Photolabile Precursors of Inositol Phosphates. Preparation and Properties of 1-(2-Nitrophenyl)ethyl Esters of *myo*-Inositol 1,4,5-Trisphosphate†

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ABSTRACT: 1-(2-Nitrophenyl)ethyl esters of D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) have been synthesized and shown to have suitable properties for use as photolabile precursors of $InsP_3$. Synthesis was accomplished by treatment of $InsP_3$ with 1-(2-nitrophenyl)diazoethane in a $CHCl_3$/water mixture. This resulted in esterification of each of the three phosphate residues in $InsP_3$, the 1-phosphate being more reactive than the 4- or 5-phosphate. Singly esterified P-1, P-4, and P-5 esters, termed P-1, P-4, and P-5 caged $InsP_3$, were isolated from the reaction mixture by anion-exchange HPLC and characterized by 500-MHz ^1H NMR spectroscopy. Each of these caged $InsP_3$ esters exists as a pair of diastereoisomers and was identified by examining the effects of pH and nitrophenyl ring current shielding on the chemical shifts of nonexchangeable inositol protons. ^1H NMR spectra of $InsP_3$ were analyzed for comparison. On photolysis the compounds released $InsP_3$ with rate constants of 175 (P-1), 225 (P-4), and 280 s^{-1} (P-5) as determined by monitoring the *aci*-nitro decay reaction at pH 7.1, 0.2 M ionic strength, 21 °C. Quantum yields determined by steady-state near-UV photolysis were 0.65 ± 0.08 for each compound. P-4 and P-5 caged $InsP_3$ were the most promising biologically inactive $InsP_3$ precursors since at concentrations up to 50 μM they did not release Ca^{2+} from smooth muscle sarcoplasmic reticulum (SR) and were not metabolized by vascular smooth muscle $InsP_3$ 5-phosphatase or bovine brain $InsP_3$ 3-kinase. In contrast, P-1 caged $InsP_3$ released Ca^{2+} from smooth muscle SR with a similar potency to $InsP_3$ and was hydrolyzed by $InsP_3$ 5-phosphatase about 4-fold more slowly than $InsP_3$ when both substrates were at 100 μM. Only P-5 caged $InsP_3$ displayed any inhibitory effects on the biological properties measured. The rate of conversion of 10 μM $InsP_3$ to $InsP_4$ by $InsP_3$ 3-kinase was decreased 2-fold in the presence of 10 μM P-5 caged $InsP_3$.

Release of biological substrates from photolabile precursors has proved to be a powerful method for introducing effectors into organized biological preparations in a controlled way [reviewed in Gurney and Lester (1987) and McCray and Trentham (1989)]. The approach depends on the synthesis of photolabile compounds that are biologically inactive and have the appropriate photochemical properties, such as high quantum yields and rapid photolysis rates. A wide variety of these compounds, commonly termed "caged" compounds, has been synthesized by derivatizing functional groups on biological substrates with 1-(2-nitrophenyl)ethyl or related protecting groups (Walker et al., 1988, and references cited therein). For instance, esterification of phosphate residues in ATP (Kaplan et al., 1978), ATP(γS) (Walker et al., 1988), GTP(β,γNH) and GTP(γS) (Dolphin et al., 1988), cAMP (Engels & Schlaeger, 1977), and cGMP (Nerbonne et al., 1984) has produced caged precursors that are potentially applicable to a broad range of biological problems.

Photolysis of caged compounds can be used to overcome delays associated with diffusion of substrates into physiological preparations; this allows time-resolved measurements to be made in such systems. The time resolution is governed by rate-limiting dark reactions that are characteristic of 2-nitrobenzyl photochemistry. Rate constants for these dark reactions can be greater than 10^4 s^{-1} providing resolution to 100 μs, although time resolutions of 1-100 ms are more common (Nerbonne, 1986; Walker et al., 1986, 1988; Tsien & Zucker, 1986; Adams et al., 1988; Karpen et al., 1988; Walker & Trentham, 1988). The concentration of the substrate formed on photolysis can be determined by HPLC analysis of the solution surrounding the physiological preparation. This concentration can be varied over a wide range by modulating the near-UV light input or by varying the concentration of the caged precursor.

We now report the preparation and characterization of three isomeric 1-(2-nitrophenyl)ethyl phosphate esters of $InsP_3$,[1] termed P-1, P-4, and P-5 caged $InsP_3$ to indicate which phosphate residue of $InsP_3$ has been esterified. Structures were determined by 500-MHz ^1H NMR. A detailed analysis of ^1H NMR spectra of $InsP_3$ was also undertaken. Photolysis of the three isomers of caged $InsP_3$ to $InsP_3$ proceeded in physiological solutions at 21 °C with rate constants of 175 s^{-1} or faster (depending on the isomer) and with high quantum yields.

$InsP_3$ has been proposed to be an intracellular messenger in a variety of cell types whose function is to mobilize Ca^{2+} from intracellular stores (Berridge & Irvine, 1984). The biological properties of the isomers of caged $InsP_3$ were established by testing their effects on Ca^{2+} release from smooth muscle SR and by examining their stability in the presence of enzymes that metabolize $InsP_3$, $InsP_3$ 5-phosphatase, and $InsP_3$ 3-kinase. The P-4 and P-5 isomers, which displayed the

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[1] Abbreviations: $InsP_3$, *myo*-inositol 1,4,5-trisphosphate; $InsP_2$, *myo*-inositol 1,4-bisphosphate; $InsP_4$, *myo*-inositol 1,3,4,5-tetrakisphosphate; PIPES, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; HDTA, 1,6-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetate; TES, *N*-[tris(hydroxymethyl)-methyl]-2-aminoethanesulfonic acid; DTT, dithiothreitol; TEAB, triethylammonium bicarbonate; ^1H NMR, proton nuclear magnetic resonance; SR, sarcoplasmic reticulum.

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least biological activity before photolysis, have been used to investigate the role of $InsP_3$ in excitation contraction coupling in muscle (Walker et al., 1987) and in the control of K^+ permeability in hepatocytes (Capiod et al., 1988).

Materials and Methods

D-*myo*-Inositol 1,4,5-trisphosphate (trilithium salt) was obtained either from Calbiochem, Behring Diagnostics, La Jolla, CA, or from Robin F. Irvine, AFRC Unit, Babraham, England. D-*myo*-[^{3}H]Inositol 1,4,5-trisphosphate (potassium salt; 5 μCi, 2–5 Ci/mmol) was purchased from NEN/Du Pont, Boston, MA. Brain calmodulin, creatine kinase, and leupeptin were obtained from Sigma Chemical Co. Saponin was purchased from ICN.

HPLC was carried out on Whatman partisil 10 SAX columns with a Waters 6000A solvent delivery system, sample injector, and UV absorbance detector at 254 nm. Gradient elutions employed two Waters 6000A pumps and a Waters M660 solvent programmer. Solvents containing ammonium phosphate were prepared by diluting 1 M $(NH_4)H_2PO_4$ and 1 M $(NH_4)_2HPO_4$ to the desired concentration with water and methanol before they were mixed to the desired pH. Conditions for achieving separations of inositol phosphates varied slightly from one column to another and with the age of each column.

^{1}H NMR spectra were obtained by using a Bruker 500-MHz spectrometer operating in the Fourier transform mode. Samples were prepared for NMR study by replacing exchangeable protons with deuterium using D_2O; rotary evaporation was used to remove water followed by two washes with D_2O (99 atom % D) before finally the samples were redissolved in 0.5 mL of D_2O (99.96 atom % D). Acetone (1%) was added as an internal reference (δ 2.225). The pH adjustments were made with 0.1 M NaOD and 0.1 M DCl. The pH values were meter readings using a Radiometer/Copenhagen pH meter and were not corrected for deuterium isotope effects.

