing instrument, using 8 keV Xe atoms for ionization and 3-nitrobenzyl alcohol or glycerol as a matrix.

Synthesis. The synthesis of photolabile caged phenylephrine derivatives involved alkylation of L-phenylephrine on its phenoxy oxygen with the photolabile caging moiety in a reactive form. In the case of *O*-(1-(2-nitrophenyl)ethyl)-phenylephrine (I), the alkylating agent was a diazonium derivative, while for the other compounds (II–IV) an alkyl bromide was used.

O-(1-(2-Nitrophenyl)ethyl)phenylephrine (I). The synthesis was carried out as previously described (Walker, 1991) with some modifications. One millimole of L-phenylephrine hydrochloride (Sigma Chemical Co., St. Louis, MO) was mixed with 7 mmol of 1-(2-nitrophenyl)diazoethane in 10 mL of dry DMSO and heated at 85 °C for 40 h. 1-(2-Nitrophenyl)diazoethane was prepared as described (Walker et al., 1989). Analytical HPLC as in Figure 2 showed that ca. 75% of phenylephrine had been converted to caged phenylephrine. The addition of 10 mol % fluoboric acid (Neeman et al., 1959) did not increase the rate or yield of the reaction. The product was extracted from the reaction mixture into $CHCl_3$ using a pH extraction procedure (Walker, 1991). The crude product was dissolved in 50% ethanol, filtered by centrifugation (0.22 μm; Costar Spin-X, Cambridge, MA), and purified by a standard procedure involving two column steps: (1) preparative HPLC on a C_{18} reverse-phase column (Whatman, Inc., Clifton, NJ) using isocratic elution with 10 mM TEAF, pH 5, 30% (v/v) methanol, and (2) cation exchange on CM-cellulose (CM-52, Whatman, Inc.) eluted with a linear gradient of 0–0.5 M TEAB. TEAB was removed by rotary evaporation, and samples were stored frozen in 50% ethanol or following lyophilization as a semicrystalline off-white powder. Yield after purification: 55–67%. ^{1}H-NMR spectra in D_2O or d_6-acetone for all four compounds (I–IV) were composites of free phenylephrine and the 2-nitrobenzyl moiety. NMR (d_6-acetone) δ 7.7–8.1 (m, 4 H, aromatic (cage group)), 6.9–7.3 (m, 4 H, aromatic (phenylephrine)), 4.8 (q, 1 H, benzylic (phenylephrine)), 4.5 (q, 1 H, benzylic (cage group)), 3.0 (br s, 3 H, NH_2, OH), 2.6 (d, 2 H, methylene), 2.3 (m, 3 H, N-methyl), 1.7 (t, 3 H, C-methyl). FAB mass spectrum: m/z = 317; calculated for $[C_{17}H_{21}N_2O_4^+]$, 317.36.

O-(2-Nitrobenzyl)phenylephrine (II). One millimole of L-phenylephrine hydrochloride was dissolved in 5 mL of dry DMSO by heating to 50 °C. Two millimole of powdered KOH was added with stirring followed by 1.5 mmol of 2-nitrobenzyl bromide. After 30–60 min, the reaction was cooled, diluted 20-fold with water, and extracted with $CHCl_3$. The $CHCl_3$ was removed by evaporation, and the crude product was dissolved in 50% ethanol and adjusted to pH 6–7 with 0.5 M HCl. Following centrifugation through a 0.22-μm filter, the product was purified by the standard procedure described for compound I. Yield after purification: 71–86%. NMR (d_6-acetone) δ 7.6–8.1 (m, 4 H, aromatic (cage group)), 6.8–7.3 (m, 4 H, aromatic (phenylephrine)), 4.9 (t, 1 H, benzylic (phenylephrine)), 4.2 (q, 2 H, benzylic (cage group)), 2.7 (d, 2 H, methylene), 2.4 (s, 3 H, N-CH_3).

O-(4,5-Dimethoxy-2-nitrobenzyl)phenylephrine (III). The same method described above for compound II was used except that 1-(bromomethyl)-4,5-dimethoxy-2-nitrobenzene was the alkylating agent. This alkylating agent was synthesized in two steps from 6-nitroveratraldehyde (Wilcox et al., 1990). Compound III was purified by preparative reverse-phase HPLC and DEAE-cellulose chromatography as for compounds I and II and was stored frozen in 50% ethanol or following lyophilization as a yellow semicrystalline solid. Yield of III after purification: 78%. NMR (d_6-acetone) δ 7.7 (s, 1 H, aromatic (cage group)), 7.6 (s, 1 H, aromatic (cage group), 6.8–7.3 (m, 4 H, aromatic (phenylephrine)), 4.9 (q, 1 H, benzylic (phenylephrine)), 4.2 (s, 2 H, benzylic (cage group)), 4.1 (d, 6 H, methoxy), 3.3 (br s, 3 H, NH_2, OH), 2.8 (d, 2 H, methylene), 2.5 (s, 3 H, N-CH_3). FAB mass spectrum: m/z = 363, 361 (– 2 H), and 345 (– H_2O) with relative intensities of 2.9:1.3:1, respectively. Calculated for $[C_{18}H_{23}N_2O_6^+]$, 363.39.

