Kinetics of Cytosolic Ca^{2+} Concentration after Photolytic Release of 1-D-*myo*-Inositol 1,4-Bisphosphate 5-Phosphorothioate from a Caged Derivative in Guinea Pig Hepatocytes

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ABSTRACT The influence of 1-D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$) breakdown by $InsP_3$ 5-phosphatase in determining the time course of Ca^{2+} release from intracellular stores was investigated with flash photolytic release of a stable $InsP_3$ derivative, 5-thio-$InsP_3$, from a photolabile caged precursor. The potency and Ca^{2+}-releasing properties of the biologically active D isomers of 5-thio-$InsP_3$ and $InsP_3$ itself were compared by photolytic release in guinea pig hepatocytes. After a light flash, cytosolic free calcium concentration ($[Ca^{2+}]_i$) showed an initial delay before rising quickly to a peak and declining more slowly to resting levels, with time course and amplitude generally similar to those seen with photolytic release of $InsP_3$. Differences were a three- to eightfold lower potency of 5-thio-$InsP_3$ in producing Ca^{2+} release, much longer delays between photolytic release and Ca^{2+} efflux with low concentrations of 5-thio-$InsP_3$ than with $InsP_3$, and persistent reactivation of Ca^{2+} release, producing periodic fluctuations of cytosolic $[Ca^{2+}]_i$ with high concentrations of 5-thio-$InsP_3$ but not $InsP_3$ itself. The lower potency of 5-thio-$InsP_3$ may be a result of a lower affinity for closed receptor/channels or a lower open probability of liganded receptor/channels. The longer delays with 5-thio-$InsP_3$ at low concentration suggest that metabolism of $InsP_3$ by 5-phosphatase may reduce the concentration sufficiently to prevent receptor activation and may have a similar effect on $InsP_3$ concentration during hormonal activation. The maximal rate of rise of $[Ca^{2+}]_i$, the duration of the period of high Ca^{2+} efflux, and the initial decline of $[Ca^{2+}]_i$ are similar with 5-thio-$InsP_3$ and $InsP_3$, indicating that $InsP_3$ breakdown is not important in terminating Ca^{2+} release. The second activation of $InsP_3$ receptors with 5-thio-$InsP_3$ and particularly the sustained periodic fluctuations of $[Ca^{2+}]_i$ indicate persistence of 5-thio-$InsP_3$, suggesting that $InsP_3$ breakdown prevents reactivation of $InsP_3$ receptors. The photochemical properties of 1-(2-nitrophenyl)-ethyl caged 5-thio-$InsP_3$ are photolytic quantum yield = 0.57 (cf. 0.65 for caged $InsP_3$) and rate of photolysis = 87 s^{-1} (half-life approximately 8 ms; cf. 3 ms for caged $InsP_3$; pH7.1; ionic strength, 0.2 M; 21°C). Caged 5-thio-$InsP_3$ at concentrations up to 360 μM did not activate $InsP_3$ receptors to produce Ca^{2+} release or block Ca^{2+} release by free 5-thio-$InsP_3$.

INTRODUCTION

The role of 1-D-*myo*-inositol 1,4,5 trisphosphate ($InsP_3$) as a second messenger releasing Ca^{2+} from intracellular stores during hormonal activation of liver and other nonexcitable cells is well established (Burgess et al., 1984; see Berridge, 1993, for a review), and the pattern of Ca^{2+} release during hormone action shows evidence of complex regulation such as well defined periodic fluctuations of $[Ca^{2+}]_i$ (Woods et al., 1986; Field and Jenkinson, 1987). Flash photolysis of caged $InsP_3$ to release a pulse of $InsP_3$ in the cytosol has been used to investigate the role of $InsP_3$ receptor kinetics in the generation of Ca^{2+} transients in guinea pig hepatocytes and has shown the presence of cooperative and autoinhibitory steps in $InsP_3$ action on Ca^{2+} stores (Ogden et al., 1990). The role of metabolic breakdown of $InsP_3$ in the kinetics of Ca^{2+} mobilization is uncertain, and stable $InsP_3$ analogues resistant to $InsP_3$ 5-phosphatase, such as 1-DL-*myo*-inositol 1,4-bisphosphate 5-phosphorothioate (DL-5-thio-$InsP_3$) (Safrany et al., 1991), have been developed to show the effects of maintained stimulation (Berridge and Potter, 1990; Payne and Potter, 1991). The $InsP_3$ receptor, however, is inactivated rapidly after activation (Finch et al., 1991) resulting in distortion of the kinetics of Ca^{2+} release unless the ligand is applied as a pulse. For this reason a photolabile caged 5-thio-$InsP_3$ (the *S*-1-(2-nitrophenyl)ethyl ester of 5-thio-$InsP_3$; Fig. 1) has been developed to permit rapid release of 5-thio-$InsP_3$ adjacent to receptors in flash photolytic experiments. The biologically active D isomer of 1-D-*myo*-inositol 1,4-bisphosphate 5-phosphorothioate (5-thio-$InsP_3$) has been used in the experiments described here.

The photochemical properties of caged 5-thio-$InsP_3$ and the properties of photolytically released 5-thio-$InsP_3$ in releasing Ca^{2+} from stores are described here in a well defined system, the guinea pig hepatocyte. Isolated hepatocytes are readily loaded with caged 5-thio-$InsP_3$ and fluorescent Ca^{2+} indicators during whole-cell patch clamp. The responses to hormonal activation and to photolytically released $InsP_3$ are well characterized. When experiments are made in Cl^--free solution, guinea pig hepatocytes have predominantly a non-desensitizing K^+ conductance activated by increases of cytosolic $[Ca^{2+}]$ above resting levels. This conductance serves as an endogenous Ca^{2+} indicator that complements the use of fluorescent Ca^{2+} probes (Capiod and Ogden, 1989b).