A 150-W xenon arc lamp was used for continuous near-UV irradiation of samples. The light was passed through a heat filter (water) and a Corning 9863 glass band pass filter (300–350 nm) before being focused onto a quartz cuvette containing the sample (1 cm path length, 0.2–1.0-mL sample volume). Flash photolysis was carried out as described previously (Walker et al., 1988) by using a frequency-doubled Candela dye laser that delivered 20–40 mJ at 320 nm in a 1-μs pulse. Absorption spectra were recorded on a Beckman DU8 spectrophotometer.

Caged $InsP_3$ Synthesis. To 2–20 mg of $InsP_3$ (3–30 μmol) in 1–2 mL of water adjusted to pH 4 with dilute HCl was added [^{3}H]$InsP_3$ to achieve the desired specific activity, typically 0.1–1.0 μCi/μmol (0.22–2.2 $\times$ 10^6 dpm/μmol). This was stirred vigorously with 2 mL of $CHCl_3$ containing 0.7 mmol of 1-(2-nitrophenyl)diazoethane for 8 h in the dark. 1-(2-Nitrophenyl)diazoethane was prepared fresh by mixing 0.178 g of the hydrazone of 2-nitroacetophenone (1.0 mmol) in 50 mL of $CHCl_3$ with 0.712 g of MnO_2 for 5 min in the dark. The red-orange solution was filtered under vacuum to remove insoluble material and rotary evaporated to remove $CHCl_3$, and the 1-(2-nitrophenyl)diazoethane was resuspended in 2 mL of $CHCl_3$ for mixing with the aqueous solution of $InsP_3$. Caution must be used during rotary evaporation because diazonium compounds can be explosive. We have experienced no such problems in over 3 years of use by avoiding prolonged evaporations and by keeping the bath temperature below 35 °C. The method of synthesis of 2-nitroacetophenone hydrazone and further details concerning the handling of 1-(2-nitrophenyl)diazoethane are described elsewhere (Walker et al., 1988).

Caged $InsP_3$ Purification. The aqueous phase of the reaction was separated from the $CHCl_3$ phase and washed three times with $CHCl_3$. Singly, doubly, and triply esterified $InsP_3$ were isolated by HPLC on a partisil 10 SAX ion-exchange column, eluted at 1.0 mL/min with a linear 30-min gradient of 0.12–0.5 M ammonium phosphate, pH 6.8, and collected in 1-mL fractions. Up to 3 μmol of caged $InsP_3$ was chromatographed in each HPLC run. (As recommended by Whatman, the system included a precolumn containing partisil 10 packing material to saturate the mobile phase with silicate ions to prevent rapid deterioration of the HPLC column at this pH.) Pooled fractions containing up to 10 μmol of caged $InsP_3$ were diluted 4–5-fold and then desalted on a DEAE-cellulose column (1.6 cm $\times$ 30 cm) that was eluted with a linear gradient of 10–500 mM TEAB. Specific activity for each peak was determined by scintillation counting (counting efficiency = 0.40) and absorption measurements [ϵ = 4240 M^{-1} cm^{-1} at λ_{265nm} (Kaplan et al., 1978)] and reported as dpm/μmol of 1-(2-nitrophenyl)ethyl groups. Starting with a specific activity of 1.5 $\times$ 10^6 dpm/μmol for $InsP_3$, the specific activity for singly caged $InsP_3$ was 1.4 $\times$ 10^6 dpm/μmol, doubly caged $InsP_3$, 7.7 $\times$ 10^5 dpm/μmol, and triply caged $InsP_3$, 4.5 $\times$ 10^5 dpm/μmol.

The singly caged $InsP_3$ was resolved into P-1, P-4, and P-5 isomers by HPLC on a partisil 10 SAX ion-exchange column eluted isocratically with 0.12 M ammonium phosphate, pH 5.4:methanol (19:1 v/v) at 2.0 mL/min. Under these conditions the three isomers could also be isolated directly from the aqueous reaction mixture without prior separation of singly caged $InsP_3$ from doubly and triply caged and uncaged $InsP_3$. The HPLC column could handle up to 10 μmol of derivatized $InsP_3$ per run. The P-1, P-4, and P-5 isomers were obtained in a ratio of 4:1:1, respectively, each with the same specific activity as the singly caged $InsP_3$ that was loaded onto the column. Singly caged $InsP_3$ prepared by photolyzing doubly and triply caged $InsP_3$ resulted in a more equal distribution of P-1, P-4, and P-5 isomers. Thus, larger quantities of the P-4 and P-5 isomers were prepared by alkylating $InsP_3$ with 1-(2-nitrophenyl)diazoethane for 24 h to give predominantly doubly and triply caged $InsP_3$ followed by partial photolysis to regenerate singly caged $InsP_3$.

Isomers collected off the HPLC column were diluted 4–5-fold and chromatographed on a DEAE-cellulose column eluted with a linear gradient of 10–500 mM TEAB to remove ammonium phosphate. As with all samples desalted in this manner, TEAB was removed by rotary evaporation followed by three methanol washes. Samples were stored as aqueous solutions at −20 °C.

Biological Measurements. All experiments with rabbit main pulmonary artery tissue were carried out in smooth muscle buffer A of the following composition: 100 mM potassium PIPES, pH 7.1; 7.9 mM $MgCl_2$; 5.5 mM ATP; 9.5 mM creatine phosphate; 50 units/mL creatine kinase; and 15 mM potassiaum HDTA (free [Mg^{2+}] = 1.5 mM, ionic strength = 0.2 M). Tension measurements were made on strips of rabbit main pulmonary artery (average dimensions, 2.8 mm $\times$ 0.36 mm $\times$ 0.12 mm) suspended on a force transducer and bathed in 45-μL troughs as described previously (Somlyo et al., 1985). Strips were permeabilized by a 15-min treatment with 50 μg/mL saponin in buffer A and then washed to remove saponin.

$InsP_3$ 5-phosphatase activity was isolated by homogenization of 50–150 mg (wet weight) of main pulmonary artery tissue

in buffer A containing leupeptin and 0.1 mM phenylmethanesulfonyl fluoride using a glass–Teflon homogenizer. Cell debris was removed by centrifugation at 600*g* for 5 min, and then the supernatant was centrifuged at 85000*g* for 2 h. Both centrifugations were carried out in a Beckman JA-17 with a GSA rotor. The pellet, which contained 40% of the activity, was resuspended in buffer A to 0.5–2 mg of protein/mL. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Typically, 0.02 mg of protein was incubated with [^{3}H]$InsP_3$ at 22 °C, the reaction was quenched by boiling for 2 min, and insoluble material was removed by centrifugation in an Eppendorf microfuge for 10 min. Samples were analyzed by HPLC on a partisil 10 SAX column eluted isocratically with 0.38 M ammonium phosphate, pH 5.4:methanol (19:1 v/v) at 2.0 mL/min. Under these conditions, elution times of various inositol phosphates were 3 min for $InsP_2$, 8 min for $InsP_3$, 5 min for P-1 caged $InsP_3$, 4 min for P-4 and P-5 caged $InsP_3$, and 2.5 min for P-1 caged $InsP_2$. $InsP_2$ cochromatographed under a variety of HPLC conditions with an authentic sample of D-*myo*-[^{3}H]inositol 1,4-bisphosphate obtained from NEN/Du Pont.

Caged $InsP_2$ was prepared on a large scale by incubating 0.4 μmol of P-1 caged $InsP_3$ with 0.3 mg (protein) of $InsP_3$ 5-phosphatase preparation in 600 μL of buffer A for 12 h at 22 °C. Caged $InsP_2$ was purified following a 10-min centrifugation in a microfuge by injecting the reaction mixture onto a partisil 10 SAX column equilibrated in 10 mM ammonium phosphate, pH 5.4:methanol (19:1 v/v), followed by a step elution with 0.2 M ammonium phosphate, pH 5.4: methanol (19:1 v/v). The product was desalted on a DEAE-cellulose column eluted with a linear gradient of 10–300 mM TEAB; 0.3 μmol was recovered. Steady-state photolysis of caged $InsP_2$ prepared in this fashion generated $InsP_2$.