O-(α-Carboxyl-2-nitrobenzyl)phenylephrine (IV). The alkylating agent, methyl α-bromo-2′-nitrophenylacetate, was synthesized in two steps from 2-nitrophenylacetic acid. The methyl ester was prepared by mixing 18.1 g (0.1 mol) of 2-nitrophenylacetic acid, 32 g (1 mol) of methanol, 200 mL of benzene, and 6 drops of concentrated H_2SO_4. Approximately 200 mL of solution was distilled off at 58 °C, and the product was purified from the residue by flash chromatography in CH_2Cl_2–hexane (1:1). Then, 9.8 g (0.05 mol) of methyl 2-nitrophenylacetate was mixed with 2% w/w benzoyl peroxide (0.196 g) in 200 mL of CCl_4. The mixture was heated to ca. 70 °C, and 9.83 g (0.055 mol) *N*-bromosuccimide was added over 90 min; the reaction was then heated under reflux for 96 h. The course of the reaction was monitored by TLC using CH_2Cl_2 as the solvent. After 24 and 48 h, fresh *N*-bromosuccinimide and benzoyl peroxide were added after the cooling and filtration of the reaction mixture. The product, methyl α-bromo-2′-nitrophenylacetate, was purified by flash chromatography using CH_2Cl_2 as solvent. Yield: 55%. NMR (d_6-acetone) δ 7.5–8.0 (m, 4 H, aromatic), 6.1 (s, 1 H, benzyl), 3.6 (s, 3 H, O-CH_3). Compound IV was synthesized by dissolving 0.1 g (0.5 mmol) of L-phenylephrine hydrochloride in 3 mL of dry DMSO (heated to 60 °C), followed by addition of 0.112 g (2 mol) of powered KOH and 0.52 g (1.9 mmol) of methyl α-bromo-2′-nitrophenylacetate and further heating at 80 °C for 4 h. Control reactions showed L-phenylephrine to be stable in the presence of KOH under the conditions used. The crude reaction was diluted into 100 mL of 50 mM TES (Sigma), pH 4.2, on ice. The pH was adjusted to 6.8, and the mixture was gravity filtered (Whatman #1). Chromatography on CM-cellulose was carried out with a linear gradient of 0–0.5 M TEAB. Treatment with 1 M NaOH at 50 °C for 20 min hydrolyzed the methyl ester to the carboxylate; hydrolysis of the methyl ester also occurred to some extent during rotary evaporation to remove TEAB. IV was isolated >99% pure by preparative HPLC on a C_{18} reverse-phase column equilibrated in 25% v/v methanol in water. Yield after purification: 61%. IV was stored frozen as an aqueous solution or following lyophilization as an off-white powder. NMR (d_6-acetone) δ 7.6–8.0 (m, 4 H, aromatic (cage group)), 6.8–7.3 (m, 4 H, aromatic (phenylephrine)), 5.2 (s, 1 H, benzylic (cage group)), 4.6 (m, 1 H, benzylic (phenylephrine)), 3.3 (br s, 3 H, NH_2, OH), 2.7 (m, 2 H, methylene), 2.5 (s, 3 H, N-methyl). The broad singlet at δ 3.3 was shifted to δ 3.7 following the addition of 50 μL of D_2O. FAB mass spectrum: m/z = 347, 345 (– 2 H) and 329 (– H_2O), with relative intensities of 5.5:3.6:1, respectively. Calculated for $[C_{17}H_{19}N_2O_6^+]$, 347.35.

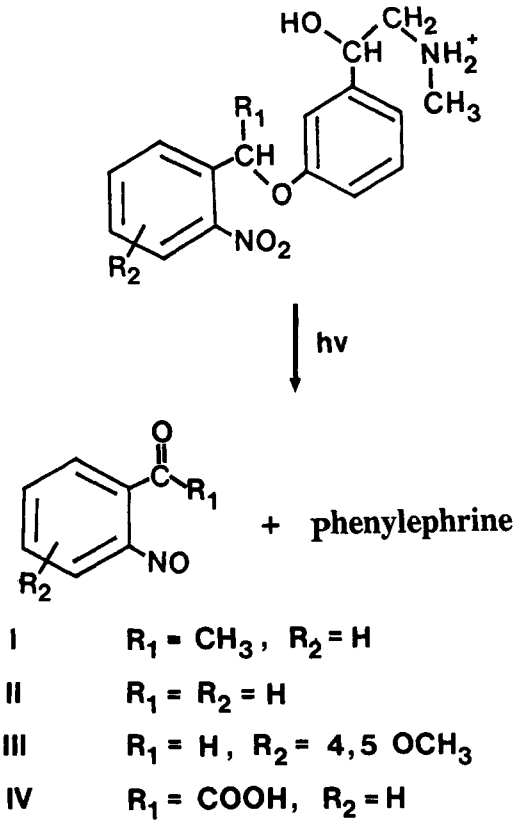
HPLC Methods. HPLC was performed with a pair of Beckman 110B pumps, 210A sample injector, and 160 absorbance detector at 254 or 280 nm. Analytical-scale HPLC was carried out with a Beckman Paritisil 10 SCX column (30 cm × 1 cm); gradient elution was controlled by Beckman System Gold software. Compounds with a net positive charge (I–III) were injected onto the column equilibrated in 0.06 M $NH_4H_2PO_4/(NH_4)_2HPO_4$, pH 5.5, 2% (v/v) methanol at 1.0 mL/min. After 5 min, a 15-min linear gradient to 0.45 M $NH_4HPO_4/(NH_4)_2HPO_4$, pH 5.5, 15% (v/v) methanol was run. The zwitterionic compound (IV) was analyzed by injection onto the SCX column equilibrated in distilled water at 1.0 mL/min. After 5 min, a 10-min linear gradient to 0.48 M $NH_4HPO_4/(NH_4)_2HPO_4$, pH 5.5, 16% (v/v) methanol was run. The column was typically conditioned by running the gradient at least once prior to sample injection. HPLC solvents were prepared by mixing solutions containing equivalent concentrations of $NH_4H_2PO_4$ and $(NH_4)_2HPO_4$ to obtain the desired pH and then diluting to the final concentration with methanol.

