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Synthesis and application of photoaffinity analogues of inositol 1,4,5-trisphosphate selectively substituted at the 1-phosphate group

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We have synthesized two photolabile arylazido-analogues of Ins(1,4,5)P_3 selectively substituted at the 1-phosphate group for determination of Ins(1,4,5)P_3-binding proteins. These two photoaffinity derivatives, namely *N*-(4-azidobenzoyl)aminoethanol-1-phospho-D-*myo*-inositol 4,5-bisphosphate (AbaIP$_3$) and *N*-(4-azidosalicyl)aminoethanol-1-phospho-D-*myo*-inositol 4,5-bisphosphate (AsaIP$_3$), bind to high affinity Ins(1,4,5)P$_3$-specific binding sites at a 9-fold lower affinity (K_d = 66 and 70 nM) than Ins(1,4,5)P_3 (K_d = 7.15 nM) in a fraction from rat pancreatic acinar cells enriched in endoplasmic reticulum (ER). Other inositol phosphates tested showed comparable (DL-*myo*-inositol 1,4,5-trisphosphothioate, K_d = 81 nM) or much lower affinities for the binding sites [Ins(1,3,4,5)P_4, K_d = 4 μM; Ins(1,4)P_2, K_d = 80 μM]. Binding of AbaIP$_3$ was also tested on a microsomal preparation of rat cerebellum [K_d = 300 nM as compared with Ins(1,4,5)P_3, K_d = 45 nM]. Ca^{2+} release activity of the inositol derivatives was tested with AbaIP$_3$. It induced a rapid and concentration-dependent Ca^{2+} release from the ER fraction [EC_{50} (dose producing half-maximal effect) = 3.1 μM] being only 10-fold less potent than Ins(1,4,5)P_3 (EC_{50} = 0.3 μM). From the two radioactive labelled analogues ([^{3}H]AbaIP$_3$ and ^{125}I-AsaIP$_3$) synthesized, the radioiodinated derivative was used for photoaffinity labelling. It specifically labelled three proteins with apparent molecular masses of 49, 37 and 31 kDa in the ER-enriched fraction. By subfractionation of this ER-enriched fraction on a Percoll gradient the 37 kDa Ins(1,4,5)P_3 binding protein was obtained in a membrane fraction which showed the highest effect in Ins(1,4,5)P_3-inducible Ca^{2+} release (fraction P_1). The other two Ins(1,4,5)P_3-binding proteins, of 49 and 31 kDa, were obtained in fraction P_2, in which Ins(1,4,5)P_3-induced Ca^{2+} release was half of that obtained in fraction P_1. We conclude from these data that the 37 kDa and/or the 49 and 31 kDa proteins are involved in Ins(1,4,5)P_3-induced Ca^{2+} release from the ER of rat pancreatic acinar cells.

INTRODUCTION

Ins(1,4,5)P_3, which is generated from PtdIns(4,5)P_2 by receptor-mediated activation of phospholipase C, releases Ca^{2+} from intracellular stores of different cells [1–4]. Specific high-affinity Ins(1,4,5)P_3-binding sites (K_d 1–5 nM) have been demonstrated in different cells [5–7], most likely associated with the endoplasmic reticulum (ER) [1,8,9]. Direct evidence that Ins(1,4,5)P_3 opens single Ca^{2+} channels has been obtained in bilayers into which vesicles from smooth-muscle sarcoplasmic reticulum had been inserted [10]. An Ins(1,4,5)P_3-specific receptor protein purified from rat cerebellum [11] mediates Ca^{2+} flux in reconstituted lipid vesicles [12]. This Ins(1,4,5)P_3 receptor protein has been immunocytochemically localized to ER in Purkinje neurons [13]. Recently, it was shown by primary structure comparison that the putative cerebellar receptor shows partial sequence identity with the skeletal-muscle ryanodine receptor [14].

Ins(1,4,5)P_3 is rapidly metabolized by a 5-phosphatase [15] and by a specific Ins(1,4,5)P_3 3-kinase [16]. Furthermore, Ins(1,4,5)P_3 has been described to bind to aldolase A [17], but the physiological importance of this interaction is not yet clear.

Studies on the Ca^{2+} release potency of different Ins(1,4,5)P_3 analogues, such as Ins(2,4,5)P_3 and *sn*-glycero(3)-1-phospho-D-*myo*-inositol 4,5-bisphosphate [Gro*P*Ins(4,5)P_2] [18] or its semi-synthetic derivatives [19] revealed that the vicinal phosphate groups at positions 4 and 5 are essential for inducing Ca^{2+} release; the monoester phosphate at the 1-position apparently enhances the affinity for the receptor (reviewed in [2,20]). The naturally occurring Ins(1,3,4)P_3 is inactive in releasing Ca^{2+} [21,22]. An esterification of Ins(1,4,5)P_3 at the 4- or 5-phosphate group ['caged' Ins(1,4,5)P_3] leads to inactivation of the Ca^{2+} releasing potential and resistance to phosphatase degradation [23].

The synthesis of an arylazido analogue of Ins(1,4,5)P_3 which labelled three proteins of 80, 50 and 27 kDa in guinea-pig macrophages has already been described [24]. However, the substituted positions have not been defined in this case, and presumably were random at all three phosphate groups. Furthermore, it has not been demonstrated that this analogue is able to release Ca^{2+}.

Owing to the structural requirements, an Ins(1,4,5)P_3 analogue for specific binding and Ca^{2+} release should be substituted at, or close to, the 1-phosphate group. Therefore we synthesized two arylazido derivatives of Ins(1,4,5)P_3 suitable for photoaffinity labelling by selectively attaching the photolabile group to the amino group of 2-aminoethanol(1)-1-phospho-D-*myo*-inositol 4,5-bisphosphate [Ae*P*Ins(4,5)P_2], which had been semi-synthetically generated from Gro*P*Ins(4,5)P_2 [19].

We report here that the two novel photolabile Ins(1,4,5)P_3 analogues, *N*-(4-azidobenzoyl)aminoethanol-1-phospho-D-

Abbreviations used: AbaIP$_3$, *N*-(4-azidobenzoyl)aminoethanol-1-phospho-D-*myo*-inositol 4,5-bisphosphate; AsaIP$_3$, *N*-(4-azidosalicyl)-aminoethanol-1-phospho-D-*myo*-inositol 4,5-bisphosphate; Ae*P*Ins(4,5)P_2, 2-aminoethanol(1)-1-phospho-D-*myo*-inositol 4,5-bisphosphate; Gca*P*Ins(4,5)P_2, glycolaldehyde(2)-1-phospho-D-*myo*-inositol 4,5-bisphosphate; Gro*P*Ins(4,5)P_2, *sn*-glycero(3)-1-phospho-D-*myo*-inositol 4,5-bisphosphate; DL-IP$_3$S$_3$, DL-*myo*-inositol 1,4,5-trisphosphothioate; ER, endoplasmic reticulum; HSAB, *N*-hydroxysuccinimidyl-4-azidobenzoic acid; NHS-ASA, *N*-hydroxysuccinimidyl-4-azidosalicylic acid; m.d.d., metal–dye detection; PAR, 4-(2-pyridylazo)resorcinol; PEI-cellulose, polyethylenimine-cellulose; EC_{50}, dose producing half-maximal effect.

myo-inositol 4,5-bisphosphate ($AbaIP_3$) and *N*-(4-azidosalicyl)aminoethanol-l-phospho-D-*myo*-inositol 4,5-bisphosphate ($AsaIP_3$), bind specifically to high-affinity binding sites in an ER fraction from rat pancreatic acinar cells and to crude rat cerebellar membranes. $AbaIP_3$ induces Ca^{2+} release from intracellular stores in rat pancreatic ER and is only 10-fold less potent than Ins(1,4,5)P_3. Furthermore, we could show that the photolabile analogue ^{125}I-$AsaIP_3$ labelled three proteins in the ER fraction as well as the InsP_3 3-kinase purified from bovine brain.