$InsP_3$ generated by hormonal stimulation releases Ca^{2+} from stores during glycogenolysis and induces KCl secretion via Ca^{2+} activation of K^+ and Cl^- ion conductances in the

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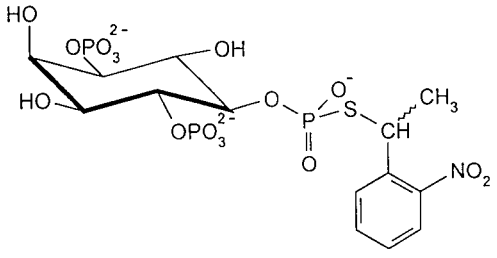

FIGURE 1 Structure of caged 5-thio-$InsP_3$.

surface membrane. Hormonally evoked Ca^{2+} release shows periodic spiking, and the role of $InsP_3$ metabolism in generating the Ca^{2+} spike characteristics may be elucidated by studying the time course of Ca^{2+} release by photolytically released 5-thio-$InsP_3$ in comparison with $InsP_3$.

MATERIALS AND METHODS

Materials

Chemicals were Analar grade from BDH or Sigma Chemical Co. (St. Louis, MO). Furaptra K-salt (Magfura-2) was from Molecular Probes (Eugene, OR). Anhydrous tetrahydrofuran was prepared by distillation from sodium benzophenone. Liquid ammonia was collected under nitrogen after distillation from sodamide (prepared in situ from sodium and ferric nitrate). 1-D-2,3,6-Tri-*O*-benzyl-*myo*-inositol 1,4-bis-(dibenzylphosphate)-5-dibenzylphosphorothioate was synthesized as described elsewhere (Desai et al., 1994). 1-(2-Nitrophenyl)ethyl bromide and 1-(2-nitrophenyl)ethyl phosphate were prepared as previously described (Corrie et al., 1992a,b). Triethylammonium bicarbonate (TEAB) buffer was prepared by bubbling CO_2 into a 1 M solution of redistilled triethylamine in water at 4°C until the pH stabilized at approximately 7.4. Phosphate buffers were made by mixing appropriate ratios of salts (e.g., K_2HPO_4 and KH_2PO_4). ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM 500 spectrometer in D_2O at pH 5.5–6.0 and referenced to internal acetone (δ 2.225). Triethylammonium salts were exchanged with Dowex 50 (Na form) before obtaining spectra.

Spectral assignments were based on a combination of single frequency decoupling experiments and interpolation from published spectra of $InsP_3$ and caged $InsP_3$ (Walker et al., 1989). ^{31}P NMR spectra were run with proton decoupling on a Bruker WM 200 spectrometer and referenced to external 85% H_3PO_4. Column fractions in TEAB buffer were augmented with D_2O (20% v/v) to provide a lock signal.

Synthesis of 5-thio-$InsP_3$ and caged 5-thio-$InsP_3$

Anhydrous ammonia (approximately 20 ml) was distilled into a solution of 1-D-2,3,6-tri-*O*-benzyl-*myo*-inositol 1,4-bis(dibenzylphosphate)-5-dibenzylphosphorothioate (125 mg, 0.1 mmol) under nitrogen in dry tetrahydrofuran (4 ml) contained in a three-neck flask fitted with a dry ice condenser. The solution was stirred, and sodium (182 mg; freshly extruded wire) was added in portions over 5 min. After an additional 10 min, the blue color was discharged by addition of solid NH_4Cl, and the ammonia was allowed to evaporate under nitrogen. The residue was diluted with a solution of acetic acid (0.42 ml) in water (10 ml) and then adjusted to pH 7.3 with 1 M KOH and diluted with water to reduce the conductivity to 3200 μS. This solution was applied to a Whatman DE52 anion exchange column (1.5 × 16 cm), and the column was washed with 10 mM TEAB (95 ml) and then eluted with a linear gradient formed from 10 and 1000 mM TEAB (each 200 ml) at a flow rate of 21 ml h^{-1}. Fractions (10.5 ml) were collected and analyzed by ^{31}P NMR spectroscopy to locate 5-thio-$InsP_3$ (see below). Fractions 12–14 were pooled, rotary evaporated (1 mm Hg), and reevaporated three times with methanol to remove excess TEAB. The residue (73 mg; 70 μmol based on the hexakis(triethylammonium) salt) was dissolved in 20 mM potassium phosphate, pH 8.0 (4.0 ml), and adjusted to pH 7.7 (20 μl of 5.5 M KOH). A portion (2 ml) of this solution was stored at −20°C, and the remainder plus rinsings (approximately 0.5 ml) from the pH electrode was treated with 1 M dithiothreitol (10 μl) and adjusted to pH 7.9. A solution of 1-(2-nitrophenyl)ethyl bromide (2.75 ml of 100 mM in dimethylformamide) was added, and the mixture was stirred in the dark at room temperature for 17 h. The solution was partitioned with diethyl ether (4 × 6 ml), and the aqueous phase was adjusted to pH 7.5 by addition of 0.5 M KH_2PO_4 (0.2 ml) and chromatographed in four equal portions on a Whatman Partisphere SAX column (catalogue No. 4621–0505) with 0.12 M ammonium phosphate, pH 5.4, plus methanol (19:1, v/v) as mobile phase at 1.0 ml min^{-1}. The flow rate was increased to 2.0 ml min^{-1} after the breakthrough peak had eluted and the major peak was collected. The pooled peak fractions from the four purification runs were adjusted to pH 7.3 with KOH, diluted fourfold with water to reduce the ionic strength, and desalted by chromatography on a Whatman DE52 anion exchange column (see above), with a linear gradient of 10–1000 mM TEAB (each 250 ml) at a flow rate of 20 ml h^{-1}. Thirty-minute fractions were collected, and fractions 18 and 19 were pooled, rotary evaporated, and reevaporated (three times) with methanol. The residue was dissolved in 0.33 mM potassium phosphate (3 ml) and stored at −20°C. The concentration of caged 5-thio-$InsP_3$ (based on ϵ_{260} 4700 M^{-1} cm^{-1}) was 3.90 mM. Fraction 20 contained a smaller quantity of the product and was processed separately to give 2.5 ml of 2.76 mM solution. The overall yield was 37%. 5-Thio-$InsP_3$ had NMR spectral parameters as follows: δ_H 4.33 (1 H, q, $J_{3,4;4,5;\text{ and H, P}}$ 9.2, H-4), 4.29 (1 H, t, $J_{1,2;2,3}$ 2.7, H-2), 4.27 (1 H, m, H-5), 4.06 (1 H, d/t, $J_{1,2}$ 2.7, $J_{1,6\text{ and H, P}}$ 9.3, H-1), 3.95 (1 H, t, $J_{1,6;5,6}$ 9.4, H-6) and 3.76 (1 H, d/d, $J_{2,3}$ 2.8, $J_{3,4}$ 9.5, H-3); δ_P 45.5 (phosphorothioate), 4.8 (phosphate), and 3.2 (phosphate).