$InsP_3$ 3-kinase was isolated from bovine brain as described by Ryu et al. (1987) with the following modifications. A sample of 130 g of tissue was homogenized in 250 mL of 20 mM TES, pH 7.1, 0.1 mM DTT, and 0.15 M sucrose with an Iona blender (2 × 20 s). The large pellet obtained from a low-speed centrifugation using a Type 42.1 rotor at 8000*g* for 15 min was discarded, and the supernatant was centrifuged with the same rotor at 70000*g* for 90 min. $InsP_3$ kinase activity was detected in the supernatant fraction by using an assay buffer of the following composition: 100 mM KCl, 50 mM PIPES titrated to pH 7.4 with KOH, 0.1 mM DTT, 20 mM $MgCl_2$, 10 mM ATP, 5 μM $CaCl_2$, 5 μM calmodulin, 10 μM [^{3}H]$InsP_3$ at 5 × 10^6 dpm/μmol, and 10 mM 2,3-diphosphoglycerate to inhibit $InsP_3$ 5-phosphatase (Downes et al., 1982). Addition of enzyme solution (0.1 mg of protein) served to initiate the reaction, which was incubated at room temperature and then quenched by boiling for 2 min. Following a 10-min centrifugation to remove insoluble material, inositol phosphates in the supernatant were analyzed by HPLC. A partisil 10 SAX column eluted isocratically with 0.65 M ammonium phosphate, pH 4.7:methanol (19:1 v/v) at 1.5 mL/min separated $InsP_2$ (void volume, 2 min), $InsP_3$ (6 min), P-1 caged $InsP_2$ (3 min), P-1 caged $InsP_3$ (5 min), P-4 and P-5 caged $InsP_3$ (4 min), and $InsP_4$ (19 min). $InsP_4$ cochromatographed with authentic [^{3}H]inositol 1,3,4,5-tetrakisphosphate obtained from NEN/Du Pont.

Results

Synthesis and Isolation of Products. 1-(2-Nitrophenyl)ethyl phosphate esters of $InsP_3$ were synthesized by mixing an aqueous solution of $InsP_3$ with an excess of the alkylating agent 1-(2-nitrophenyl)diazoethane in $CHCl_3$. In this mixed solvent system with the aqueous phase adjusted to pH 4–5, the reaction is specific for weakly ionizing phosphate oxygens (Walker et al., 1988). Figure 1A shows the initial separation of the aqueous phase of the reaction mixture into three fractions by anion-exchange HPLC. Steady-state near-UV irradiation of material in each fraction regenerated $InsP_3$. The ratio of 1-(2-nitrophenyl)ethyl groups (A_{265nm}) to $InsP_3$ molecules (dpm) served to identify these products as singly, doubly, and triply esterified $InsP_3$. Reaction for 5 h favored formation of singly esterified $InsP_3$, which represents a mixture of compounds derivatized on the 1-, 4-, and 5-phosphate residues (I–III). Reaction for 24 h favored formation of $InsP_3$ derivatized on all three phosphate residues (IV). No product containing more than three 1-(2-nitrophenyl)ethyl groups was detected. In control reactions 1-(2-nitrophenyl)diazoethane

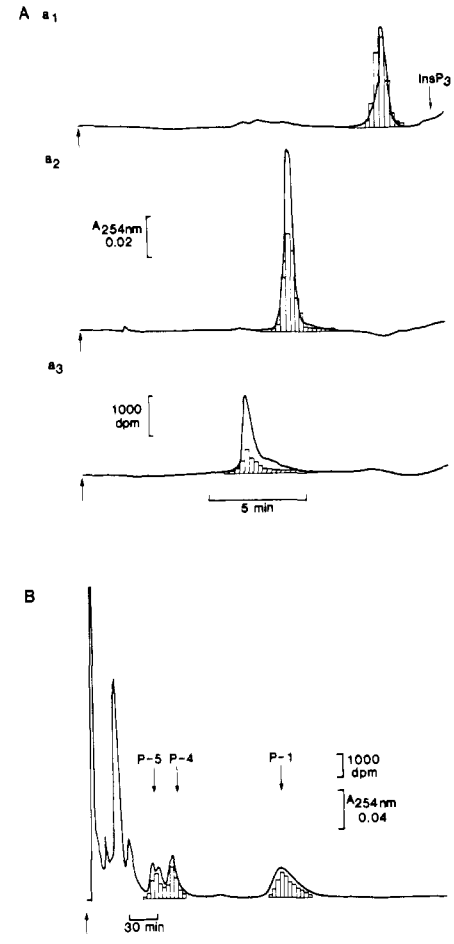
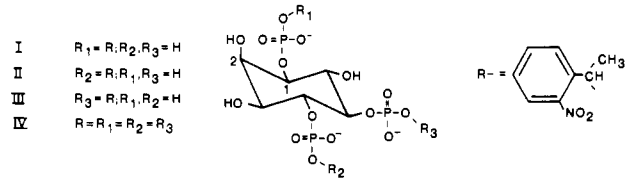


FIGURE 1: (A) HPLC traces of purified singly, doubly, and triply caged $InsP_3$ previously isolated from the reaction mixture by preparative HPLC under similar conditions. Aliquots were injected onto a partisil 10 SAX column equilibrated with 0.12 M ammonium phosphate, pH 6.8, and eluted with a 1.0 mL/min linear 15-min gradient to 0.6 M ammonium phosphate, pH 6.8. Absorbance at 254 nm was monitored (solid lines), and 0.25-mL fractions were collected and counted (bars). (a_1) 0.1 μmol of singly caged $InsP_3$; (a_2) 0.1 μmol of doubly caged $InsP_3$; (a_3) 0.04 μmol of triply caged $InsP_3$. The retention time of $InsP_3$ is indicated in (a_1). (B) HPLC trace showing preparative scale separation of P-5, P-4, and P-1 caged $InsP_3$ without prior purification of singly caged $InsP_3$. Unfractionated caged $InsP_3$ (0.5 μmol) was injected onto a partisil 10 SAX column equilibrated at 2.0 mL/min in 0.12 M ammonium phosphate, pH 5.4:methanol (19:1 v/v). Absorption at 254 nm (solid lines) was monitored, and 200 μL from each 2-mL fraction was counted (bars). Two incompletely resolved peaks of radioactivity corresponding to doubly and triply caged $InsP_3$ eluted within the first 60 min. Then singly caged $InsP_3$ isomers eluted in the order shown in a ratio of 1:1:2. The appearance of the P-5 isomer as a doublet is attributed to the presence of diastereoisomers.



did not alkylate the hydroxyl functions of inositol under the conditions used.

The three singly esterified $InsP_3$ isomers, I–III, were separated in a high resolution HPLC system as described by Irvine et al. (1985) (data not shown), or by HPLC using isocratic elution (Figure 1B). The elution profile in Figure 1B shows a doublet (two peaks of equal intensity) ahead of a larger single peak with a peak ratio that depended on the method of preparation of singly caged $InsP_3$. Alkylation of $InsP_3$ with 1-(2-nitrophenyl)diazoethane gave the three isomers in an approximate ratio of 1:1:4. The predominant isomer formed was identified as the P-1 ester by the ^1H NMR analysis presented below, indicating that the 1-phosphate is more reactive than the 4- or 5-phosphate. A more equal distribution of the three isomers was obtained by alkylating for 24 h to give predominantly doubly and triply esterified $InsP_3$ followed by partial near-UV irradiation to regenerate singly esterified $InsP_3$. In a variety of experiments, the ratio of isomers prepared in this fashion was approximately 1:1:2 as in Figure 1B. This altered ratio indicates that photochemical removal of 1-(2-nitrophenyl)ethyl groups from doubly and triply esterified $InsP_3$ is less selective for a particular phosphate residue than is alkylation of $InsP_3$.