Preparative HPLC was carried out using a 2.2 cm × 25 cm ODS-3 Magnum 20 column (Whatman, Inc.). The column was equilibrated with the eluting solvent [10 mM TEAF, pH 5.5, 30% (v/v) methanol] for 16 h at 1 mL/min. Samples of up to 2 mL were injected and eluted isocratically at 8 mL/min. Fractions were collected at 1.5-min intervals.

Photolysis. Steady-state photolysis was accomplished by repetitive flashes from a xenon arc lamp (Rapp & Guth, 1988) filtered to pass ca. 300–350-nm light (UG-11, Schott; 305 cutoff, Oriel). Aqueous samples were photolyzed in a quartz cuvette [0.3 cm × 1 cm (path length)] or for concentrations >0.5 mM in a specially designed 18-μL quartz chamber with a light path of 0.1 cm. Laser flash photolysis and detection of optical signals was carried out with a frequency doubled ruby laser (80–100 mJ; 347 nm) as described by McCray et al. (1980) and Walker et al. (1988).

Smooth Muscle Preparations. Strips of vascular smooth muscle, approximately 200 μm in width and 2–3 mm in length, were prepared from the caudal artery of male Sprague–Dawley rats. The artery was exposed, ligated, and removed, then it was pinned out on a silgard-coated petri dish and covered with oxygenated modified Krebs solution (116.7 mM NaCl, 4.5 mM KCl, 1.9 mM $CaCl_2$, 2.4 mM Na_2SO_4, 2.25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 5 mM glucose, 25 mM HEPES, pH 7.1, 25 °C). The artery was opened with a single cut along its length, and 200-μm-wide strips were cut perpendicular to its length. It was not possible to separate individual layers of smooth muscle in this tissue.

T-shaped aluminium foil clips were folded around each end of the strip, and the loop in the end of the T-clip was hitched to a stainless steel hook attached to an apparatus, similar to that described by Barsotti and Ferenczi (1988). The hooks positioned the strip in one of a series of 15-μL troughs cut into a temperature-regulated stainless steel block. The troughs were raised and lowered pneumatically within 150 ms. At the front end of the block a trough was fashioned from two perpendicular walls of a quartz cuvette so that the fiber could be illuminated from the side or from below. One hook was epoxied to a silicon strain guage (Akers #801, Norway) and the other to a stationary mount. The signal for the strain guage was amplified and sent to several analog and digital recording devices. Photolysis was effected by a single pulse of laser light (50 ns, 80–200 mJ, 347 nm) produced by frequency doubling the output of a Q-switched pulsed ruby laser (Laser Applications, Inc., Winter Park, FL). Glutathione



I $R_1 = CH_3$, $R_2 = H$

II $R_1 = R_2 = H$

III $R_1 = H$, $R_2 = 4,5\ OCH_3$

IV $R_1 = COOH$, $R_2 = H$

FIGURE 1: Structures and proposed photolysis products of four 'caged' phenylephrine compounds.

(Sigma; 10 mM) was included in photolysis solutions to minimize tissue damage due to photolysis byproducts.

Statistical Analysis of Force Traces. An analysis of variance was conducted for the measurements of lag time and rise time. α for each analysis was adjusted to $p = 0.025$, using the Bonferroni correction factor to minimize the probability of type-I errors, resulting from measuring both variables from the same strips of artery. In the event of significance, mean contrasts were conducted using the orthogonal F statistic. α was adjusted to $p = 0.007$ in accordance with the number of mean contrasts conducted for each variable.

RESULTS

Figure 1 shows the structures of the four photolabile derivatives of L-phenylephrine that were synthesized. Each contains a 2-nitrobenzyl moiety attached via an ether linkage to the single phenolic oxygen of phenylephrine. Compounds I–IV differ only in substituents on the photolabile group.

Absorbance Properties. The UV–visible absorbance spectra of compounds I, II, and IV showed a maximum around 270 nm (Table I). The 4,5-dimethoxy compound (III) was characteristically red-shifted, displaying a maximum around 330 nm (Table I). Free phenylephrine has an absorbance peak at 270 nm at pH 7; however, when the pH is raised to 12, the absorbance peak is at 293 nm. The maximum at 293 nm is due to the formation of the phenoxide anion. In contrast, absorbance spectra of compounds I–IV were the same at pH 7 and at pH 12. This is consistent with all four compounds being derivatized on the phenolic oxygen. Compounds I–III were soluble in chloroform at high pH, an observation that is also compatible with alkylation of the phenoxy group. Phenylephrine is not chloroform soluble at high pH because of ionization of the phenolic hydroxyl group. Absorbance

Table I: Spectral and Photochemical Properties[a]

compd	λ_{max}, nm	ϵ^b, M^{-1} cm^{-1}	$t_{1/2}{}^c$ ms	Φ^d	Φ^e
I	272	6,200	300	0.11	0.11
II	272	6,200	nd	0.05	nd
III	330	5,000	2.8	nd	0.13
IV	270	6,000	0.35	0.28	0.21

[a] Structures in Figure 1. UV–Vis absorption spectra were recorded in 100 mM TES pH 7. [b] Molar extinction coefficients were estimated as the sum of contributions from free phenylephrine (2000 M^{-1} cm^{-1} at 270; 0 at 330 nm) and the caging group. [c] *aci*-Nitro decay half-times were determined by flash photolysis at pH 7, 22 °C. Quantum yields, Φ, were determined as in Figure 2 with a xenon flash lamp,[d] or by using 347-nm irradiation from a frequency doubled ruby laser.[e] A 2.4-fold greater absorption of 347 nm light was accounted for in calculating Φ for III. Errors in $t_{1/2}$ and Φ values are estimated to be ≤ 6%.

spectra of compounds I–IV *after photolysis* showed an absorbance increase throughout the range of 300–500 nm, while at pH 12, a peak at 293 nm revealed the presence of the phenoxide of free phenylephrine (not shown).