Caged 5-thio-$InsP_3$ had the following NMR spectrum in which most signals are split into pairs of equal intensity because of the presence of two diastereoisomers (designated A and B) that arise because of the racemic center in the cage moiety. When assignments to individual diastereoisomers could not be made unambiguously, signals are reported without assignment to either isomer: δ_H 7.88–7.90 (2 H, m, Ar-H3,6); 7.71 and 7.72 (1 H, 2 overlapped t/d, J_{ortho} 7.6, J_{meta} 1.2, Ar-H5), 7.44–7.49 (1 H, m, Ar-H4), 5.05–5.08 (1 H, m, ArCH), 4.23 and 4.26 (1 H, 2 × t, $J_{1,2;2,3}$ 2.8, H-$2_{B\text{ and }A}$), 4.13 and 4.21 (1 H, 2 × q, $J_{3,4;4,5;\text{ and H,P}}$, 8.8, H-$4_{B\text{ and }A}$), 3.91 and 4.06 (1 H, 2 × q, $J_{4,5;5,6;\text{ and H, P}}$ 9.6, H-$5_{B\text{ and }A}$), 3.86 and 3.93 (1 H, 2 × d/t, $J_{1,2}$ 2.9, $J_{1,6\text{ and H, P}}$ 9.0, H-1), 3.68 and 3.83 (1 H, 2 × t, H-6), 3.66 and 3.71 (1 H, 2 × d/d, H-$3_{B\text{ and }A}$), 1.74 (3 H, d, J 7, CH_3); δ_P 21.65 and 22.00 (P_5), 5.0 (P_1), 4.8, and 4.9 (P_4).

Photolysis kinetics and product quantum yield of caged 5-thio-$InsP_3$

The kinetics were analyzed in an absorption spectrophotometer coupled to a Candela Model 1050 dye laser as described by Walker et al. (1988). The solution for photolysis was prepared in internal solution (see above) with 0.2 mM caged 5-thio-$InsP_3$ instead of 0.5 mM furaptra. The experiments were performed at 20°C, and the rate of decay of the single exponential signal at 406 nm (which has previously been shown to monitor the rate-determining step in photorelease of caged ATP (Walker et al., 1988)) was 87 s^{-1}.

For determination of the product quantum yield (Q_p) a solution containing 0.3 mM 1-(2-nitrophenyl)ethyl phosphate, 0.25 mM caged 5-thio-$InsP_3$, and 1 mM dithiothreitol in 0.1 M potassium phosphate, pH 7.0, was irradiated in a 4- × 4-mm quartz cell with light from a mercury arc lamp that passed through a Beckman 18A filter (290–400 nm) before illuminating the cell. The irradiated samples were analyzed by reverse phase HPLC (Merck Lichrocart RP8 column; catalogue No. 50832) with 20 mM potassium phosphate, pH 5.6, as mobile phase at a flow rate of 1.5 ml min^{-1}. Retention times were 0.95 and 6.6 min for the caged 5-thio-$InsP_3$ and caged phosphate, respectively. The extent of conversion of each compound was

calculated from the decrease in areas of the peaks from the chromatogram. By comparison of the relative conversion of the two compounds with the known Q_p of 0.54 for 1-(2-nitrophenyl)ethyl phosphate (Kaplan et al., 1978), the Q_p for caged 5-thio-$InsP_3$ was calculated to be 0.57.

Cell preparation and solutions

Guinea pig hepatocytes were isolated by perfusion with collagenase followed by mechanical dispersion (Capiod and Ogden, 1989a,b). Cells in suspension were plated onto 40-mm quartz coverslips precoated with collagen in Williams medium E and kept at 37°C in an atmosphere of CO_2 (5%) in air. Whole-cell recordings were made after 2–5 h. Experiments were done in Cl^--free solutions with gluconate ions as substitute. External solution contained (mM): sodium gluconate, 145; potassium gluconate, 5.6; $CaSO_4$, 5; $MgSO_4$, 1.2; Hepes, 8. Internal solution contained (mM): potassium gluconate, 153; ATP-Na_2, 3; $MgSO_4$, 3; Hepes, 8; furaptra, 0.5; and concentrations of caged $InsP_3$ or caged 5-thio-$InsP_3$ as required. All the solutions were buffered at pH 7.3 and 0.2-μm Millipore filtered. Experiments were made at room temperature (approximately 27°C).