Each isomer of singly esterified $InsP_3$ exists as a pair of diastereoisomers due to the presence of both configurations of the chiral 1-(2-nitrophenyl)ethyl group. This was demonstrated experimentally in the ^1H NMR studies discussed below. The fact that P-5 runs as a double peak in Figure 1B is attributed to a partial resolution of diastereoisomers by HPLC.

^1H NMR Characterization. All of the peaks in the ^1H NMR spectra of the biologically important compounds $InsP_2$, $InsP_4$, and the 1,3,4-isomer of $InsP_3$ have been assigned previously (Shibata et al., 1984; Lindon et al., 1986, 1987; Cerdan et al., 1986). However, earlier work on the 1,4,5-isomer of $InsP_3$ indicated nearly complete overlap of the H-1, H-5, and H-6 signals, and this seemed to limit the usefulness of ^1H NMR for $InsP_3$ structural studies. Figure 2A shows that the six inositol ring protons of $InsP_3$ can in fact be resolved by recording spectra at pH 5.4 on a 500-MHz instrument. Peak assignments are straightforward taking into consideration (1) that all protons are axial except H-2 and thus the signals from H-2 and its neighboring protons, H-1 and H-3, display a characteristic axial–equatorial coupling constant of about 3 Hz, while the axial–axial coupling constant is about 9 Hz (Shibata et al., 1984; Lindon et al., 1986); and (2) that phosphorus of the phosphate residue couples to ring protons with a coupling constant of about 9 Hz only if the phosphate and the proton are bonded to the same carbon (Shibata et al., 1984). Assignments derived from such an analysis were confirmed by proton homonuclear spin-decoupling experiments.

The chemical shifts of all six nonexchangeable $InsP_3$ protons are pH dependent. However, those adjacent to titrating phosphate residues, H-1, H-4, and H-5, are the most influenced by pH, decreasing by greater than 0.10 ppm over the pH range 5–9.5 (Figure 3A). The chemical shifts for H-1, H-4, and H-5 change over the entire range of nearly 5 pH units, and this non-Henderson–Hasselbach behavior indicates that the three phosphate residues interact significantly during protonation. It seems unlikely that the titration curves are being influenced by divalent metal ions since addition of 1 mM EDTA had no effect on the spectra of $InsP_3$ at several pH values. No large changes in proton–proton coupling constants as a function of pH over the pH range 5–9.5 were noted, indicating the absence of a pH-dependent ring inversion similar to that observed for phytic acid ($InsP_6$) (Isbrandt & Oertel, 1980).

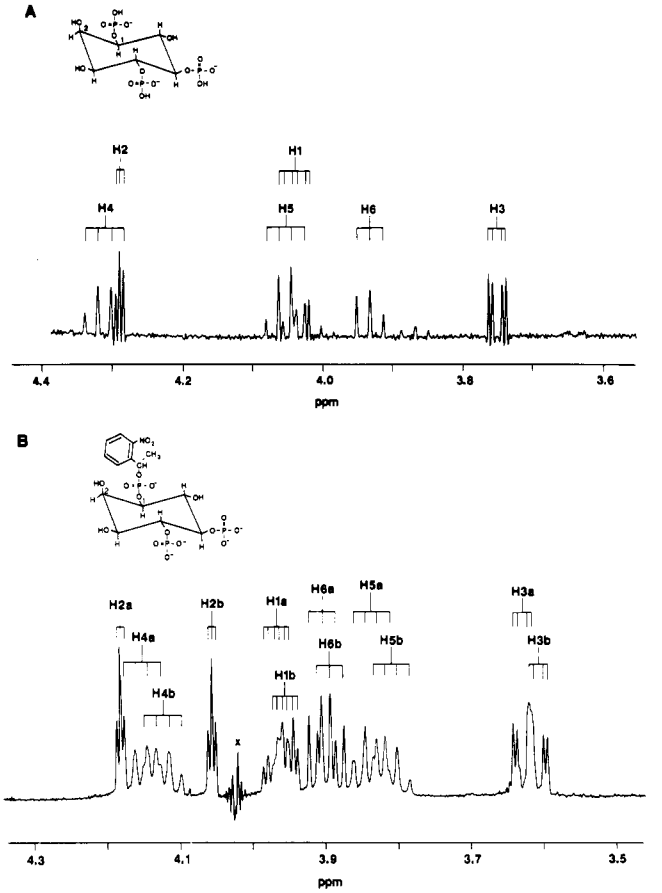


FIGURE 2: (A) Resolution-enhanced 500-MHz ^1H NMR spectrum of $InsP_3$, recorded from a sample containing 2 mM $InsP_3$ in D_2O adjusted to pH 5.4 with DCl, 25 °C. Minor unassigned peaks at 4.00, 3.85, 3.87, and 3.89 ppm are likely to be hydrolysis products of $InsP_3$. (B) 500-MHz NMR spectrum of P-1 caged $InsP_3$, recorded from a sample of material purified as in Figure 1B labeled P-1, at 10 mM in D_2O adjusted to pH 10.5 with NaOD, 25 °C. Peaks marked by an × are an instrument artifact.

^1H NMR spectra for each of the three isomers purified as in Figure 1B included four protons in the aromatic region, one benzyl proton, three methyl protons, and six inositol ring protons. Integration of these signals was consistent with one 1-(2-nitrophenyl)ethyl group per inositol ring for each isomer. An added complexity in the spectra for the 1-(2-nitrophenyl)ethyl esters compared to the spectrum of $InsP_3$ arises from the presence of diastereoisomers. The 1-(2-nitrophenyl)ethyl group contains a chiral center at the benzyl carbon, and esterification with 1-(2-nitrophenyl)diazoethane introduces both configurations (*R* and *S*) into the molecule in approximately equal amounts. The resulting NMR spectra of the P-1, P-4, and P-5 isomers are in each case composites of two similar spectra for the two diastereoisomers. Figure 2B shows the inositol proton region for the P-1 isomer. The signals in this spectrum were assigned by comparison with the $InsP_3$ spectrum and by proton spin decoupling experiments. Each inositol ring proton in the two diastereoisomers results in two signals (labeled a and b in Figure 2B) with identical multiplicities but slightly different chemical shifts. Diaste-

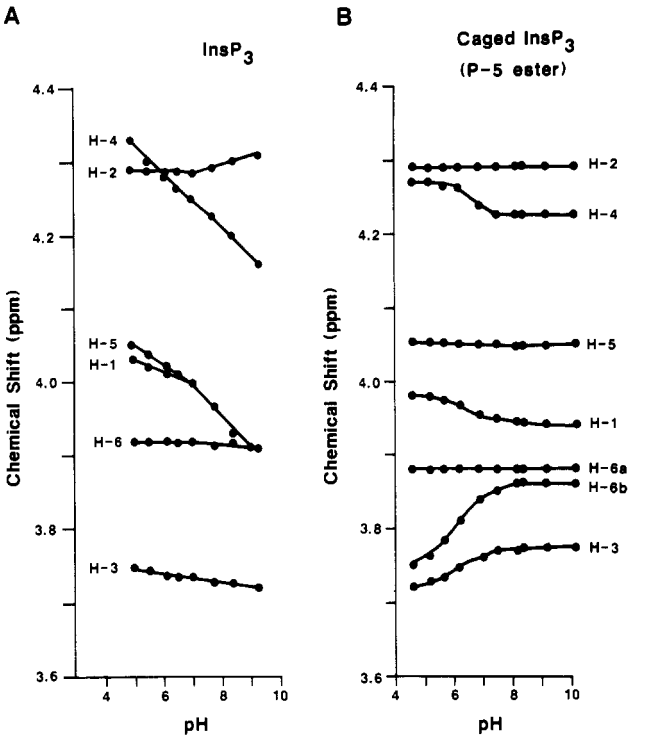


FIGURE 3: pH titrations of the ^1H chemical shifts of the *myo*-inositol ring protons in (A) $InsP_3$ and (B) P-5 caged $InsP_3$. Spectra were recorded initially at high pH and then at intermediate and low pH values following titration with DCl. The chemical shift changes were reversible as shown by recording a spectrum after returning to high pH.

reomeric pairs of proton signals that are well resolved (≥0.03 ppm difference) are included in Table I for all three isomers. The assignments for the inositol ring protons of the P-4 and P-5 isomers (Table I) were made in an analogous manner to those for P-1.

