Measurement of picomole amounts of any inositol phosphate isomer separable by h.p.l.c. by means of a bioluminescence assay

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An assay is described which allows the determination of the mass of any individual inositol phosphate (Ins$P$) isomer by combining a popular h.p.l.c. separation method with simple desalting, dephosphorylation and final measurement of Ins liberated using an inositol dehydrogenase–NADH-linked bioluminescence reaction. The limit of sensitivity of this assay is about 1 pmol of Ins, routinely 5 pmol. About 40 mg wet wt. of guinea pig small intestine longitudinal smooth muscle contains 5 pmol of Ins(1,4,5)P_3. For Ins(1,3,4)P_3 or Ins(1,3,4,5)P_4 slightly more smooth muscle is needed, and for major isomers of InsP_1 or InsP_2 10 mg wet wt. or less of tissue can be used. A 35 mm tissue culture plate with a confluent layer of rat fibroblasts contains about 30 pmol of Ins(1,4,5)P_3. The method was applied to the measurement of the masses of Ins1P_1, Ins4P_1, Ins(1,4)P_2, Ins(1,3,4)P_3, Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 and InsP_5. The h.p.l.c. elution profiles of radiolabelled Ins$P$s generated from [^{32}P]P_i-labelled human erythrocytes, [^{3}H]Ins-labelled cultured rat fibroblasts and [^{3}H]Ins-labelled smooth muscle fragments from guinea pig small intestine were compared with the h.p.l.c. elution profiles of their masses.

INTRODUCTION

Measurement of the changes in inositol phospholipid turnover during receptor stimulation in a wide variety of cells has implicated one isomer, Ins(1,4,5)P_3, as a putative second messenger causing the release of Ca^{2+} from internal stores of the sarcoplasmic reticulum (for reviews see Berridge, 1987; Berridge & Irvine, 1989). The isomers of inositol phosphates (Ins$P$s) which make up the metabolic cycle generating and removing this second messenger have mainly been investigated using radio-active isotopes followed by h.p.l.c. separation. Although many of the Ins$P$ isomers may serve only as metabolic intermediates (Shears, 1989), others may have modulatory properties (Morris et al., 1987) or may act as extracellular signals (Vallejo et al., 1987). When using radioactive tracers (i.e. [^{3}H]Ins or [^{32}P]P_i) to estimate changes in the amounts of Ins$P$s, tissues should be labelled to isotopic equilibrium so that changes in the radio-activity of an individual Ins$P$ reflect changes in its mass, rather than in its specific radioactivity (SA). Labelling tissues with [^{3}H]Ins requires long incubation periods which can result in decreased agonist responses [e.g. in platelets (Rittenhouse & Sasson, 1985). [^{32}P]P_i labelling results in labelled nucleotides and other phosphorylated compounds which interfere with the analysis of [^{32}P]P_i-containing Ins$P$s (Dangelmaier et al., 1986). To achieve true isotopic equilibrium it is necessary to incubate cells for several days with the radiolabel (Horstman et al., 1988) or to use Ins-depleted cells with prolonged (days) periods of incubation (Stephens et al., 1989a). These methods are only suitable for cultured cells and not for fresh tissue. Depleting tissues of Ins cause changes which alter the size of responses to agonists. Finally, since a number of phosphoinositide pools may exist (King et al., 1987; Monaco, 1987; Gascard et al., 1989) which can be preferentially labelled, the SAs of individual Ins$P$ isomers may alter upon agonist stimulation. Hence the only suitable method for determining the amounts of Ins$P$s present during receptor stimulation is to measure their masses.

Several methods for mass measurement of Ins$P$s have been

developed (for reviews see Dean & Beavan, 1989; Palmer & Wakelam, 1989; Shears, 1989). Some of these methods have been successfully applied to eluates from AG1X8 anion-exchange resin (Rittenhouse & Sasson, 1985; Shayman & Kirkwood, 1987; Shayman et al., 1987; Heathers et al., 1989), although this does not allow separation of the individual isomers of InsP_3. An enzyme-linked determination of Ins$P$s using fluorimetric measurements (Macgregor & Matschinsky, 1986) was applied to eluates from AG1X8 anion-exchange columns by Shayman et al. (1987). This technique has also been used by Stephens & Downes (1990) on eluates from h.p.l.c. after extensive desalting. Tarver et al. (1987), using an isotopic dilution assay with Ins(1,4,5)P_3 3-kinase, measured the amount of Ins(1,4,5)P_3 and deduced the amount of Ins(1,3,4)P_3 present after applying the technique of Rittenhouse & Sasson (1985), which measured total InsP_3s. Other studies have calculated the mass of Ins(1,4,5)P_3 indirectly from the SA of [^{32}P]ATP (Burgess et al., 1984; Horstman et al., 1988). A few methods have been developed which can be applied to eluates from h.p.l.c. analysis (Meek, 1986; Portilla & Morrison, 1986; Mayr, 1988; Heathers et al., 1989). Many of these methods, however, are not suitable for multi-sample analysis and are not available for routine application in the majority of laboratories. Several binding assays have been developed specifically for the measurement of the mass of Ins(1,4,5)P_3 (Bradford & Rubin, 1986; Challiss et al., 1988; Bredt et al., 1989; Palmer et al., 1989).