Whole-cell recordings

Standard tight-seal whole-cell patch clamp recording techniques were used to voltage clamp and internally perfuse single hepatocytes (Hamill et al., 1981). Patch pipettes were made from pyrex glass with a microfilament insertion, and the tips were covered with a wax comprising parafilm (40%) and mineral oil (60%). Whole-cell currents at constant holding potential, usually 0 mV, were recorded on FM tape. Guinea pig hepatocytes have small leakage conductance before activation and no voltage-gated conductance. The Ca^{2+}-activated K^+ conductance appears as an outward current of 0.5–1.5 nA amplitude at 0 mV.

Fluorescence measurements and flash photolysis

Furaptra K^+ salt and the caged compounds were introduced into the cell by diffusion from the patch pipette, and 5–6 min were required for equilibration of the furaptra fluorescence signal. Microspectrofluorimetry was on a Nikon TMD microscope with 40× 1.3 NA C-fluor objective. Excitation light (400–440 nm) was from a xenon arc lamp, and light emitted from the patch-clamped cell was defined by a rectangular diaphragm and taken to a photon-counting photomultiplier via a 490-nm long-pass filter. Photon pulses were counted in a Tecmar interface with PTI software and in parallel were converted to an analog signal by a Cairn Research photon-counting module and recorded on FM tape.

Furaptra was used at single excitation and emission wavelengths centered at 420 and 510 nm. The fluorescence is almost completely quenched by high $[Ca^{2+}]$, permitting ready calibration of the fluorescence to $[Ca^{2+}]$ (Konishi et al., 1991; Ogden et al., 1995). Briefly, because of the complete quench of furaptra fluorescence by high $[Ca^{2+}]$, the intrinsic fluorescence of the cell was taken as the fluorescence at saturating free $[Ca^{2+}]$, F_{Camax}. The dissociation constant, K, for Ca^{2+}-furaptra was estimated at 21°C in cuvette measurements as 48 μM, with citrate buffers containing 0.5 mM free Mg^{2+} at the experimental ionic strength. The free $[Ca^{2+}]_i$ in unstimulated guinea pig hepatocytes has been estimated as 0.2 μM (Burgess et al., 1984), and therefore the fluorescence under resting conditions can be used as a good estimate of F_{Camin}, the fluorescence at 0 $[Ca^{2+}]$, once the pipette/cell concentrations have equilibrated in whole-cell recording. The free $[Ca^{2+}]$ during a response was calculated from fluorescence F with the relation

$$[Ca^{2+}]_i = K(F_{Camin} - F)/(F - F_{Camax}) \quad (1)$$

The Ca^{2+}-dependent K^+ conductance has a range of activation by Ca^{2+} ions of 0.3–1.5 μM and a maximal open probability of 0.9 (Capiod and Ogden, 1989b). It is not activated at $[Ca^{2+}]_i$ less than 0.3 μM and provides a good index of low free $[Ca^{2+}]_i$ in resting cells. Cells with any degree of activation of the K^+ conductance before $InsP_3$ stimulation were not used. The conductance was also used as an index of $[Ca^{2+}]_i$ during experiments.

Photolysis was produced by a 1-ms pulse from a xenon arc flashlamp (Rapp and Guth, 1988) focused through a UG11 filter (280–360 nm) from 4 cm above the cell to produce a 4–5 mm spot. Photolytic conversion of caged ATP in the microscope was measured by high pressure liquid chromatography as described previously (Ogden et al., 1990) and the value used to calculate the photolytic conversion of caged 5-thio-$InsP_3$ by multiplying with the ratio of the quantum yields of caged thio-$InsP_3$ to caged ATP, i.e., 0.57/0.63, determined under the same ionic conditions.

The output of the flashlamp was adapted for most experiments to be adjustable in the range producing 6–14% photolysis of caged ATP by adjusting the voltage on the capacitor bank. The coefficient of variation of photolysis of caged ATP determined in a series of experiments was 10%, indicating the degree of reproducibility of photolysis in each cell. The optical artifact arising from the UV pulse was minimized (4–8 ms) by use of quartz coverslips and UV block oil between the coverslip and objective (Carter and Ogden, 1992; Ogden et al., 1993).

RESULTS

It is important that caged 5-thio-$InsP_3$ itself does not release Ca^{2+} at concentrations needed to generate sufficient free 5-thio-$InsP_3$ to produce activation. To test this, the Ca^{2+} activated K^+ conductance was monitored immediately on establishing a whole-cell recording to see, first, whether the cell had an initial low resting $[Ca^{2+}]$ suitable for experimentation and, second, to detect activation of Ca^{2+} release as the caged 5-thio-$InsP_3$ diffused into the cytosol, before the Ca^{2+} indicator was present. Caged 5-thio-$InsP_3$ produced no increase in $[Ca^{2+}]_i$ as judged by failure to activate the K^+ conductance at concentrations tested up to 360 μM, i.e., approximately 200 times the lowest concentration of 5-thio-$InsP_3$ that produced an increase in $[Ca^{2+}]_i$ after photolysis (see below).