MATERIALS AND METHODS

Materials

N-Hydroxysuccinimidyl 4-azidobenzoate (HSAB) and Tes were obtained from Sigma (Munich, Germany), *N*-hydroxysuccinimidyl 4-azidosalicylic acid (NHS-ASA) and Iodo-beads were from Pierce (Heidelberg, Germany), *N*-hydroxysuccinimidyl 4-azido[3,5-^{3}H]benzoate and [^{3}H]Ins(1,4,5)P_3 (17–20 Ci/mmol) were purchased from New England Nuclear (NEN, Bad Homburg, Germany), DL-IP_3S_3 as triethylammonium salt (pure as stated by ^{31}P n.m.r. [25]), was obtained from Dr. B. V. L. Potter, Department of Chemistry, University of Leicester, Leicester LE1 7RH, U.K., [^{14}C]methylated protein standards, Na^{125}I and Amplify were from Amersham Buchler (Braunschweig, Germany), protein standards for SDS/PAGE and Q-Sepharose (fast flow) were obtained from Pharmacia (Freiburg, Germany). All other reagents were of the highest purity available and were obtained from Merck (Darmstadt, Germany) or Riedel de Haen (Seelze, Germany).

Synthesis of Ae*P*Ins(4,5)P_2

This starting compound for the synthesis of both photolabile compounds was prepared from glycolaldehyde(2)-l-phospho-D-*myo*-inositol 4,5-bisphosphate [Gca*P*Ins(4,5)P_2] by reductive amination. The preparation of the latter compound, the details of the Schiff-base formation, reduction and the purification by Dowex-1 chromatography were described previously [19]. For the amination, ammonium acetate, pH 6.0, and Gca*P*Ins(4,5)P_2 were mixed at final concentrations of 0.2 M and 4 mM respectively. The yield of Ae*P*Ins(4,5)P_2 was 65%. The reductive amination product was purified by Dowex-1 chromatography as described previously [19]. A further chromatography under identical conditions resulted in a product which was free from detectable side-products as deduced from h.p.l.c. analysis (see under 'Analytical methods' below). H.p.l.c. analysis after alkali hydrolysis (see under 'Analytical methods' below) resulted in a mixture of 78% Ins(1,4,5)P_3 and 22% Ins(4,5)P_2, as expected for a compound with intact l-phosphodiester group and inositol ring structure. The carrier-free ammonium salt was dissolved in water and stored frozen or freeze-dried (−30 °C) under N_2.

Synthesis of $AbaIP_3$ and $AsaIP_3$

Synthesis of $AbaIP_3$ and $AsaIP_3$ was carried out in the dark or under dim red light at room temperature as follows.

To 40 μl of AePIns(4,5)P_2 (236 nmol) 390 μl of dimethylformamide, 130 μl of distilled water and 15 μl of 0.8 M-triethylammonium acetate buffer, pH 8.0, were added. Then 40 μl of HSAB (4 μmol) was added to the mixture. The reaction was allowed to proceed for 16 h with gentle rotation of the tube, stopped by the addition of 15 μl of 1.0 M-acetic acid and diluted with 2.4 ml of 5 mM-ammonium acetate/10% (v/v) ethanol, pH 5.0. The solution was then applied to a column (1.3 cm × 0.5 cm) of Q-Sepharose equilibrated with 5 mM-ammonium acetate/10% (v/v) ethanol, pH 5.0. $AbaIP_3$ was eluted with a step gradient (6 ml each) of 0.005 M-, 0.5 M- and 1.0 M-ammonium acetate in 10% (v/v) ethanol, pH 5.0, at a flow rate of 40 ml/h, and 0.6 ml fractions were collected. Aliquots of the fractions were measured for their phosphate content (see below). The fractions containing $AbaIP_3$, which were eluted with 1.0 M-ammonium acetate, were pooled and repeatedly freeze-dried from water to remove ammonium acetate. Ae*P*Ins(4,5)P_2 that had not reacted and some Ins(1,4,5)P_3, generated by hydrolysis of Ae*P*Ins(4,5)P_2, were eluted with the 0.5 M-buffer. The residue was dissolved in water/10% (v/v) ethanol and stored under N_2 at −30 °C. The yield of $AbaIP_3$ was 65–70% with a purity > 99.0%, as judged by h.p.l.c. analysis (Fig. 2*b* below). H.p.l.c. analysis of the KOH hydrolysate of $AbaIP_3$ revealed a mixture of 78% Ins(1,4,5)P_3 and 22% Ins(4,5)P_2 (Fig. 2*c* below).

The synthesis of the other photoaffinity analogue, $AsaIP_3$, was carried out using 4 μmol of NHS-ASA instead of HSAB, essentially as described for $AbaIP_3$. The yield of this compound was 56–60% and the purity > 99% as judged by h.p.l.c..

Synthesis of ^{3}H-$AbaIP_3$

Non-radioactive HSAB (5 μl) dissolved in dimethylformamide was added to 1 ml of [^{3}H]HSAB (1 mCi; 19–21 nmol) in propan-2-ol to give 40 nmol of the *N*-hydroxysuccinimidyl ester. The solution was cooled on ice and the volume reduced to about 10 μl under a stream of N_2. To this solution 35 μl of dimethylformamide, 3 μl of 0.8 M-triethylammonium acetate, pH 8.0, and 10 μl of AePIns(4,5)P_2 (59 nmol) were added and incubated for 16 h with gentle rotation of the tube. The reaction was stopped by the addition of 5 μl of 1.0 M-acetic acid, diluted with 750 μl of 5 mM-ammonium acetate/10% (v/v) ethanol, pH 5.0, and loaded on to a column (0.6 cm × 0.5 cm) of Q-Sepharose. The column was washed first with 3 ml of the dilution buffer and then with 3 ml of 0.3 M-ammonium acetate (pH 5.0)/10% (v/v) ethanol. [^{3}H]$AbaIP_3$ was eluted with 7 ml of a linear gradient from 0.3 to 1.0 M-ammonium acetate/10% ethanol (v/v), pH 5.0. Fractions (0.5 ml each) were collected at a flow rate of 35 ml/h, and 2 μl aliquots of the fractions were counted for radioactivity. The [^{3}H]$AbaIP_3$ which was eluted at about 0.95 M-ammonium acetate, was pooled and freeze-dried three times from water to remove ammonium acetate. The dried residue was dissolved in water/10% ethanol and stored under N_2 at −30 °C. The yield of [^{3}H]$AbaIP_3$ (sp. radioactivity: 24.5 Ci/mmol) varied between 1.4 and 2.0%, as calculated from the recovery of radiolabelled material (setting 1 mCi to 100%). The radiochemical purity of the purified product was > 99% as deduced from the data in Fig. 2(*d*).