Quantum Yields for Photoconversion. Quantitation of the extent of photolysis was carried out by HPLC analysis. Phenylephrine and compounds I–IV were separated and identified by use of a cation exchange column (Figure 2). Compound IV interacted poorly with this column because of its nearly zero net charge. (At pH 5, both the amine and the carboxyl group are expected to be nearly fully ionized.) As shown in Figure 2A, compound IV eluted from the cation exchange column considerably earlier than phenylephrine. Representative of the other three compounds (I–III), Figure 2A (traces c and d) shows that II interacted more strongly with the cation exchange column than did phenylephrine. The elution properties of all four compounds are readily explained on the basis of charge and the ability of I–III to adsorb to the column via hydrophobic interactions. Good separation of the photosensitive compounds, free phenylephrine, and photolysis byproducts permitted a quantitative analysis of the extent of photoconversion.

In Figure 2B, the extent of photolysis of each compound was determined and compared with caged phosphate, which was mixed in equal concentration with each caged phenylephrine compound prior to photolysis. Caged phosphate has a known photochemical quantum yield of 0.54 (Kaplan et al., 1978), and its photoproduct, inorganic phosphate, was detected by a colorimetric assay (Lanzetta et al. 1979). Quantum yields for the new compounds were determined by the extent of photolysis relative to caged phosphate (i.e., the ratio of slopes in Figure 2B multiplied by 0.54). This approach was not valid for compound III because of its higher efficiency than caged phosphate in absorbing the irradiating light. For this reason, the same experiment was carried out using photolysis at 347 nm, and the difference in absorbance between III and caged phosphate at 347 nm (2.4-fold) was taken into account in calculating the quantum yield of III (Table I). Quantum yields estimated from broad band irradiation at 300–350-nm and at 347 nm were similar (Table I) and showed that compound IV was the most photosensitive of the phenylephrine derivatives. Control experiments showed that L-phenylephrine itself was not sensitive to UV-light under the conditions of photolysis used here.

In a preliminary account of the properties of compound I (Walker & Trentham, 1988), a quantum yield of 0.4 was reported. This overestimate was likely to be due to the use of a reverse-phase HPLC column which did not adequately resolve free phenylephrine from other photoproducts near the void volume of the column.

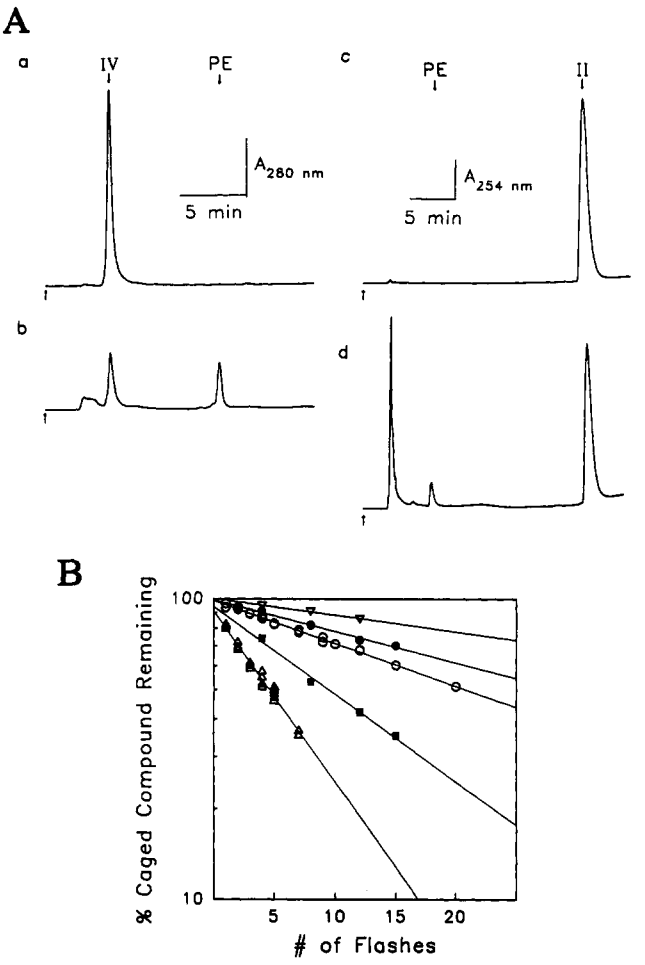


FIGURE 2: HPLC analysis of photoproducts. (A) A solution of 1 mM α-carboxyl caged phenylephrine (IV) was analyzed by cation exchange chromatography (see Experimental Procedures) before (a) and after (b) six flashes from a xenon arc lamp. A solution of 1 mM 2-nitrobenzyl caged phenylephrine (II) was analyzed similarly (see Experimental Procedures) before (c) and after (d) 12 flashes from a xenon arc lamp. (B) The extent of photolysis of compounds I–IV was determined by HPLC analysis as in A. Each compound was mixed with an equal concentration of caged P_i ([1-(2-nitrophenyl)-ethyl]phosphate) in 50 mM TES, pH 7, 10 mM DTT and then exposed to an increasing number of flashes. Half of the solution was injected onto the HPLC and half was analyzed for free phosphate by the method of Lanzetta et al. (1979). Regression lines give percent of photolysis per flash: I (●) 2.1%, II (▽) 0.8%, III (○) 2.3%, IV (■) 5.2%, and caged P_i (△) 10%.