Once the fluorescent indicator was equilibrated in the cell, changes in $[Ca^{2+}]_i$ were monitored with both the fluorescent Ca^{2+} indicator furaptra (500 μM) and the Ca^{2+}-activated K^+ conductance as outward whole-cell current at 0 mV membrane potential. Responses to release of 1.1, 11, and 27 μM 5-thio-$InsP_3$ in three different cells are shown in Figs. 2 and 3, and responses to low (0.7 μM) and high (7 μM) concentrations of $InsP_3$ in Fig. 4. The changes in $[Ca^{2+}]_i$ and K^+ conductance shown in the traces in Figs. 2, 3, and 4 can be divided into three time components: (1) an initial delay between 5-thio-$InsP_3$ or $InsP_3$ release and the rise of $[Ca^{2+}]$, (2) a period of rapid efflux from stores producing a high $d[Ca^{2+}]/dt$ and fast conductance change, and (3) an abrupt change to a negative slope $d[Ca^{2+}]/dt$ as a result of net Ca^{2+} loss from the cytosol. The general characteristics of the response can be summarized with reference to Figs. 2, 3, and 4. At high concentrations of 5-thio-$InsP_3$ or $InsP_3$, the delay was shorter and maximal $d[Ca^{2+}]/dt$ faster than at low concentrations. At low (i.e., up to 3.2 μM) 5-thio-$InsP_3$ concentrations, the decline of $[Ca^{2+}]$ showed a large monotonic component and evidence of reactivation of Ca^{2+} release in all but 1 cell. At high concentrations, clear evidence of prolonged Ca^{2+} release followed an initial monotonic decline. With $InsP_3$, prolonged activation of Ca^{2+} release after the initial decline was less marked and seen only at high concentrations. The results with 5-thio-$InsP_3$ from 22 cells are summarized in Table 1. Data are given for the first flash only

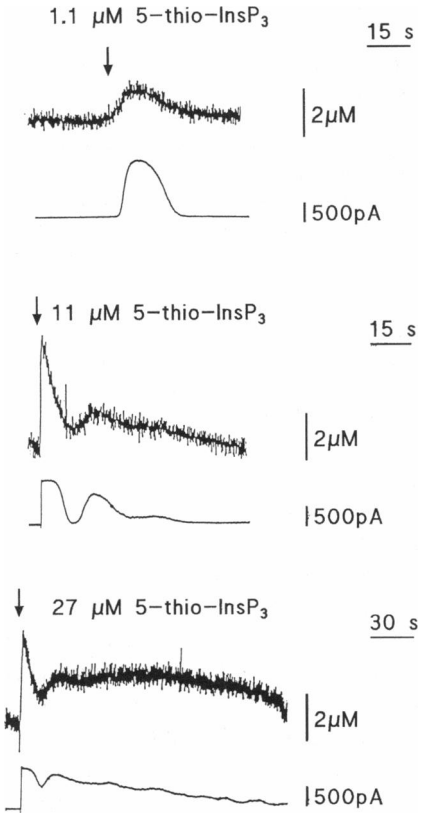

FIGURE 2 Flash photolysis of caged 5-thio-$InsP_3$ in guinea pig hepatocytes. Free $[Ca^{2+}]_i$ measured with furaptra fluorescence (upper traces) and whole-cell Ca^{2+}-activated K^+ current at 0 mV (lower traces) in three hepatocytes after photolysis releasing: (*A*) 1.1 μM, (*B*) 11 μM, and (*C*) 27 μM 5-thio-$InsP_3$ at the times indicated by the arrows.

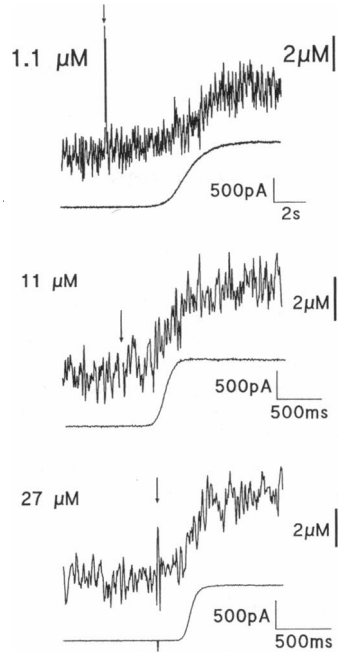

FIGURE 3 Expanded records illustrating the delay and initial rise of $[Ca^{2+}]$ after release of 1.1, 11, and 27 μM 5-thio-$InsP_3$ at the times indicated by the arrows. Data are as in Fig.2.

in each cell because of the evidence of accumulation of 5-thio-$InsP_3$ with subsequent flashes (see below).

The potency ratio of 5-thio-$InsP_3$ to $InsP_3$ is best estimated from these data as the lowest concentrations that produce detectable Ca^{2+} release. Two of six cells tested with 1.1 μM 5-thio-$InsP_3$ produced a small, slow rise of $[Ca^{2+}]_i$. $InsP_3$ released by photolysis to produce a cytosolic concentration of 0.4 μM reproducibly evoked a small increase of $[Ca^{2+}]_i$ in guinea pig hepatocytes (Ogden et al., 1990, and work in progress). Thus, the low concentration potency ratio of 5-thio-$InsP_3$ to $InsP_3$ is at least 1.1:0.4, i.e., at least three times less potent. Reproducible responses were obtained with 2.5 μM 5-thio-$InsP_3$, giving a sixfold lower potency than $InsP_3$.