Radioiodination of $AsaIP_3$

The iodination of $AsaIP_3$ was carried out using a modified solid-phase method [26]. $AsaIP_3$ (5 nmol) in 130 μl of 0.1 M-triethylammonium acetate, pH 8.5 (incubation buffer), was rapidly mixed with 0.80 mCi of Na^{125}I (20 nmol) in 70 μl of incubation buffer. The iodination was started with one Iodo-bead, which had been washed with water (3 ml) and incubation buffer (3 × 1 ml). After a 4 h incubation in the dark at room temperature the Iodo-bead was removed and washed with incubation buffer containing 30% (v/v) ethanol (2 × 200 μl). The wash solution was combined with the reaction mixture, 400 μl of 0.1 M-NaI in incubation buffer were added and the crude radioiodinated product loaded on to a Q-Sepharose column (0.6 ml), equilibrated with 0.1 M-ammonium acetate (pH 5.0)/10% (v/v) ethanol/0.1 M-LiCl (equilibration buffer). The column was first washed with the equilibration buffer (6 ml), then with 1.2 M-LiCl in equilibration buffer (12 ml) and eluted with 0.1 M-ammonium acetate (pH 5.0)/40% (v/v) ethanol/1.2 M-LiCl (3 ml). Most of the radioactivity which was eluted in a single peak with the last eluent was pooled, the volume reduced to 200 μl by freeze-drying and applied to a column

(0.5 cm × 17 cm) of Bio-Gel P2 for desalting. The radiochemical purity of the products eluted from this column in a broad peak was assessed by t.l.c. and autoradiography. Besides the desired product a small amount (< 10%) of a dephosphorylated derivative ($AsaIP_2$) incompletely removed by the preceding anion-exchange chromatography was detected in the earlier-eluted fractions. By pooling only the later-eluted fractions this contamination could be reduced to less than 4%. A very small radioactive spot migrating somewhat slower than $AsaIP_3$ might be indicative of a small amount of doubly iodinated $AsaIP_3$. The pooled fractions were twice freeze-dried from water to remove the ammonium acetate. The purified ^{125}I-$AsaIP_3$, with a specific radioactivity of 38 Ci/mmol, was stored at −30 °C in water/10% ethanol.

Analytical methods

Total phosphate was determined according to the method of Lanzetta *et al.* [27], with minor modifications. Aliquots (45 μl) of the fractions from the Q-Sepharose chromatography of the non-radioactive synthesis were dried at 110 °C for 30 min in borosilicate tubes and then hydrolysed with 50 μl of 1.5 M-H_2SO_4 by heating at 160 °C for 75 min. After cooling to room temperature samples were adjusted to 150 μl with water. To 10 μl of the sample, 100 μl of a dye solution [0.045% Malachite Green hydrochloride/4.2% (w/v) ammonium heptamolybdate in 4 M-HCl/2% (w/v) Tergitol NP-10; 147:49:4, by vol.] was added in a microtitre well and the dye reaction stopped after 1 min by the addition of 20 μl of 34% (w/v) trisodium citrate solution. After vortex-mixing for 1 min the absorbance was measured at 565 nm in a microtitre reader (Multiscan Mark II; Flow Laboratories).

For alkali hydrolysis 2.5 μl of $AbaIP_3$ or $AsaIP_3$ were mixed with 10 μl of 5 M-KOH and 37.5 μl of water and hydrolysed by incubation at 100 °C for 45 min. The cooled sample was acidified with 20 μl of 1.0 M-acetic acid, diluted to 1 ml with water and applied to a Mono-Q column (5 mm × 30 mm) for h.p.l.c. analysis.

The purity and composition of the photoaffinity derivatives were analysed by h.p.l.c. with the metal–dye-detection (m.d.d.) method as described in [28,29]. The acidic elution system with an upward-concave gradient from 0.2 to 500 mM-HCl containing 14 μM-YCl_3 (10 min, 2 ml/min) was used to separate the inositol phosphates. The solution for post-column detection of the phosphorylated compounds contained 1.6 M-triethanolamine (adjusted to pH 9.0 with HCl) and 0.2 mM-PAR, and was applied at a flow rate of 1.0 ml/min. The radiochemical purity of labelled analogues was also assessed by t.l.c. separation on polyethyleneimine (PEI)-cellulose in 0.8 M-triethylammonium hydrogen carbonate, pH 7.5.

Photoaffinity labelling with ^{125}I-$AsaIP_3$

A fraction enriched in ER (11000 *g* 'fluffy layer') was prepared from rat pancreatic acinar cells as described in [30]. For further purification the ER fraction was applied to a Percoll gradient as described in [31]. The ER fraction or the Percoll-gradient-derived fractions (500 μg of protein) were incubated with 30 nM-^{125}I-$AsaIP_3$ for 6 min at 4 °C in the presence or absence of 50 μM-DL-IP_3S_3 in a total volume of 300 μl containing (final concentrations given) 50 mM-Tes, 50 mM-KCl, pH 8.2 (buffer B). The samples were then irradiated for 2 min with a u.v. lamp (VL-100 C; 254 nm; 100 W; Herolab, St. Leon-Rot, Germany) at 0 °C and a distance of 5 cm. The irradiated membranes were precipitated with ice-cold 10% (w/v) trichloroacetic acid (final concn.) and centrifuged at 10000 *g* for 15 min. The pellets were washed twice with 4% trichloroacetic acid, dissolved in SDS sample buffer and subjected to SDS/PAGE. The gel was dried and exposed to Kodak XAR-5 film at −70 °C.

Isolation of Ins(1,4,5)P_3 3-kinase and photolabelling with ^{125}I-$AsaIP_3$

Ins(1,4,5)P_3 3-kinase was isolated from bovine brain. The purification was done mainly as described by Takazawa *et al.* [32]. Essential modifications of the method allowed a purification of 20000-fold of an 80 kDa polypeptide which is associated with Ins(1,4,5)P_3 3-kinase activity (phosphorylation of 5 μmol of Ins(1,4,5)P_3/min per mg at pH 7.5 and 37 °C). A detailed description of the purification is available from M.N.-S or G.W.M. on request). For photolabelling, 4 μg of purified Ins(1,4,5)P_3 3-kinase was incubated in 200 μl containing 10 mM-triethanolamine, 100 mM-KCl, 1 mM-EGTA, 1.2 mM-$CaCl_2$, 50 μM-ATP, 2 mM-$MgCl_2$, 0.1 μM-calmodulin, 0.1% Triton X-100, pH 7.5, for 1 min at 25 °C in the presence or absence of 800 μM-Ins(1,4,5)P_3 or of 500 μM-DL-IP_3S_3 with 0.5 μM-^{125}I-$AsaIP_3$. After irradiation for 1 min, the samples were treated as described above for ER.

Binding/displacement experiments

Binding of Ins(1,4,5)P_3 and its displacement by other inositol phosphates and by our analogues was performed as described by Worley *et al.* [33]. [^{3}H]Ins(1,4,5)P_3 binding to the ER fraction of acinar cells from rat pancreas and to rat cerebellar membranes was measured in duplicate in a total volume of 0.4 ml of buffer B. The assays contained 500 μg of membrane protein, 3 nM-[^{3}H]Ins(1,4,5)P_3 and different inositol phosphate analogues as indicated in the legend to Fig. 4 (below). After incubation for 8 min at 4 °C, samples were centrifuged at 10000 *g* for 5 min (Beckman Microfuge 12) and the supernatant was discarded.