The separation of labelled Ins$P$s has been most successful using the h.p.l.c. method of Irvine et al. (1985) or modifications of this method (Batty et al., 1985). Peaks are sharper using this technique in the presence of phosphate than in its absence, so that the most popularly used eluate for analysis of Ins$P$s has been either ammonium formate buffered with phosphoric acid (Irvine et al., 1985) or ammonium phosphate buffers (Dean & Moyer, 1987). The measurement of the mass of Ins$P$s in eluates of this type has been very difficult, especially for the more highly phosphorylated Ins$P$s, due to the presence of high concentrations of ions, particularly phosphates. This has led to the development of laborious desalting techniques (Dean & Moyer, 1988; Stephens

et al., 1988) which are not suitable for multiple sample analysis. Portilla & Morrison (1986), using this h.p.l.c. method, circumvented the problem by using acid hydrolysis of the Ins*P*s, as dephosphorylation using alkaline phosphatase (alk.P) is inhibited by phosphate.

The method that we describe here is suitable for multiple sample analysis from h.p.l.c. eluates which contain phosphate ions or potentially any other buffer ion contaminant. It comprises three essential steps after h.p.l.c. analysis: (1) simple desalting, (2) dephosphorylation with alk.P, and (3) oxidation, reduction and measurement using an D-*myo*-inositol dehydrogenase (IDH): NADH-linked bioluminescence reaction for Ins. This method fulfils all the requirements for a non-radiometric microanalysis: it is potentially applicable to and specific for any Ins*P* isomer and it is suitable for multi-sample analysis.

MATERIALS AND METHODS

Enzymes

IDH (EC 1.1.1.18; Sigma 1-0255) and alk.P type VII-N (EC 3.1.3.1; Sigma P-2276) were purchased from Sigma Chemical Co. IDH was dissolved in 20 mM-KH_2PO_4, pH 7.0, at 500 munits/ml and stored at -20 °C in 600 μl aliquots. It was stable for at least 9 months. Batch no. 117F 6812 was used throughout the experiments described here. Each batch needed to be tested in the coupled assay to give optimal conditions of low background light emission in the absence of Ins, but with a maximum light signal in the presence of 2.5–200 pmol of Ins. Alk.P was kept at 4 °C. Before use, the ammonium sulphate solution in which it was suspended was removed by centrifugation and the protein pellet was immediately resuspended in 50 mM-Tris/HCl, pH 9.0, and used.

NADH: FMN oxidoreductase (OR; EC 1.6.8.1) and bacterial luciferase (BL; EC 1.14.14.3) (both from *Photobacterium fischeri*) were obtained from Boehringer and made up according to the manufacturer's recommendations. OR was stored wrapped in foil in 20 μl aliquots at 4 °C. BL was stored wrapped in foil in 62 μl aliquots at -20 °C. During preparation they were kept sterile on ice and were covered with foil.

Other compounds

Ins, Ins1P_1, Ins(1,4,5)P_3, carbachol, FMN, decanal, fatty-acid-free BSA, sodium pyrophosphate and Q-Sepharose (fast flow) were obtained from Sigma Chemical Co. Ins, Ins1P_1 and Ins(1,4,5)P_3 standards were made up in water and stored at -20 °C. They were stable on refreezing for at least 3 months. Portions of 100 mM-sodium pyrophosphate, (made pH 9.0 with KOH) were kept sterile at 4 °C. Decanal was stored at -20 °C as a 2 mg/ml solution in 95% ethanol (AnalaR, BDH). FMN was made up in water fresh for each batch of monitoring reagent.

NAD^+ and NADH were obtained from Boehringer. A 5 mM-NAD^+ solution was made up fresh, buffered to pH 7.0 with $NaHCO_3$, covered in foil and kept on ice throughout an assay. Portions of 7.5 μM-NADH in water were stored covered in foil at -20 °C and were used for internal standard determination.

[³H]Ins (SA 19Ci/mmol; Amersham TRK 911), [³H]Ins-(1,4,5)P_3 (SA 20.4 Ci/mmol; Amersham TRK 999) and [³²P]P_i (carrier-free; Amersham PBS 13) were obtained from Amersham International. [³H]Ins contained a PT6 tablet which absorbs radiolysis products, so that no purification step was necessary before use. Radiolysis products could not be detected using h.p.l.c.

Thrombin was obtained from Armour Pharmaceutical Company. All other chemicals were obtained from BDH and were at least AnalaR grade.