Photolysis Rates and the aci-Nitro Intermediate. Photolysis rates for the new compounds were analyzed by monitoring the formation and decay of a presumed *aci*-nitro intermediate in the photolysis reaction. Laser pulse photolysis of I–IV induced a rapid increase in absorbance around 400 nm, followed by an approximately exponential decrease toward the initial absorbance (Figure 3). No absorbance changes were observed when solutions of L-phenylephrine were photolyzed. Similar transients have been observed with a variety of 2-nitrobenzyl compounds and have been interpreted to represent an *aci*-nitro species [reviewed in McCray and Trentham (1989)]. Most importantly, in at least two cases, the rate of decay of this species has been correlated with the rate of release of the biological substrate (Walker et al., 1988; Wootton & Trentham, 1989). Flash photolysis studies with compound I revealed a spectral transient with an absorbance spectrum that peaked at around 400 nm (not shown) similar to caged ATP (McCray et al., 1980) and caged carbachol (Walker et al., 1986). For compound I, the decay of this intermediate was slow, 2–3 s^{-1} (Figure 3A, trace a). Com-

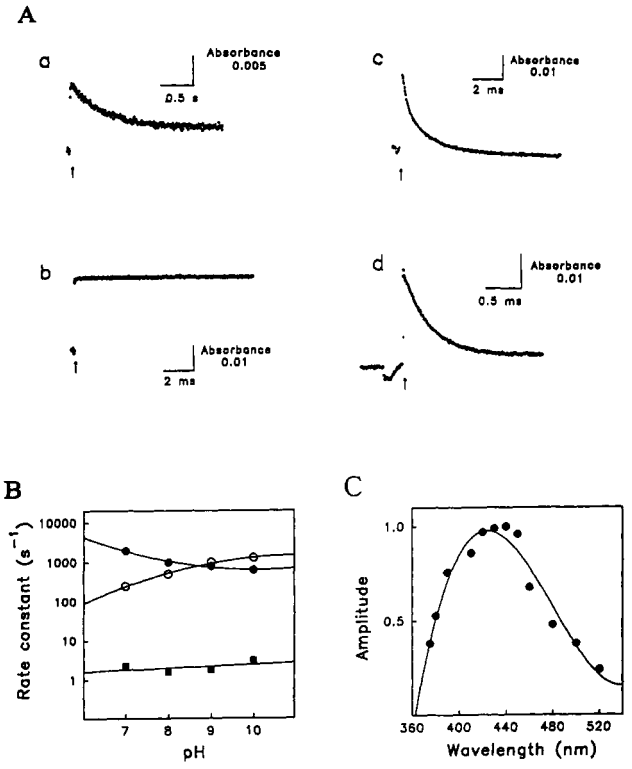


FIGURE 3: Rates of photolysis determined by laser pulse photolysis at 22 °C. (A) Aqueous solutions containing caged phenylephrine were irradiated by a single laser pulse (80–100 mJ; 347 nm) at the arrow. (a) 1 mM [1-(2-nitrophenyl)ethyl]phenylephrine (I) in 100 mM Tris, pH 8.0. (b) 1 mM (4,5-dimethoxy-2-nitrobenzyl)-phenylephrine (III) in 100 mM Tris, pH 8.0, without thiol. (c) 1 mM compound III in 100 mM Tris, pH 8.0, with 100 mM DTT. (d) 0.5 mM (α-carboxyl-2-nitrobenzyl)phenylephrine (IV) in 100 mM Tris, pH 8.0, without thiol. 10 mM DTT had no detectable effect on transients in (a) and (d). (B) Photolysis rate versus pH. The *aci*-nitro decay rates were obtained by fitting the data to single exponentials using a nonlinear least-squares method. pH was buffered at 7.0 with 100 mM TES or phosphate, at 8.0 with 100 mM Tris or bicine, at 9.0 with 100 mM CHES or borate, and at 10.0 with 100 mM CHES or carbonate. Solid lines illustrate the trend of the data. I (■), III (○), and IV (●). (C) Absorption spectrum of the *aci*-nitro intermediate of *O*-(α-carboxyl-2-nitrobenzyl)phenylephrine (IV). Flash photolysis was carried out as in panel A, and the ampltiude of the absorbance change normalized to the maximum absorbance is plotted versus the wavelength being monitored. Absorption maxima occurred at 435 nm for IV, at 390 nm for II (not shown), and at 420 nm for III (not shown).

pound II was not investigated in detail because the amplitude of the spectral signal was small (consistent with a poor quantum yield), which made reliable analysis difficult.

In the case of the dimethoxy compound (III), decay of the *aci*-nitro signal was observed only in the presence of a thiol compound such as DTT (Figure 3A, traces b and c). This is probably because the absorbance of the dimethoxy *aci*-nitro species is similar in this wavelength range to the main photolytic byproduct (Figure 1 for structures), but reaction of the photolytic byproduct with DTT decreases its absorbance in this range (Goldman et al., 1982). The rate of decay measured for compound III was dependent on the concentration of DTT consistent with a bimolecular reaction between the byproduct and DTT (McCray & Trentham, 1989). The rate constant of 220 s^{-1} obtained from Figure 3A at 100 mM DTT represents a lower limit for the rate of decay of the *aci*-nitro species. It is apparent that the 4,5-dimethoxy groups on the 2-nitrobenzyl nucleus significantly increased the photolysis rate as has been observed with caged cyclic AMP (Nerbonne et al., 1984).

Flash photolysis of compound IV revealed an apparent *aci*-nitro intermediate that displayed an absorbance peak at 435 nm (Figure 3C). The *aci*-nitro signal for compound IV decayed approximately 1000 times faster than compound I and some 10-fold faster than the lower limit estimate for compound III (Figure 3, Table I). Compound IV photolyzed with a half-time of 350 μs in buffers that were close to physiological (100 mM ionic strength, pH 7.0, 22 °C).