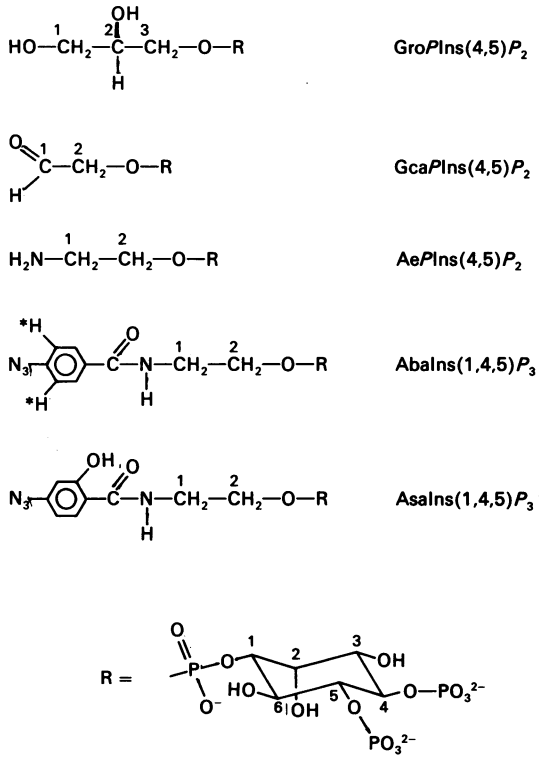


Fig. 1. Structures of the substituents at the 1-phosphate group of Ins-(1,4,5)P_3 in the synthetic route of $AbaIP_3$ and $AsaIP_3$

The carbon atoms of the substituents are numbered. The hydrogen atoms at the benzol ring marked with an asterisk denote the positions of the ^{3}H label.

The tips of the centrifugation tubes were cut into vials with 4 ml of scintillant (Rotiszint 22X) and, after vortex-mixing, the radioactivity was counted. Non-specific binding was determined in the presence of 2 μM-Ins(1,4,5)P_3.

Ca^{2+} release experiments

Ca^{2+} release with Ins(1,4,5)P_3 or AbaIP$_3$ was measured in pancreatic ER membrane vesicles or in the Percoll gradient fractions P_1 and P_2 using a Ca^{2+}-selective electrode as previously described [34]. Membrane vesicles (1 mg of protein) were incubated at 30 °C in 2 ml of buffer containing (in mM): KCl 120; $MgCl_2$ 6; Hepes 25; potassium phosphate 1.2; potassium pyruvate 5; potassium succinate 5; NaN_3 10; ATP 5; phosphocreatine 10; creatine kinase 10 units/ml; antimycin A 0.01 and oligomycin 0.005 (pH 7.0, adjusted with KOH). The inositol phosphates were added after the Ca^{2+} uptake had reached a steady state (20–30 min).

Other methods

Protein was determined by the method of Bradford [35], with BSA as the standard. SDS/PAGE was performed by the method of Laemmli [36] and gels were stained either with Serva Blue 250-R or with 250-G.

RESULTS

Synthesis of AbaIP$_3$ and AsaIP$_3$

For synthesis of an Ins(1,4,5)P_3 analogue suitable for photoaffinity labelling we chose HSAB, a hetero-bifunctional photoactivatable compound, originally used for photoaffinity labelling of peptide-hormone-binding sites [37]. This reagent has the advantage of being available both in non-radioactive and doubly tritiated form. An additional photolabile derivative of Ins(1,4,5)P_3, AsaIP$_3$, was synthesized by using NHS-ASA instead of HSAB. This label can be easily radioiodinated and was mainly used for photoaffinity experiments because of the markedly higher specific radioactivities achieved. The structures of AbaIP$_3$ and AsaIP$_3$ and their precursors in the synthetic route are shown

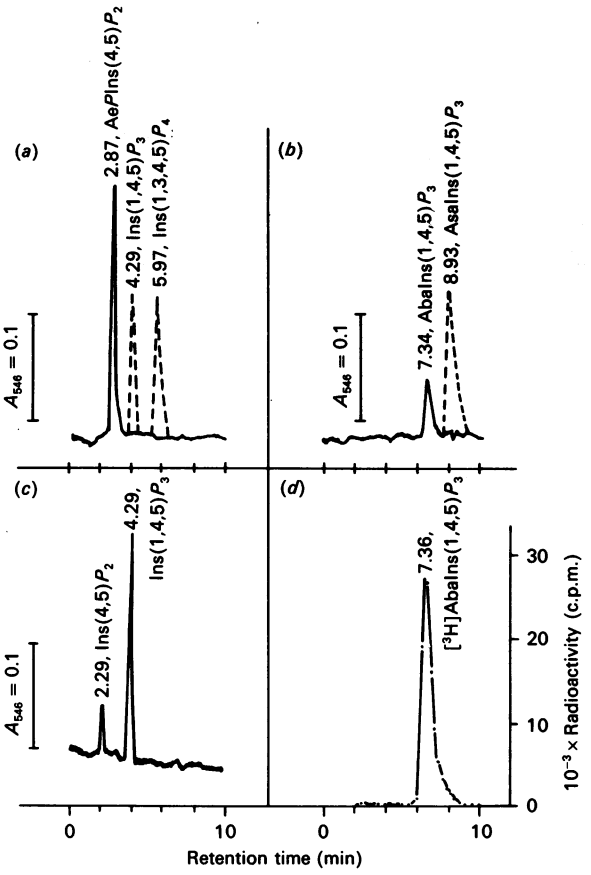


Fig. 2. H.p.l.c.–m.d.d. analysis of AbaIP$_3$, AsaIP$_3$ and other inositol phosphates

The separation of the inositol phosphates on h.p.l.c. was performed as described in the Materials and methods section. The exponential gradient of HCl started at zero retention time, and the absorbance was recorded at 546 nm in a cell with a pathlength of 5 mm. Baseline-subtracted h.p.l.c. separation profiles are shown, and retention times of the various inositol phosphates are given at the peaks in the chromatograms. (*a*) Chromatogram of 5 nmol of Ae*P*Ins(4,5)P_2. The broken-line peaks represent the elution positions of 1 nmol of Ins(1,4,5)P_3 (retention time 4.29 min) and 1 nmol of Ins(1,3,4,5)P_4 (5.97 min) respectively. (*b*) Analysis of 4 nmol of AbaIP$_3$ after purification by Q-Sepharose chromatography. The broken-line peak at retention time 8.93 min indicates the elution position of AsaIP$_3$ (10 nmol). Note that the two photoaffinity analogues of Ins(1,4,5)P_3 are eluted in broad peaks, in contrast with the other inositol phosphates. (*c*) Chromatogram of the neutralized KOH hydrolysate of 4 nmol of AbaIP$_3$. The peaks at retention time 2.29 and 4.29 min correspond to Ins(4,5)P_2 and Ins(1,4,5)P_3 respectively. (*d*) Analysis of 3 pmol of [^{3}H]AbaIP$_3$ after purification by Q-Sepharose chromatography. The radioactivity was determined in fractions collected at 0.25 min intervals.