The duration of the delay between photolytic release of 5-thio-$InsP_3$ and rise of the $[Ca^{2+}]$ or conductance traces decreased from a mean of 4 s at low concentration to a minimum of 30 ms at high concentration (mean, 67 ms; Table 1). The delay after photolytic release of $InsP_3$ decreases in duration from 0.5–1 s at low concentration (Ogden et al., 1990) to 20 ms at high concentration (Ogden et al., 1991). Thus, the delays with low concentrations of 5-thio-$InsP_3$ are much longer than those seen with low concentrations of $InsP_3$ but are reduced by a similar factor with release of high concentrations.

The maximal rate of rise of $[Ca^{2+}]_i$ in the cytosol is proportional to the Ca^{2+} efflux from stores and provides a measure of the activation of $InsP_3$ receptors, although it is subject to large cell-to-cell variability, possibly because of differences in $InsP_3$ receptor density/unit cytosolic volume. At low concentration of 5-thio-$InsP_3$, the maximal rate of rise was approximately 1–2 μM/s and increased to a mean of 8.4 μM/s at high concentration. Similar values, also with a wide range at each concentration in different cells, are found with low and high concentrations of $InsP_3$ (T. Capiod and D. C. Ogden, unpublished results). The duration of the period of high Ca^{2+} efflux, when $d[Ca^{2+}]_i/dt$ is maximal and relatively constant in each response, was 2 s at low concentration and 500 ms at high concentration. These represent the periods of high open probability of $InsP_3$ receptors and are similar to the values found with low and high concentrations of $InsP_3$ (T.Capiod and D. C. Ogden, unpublished results).

After the peak $[Ca^{2+}]$, which ranged from 0.8 to 5.5 μM in different cells, $[Ca^{2+}]$ declined initially monotonically with a half-time of 5.6 ± 3.6 s (mean ± SD; n = 9; mean rate, −0.28 ± 0.16 μM/s). In 19 of 22 cells the response showed a second elevation of $[Ca^{2+}]$, with a lower rate of rise than the initial response and occurring approximately 10 s later (Fig. 2). In only 1 cell tested, at low (1.1 μM Fig. 2) 5-thio-$InsP_3$, was a monotonic decline to initial baseline seen. This contrasts with data obtained with $InsP_3$ itself, which showed little evidence of prolonged or secondary activation except at high $InsP_3$ concentration. In 3 cells with 5-thio-$InsP_3$, repetitive fluctuations of $[Ca^{2+}]$ were seen. In the example shown in Fig. 5, a train of 13 spikes of $[Ca^{2+}]_i$ was initiated by release of 18 μM 5-thio-$InsP_3$. The period between spikes was 20 s, and the amplitude and rate of rise declined progressively. The decline of the K^+ conductance to zero between spikes indicates that the free $[Ca^{2+}]$ adjacent

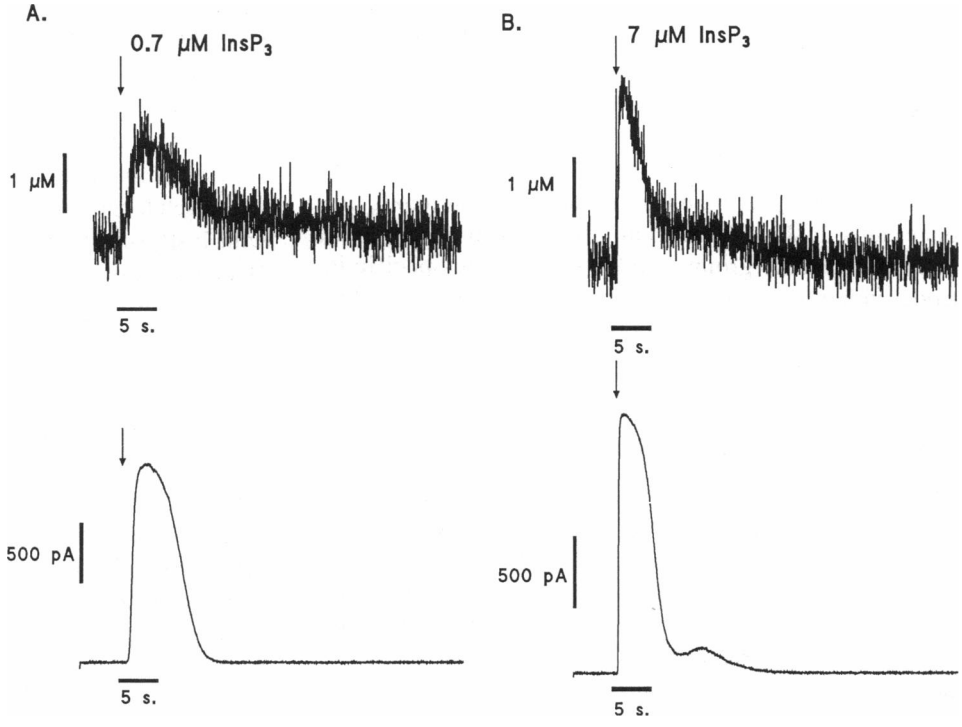

FIGURE 4 Flash photolysis of caged $InsP_3$ in guinea pig hepatocytes. Responses to release of (*A*) 0.7 μM $InsP_3$ and (*B*) 7 μM $InsP_3$ in different cells. Upper traces show free $[Ca^{2+}]$ measured with 500 μM furaptra, lower traces outward current a result of activation of K^+ conductance by Ca^{2+} at 0 mV in Cl^--free solution. Photolysis by 1-ms flash at the times indicated by the arrows.

to the membrane has fallen below 0.3 μM. The prolonged or repetitive activation suggests that 5-thio-$InsP_3$ persists in the cytosol.