The pH dependence of the $InsP_3$ ^1H NMR spectrum offers a simple and general means of identifying esterified phosphate groups of $InsP_3$. Phosphate esterification would be expected to eliminate or greatly reduce the characteristic pH sensitivity of the proton bonded to the same carbon as the modified phosphate residue. Figure 3B shows a pH titration for the caged $InsP_3$ isomer that elutes from the HPLC column first. Of the three protons directly adjacent to phosphate residues, only the H-5 signal has lost its characteristic pH sensitivity, indicating that this isomer is esterified on the 5-phosphate. Several other features of this pH titration are worth noting. First, the pH sensitivities of H-3 and H-6, which are not directly linked to a phosphate group, increased significantly. Second, the phosphates titrated in a noninteracting manner, giving rise to sigmoidal curves resembling simple Henderson–Hasselbach curves with inflection points around pH 6.2–6.5 (Figure 3B). This is within the range of pK_a values expected for phosphates (*Spec. Publ.—Chem. Soc.*, 1964). Of the three caged $InsP_3$ isomers, this is the only one to show simple ionization behavior. This is consistent with its being esterified on the 5-phosphate residue, as in this molecule the titrating phosphates would be as far away from one another as possible.

The results of pH titrations carried out on the other isomers are summarized in Table I. Again, the identities of these compounds were revealed by the loss of pH sensitivity of the proton adjacent to the modified phosphate, H-4 for the second isomer off the HPLC column of Figure 1B and H-1 for the third isomer. Titration curves for both the P-1 and P-4 esters (data not shown) display non-Henderson–Hasselbach behavior

Table I: ^1H NMR Chemical Shifts of $InsP_3$ and Caged $InsP_3$ Isomers

		chemical shift[a] (ppm)				pH titration,[b] chemical shift difference (ppm)			
proton		$InsP_3$	P-1	P-4	P-5	$InsP_3$	P-1	P-4	P-5
H-1		4.03	3.96	3.95	3.97	−0.11	0	−0.06	−0.04
H-2	a		4.18	4.23			0	+0.11	
		4.29			4.29	+0.01			0
	b		3.98	4.20			+0.07	+0.14	
H-3	a			3.69			+0.03		
		3.75	3.60		3.72	−0.02		+0.04	+0.04
	b			3.66			−0.03		
H-4		4.31	4.23	4.25	4.26	−0.14	−0.09	−0.02	−0.04
H-5		4.05	3.96	4.01	4.05	−0.13	−0.14	−0.12	0
H-6	a				3.87				0
		3.93	3.87	3.86		+0.02	+0.03	+0.03	
	b				3.75				+0.10
benzyl	a			6.02	6.06			−0.03	−0.07
			5.90				0		
	b			5.97	5.98			−0.04	−0.02

[a] Chemical shift values were taken from the center of multiplets assigned to each proton; spectra were recorded at pH 5.4, 25 °C. Due to the presence of diastereoisomers, two chemical shift values, designated a and b, are listed for those protons that were separated by ≥0.03 ppm. Otherwise, chemical shift values represent the average of the two diastereoisomers. Peaks labeled *a* are arbitrarily assigned to the more downfield resonance of a diastereoisomeric pair and, as a result, all peaks labeled *a* are not necessarily from the same diastereoisomer. [b] Under the heading 'pH titration', values represent the difference between chemical shifts at high and low pH, 25 °C. Precise pH values for the four compounds were as follows: $InsP_3$, 10.5 and 5.4; P-1, 10.5 and 5.8; P-4, 10.8 and 5.5; P-5, 10.2 and 4.6. A positive number indicates an increase in chemical shift as the pH is increased.

similar to that for $InsP_3$ and indicate that the 1- and 5-phosphates interact in the P-4 ester and that the 4- and 5-phosphates interact in the P-1 ester. Secondary effects are also observed, esterification of P-4 altering the pH effects on the chemical shifts of H-2 and H-3, and esterification of P-1 altering the pH effects on the H-2 signals (Table I).

The benzyl proton of the 1-(2-nitrophenyl)ethyl protecting group gives rise to a pair of signals from diastereoisomers for the P-4 and P-5 esters but not for the P-1 ester (Table I). Chemical shifts for the benzyl proton of P-4 and P-5 are also pH dependent (Table I). These effects are likely to be the result of the close proximity through space of the 1-(2-nitrophenyl)ethyl phosphoryl moiety to a weakly ionizing phosphate residue, a situation that exists for both the P-4 and P-5 isomers but not for the P-1 isomer.

A comparison of the inositol ring proton chemical shifts before and after esterification at low pH (Table I) reveals the shielding effects of the 1-(2-nitrophenyl)ethyl ring. In each case the proton signal most affected is not on the carbon containing the modified phosphate residue but on a carbon adjacent to the modified phosphate. For instance, esterification of the 5-phosphate has the largest shielding effect on H-6 signals, esterification of the 4-phosphate on H-3 (and H-2) signals, and esterification of the 1-phosphate on H-2 signals. These shielding effects occur mainly on only one side of the modified phosphate residue and reflect the stereospecific shielding from the aromatic ring currents of this nitrophenyl ring. Thus, the shielding effects of the 1-(2-nitrophenyl)ethyl group also reveal its position in the molecule albeit less directly than by the pH analysis described above (Figure 3; Table I). Taken together, these ^1H NMR analyses serve to identify the P-5, P-4, and P-1 esters of $InsP_3$ that were isolated from the alkylation reaction mixture as in Figure 1B.

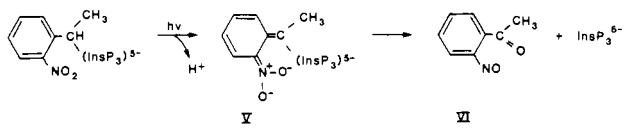
Photochemical Properties. Near-UV irradiation of 1-(2-nitrophenyl)ethyl phosphate esters of $InsP_3$ at neutral pH

Table II: Photochemical Properties of Caged $InsP_3$[a]

	Q_p[b]	k[c] (s^{-1})
singly caged $InsP_3$		
P-1 isomer (I)	0.65	175
P-4 isomer (II)	0.65	225
P-5 isomer (III)	0.65	280
triply caged $InsP_3$ (IV)	0.6	245
caged ATP	0.63	90

[a] Measured under standard conditions at 21 °C: 140 mM KCl, 3 mM $MgCl_2$, 100 mM TES adjusted to pH 7.1 with KOH. [b] Q_p represents the quantum yield determined by steady-state photolysis of each compound mixed with caged ATP, followed by HPLC separation of caged compounds and products. The extent of conversion was normalized to 0.54, which is the Q_p value for caged P_i (Kaplan et al., 1978). [c] k is the first-order rate constant obtained by least-squares analysis of the *aci*-nitro decay reaction monitored at 380 nm. k values decreased 10-fold at pH 8.1; 3 mM $MgCl_2$ reduced the k value by 2-fold for caged ATP photolysis (Walker et al., 1988) but had no effect on caged $InsP_3$ photolysis.

initiates a photochemical reaction that produces a proton, 2-nitrosoacetophenone, and $InsP_3$.