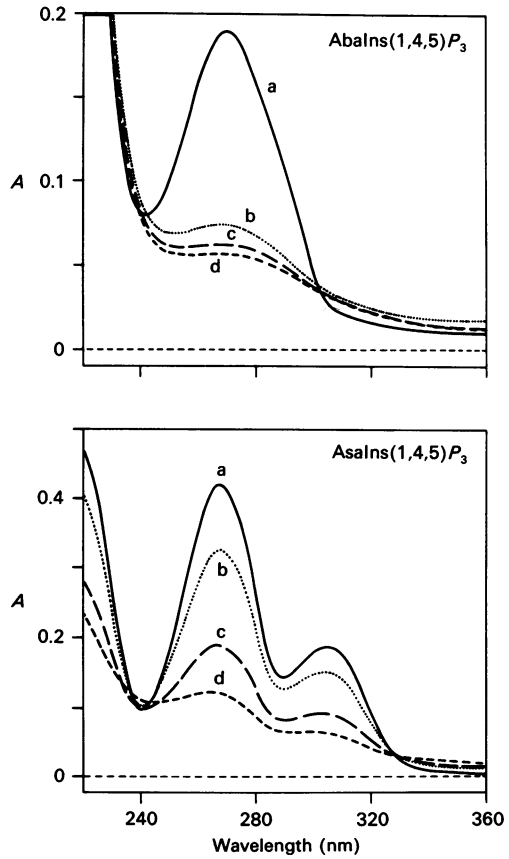


Fig. 3. Photodecomposition of AbaIP$_3$ and AsaIP$_3$

Aqueous solutions of AbaIP$_3$ (3 μM) or AsaIP$_3$ (10 μM) were exposed to various times of irradiation at 254 nm as described in the Materials and methods section. a, Control; b, 5 s; c, 15 s; and d, 30 s. The samples were then placed in a 1 ml quartz cuvette (1 cm pathlength) and the absorbance spectra were recorded.

in Fig. 1. The h.p.l.c. profiles of AbaIP$_3$ and AsaIP$_3$ obtained after purification by Q-Sepharose chromatography and KOH hydrolysis are depicted in Fig. 2. They show that free Ins(1,4,5)P_3 is not present. The percentage distribution between Ins(1,4,5)P_3 and Ins(4,5)P_2 as products of the alkali hydrolysis of AbaIP$_3$ and AsaIP$_3$ indicated that the l-phosphodiester group and the inositol ring have been conserved under the conditions of the synthesis. AbaIP$_3$ when stored for 4 months at −30 °C revealed no detectable decomposition products on h.p.l.c. analysis (results not shown).

The absorption spectra of 3 μM-AbaIP$_3$ or 10 μM-AsaIP$_3$ after u.v. irradiation at 254 nm for different periods are shown in Fig. 3. The non-irradiated AbaIP$_3$ shows a strong absorption with a maximum at 269 nm which almost completely disappeared after 5 s of irradiation. Non-irradiated AsaIP$_3$ shows two absorption maxima at 267 and 305 nm, both of which declined rapidly after irradiation. From the molar absorbance coefficients of 4-azidobenzoylglycine and 4-azidosalicylglycine molar ratios of the substituents to Ins(1,4,5)P_3 of 1.0:0.96 and 1.0:0.98 respectively were calculated.

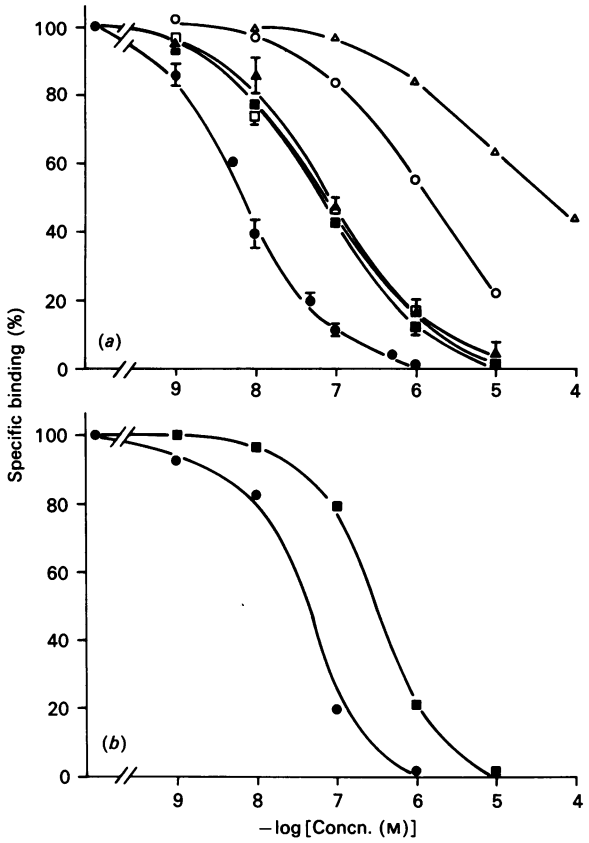


Fig. 4. Displacement of [^{3}H]Ins(1,4,5)P_3 from pancreatic ER (*a*) and from cerebellar membranes (*b*) by inositol phosphates

The concentration of [^{3}H]Ins(1,4,5)P_3 was 3 nM, and the specific binding was estimated as described in the Materials and methods section. (*a*) The displacement curves for Ins(1,4,5)P_3 (●), AbaIP$_3$ (■), AsaIP$_3$ (□), DL-IP$_3$S$_3$ (▲), Ins(1,3,4,5)P_4 (○) and Ins(1,4)P_2 (△) in pancreatic ER. The data are means ± S.E.M. for three independent experiments [except for Ins(1,3,4,5)P_4 and Ins(1,4)P_2 for which there were only two experiments] and expressed as percentages of control specific binding. (*b*) The competition curves in rat cerebellar membranes for Ins(1,4,5)P_3 (●) and AbaIP$_3$ (■). The data are mean values from two independent experiments. 100% specific Ins(1,4,5)P_3 binding refers to 85–106 fmol/mg of protein in pancreatic ER and 920–1250 fmol/mg of protein in cerebellar membranes.

Binding of [^{3}H]Ins(1,4,5)P_3 and displacement by different inositol phosphates

The specificity of AbaIP$_3$ and AsaIP$_3$ binding to Ins(1,4,5)P_3-binding sites was determined in competitive binding experiments performed with Ins(1,4,5)P_3 and other inositol phosphates in a microsomal fraction from rat pancreatic acinar cells enriched in endoplasmic reticulum (Fig. 4*a*) and in a microsomal preparation of rat cerebellum (Fig. 4*b*). As shown in Fig. 4(*a*), AbaIP$_3$ binds specifically to Ins(1,4,5)P_3-binding sites with a K_d value of 66 ± 2.7 nM (mean ± S.E.M., $n = 3$) as determined from the competition of 3 nM-[^{3}H]Ins(1,4,5)P_3 at pH 8.2. This value is about 9-fold lower than the K_d value of 7.15 ± 0.62 nM (mean ± S.E.M., $n = 3$) for Ins(1,4,5)P_3 and very close to the K_d of DL-IP$_3$S$_3$ (81 ± 5.9 nM), a recently synthesized phosphatase-resistant Ca^{2+}-mobilizing analogue of Ins(1,4,5)P_3 [38]. AsaIP$_3$ binds to Ins(1,4,5)P_3-specific binding sites with the same affinity (K_d = 70 ± 3.5 nM) as AbaIP$_3$. The metabolic compounds of Ins(1,4,5)P_3, namely Ins(1,3,4,5)P_4 and Ins(1,4)P_2, showed very low affinities for the binding site, with K_d values of 4 μM and 80 μM respectively. The shapes of the displacement curves for AbaIP$_3$ and AsaIP$_3$, as well as those for Ins(1,3,4,5)P_4 and DL-IP$_3$S$_3$, and their parallelism with the curve for Ins(1,4,5)P_3, are consistent with competitive interaction at the Ins(1,4,5)P_3-specific binding site. Fig. 4(*b*) shows that AbaIP$_3$ also binds to a microsomal fraction of rat cerebellum. Its affinity (K_d = 300 nM) is about 7-fold lower than that of Ins(1,4,5)P_3 (K_d = 45 nM).