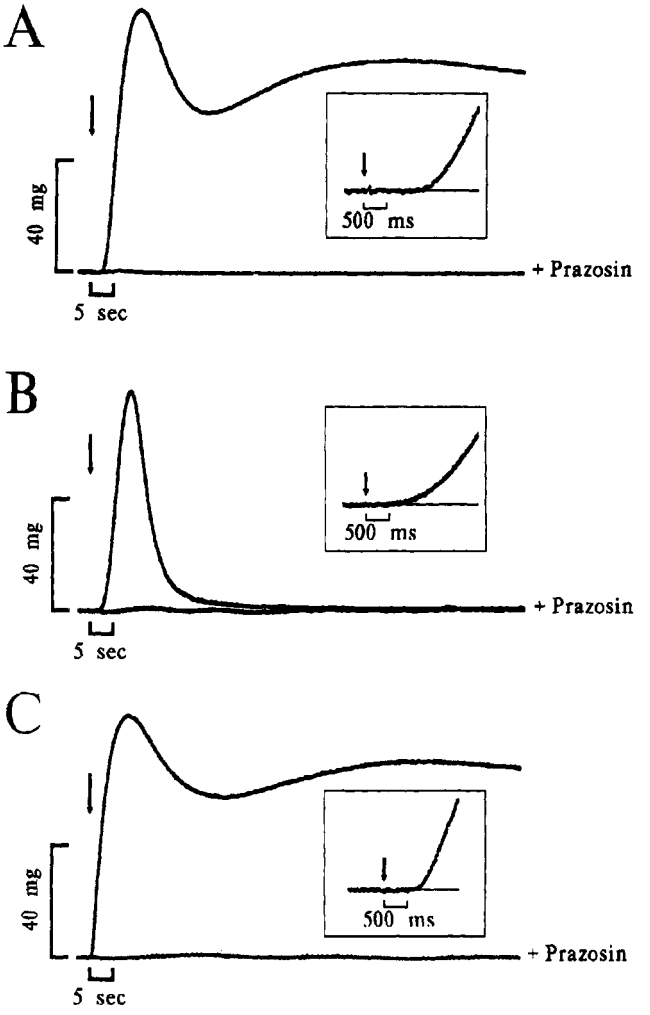


FIGURE 4: Force development in rat caudal artery smooth muscle in response to (A) 50 μM phenylephrine, (B) laser photolysis of 250 μM compound I, and (C) laser photolysis of 100 μM compound IV. Respnoses were measured in Krebs buffer containing 10 mM glutathione, pH 7.1, 25 °C. Free phenylephrine concentrations for B and C were estimated to be ≥10 μM. Responses were blocked by 5-min preincubation with 1 μM prazosin. Insets show faster time base records, and arrows indicate the time of phenylephrine addition or of the laser pulse.

Biological Properties. Bath application of 50 μM phenylephrine elicited contractions of rat caudal artery smooth muscle tissues. Contractions consisted of an initial transient phase followed by a phase of force maintenance (tonic phase) (Figure 4A). The dose–response relationships for both phases are illustrated in Figure 5A. There was no detectable augmentation or inhibition of force development during either the transient or tonic phases of a phenylephrine-induced contraction in the presence of compound IV (Figure 5A). This was true whether the tissue was preincubated with compound IV for several minutes before exposure to phenylephrine or whether the tissue was exposed to compound IV and phenylephrine simultaneously.

Photolysis of IV initiated contractions of smooth muscle from rat caudal artery (Figure 4C), and contractions were fully blocked by the α_1-adrenergic antagonist prazosin. The

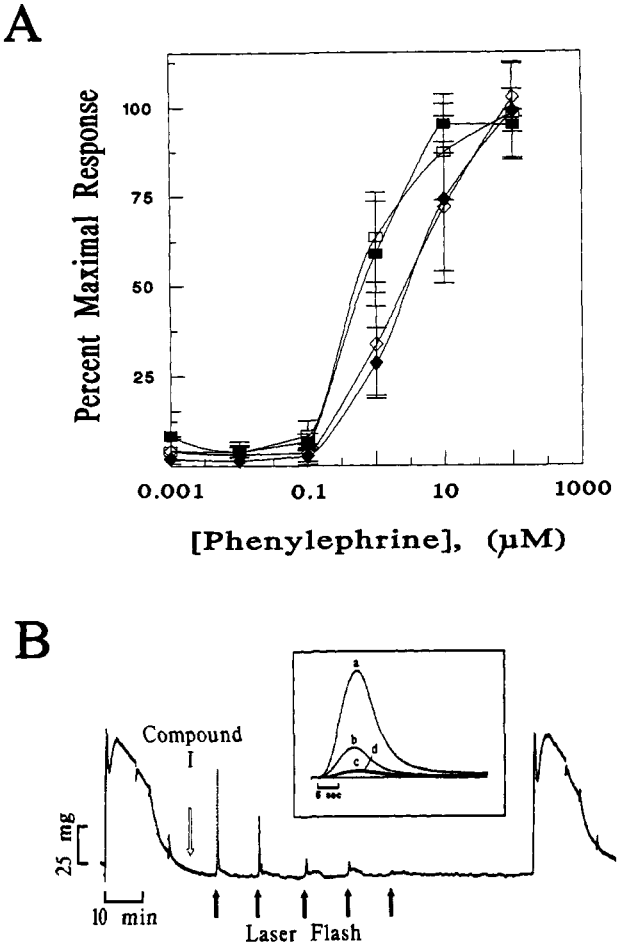


FIGURE 5: (A) Phenylephrine dose–response relationships in rat caudal artery. Control curves with bath-applied phenylephrine for phasic (□) and tonic (◇) contractions as in Figure 4. Effects of 250 μM compound IV (preincubated for 3 min) on phasic (■) and tonic (◆) contractions elicited by bath-applied phenylephrine. Responses were normalized to force developed at 50 μM phenylephrine. Values are mean ± sem of four measurments. (B) Removal of caged phenylephrine from rat caudal artery, 25 °C. A response to 50 μM phenylephrine and its subsequent washout is shown at the beginning of the chart recording. The tissue was then incubated in 500 μM compound I for 3 min (open arrow). The attempted washout of compound I involved 3 × 2 min washes with Krebs buffer and was followed by a laser flash (filled arrow); the rinse–flash sequence was repeated 5 times until the response was eliminated. Another 50 μM phenylephrine contraction was recorded at the end of the experiment. Bar is 10 min. Inset: fast time base records of laser-induced contractions.