For biological studies the important photochemical parameters are the rate of $InsP_3$ formation and the caged $InsP_3$ quantum yield, Q_p (defined as the ratio of $InsP_3$ molecules formed to photons absorbed). The rate constant for the rate-limiting step of the above reaction was determined by monitoring two processes spectrophotometrically: decay of the *aci*-nitro intermediate, V, and formation of 2-nitrosoacetophenone (VI), both of which occur concomitantly with $InsP_3$ release (see Discussion). The *aci*-nitro species is detected when photolysis is monitored at 380 nm (Figure 4A), while 2-nitrosoacetophenone is detected at 740 nm (Walker et al., 1986, 1988). It should be noted that the small extinction coefficient of the 740 nm signal (ϵ_{740nm} = 50 M^{-1} cm^{-1}) makes the rate constant associated with 2-nitrosoacetophenone formation more difficult to measure accurately than that associated with the *aci*-nitro signal. However, both signals are single exponentials and give similar values for rate constants of caged $InsP_3$ photolysis. The P-5 isomer displayed the highest rate constant, 280 s^{-1} at pH 7.1, 0.2 M ionic strength, 21 °C, although all three caged $InsP_3$ isomers photolyzed at least 2 times faster than caged ATP (Table II). Part of this difference is probably due to the presence of Mg^{2+}, which binds to caged ATP and slows its photolysis kinetics but has no apparent effect on caged $InsP_3$ photolysis at physiological concentrations. Rate constants for caged $InsP_3$ photolysis were markedly pH dependent, increasing linearly with $[H^+]$ over the pH range 6–9, as has been observed for caged ATP photolysis (McCray et al., 1980).

The quantum yield for $InsP_3$ release, Q_p, was determined by comparing the amount of $InsP_3$ formed to the amount of ATP formed when caged $InsP_3$ and caged ATP were mixed together in aqueous buffer and exposed to continuous near-UV light for up to 4 min (Figure 4B). Q_p values are calculated from the slopes of semilogarithm plots by normalizing to the value of 0.54 for caged P_i, which has been determined independently by use of a chemical actinometer (Kaplan et al., 1978). Q_p values for the three isomers of caged $InsP_3$ determined by this method were 0.65 (Table II), similar to the value for caged ATP (0.63). Q_p values were independent of pH over the range pH 6–9. Within experimental accuracy (~10%), conversion of caged $InsP_3$ to $InsP_3$ and caged ATP

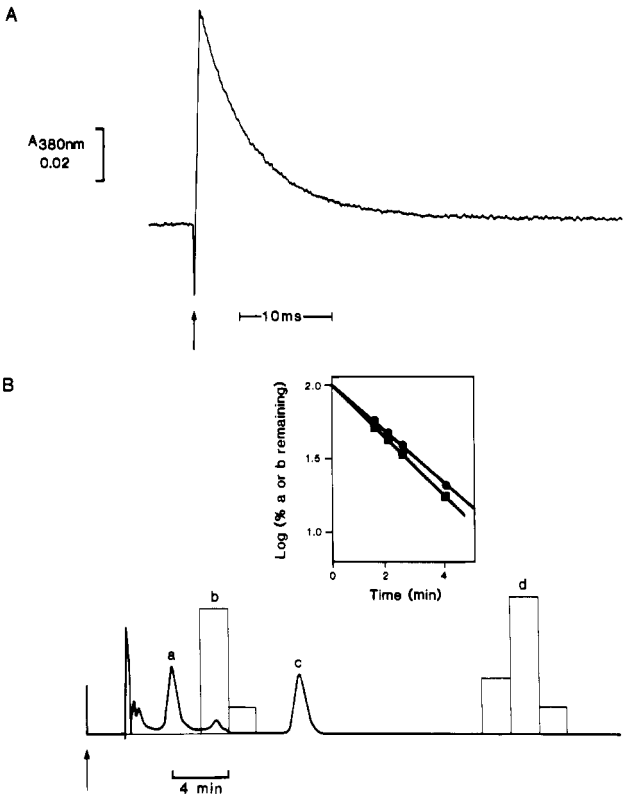


FIGURE 4: (A) Kinetics of P-1 caged $InsP_3$ photolysis. A 50-mJ laser pulse at 320 nm is directed onto a solution containing 1 mM P-1 caged $InsP_3$, 1.5 mM $MgCl_2$, 140 mM KCl, and 100 mM TES adjusted to pH 7.1 with KOH, 21 °C. The reaction was monitored orthogonal to the laser pulse with an absorption spectrophotometer at 380 nm. The arrow marks the time of the laser pulse. Rate constants were derived by least-squares analysis. (B) HPLC trace (solid continuous line, absorbance at 254 nm; bars, tritium) showing the separation of $InsP_3$ (d), P-4 caged $InsP_3$ (b), ATP (c), and caged ATP (a), for quantum yield measurements. Mixtures of 100 μM caged ATP and 100 μM caged $InsP_3$ were photolyzed, and then aliquots were chromatographed by HPLC on a partisil 10 SAX column eluted isocratically with 0.3 M ammonium phosphate, pH 5.4:methanol (19:1 v/v). (Inset) Semilogarithm plot of the time course of steady-state photolysis of caged $InsP_3$ (■) and caged ATP (●). Q_p was derived from the slope after normalization to Q_p = 0.54 for caged P_i. Error limits were estimated to be ±12% and are due principally to the ±10% estimated error in the Q_p value for caged P_i (Walker et al., 1988).

to ATP also occurred to the same extent in skinned muscle fibers following a single 50-ns laser pulse at 347 nm (Goldman et al., 1986; Walker et al., 1987).

Biological Properties. The physiological response of a permeabilized smooth muscle tissue to $InsP_3$ is shown in Figure 5. Tension development, which depends on preloading the SR with Ca^{2+} and is abolished by disruption of the SR with Triton X-100 (Somlyo et al., 1985), was used as an indirect measure of SR Ca^{2+} release. At a concentration of 10 μM, P-1 caged $InsP_3$ induced tension responses that were roughly equivalent to full $InsP_3$ responses (Figure 5A). In contrast, a mixture of P-4 and P-5 caged $InsP_3$ had no effect on tension at concentrations up to at least 50 μM (Figure 5B), and 50 μM P-5 caged $InsP_3$ alone also did not induce a tension response (Figure 5C). Finally, neither P-4 nor P-5 caged $InsP_3$ antagonized the $InsP_3$-induced Ca^{2+} release as found by testing mixtures of the caged compounds with 1–10 μM $InsP_3$ (data not shown). Similar results concerning the Ca^{2+} releasing activity of P-1, P-4, and P-5 caged $InsP_3$ were obtained with tension measurements in a guinea pig portal vein smooth muscle preparation (Somlyo et al., 1988) and with electrophysiological measurements in hepatocytes (Capiod et al., 1988).

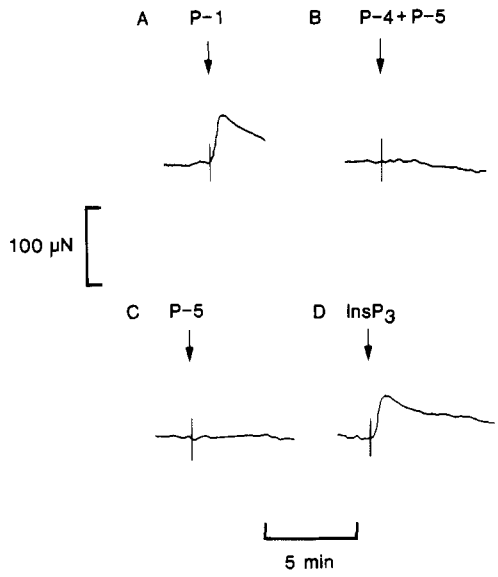


FIGURE 5: Effects of caged $InsP_3$ isomers on Ca^{2+} release measured by tension development in permeabilized vascular smooth muscle bundles. (A) 10 μM P-1 caged $InsP_3$; (B) 50 μM caged $InsP_3$ containing equal amounts of P-4 and P-5 isomers; (C) 50 μM P-5 caged $InsP_3$; (D) 30 μM $InsP_3$. At the arrows, muscle bundles suspended on a force transducer were transfered into 45-μL troughs containing caged $InsP_3$ in smooth muscle buffer A (see Materials and Methods) at 22 °C.