Ins(1,4,5)P_3- and AbaIP$_3$-induced Ca^{2+} release

To investigate whether the photosensitive compound AbaIP$_3$ could induce Ca^{2+} release from non-mitochondrial intracellular stores, Ca^{2+} release in response to AbaIP$_3$ or Ins(1,4,5)P_3 was determined with a Ca^{2+}-sensitive electrode in fractions enriched in ER (11000 *g* 'fluffy layer'; Percoll-gradient-derived fractions P_1 and P_2; Fig. 5). As shown in Fig. 5(*a*), ATP-dependent Ca^{2+} uptake into the fluffy-layer vesicles lowered the free Ca^{2+}-concentration from 1 μM to about 390 nM. For measurement of the maximal Ca^{2+} release induced by the different Ins(1,4,5)P_3 concentrations, vanadate (10^{-4} M) was added in the steady state to inhibit Ca^{2+} re-uptake. Addition of AbaIP$_3$ induced a concentration-dependent release of the accumulated Ca^{2+}. The response was rapid, and the Ca^{2+} released at the highest applied concentration of 10 μM-AbaIP$_3$ amounted to 84% of the Ca^{2+} released with the maximally effective concentration of Ins(1,4,5)P_3 (5 μM). The concentration of AbaIP$_3$ required for half-maximal Ca^{2+} release was 3.1 μM, as estimated from the experiment shown in Fig. 5 (inset). The EC_{50} (dose producing half-maximal effect) value for Ins(1,4,5)P_3 was 0.3 μM. These results clearly demonstrate that the photoaffinity analogue of Ins(1,4,5)P$_3$, AbaIP$_3$, is a full agonist and only 10-fold less potent than Ins(1,4,5)P_3 in releasing Ca^{2+} from non-mitochondrial intracellular stores. Further purification of the fluffy-layer fraction on a Percoll gradient showed that Ins(1,4,5)P_3-induced Ca^{2+} release was only observed in the lighter fractions P_1 and P_2 (Fig. 5*b*), whereas fractions P_3–P_5 showed no effect on Ca^{2+} release after addition of Ins(1,4,5)P_3 (results not shown), when Ca^{2+} uptake into the vesicles was performed in the presence of 10^{-4} M vanadate. The Ins(1,4,5)P_3-induced Ca^{2+} release was most effective in fraction P_1, whereas in fraction P_2 Ca^{2+} release was only half of that obtained in P_1 (Fig. 5*b*).

Photoaffinity labelling of ER

For photoaffinity labelling of the high-affinity Ins(1,4,5)P_3-binding protein(s) from the ER-enriched fractions (11000 *g* 'fluffy layer', Percoll-gradient-derived vesicle fractions P_1 and P_2) from rat pancreatic acinar cells we used the ^{125}I-labelled

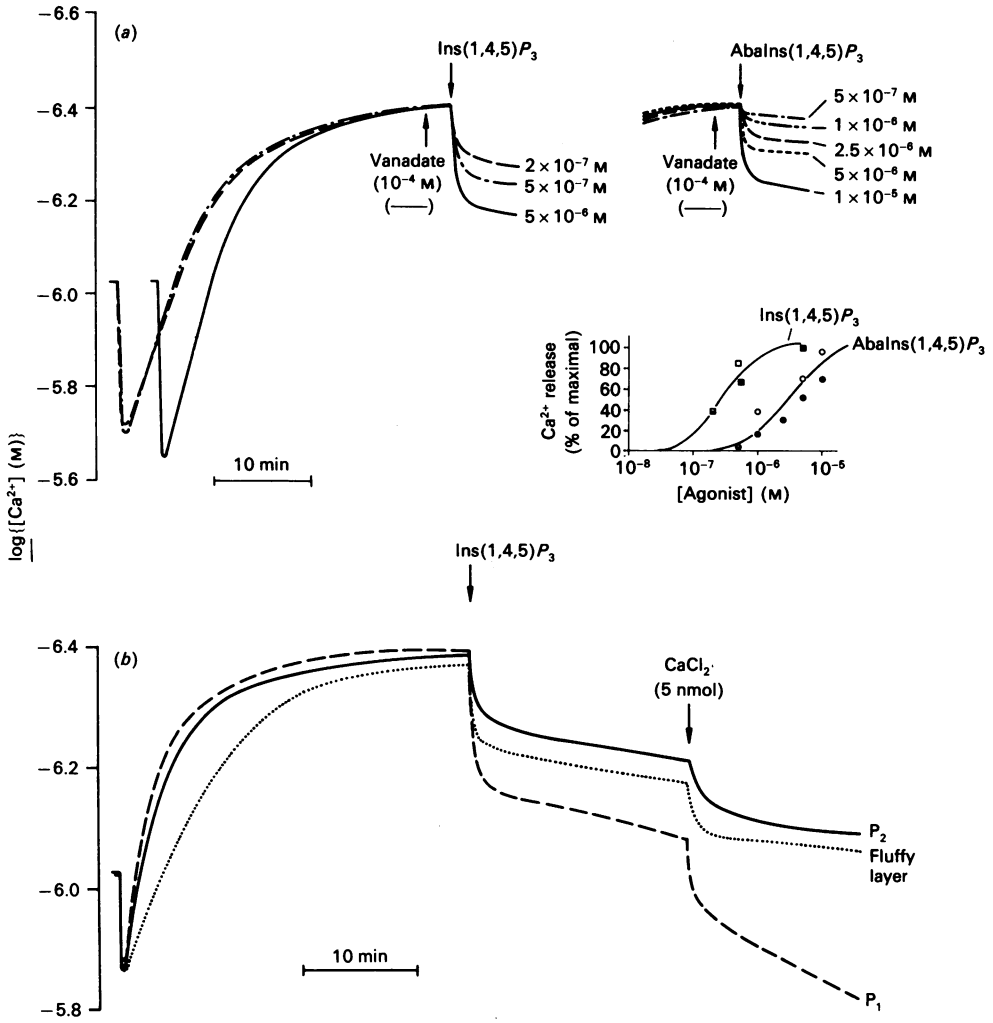


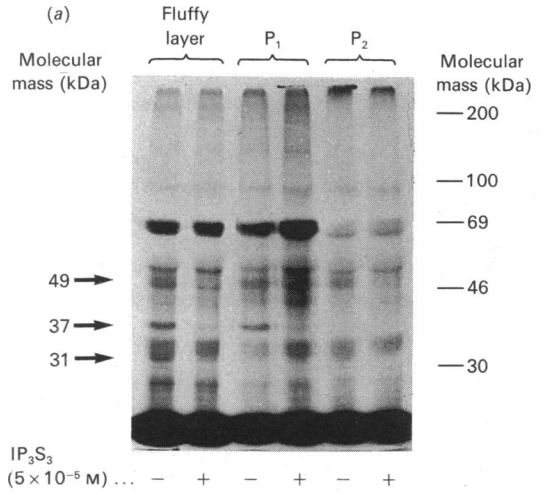
Fig. 5. (*a*) Concentration-dependent Ca^{2+} release of Ins(1,4,5)P_3 and AbaIP$_3$ from rat pancreatic ER and (*b*) Ins(1,4,5)P_3-induced Ca^{2+} release in microsomal vesicle fractions (11000 *g* 'fluffy layer', Percoll fractions P_1 and P_2)