time course of contraction at 25 °C showed a latency period of 0.93 ± 0.09 s prior to the onset of contraction, and then force developed with a 10–90% rise time of 3.56 ± 0.17 s (Table II). Rapid immersion into 50 μM phenylephrine produced a very similar contraction (Figure 4A) but resulted in slower kinetics of force development including at least a 50% increase in both the latency and rise time (Table II).

Photolysis of compounds I–III produced contractions that consisted mainly of an initial transient phase (Figure 4B). Force declined to near resting levels and no sustained contractile phase developed (compare panels A and B in Figure 4). The amount of force produced during the transient phase was maximal, consistent with the photorelease of ≥10 μM phenylephrine, but the force generated during the tonic phase was reduced by >90%. Under conditions which produced ≥10 μM phenylephrine, values for lag times and rise times were not significantly different ($p > 0.1$) when measured with any of the four caged phenylephrine compounds (Table II).

Table II: Kinetics of Force Development following Caged Phenylephrine Photolysis[a]

compd	latency,[b] s	rise time,[c] s	n
I	1.15 ± 0.06	3.03 ± 0.06	7
II	1.48 ± 0.11	3.59 ± 0.35	7
III	1.30 ± 0.08	3.28 ± 0.10	6
IV	0.93 ± 0.09	3.56 ± 0.17	7
phenylephrine	1.89 ± 0.15	4.70 ± 0.49	10

[a] Maximal responses were obtained in rat caudal artery at 25 °C in Krebs buffer using concentrations of caged phenylephrine that gave a minimum of 10 μM free phenylephrine on photolysis. [b] The time between laser pulse and the onset of force development was determined by visual inspection of digitized traces (mean ± sem). [c] Time taken for force to rise from 10% to 90% of maximal (mean ± sem).

Thus, compounds I–III gave normal transient responses, but the tonic phase of contraction appeared to be selectively inhibited.

The inhibition was further characterized by competition experiments using compounds I–III and bath application of phenylephrine. Compounds I–III had no detectable effects on the phenylephrine dose–response curves (for either the transient or tonic phase) when the tissue was exposed to phenylephrine and inhibitor (i.e., I–III) simultaneously. Inhibition of tonic phases of phenylephrine contractions was observed only if the tissue was preincubated with I, II, or III for 3 min or more prior to activation by phenylephrine. Inhibition is not likely to be competitive in nature as it could not be overcome by higher concentrations of phenylephrine, and contractions elicited by KCl depolarization were also inhibited by preincubation with compounds I–III (not shown).

Another observation regarding the biological properties of compounds I–III was the difficulty encountered in washing them out of the smooth muscle tissues. This is illustrated by the experiment shown in Figure 5B. After incubation of the preparation with compound I (500 μM, 3 min), washing the preparation with Krebs ringer did not eliminate the response to a laser flash. The wash procedure involved three exchanges with fresh Krebs buffer in the 15-μL bathing solution at 2-min intervals. Further washes progressively decreased the contractile response to laser pulses (Figure 5B, inset). All responses were blocked by 1 μM prazosin, indicating that they were due to the release of phenylephrine from caged phenylephrine and not due to artifacts associated with the laser flash. Contractile responses to the laser flash were not observed if the tissue had never been exposed to a caged phenylephrine compound. Similar results were obtained with compounds II and III. Incubation with high concentrations of compounds I–III appears to lead to partitioning of the compounds into nonspecific sites in the smooth muscle tissue which makes rapid washout difficult. In contrast to compounds I–III, compound IV was readily washed out of the smooth muscle preparations.

Contractions initiated by photolyis of compound IV gave a dose–response relationship that matched reasonably well with control phenylephrine dose–response curves (not shown). The biological properties of compounds I–IV, and the contractile responses observed on laser photolysis, were not unique to the rat caudal artery. Similar results were obtained with guinea pig portal vein and rabbit portal vein smooth muscle tissues.

DISCUSSION

The goal of this work was to develop photolabile caged derivatives of phenylephrine for time-resolved studies of α-adrenergic mechanisms. In general, the criteria for a good

caged molecule include: (1) little or no biological activity prior to photolysis, and (2) rapid and efficient release of the biological molecule from the chemical cage. Significant progress toward satisfying these criteria for a caged phenylephrine compound has now been made.

The results of this study show that the α-adrenergic agonist phenylephrine can be easily and selectively alkylated on its phenolic oxygen, permitting a variety of different photolabile 2-nitrobenzyl groups to be introduced at that position. Phenylephrine was initially derivatized with the 1-(2-nitrophenyl)ethyl protecting group (Walker & Trentham, 1988) because of its wide application in a variety of caged compounds [reviewed by McCray and Trentham (1989)]. While the resulting caged compound (I, Figure 1) photolyzed to free phenylephrine with near-UV irradiation, its photolysis rate at 2 s^{-1} was on the low end of the range observed with other 2-nitrobenzyl compounds and possibly too slow for studies of α-adrenergic mechanisms (Walker & Trentham, 1988; but see Somlyo et al., 1988). Moreover, the compound was found to possess undesirable biological properties (see below), and so the need arose to examine other photosensitive protecting groups.