The time courses of enzymatic hydrolysis of $InsP_3$ and P-1, P-4, and P-5 caged $InsP_3$ are illustrated in Figure 6A. This phosphatase activity was isolated from homogenates of rabbit main pulmonary artery and characterized to determine its specificity. The enzyme is Mg^{2+} dependent, has a K_m for $InsP_3$ of 20 μM, and is inhibited by millimolar concentrations of 2,3-diphosphoglycerate. The only product of $InsP_3$ hydrolysis is the 1,4-isomer of *myo*-inositol bisphosphate, and this $InsP_2$ is not further hydrolyzed. Thus, although this is not a pure preparation of $InsP_3$ 5-phosphatase, the activity is specific for the 5-phosphate of $InsP_3$ and displays many of the properties of $InsP_3$ 5-phosphatases from other tissues (Downes et al., 1982). As shown in Figure 6A, the P-1 ester of $InsP_3$ was hydrolyzed about 4-fold more slowly than $InsP_3$, while the P-4 and P-5 esters were not detectably hydrolyzed. Competition studies showed that P-4 and P-5 caged $InsP_3$ did not decrease the rate of $InsP_3$ hydrolysis at concentrations up to 50 μM. Thus, P-4 and P-5 caged $InsP_3$ do not seem to interact with $InsP_3$ 5-phosphatase as substrates or inhibitors. P-4 and P-5 caged $InsP_3$ were similarly resistant to hydrolysis and noninhibitory toward the $InsP_3$ 5-phosphatase activity associated with permeabilized vascular smooth muscle strips (Walker et al., 1987).

A large-scale treatment of P-1 caged $InsP_3$ with the phosphatase allowed 0.3 μmol of the only hydrolysis product to be isolated and characterized (see Materials and Methods). The product retained the 1-(2-nitrophenyl)ethyl group and photolyzed to $InsP_2$. Thus, the enzyme activity selectively removed the 5-phosphate of P-1 caged $InsP_3$.

The time course of conversion of 10 μM $InsP_3$ to $InsP_4$ by a soluble extract of bovine brain is shown in Figure 6B. This enzyme activity is stimulated by Ca^{2+}–calmodulin, has a K_m for $InsP_3$ of approximately 1 μM, and specifically phosphorylates the 3-position of $InsP_3$ as described by others (Irvine et al., 1986; Ryu et al., 1987). Although each isomer of caged $InsP_3$ has a free 3-hydroxyl group, none was detectably phosphorylated to an $InsP_4$ ester by this enzyme activity (Figure 6B). Furthermore, P-1 and P-4 caged $InsP_3$ at concentrations up to 50 μM did not inhibit the initial rate of

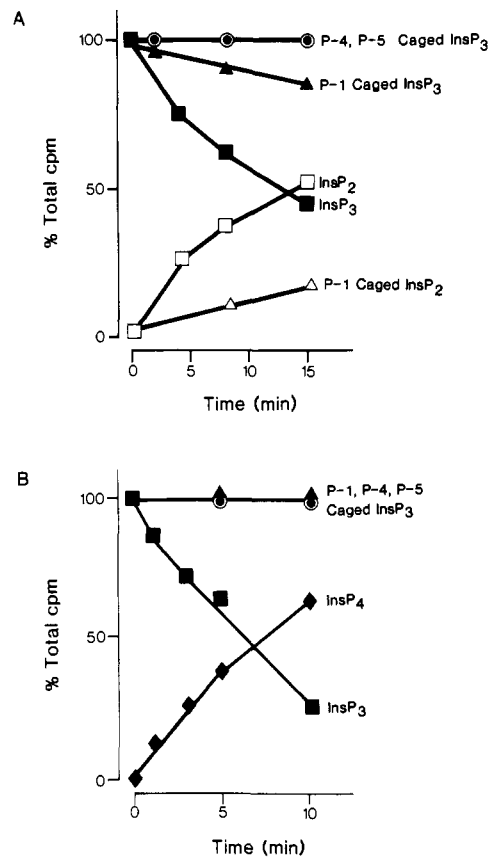


FIGURE 6: (A) Time courses of $InsP_3$ 5-phosphatase hydrolysis of $InsP_3$ (■), P-1 caged $InsP_3$ (▲), P-4 caged $InsP_3$ (○), and P-5 caged $InsP_3$ (●), each at an initial concentration of 100 μM in smooth muscle buffer A (see Materials and Methods) at 22 °C. The product of $InsP_3$ hydrolysis was $InsP_2$ (□) and of P-1 caged $InsP_3$ was P-1 caged $InsP_2$ (△). (B) Effects of $InsP_3$ 3-kinase on P-1 (▲), P-4 (○), and P-5 caged $InsP_3$ (●), each at a concentration of 40 μM in kinase assay buffer (Materials and Methods) at 22 °C. Conversion of 10 μM $InsP_3$ (■) to $InsP_4$ (◆) by the kinase preparation served as a control. Progress of both enzymatic reactions was monitored by HPLC with each injection containing 7×10^3 dpm of tritium (counting efficiency = 0.32). The detection limit was approximately 300 dpm, equivalent to 5% hydrolysis or phosphorylation of caged $InsP_3$. Data points for P-1, P-4, and P-5 caged $InsP_3$ overlap and do not deviate from 100% total cpm. Standard deviations for duplicate samples were ±6%.

conversion of 10 μM $InsP_3$ to $InsP_4$. Under the same conditions, the P-5 ester inhibited the initial rate of $InsP_4$ formation by 2-fold at a concentration of 10 μM.

Discussion

By use of a convenient method developed for esterifying weakly ionizing phosphate residues in nucleotides (Walker et al., 1988), $InsP_3$ was esterified on each one of its three phosphate residues with a photolabile 1-(2-nitrophenyl)ethyl group. The resulting P-1, P-4, and P-5 caged $InsP_3$ isomers were separated, and their structures were elucidated by high-field ^1H NMR measurements. The nonexchangeable inositol ring protons of $InsP_3$ were influenced by the ionization state of its phosphate residues so that, at an appropriate pH, all six protons could be resolved and assigned. The characteristic pH dependence of the $InsP_3$ spectra also provided a general means for identifying which phosphate residue in the three isomers of caged $InsP_3$ was esterified. Our NMR results are fully consistent with the interpretation of Lindon et al. (1986) that $InsP_3$ assumes a chair conformation in aqueous solution where the 3- and 6-hydroxyls and the 1-, 4-, and 5-phosphates are all equatorial and the 2-hydroxyl is axial. The P-1, P-4, and P-5 phosphate esters of $InsP_3$ exist in so-

lution in similar chair conformations. The observed differences in biological properties are not likely to result from unfavorable ring conformations but are probably the result of introducing a bulky ester group and masking a negative charge on one of the phosphate residues.