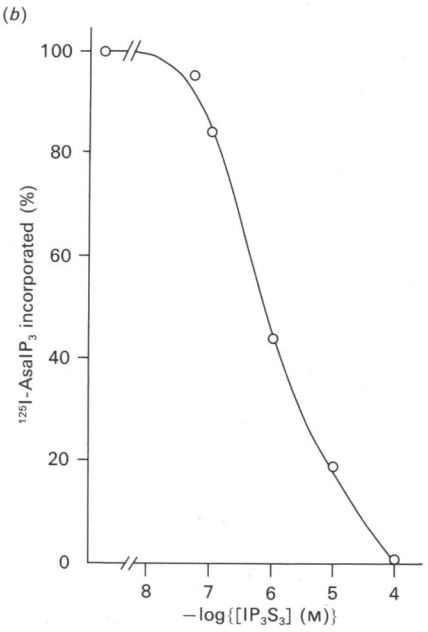
ER (1 mg of protein) was incubated at 30 °C in 2 ml of the medium given in the Materials and methods section. Free Ca^{2+} concentrations in the medium were measured with the Ca^{2+}-selective electrode. Vanadate (10^{-4} M) was added in the steady-state 2 min before the addition of the inositol phosphates to inhibit the Ca^{2+} re-uptake. The release of Ca^{2+} evoked by increasing concentrations of Ins(1,4,5)P_3 or AbaIP$_3$ after 2 min is shown. The data are from one experiment which is representative of three other experiments. The inset shows dose–response curves of Ins(1,4,5)P_3- (□, ■) and AbaIP$_3$- (○, ●) induced Ca^{2+} release from rat ER. The experimental conditions were as described above. The values given are from two independent experiments. (*b*) For each fraction 1 mg of protein was incubated under the experimental conditions described above, except that the medium contained 10^{-4} M-vanadate. Ins(1,4,5)P_3 (5 μM) and $CaCl_2$ (5 nmol) were added to the medium where indicated. The data are from one experiment representative of five others.

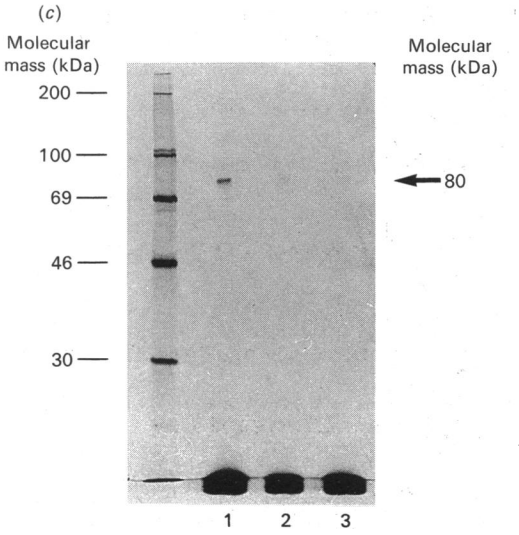
AsaIP$_3$ (see the Materials and methods section). Incubation of the ER fractions with 30 nM-^{125}I-AsaIP$_3$, and subsequent photoirradiation resulted in the labelling of several proteins, as shown in the autoradiogram obtained after SDS/PAGE of the photolysed samples (Fig. 6*a*, lane 1). The presence of an excess amount of DL-IP$_3$S$_3$ (5×10^{-5} M) during the incubation and photoirradiation almost completely abolished the labelling of proteins with apparent molecular masses of 37 and 49 kDa and partially that of a 31 kDa protein (Fig. 6*a*, lane 2). The presence of 10^{-4} M-Ins(1,4,5)P_3 during incubation and photolysis also protected these proteins from labelling with ^{125}I-AsaIP$_3$ (results not shown). The predominantly labelled protein banding at 68 kDa is very likely to be albumin, as photolabelling in the presence of 50 μg of albumin resulted in greatly enhanced incorporated radioactivity in this band (results not shown). The Ins(1,4,5)P_3-binding proteins could be separated by further subfractionation of the ER fraction on a Percoll gradient (Fig. 6*a*, lanes 3–6). In fraction P_1 only the 37 kDa protein was specifically labelled (Fig. 6*a*, lanes 3 and 4). The Ins(1,4,5)P_3-binding proteins with apparent molecular masses of 31 and 49 kDa were obtained in fraction P_2 of the Percoll gradient (Fig. 6*a*, lanes 5 and 6). The labelling of the 37 kDa protein with ^{125}I-AsaIP$_3$ was specifically inhibited by DL-IP$_3$S$_3$ in a concentration-dependent manner. Counting of the incorporated radioactivity in the gel slices of the 37 kDa-protein area revealed that photolabelling in the presence of 10^{-4} M-DL-IP$_3$S$_3$ totally prevented the labelling of the 37 kDa protein, and the half-maximal effect (EC_{50}) was achieved with 7.5×10^{-7} M-DL-IP$_3$S$_3$ (Fig. 6*b*).

Photoaffinity labelling of Ins(1,4,5)P_3 3-kinase

The photoaffinity analogue ^{125}I-AsaIP$_3$ was used to label purified Ins(1,4,5)P_3 3-kinase. Incubation of the kinase with the photolabel for 1 min and subsequent photoirradiation resulted in the labelling of a protein with an apparent molecular mass of







80 kDa (Fig. 6*c*, lane 1), which has been identified as Ins(1,4,5)P_3 3-kinase (see the Materials and methods section; results not shown). The labelling of the 80 kDa protein was completely inhibited by 500 μM-DL-IP_3S_3 (Fig. 6*c*, lane 3) and to a lesser extent by 800 μM-Ins(1,4,5)P_3 present in the incubation buffer and during photoirradiation (Fig. 6*c*, lane 2).

DISCUSSION

It has been demonstrated that derivatives of Ins(1,4,5)P_3 containing different cationic and amphipathic substituents at the 1-phosphate group can release Ca^{2+} [19]. We therefore designed a route for the synthesis of a photoaffinity analogue of Ins(1,4,5)P_3 by selectively attaching the photolabile group to the 1-phosphate position. The developed synthesis for $AbaIP_3$ and $AsaIP_3$ has several advantages: (i) The starting material [Gro*P*-Ins(4,5)P_2] has already the desired D-configuration. So tedious separation of racemic reaction products are avoided. (ii) The derivatization is position-selective and performed only by simple chemical reactions. (iii) Without laborious protection and deprotection techniques of the vicinal 4,5-phosphate groups, the Ca^{2+}-release activity is almost fully retained in the analogue. (iv) No cationic charges are introduced into the substituent close to the 1-phosphate group. This latter point is important, as cationic charges in substituents at the 1-phosphate group further reduce the biological activity of Ins(1,4,5)P_3 analogues [19], presumably via a reduced binding affinity. Our synthetic route is being successfully used to synthesize other versatile affinity analogues of Ins(1,4,5)P_3.

The reason for the very low yields in the synthesis of [^{3}H]$AbaIP_3$ as compared with the non-radioactively labelled substance is unknown at present. We assume that radiolabelling of HSAB makes this ester more sensitive to hydrolysis or oxidation by solute contaminants during the course of the volume reduction (S. Hurt, personal communication). The assumption is based on the fact that a synthesis performed only with non-radiolabelled HSAB under conditions identical with those described for ^{3}H-$AbaIP_3$, gave about 5-fold higher yields of $AbaIP_3$.