Additional evidence of the stability of 5-thio-$InsP_3$ was from the response to a second flash in the same cell. In three experiments, 1.1 μM 5-thio-$InsP_3$ released by the first flash produced no response. When the cells were tested again with release of the same concentration of 5-thio-$InsP_3$, after 60 s or longer, in each case a response comprising an increase in $[Ca^{2+}]_i$ and K^+ conductance was seen with a mean delay of 5.2 s (range, 2.6–16 s), mean rate of rise of 1.0 μM/s (0.2–2 μM/s), and peak $[Ca^{2+}]$ of 2.4 μM (0.8–2.6 μM).

The possibility that caged 5-thio-$InsP_3$ might antagonize the actions of 5-thio-$InsP_3$ itself was tested by comparing results of experiments in which approximately equal concentrations of 5-thio-$InsP_3$ were released but in some cases with high (180 μM) and in others low (60 μM) concentrations of caged 5-thio-$InsP_3$ present. At 60 μM caged 5-thio-$InsP_3$, release of approximately 11 μM 5-thio-$InsP_3$ by a single flash produced a mean delay of 190 ms and $d[Ca^{2+}]_i/dt$ of 6.1 μM/s in three cells, compared with values of 150 ms and 7.6 μM/s in three cells with 10 μM released from a 180 μM cage. Thus, the presence of 170 μM caged 5-thio-$InsP_3$ produces little if any more block than 50 μM, suggesting that competition by caged 5-thio-$InsP_3$ for $InsP_3$ receptor sites is not significant.

TABLE 1 5-Thio-$InsP_3$ results

[5-Thio-$InsP_3$] (μM)	*n*	Delay (ms)	Maximal slope (μM/s)	$[Ca^{2+}]_i$ increase (μM)
1.1	2/6	2500, 5500	1.0, 1.7	2.0, 4.0
2.5 and 3.2*	5/5	890 ± 200	1.9 ± 0.1	2.0 ± 0.2
11	6/6	170 ± 40	6.9 ± 1.3	3.6 ± 0.6
18	2/2	180, 240	2, 13	2, 4.5
27	3/3	67 (30–130)	8.4 (7.2–9.1)	6.2 (4–9)

n, number of cells responding to first flash/number tried. Results are expressed as mean ± sem, mean (range), or individual values.
*Data obtained at these two concentrations were combined.

DISCUSSION

Synthesis of 5-thio-$InsP_3$ and caged 5-thio-$InsP_3$

The synthesis of racemic *myo*-inositol 1,4-bisphosphate 5-phosphorothioate has been described previously (Noble et al., 1992). In the present work, the isomer with the natural configuration has been prepared by using sodium-liquid ammonia to cleave nine benzyl groups from the fully protected

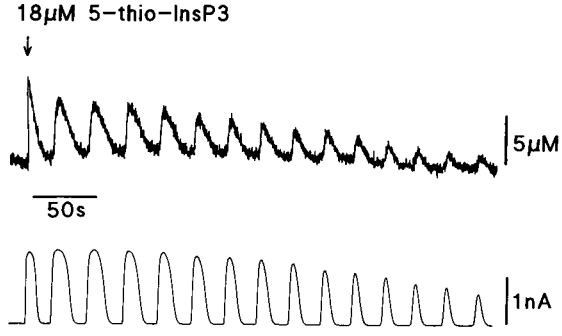

FIGURE 5 Sustained fluctuations of $[Ca^{2+}]$ (upper trace) and of Ca^{2+}-activated K^+ current (lower trace) at 0 mV membrane potential in a guinea pig hepatocyte after photolytic release of 18 μM 5-thio-$InsP_3$ at the time indicated by the arrow.

precursor 1-D-2,3,6-tri-*O*-benzyl-*myo*-inositol 1,4-bis-(dibenzylphosphate)-5-dibenzylphosphorothioate (Desai et al., 1994). Analogous cleavage of fully benzylated precursors has been reported in synthesis of racemic 1,4,5-*myo*-inositol trisphosphorothioate and 4,5-bisphosphate-1-phosphorothioate (Dreef et al., 1991). In contrast to the complex caging/partial uncaging strategy required to produce caged $InsP_3$ (Walker et al., 1989), the presence of the phosphorothioate group enables simple, site-specific introduction of the caging moiety, as the reaction of 5-thio-$InsP_3$ with 1-(2-nitrophenyl)ethyl bromide occurs only at the sulfur atom (cf. Corrie et al., 1992a).

Potency of 5-thio-$InsP_3$ relative to $InsP_3$

The potency of 5-thio-$InsP_3$ relative to $InsP_3$ is given here as the ratio of concentrations that produce a detectable increase of $[Ca^{2+}]$ when compared in several cells. A more rigorous measure would be the concentrations required to produce a Ca^{2+} efflux rate, measured by peak $d[Ca^{2+}]/dt$, of 50% of the maximum recorded at high $[InsP_3]$. This measure is impractical in the present experiments because of cell-to-cell variability in $d[Ca^{2+}]/dt$, a result of factors such as differing densities/unit volume of $InsP_3$ receptors, and because the accumulation of 5-thio-$InsP_3$ from one flash to the next prevents the two-flash experiments that are possible with more labile $InsP_3$. The concentrations of the 1-D isomer of 5-thio-$InsP_3$ required to produce a small increase of $[Ca^{2+}]_i$ when released by flash photolysis were 1.1–2.5 μM, three to six times higher (i.e., lower potency) than the 1-D isomer of $InsP_3$ (0.4 μM; Ogden et al., 1990), which produced small responses in the same conditions. In a parallel series of experiments to those reported here, flash photolytic release of 5-thio-$InsP_3$ was used to test whether $InsP_3$ metabolism was reponsible for the low potency of $InsP_3$ observed in cerebellar Purkinje cells as compared with hepatocytes (see Khodakhah and Ogden, 1993). The low concentration potency of 5-thio-$InsP_3$ relative to $InsP_3$ was the same, requiring 70 and 9 μM, respectively, suggesting that metabolism of $InsP_3$ is not responsible for the low sensitivity of Purkinje cells (Khodakhah and Ogden, 1995). Safrany et al. (1991) obtained EC_{50} values of 0.8 μM for DL-5-thio-$InsP_3$ as compared with 0.11 μM for $InsP_3$, measuring steady-state Ca^{2+} depletion from stores in permeabilized neuroblastoma cells.