Biological Activity of Caged Phenylephrine Compounds. The four derivatives examined here displayed no agonist nor antagonist effects when smooth muscle tissues were exposed to the test compound and phenylephrine simultaneously. Therefore, I–IV probably do not interact with the α-adrenergic receptor. However, when preequilibrated with compounds I–III, the tonic phase of contraction was inhibited whether phenylephrine was introduced by photorelease or by bath application. Inhibition appeared to correlate with adsorption or partitioning of the caged phenylephrine compounds into smooth muscle cell membranes. Recovery of the tonic phase occurred following a complete washout of compounds I–III, which was facilitated by extensive washing and irradiation. It is clear that compounds I–III are not strictly compatible with the first criterion for a useful caged compound, namely, no biological activity before photolysis.

The α-carboxyl-2-nitrobenzyl protecting group was successfully used in the development of caged carbachol where it helped to eliminate biological side effects before photolysis (Milburn et al., 1989). It has also been used in the development of caged glycine compounds (Billington et al., 1992). When used to prepare caged phenylephrine (compound IV), the negatively charged carboxylate group on the 2-nitrobenzyl moiety increased the water solubility of caged phenylephrine, prevented nonspecific binding to smooth muscle tissues, and eliminated deleterious biological side effects.

Other Benefits of Compound IV. Compound IV appears to meet the requirements outlined earlier for a useful caged molecule. In addition to its lack of biological activity, the photolysis rate of IV was the fastest of the four compounds investigated, providing a sub-millisecond time resolution for photolysis experiments with caged phenylephrine. The quantum yield of IV was the highest of the four compounds. In practical terms, the efficiency of photolysis was such that up to 24% of compound IV was converted to free phenylephrine with a single 200 mJ flash from a frequency-doubled ruby laser at 347 nm.

Analysis of α-adrenergic mechanisms in a variety of physiological tissues can now be achieved in a more quantitative manner. In rat caudal artery smooth muscle, the delay of 0.93 ± 0.09 s observed prior to the onset of force development (Figure 4C; Table III) is clearly due to biological processes and not the result of diffusional delays or slow photochemical release of phenylephrine. Similar delays and rise times were observed in portal vein and pulmonary artery smooth muscles, indicating that this time course reflects a general property of α-adrenergic agonist stimulation, at least in vascular smooth muscle.

Results in Table II showed that responses to bath application of 50 μM phenylephrine were probably slowed by diffusion. That diffusion occurred so slowly, requiring 1–2 s, is likely the result of the multicellular nature of the preparation, in which compounds in the bath need to diffuse through unstirred layers of glycocalyx and possibly deep down inside several layers of cells to reach receptor sites. In a previous study, bath application of 100 μM of phenylephrine gave a similar contraction time course to that observed following photolysis of compound I (Somlyo et al., 1988). It was concluded that both methods overcame diffusional delays and revealed the intrinsic response of the tissue to phenylephrine. The present results with compound IV confirm this conclusion. Compound IV is expected to be more useful than compound I in future studies because of its improved biological and photochemical properties. The earliest events following α-adrenergic receptor stimulation such as G-protein activation, ion channel gating, and second messenger formation will be more amenable to investigation with compound IV. It will also permit characterization of the kinetics of contraction in smooth muscles such as vas deferens which have been reported to show a shorter latency (<100 ms) between α-stimulation and force development (Amobi & Smith, 1987). Compound IV will also circumvent the problem of partitioning (and presumably accumulation) of caged phenylephrine compounds at the membrane surface, which creates difficulties in establishing the concentration of phenylephrine produced near the receptor sites on photolysis. Thus, compound IV will permit the dependence of the contraction time course on phenylephrine concentration to be evaluated.

Mechanism of Photolysis. Previous work has shown that phenolic oxygens can be protected with 2-nitrobenzyl groups and deprotected by near-UV irradiation (Amit et al., 1976; Walker & Trentham, 1988; Ware et al., 1989; Mitchison, 1989). The precise mechanism of photolysis of this class of compounds is not known. In the present work, detection of absorbance transients with properties similar to well-characterized *aci*-nitro intermediates (McCray et al., 1980) suggests dark reactions which are similar to those proposed for the more extensively investigated caged phosphate compounds (Walker et al., 1988; McCray & Trentham, 1989). The *aci*-nitro species detected with compound IV had properties similar to caged carbachol (Milburn et al., 1989) and caged glycine (Billington et al., 1992), including an absorption maximum around 435 nm and a rapid decay rate (>900 s^{-1} at pH 7); these compounds each utilized the α-carboxyl-2-nitrobenzyl protecting group. Caged phenylephrine photolysis rates were not markedly pH dependent (Figure 3B), in contrast to caged phosphate compounds whose photolysis rates were acid-catalyzed (McCray et al., 1980; Walker et al., 1988), and caged carbamate and amine compounds that appear to be acid- and base-catalyzed (Walker et al., 1986; Billington, 1992).

Biological molecules related to phenylephrine now become good candidates for the development of photolabile precursors. Catecholamines such as noradrenaline, adrenaline or the β-selective agonist isoproterenol should be readily synthesized by applying the techniques developed here. Recently, fluorescein and related probes have been derivatized with photolabile 2-nitrobenzyl groups on phenolic oxygens, a modi-

fication that results in a dramatic quenching of the fluorescence (Ware et al., 1986). Photolysis gives rise to an increase in fluorescence that can be used to monitor movement of macromolecules within cells (Mitchison, 1989). The new α-carboxyl 2-nitrobenzyl protecting group may be useful in such studies because of its beneficial photochemical properties and its greater water solubility than the conventional 2-nitrobenzyl group.

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