We could demonstrate that $AbaIP_3$ or $AsaIP_3$ are potent analogues of the intracellular messenger Ins(1,4,5)P_3. This type of photoaffinity analogue specifically binds to Ins(1,4,5)P_3-binding sites in pancreatic endoplasmic reticulum and cerebellar membranes and is able to specifically label Ins(1,4,5)P_3 3-kinase. The results of the h.p.l.c. analysis of purified $AbaIP_3$ rule out the

Fig. 6. Photoaffinity labelling with ^{125}I-$AsaIP_3$

(*a*) Labelling of the ER-enriched fractions from pancreatic acinar cells. Vesicle fractions (500 μg of protein) were incubated as described in the text with 3×10^{-8} M ^{125}I-$AsaIP_3$ in the presence or absence of 5×10^{-5} M-DL-IP_3S_3 and then irradiated. Protein separation and autoradiography were as described in the Materials and methods section. Molecular masses of ^{14}C-labelled standard proteins are given in the right-hand lane. The results shown are representative for three similar experiments. (*b*) Inhibition of photoaffinity labelling with ^{125}I-$AsaIP_3$ of the 37 kDa protein with increasing concentrations of DL-IP_3S_3. The values are mean values from two independent experiments and expressed as a percentage of the radioactivity (c.p.m.) incorporated into the gel slice of the 37 kDa protein minus the amount incorporated into gel slices not exhibiting discrete labelling (background labelling). The '100%' value refers to the radioactivity incorporated in the absence of DL-IP_3S_3 (100% = 364 c.p.m.). (*c*) Photoaffinity labelling of purified Ins(1,4,5)P_3 3-kinase. A portion (4 μg) of protein was incubated with 5×10^{-7} M-^{125}I-$AsaIP_3$ (control, lane 1) or with addition of 8×10^{-4} M-Ins(1,4,5)P_3 (lane 2) or of 5×10^{-4} M-DL-IP_3S_3 (lane 3) as described in the Materials and methods section. Molecular masses of [^{14}C]methylated marker proteins are given in the left-hand lane.

possibility that the displacement of [^{3}H]Ins(1,4,5)P_3 binding had resulted from contamination with Ins(1,4,5)P_3. A disadvantage of the photoaffinity analogue is its greater hydrophobicity as compared with Ins(1,4,5)P_3. This may lead to altered binding characteristics in terms of additional hydrophobic forces. The greater hydrophobicity of photoaffinity derivatives of the aryl-azido type has to be dealt with in any use of these structural analogues of natural compounds [e.g. 39]. In particular in crude extracts from cells or tissues containing numerous proteins with hydrophobic sites neighboured by cationic residues unspecific labelling will be obtained besides the specific labelling of the Ins(1,4,5)P_3 binding proteins. Therefore care should be taken in stating that all proteins labelled in crude extracts with such an analogue are Ins(1,4,5)P_3 'receptor' proteins. Even the displacement of the label by relatively high concentrations of DL-IP_3S_3 or Ins(1,4,5)P_3 is not a stringent proof of such a statement, since this phenomenon could also occur in a non-specific amphipathic–cationic binding site. Hence additional proof is required, such as the demonstration of biological activity or 'specific' labelling by a structurally quite different affinity analogue, optimally one devoid of such a hydrophobic side group.

Photoaffinity labelling of ER fractions from pancreatic acini indicates the existence of three 'specifically' labelled Ins(1,4,5)P_3-binding proteins of lower M_r (49, 37 and 31 kDa respectively). For protection of ER proteins from photolabelling, a higher concentration of Ins(1,4,5)P_3 had to be used than of DL-IP_3S_3. The higher potency of DL-IP_3S_3 to protect proteins from labelling may be attributed to the metabolic stability of DL-IP_3S_3 [38,40]. Photoaffinity labelling of purified Ins(1,4,5)P_3 3-kinase was also more effectively inhibited by DL-IP_3S_3 than by Ins(1,4,5)P_3. The obvious binding of DL-IP_3S_3 to Ins(1,4,5)P_3 3-kinase contrasts with the observation of Taylor *et al.* [38] that DL-IP_3S_3 does not compete with Ins(1,4,5)P_3 for phosphorylation when present in a 10-fold molar excess. This discrepancy may result from the different DL-IP_3S_3 concentrations used, since we added DL-IP_3S_3 at a 1000-fold molar excess.

The fact that the Percoll gradient fraction P_1 contains only one specifically labelled Ins(1,4,5)P_3-binding protein with an apparent molecular mass of 37 kDa and that this fraction shows the highest Ca^{2+} release induced by Ins(1,4,5)P_3 suggests that the 37 kDa protein might be involved in Ca^{2+} release. However, in spite of the apparent absence of the 37 kDa protein in fraction P_2, Ins(1,4,5)P_3 induces Ca^{2+} release from this fraction. We do not know the relationship between the size of the Ca^{2+} pools from which Ins(1,4,5)P_3 releases Ca^{2+} and the concentration of Ins(1,4,5)P_3 receptors in the membrane of these Ca^{2+} pools in both fractions. If the ratio of Ca^{2+} pool size to Ins(1,4,5)P_3 receptor concentration was high, minor amounts of Ins(1,4,5)P_3 receptors which might not be detected by photoaffinity labelling could exert an effect on Ca^{2+} release. The question therefore remains open as to whether the 37 kDa protein in fraction P_1 or the 49 kDa and 31 kDa proteins in P_2, or all three of them, are involved in Ins(1,4,5)P_3-induced Ca^{2+} release.

The parallelism and the shape of the displacement curves suggest that our photoaffinity analogues interact with Ins(1,4,5)P_3-specific binding sites in a competitive manner. AbaIP$_3$ and AsaIP$_3$ show the same affinity to these Ins(1,4,5)P_3-binding sites as DL-IP_3S_3. Since DL-IP_3S_3 is a synthetic racemic mixture of D and L enantiomers [25] and a strict stereoselectivity of the Ins(1,4,5)P_3 receptor for the D-Ins(1,4,5)P_3 enantiomer had been shown [40], the true K_d for D-IP_3S_3 may be only 40.5 nM and not 81 nM. The affinity of DL-IP_3S_3 to the Ins(1,4,5)P_3-specific binding sites of ER from pancreatic acinar cells is similar to that described for high-affinity Ins(1,4,5)P_3-binding sites in hepatocytes (K_d = 121 nM) [40], but higher, as described for rat cerebellum (K_d = 2 μM) [41].

AbaIP$_3$ is a full agonist which mobilizes Ca^{2+} from non-mitochondrial intracellular stores in pancreatic ER only 10-fold less potently than Ins(1,4,5)P_3. This correlates well with the difference in the binding affinities (K_d values) of Ins(1,4,5)P_3 and AbaIP$_3$ for Ins(1,4,5)P_3-specific binding sites.

Our results show that the 1-phosphate group of Ins(1,4,5)P_3 can be modified with minor loss in biological activity and affinity to Ins(1,4,5)P_3-binding sites. These observations correlate with the prediction that the 1-phosphate group of Ins(1,4,5)P_3 increases the affinity for the receptor and that the *vic*-4- and -5-phosphate group are essential for Ca^{2+} release [2,20]. AbaIP$_3$ and AsaIP$_3$ therefore should be useful tools for isolation of the Ins(1,4,5)P_3 receptor proteins associated with Ca^{2+} release and should help in the investigation of the biological aspects of the Ins(1,4,5)P_3 signalling pathway.

Furthermore, these compounds are potentially important for specific labelling and structural identification of the specific Ins(1,4,5)P_3-binding sites of cellular 'receptor' proteins such as the Ins(1,4,5)P_3-sensitive Ca^{2+}-release channel protein from brain [14,42] or enzymes which metabolize this compound, such as Ins(1,4,5)P_3 3-kinase (the present results) or InsP_3 5-phosphatase.

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