Esterification of the 1-phosphate group of $InsP_3$ with the 1-(2-nitrophenyl)ethyl group had only minor effects on its biological activity. This is consistent with results obtained with P-1 glyceryl-$InsP_3$, which, like $InsP_3$, induced Ca^{2+} release in insulinoma cells (Williamson et al., 1985) and was a substrate for the $InsP_3$ 5-phosphatase of human erythrocytes (Downes et al., 1982). Even removal of the 1-phosphate did not greatly reduce the Ca^{2+} mobilizing activity of $InsP_3$ in permeabilized Swiss 3T3 cells, whereas its activity was lost on removal of the 4- or 5-phosphate groups (Irvine et al., 1984). Our observation that esterification of either the 4- or 5-phosphate residues decreased Ca^{2+} releasing potency by at least 100-fold is consistent with the idea that vicinal 4,5-phosphate groups are major structural determinants of the Ca^{2+} mobilizing activity of inositol phosphates. Modification of the 4- or 5-phosphate also produces analogues that are resistant to hydrolysis by $InsP_3$ 5-phosphatase and are not substrates for $InsP_3$ 3-kinase. The P-5 1-(2-nitrophenyl)ethyl ester of $InsP_3$ is, however, an inhibitor of the soluble $InsP_3$ 3-kinase of bovine brain, although it is not yet clear whether inhibition is due to one or both diastereoisomers. Esterification of the 1-phosphate residue could be exploited in preparing fluorescent-labeled $InsP_3$ or photoaffinity labels (Hirata et al., 1985) that would bind to $InsP_3$ receptors and $InsP_3$ 5-phosphatases.

The efficiency of conversion of caged $InsP_3$ to $InsP_3$ is related to the product quantum yield, Q_p. The fraction converted on pulse photolysis depends on many factors, including the incident photon intensity, wavelength, and duration of the flash, and values can be obtained that are less than (Walker et al., 1987) or greater than Q_p (Ferenczi et al., 1984). Thus, in physiological experiments with caged $InsP_3$, it is necessary to determine empirically the extent of conversion, for instance, by HPLC analysis of samples photolyzed under the experimental conditions being used. The relationship between the Q_p values measured here and the percentage conversion in a single laser pulse is discussed elsewhere (Walker et al., 1988).

The rate of $InsP_3$ release on photolysis of caged $InsP_3$ is difficult to measure directly because an assay specific for $InsP_3$ and offering a millisecond time resolution is not available. The rate of caged $InsP_3$ photolysis was therefore measured indirectly by monitoring the formation and decay of an *aci*-nitro intermediate at 380 nm and the formation of the main photolytic byproduct, 2-nitrosoacetophenone, at 740 nm. The interpretation of these spectral signals and their relationship to $InsP_3$ release is based on an analysis of the mechanism of caged ATP photolysis (Walker et al., 1988). Caged ATP photolyzes in two kinetically resolvable steps. In the first, photon absorption followed by release of a proton (monitored by using a pH indicator) and formation of the presumed *aci*-nitro intermediate occur within 10 μs. In the second, the *aci*-nitro intermediate decays concomitantly with release of ATP (monitored by actomyosin dissociation) and formation of 2-nitrosoacetophenone. $InsP_3$ is likely to be released from caged $InsP_3$ by a similar mechanism, and so the analogous signals at 380 and 740 nm are used to evaluate caged $InsP_3$ photolysis. According to this analysis, laser pulse photolysis releases $InsP_3$ from all three isomers of caged $InsP_3$ with $t_{1/2}$ values ranging from 2 to 4 ms under conditions that are close to physiological (1.5 mM free Mg^{2+}, pH 7.1, 0.2 M ionic strength, 21 °C).

One possible drawback in the use of caged $InsP_3$ in physiological experiments is that the photolytic byproducts, a proton and 2-nitrosoacetophenone, might either induce a response or cause damage to the preparation. The most convincing control experiments depend on the use of caged $InsP_2$, which on photolysis releases the same byproducts as caged $InsP_3$ but generates the biologically inactive inositol phosphate $InsP_2$. This also controls against the possibility of laser flash artifacts. The demonstration of reproducible biological responses to caged $InsP_3$ photolysis in the same preparation is also a strong indication that the preparation is not being damaged during the experiment. The potentially deleterious effects of 2-nitrosoacetophenone can be greatly reduced or eliminated by including a thiol in the photolysis solutions (Kaplan et al., 1978; Goldman et al., 1984; Walker et al., 1988).

The P-4 and P-5 1-(2-nitrophenyl)ethyl esters of $InsP_3$, by virtue of their advantageous photochemical and biological properties, represent useful new tools for initiating $InsP_3$ responses in cellular and other organized biological preparations with a time resolution of a few milliseconds. The compounds are water soluble and do not readily pass through biological membranes. This property restricts the compounds to aqueous compartments within cells and is likely to prevent their concentration within membranes or at membrane surfaces. Thus far, these compounds have provided time-resolved measurements of muscle contraction in vascular smooth and skeletal muscle preparations (Walker et al., 1987), the elevation of a fertilization envelope in sea urchin eggs (C. Petcelt, M. Hafner, and J. W. Walker, unpublished observations), and activation of K^+ channels in hepatocytes (Capiod et al., 1988). For introducing caged $InsP_3$ into cell interiors, these investigations have relied on saponin permeabilization of cells, microinjection, and internal perfusion from a patch-clamp pipette, respectively. Besides its use as a kinetic tool, caged $InsP_3$ holds promise in future investigations that could benefit from having control over when (such as during different phases of a cell cycle) and where $InsP_3$ is released within a cell.

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Registry No. I, 119147-19-2; II, 119147-20-5; III, 119147-21-6; IV, 119147-22-7; VI, 25798-61-2; $InsP_3$, 85166-31-0; $InsP_3$ 3-kinase, 106283-10-7; $InsP_3$ 5-phosphatase, 106283-14-1; Ca, 7440-70-2; 1-(2-nitrophenyl)diazoethane, 114119-94-7; 2-nitroacetophenone hydrazone, 116271-34-2.

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Mechanism of Action of *Escherichia coli* Exonuclease III†

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ABSTRACT: Exonuclease III is the major apurinic/apyrimidinic (AP) endonuclease of *Escherichia coli*, accounting for more than 80% of the total cellular AP endonuclease activity. We have shown earlier that the endonucleolytic activity of exonuclease III is able to hydrolyze the phosphodiester bond 5′ to the urea *N*-glycoside in a duplex DNA [Kow, Y. W., & Wallace, S. S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8354-8358]. Therefore, we were interested in studying the mechanism of action of the endonucleolytic activity of exonuclease III by preparing DNA containing different base lesions as well as chemically modified AP sites. When AP sites were converted to *O*-alkylhydroxylamine residues, exonuclease III was able to hydrolyze the phosphodiester bond 5′ to *O*-alkylhydroxylamine residues. The apparent K_m for different *O*-alkylhydroxylamine residues was not affected by the particular *O*-alkylhydroxylamine residue substituted; however, the apparent V_{max} decreased as the size of the residue increased. On the basis of a study of the substrate specificity of exonuclease III, a modification of the Weiss model for the mechanism of action of exonuclease III is presented. Furthermore, a temperature study of exonucleolytic activity of exonuclease III in the presence of Mg^{2+} showed discontinuity in the Arrhenius plot. However, no discontinuity was observed when the reaction was performed in the presence of Ca^{2+}. Similarly, no discontinuity was observed for the endonucleolytic activity of exonuclease III, in the presence of either Ca^{2+} or Mg^{2+}. These data suggest that, in the presence of Mg^{2+}, exonuclease III exists in two temperature-dependent conformations. The transition temperature for the conformation change occurred at 25 °C, and in the presence of Ca^{2+}, only one rate-limiting step was observed.

Apurinic/apyrimidinic (AP) sites are common intermediates in the repair of most DNA base damages (Loeb & Preston, 1986), and they are readily generated by a number of chemical and physical agents (Loeb & Preston, 1986; Teoule, 1987). Due to the instability of the N-glycosylic bond, depurination can occur at a significant rate under physiological conditions (Lindahl & Nyberg, 1972; Loeb & Preston, 1986). AP sites have been shown to be in vitro blocks to DNA synthesis

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