Caged 5-thio-$InsP_3$ neither released Ca^{2+} nor blocked the action of 5-thio-$InsP_3$ at concentrations up to 360 μM, covering the range useful experimentally with 5–50% photolysis.

Time course of the $[Ca^{2+}]_i$ change evoked by photolytic release of 5-thio-$InsP_3$

The pattern seen in the kinetics of Ca^{2+} release by 5-thio-$InsP_3$ is similar to that found with $InsP_3$ itself (Figs. 2, 3, and 4). There is first a delay that decreases in duration at high concentration, a period of rapid efflux lasting 0.5–2 s with the rate $d[Ca^{2+}]/dt$ higher at high concentration, and then a sharp transition to negative $d[Ca^{2+}]/dt$ and slower recovery toward basal $[Ca^{2+}]$. A reactivation of Ca^{2+} release of variable intensity occurs with 5-thio-$InsP_3$ and in some cells with high concentration of $InsP_3$.

The delay after the flash before a detectable rise of $[Ca^{2+}]_i$ seen with minimally effective concentrations of 5-thio-$InsP_3$ was much longer (mean, 4 s at 1.1 μM) than the delays seen with $InsP_3$ (mean, 0.68 s at 0.4 μM; T. Capiod and D. C. Ogden, manuscript in preparation). The shorter delay with $InsP_3$ suggests that breakdown by 5-phosphatase may increase the concentrations needed to evoke a response and that lower concentrations of $InsP_3$ acting with longer delays may be effective if 5-phosphatase were inhibited. This provides evidence that the 5-phosphatase may modify hormonal responses by reducing $InsP_3$ concentrations during hormone action. Also, the potency ratio of $InsP_3$ to 5-thio-$InsP_3$ may be greater than that given here if 5-phosphatase were not active. At high concentrations of $InsP_3$ and 5-thio-$InsP_3$, the delays are short, <20 and 60 ms, respectively, and the rates of photolysis (half-times of 3 and 8 ms, respectively) may be partially rate limiting in receptor activation. The origin of the delay with $InsP_3$ and 5-thio-$InsP_3$ is uncertain. It may be a result of cooperativity in $InsP_3$ binding, the structural and some flux data (Meyer et al., 1988) suggesting four bindings per activation, or to a positive cooperative interaction with cytosolic Ca^{2+} (Iino, 1990; mechanisms reviewed by Ogden et al., 1993). The data presented here do not distinguish between these mechanisms.

The rate of rise of cytosolic $[Ca^{2+}]$ increases with 5-thio-$InsP_3$ concentration, with a similar maximal value to that seen with $InsP_3$ and with a similar duration of the period of high efflux, 2 s at low and 0.5 s at high concentration. The peak Ca^{2+} concentrations attained with 5-thio-$InsP_3$ are also similar to those with $InsP_3$. The absence of any difference suggests that $InsP_3$ breakdown is not important in determining the kinetics of this phase of the $[Ca^{2+}]$ response.

The short period of high $d[Ca^{2+}]/dt$ and sharp decline of $[Ca^{2+}]_i$ after the peak indicates that efflux from stores is very quickly terminated, even though 5-thio-$InsP_3$ or $InsP_3$ is still present. This supports earlier conclusions based on twin pulse experiments with $InsP_3$ (Ogden et al., 1990) that there is an inactivation process, probably via elevated $[Ca^{2+}]_i$.

With 5-thio-$InsP_3$, there was evidence of reactivation of Ca^{2+} release after the initial decline even with low concentrations, whereas evidence of reactivation was seen only at high concentrations of $InsP_3$ itself. Repeated activation of Ca^{2+} release has been seen in hepatocytes during continuous perfusion of 1,4,5-$InsP_3$ and 2,4,5-$InsP_3$ at high concentration from the whole-cell patch pipette (Capiod et al., 1987; Ogden et al., 1990) and was advanced as evidence that the properties of the $InsP_3$ receptor are important in producing the periodic fluctuations of $[Ca^{2+}]$ seen in guinea pig hepatocytes with hormonal stimulation. The prolonged periodic fluctuations reported here after a single pulse of 5-thio-$InsP_3$ provide additional evidence that cyclic activation and inactivation of the $InsP_3$ receptor may occur with a steady $InsP_3$

concentration, with spike interval determined by the 20-s recovery time from Ca^{2+} inactivation (Ogden et al., 1990).

In summary, the results with photorelease of 5-thio-$InsP_3$ show that breakdown of $InsP_3$ is a significant factor in determining the time course and concentration dependence of the initial activation of Ca^{2+} release and is essential to prevent reactivation of the Ca^{2+} release mechanism once $InsP_3$ production has declined. They also show that $InsP_3$ metabolism does not play a part in terminating the period of activation of $InsP_3$ receptors, that this happens by a different mechanism, and that reactivation of receptors during hormone-induced Ca^{2+} fluctuations may occur without fluctuations in the $InsP_3$ concentration.

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