Krebs–Ringer bicarbonate (KRB) buffer solution was gassed throughout with O_2/CO_2 (19:1) and was of the following composition (final, mM): NaCl, 120; KCl, 5.9; $NaHCO_3$, 15.4; NaH_2PO_4, 1.2; glucose, 11.5; $MgCl_2$, 1.2; $CaCl_2$, 2.5.

Distilled deionized water was further purified using a MilliQ reagent-grade water purification system and was used throughout all procedures. All stored solutions were sterilized by passing through a 0.2 μm-pore-size flowpore filter unit. All tubes and bottles were acid-washed (500 mM-HCl, filtered), MilliQ-rinsed and autoclaved where possible. This was to avoid high and variable backgrounds due to dust particles and micro-organisms (Stanley, 1974). Berthold bioluminometer tubes were rinsed and dried just before use (not suitable for autoclaving).

A Berthold Biolumat 9500 luminescence analyser using 12 mm × 47 mm cuvettes (Berthold) was used, linked to a BBC microcomputer.

Labelling of guinea pig small intestine

A female guinea pig (250–350 g) was killed by cervical dislocation and exsanguination. Longitudinal smooth muscle strips (approx. 1 g original wet wt., equivalent to approx. 25 mg of protein) were removed (using a method similar to that of Paton & Zar, 1968) and placed in pre-gassed KRB at 37 °C. After three rapid washes in fresh KRB, 400 μm fragments were made by cross-chopping the strips using a McIlwain tissue chopper. The fragments were transferred to a clean pot containing 10 ml of KRB, gassed, capped and incubated at 37 °C for 20 min. After this the fragments were washed once with 10 ml of KRB and then incubated for 3 h at 37 °C in 1 ml of KRB containing 50 μCi of [³H]Ins (SA 19 Ci/mmol, which therefore contains 50 μM unlabelled Ins), with gassing every 20 min. At the end of 3 h, excess KRB was removed and the fragments were washed with 4 × 10 ml of fresh KRB. The smooth muscle fragments were resuspended in fresh KRB and divided into 250 μl portions to give approx. 2.5 mg of protein/tube (about 100 mg of tissue/tube). The effects of carbachol were studied at 37 °C by the rapid addition of 250 μl of KRB and 200 μM-carbachol (100 μM, final). A 500 μl aliquot of ice-cold 20% (w/v) trichloroacetic acid was added 5 s later. EDTA (100 mM, 50 μl) was then added to the tubes and they were vortex-mixed and placed on ice for 20 min. Tubes were centrifuged at 4000 g for 10 min. The supernatant was removed and the trichloroacetic acid was extracted by the addition of 4 × 5 ml water-saturated diethyl ether; the residual ether was removed using N_2 and the pH was adjusted to 7.0 with 1 M-NaOH. The pellet was stored for protein determination, which was carried out using the method of Lowry *et al.* (1951). Samples were assayed by h.p.l.c. within 7 days. In order to obtain 5 pmol of Ins(1,4,5)P_3 approx. 40 mg of tissue (wet wt.) was required. This is similar to that required for radioactive isotope experiments. However, because we wished to compare the elution profiles of radioactivity (d.p.m.) and mass from h.p.l.c. runs, for these experiments it was necessary to use 400 mg (wet wt.).

Labelling of human erythrocytes

[4-³²P]Ins(1,4)P_2 ([³²P]InsP_2) and [4,5-³²P]Ins(1,4,5)P_3 ([³²P]InsP_3) were prepared from [³²P]P_i-labelled human erythrocyte membranes essentially as described by Downes & Michell (1981). Briefly, 60 ml of packed erythrocytes was labelled for 3 h at 37 °C with 3 mCi of [³²P]P_i, followed by lysis and thorough washing to remove contaminating haemoglobin. The [³²P]P_i-labelled erythrocyte ghosts were then incubated in the presence of 1 mM-Ca^{2+} at 37 °C for 30 min, thus activated phospholipase C to produce [³²P]InsP_2 and [³²P]InsP_3. After removal of the membranes the labelled Ins*P*s were separated from each other

using either (1) elution with a solution of LiCl from an AG1X8 anion-exchange column (chloride form) followed by freeze-drying and ethanol extraction to remove the LiCl, or (2) elution from Q-Sepharose columns using HCl followed by freeze-drying to remove the acid. $[4,5-^{32}P]Ins(1,3,4,5)P_4$ ($[^{32}P]InsP_4$) was prepared from $[^{32}P]InsP_3$ by incubation with a 2% (w/v) homogenate of rat brain, essentially as described by Irvine *et al.* (1986), except that 10 mM-2,3 bisphosphoglycerate was included.

Labelling of cultured fibroblasts

Rat fibroblasts were prepared as previously described (Freshney, 1983). Cultures were maintained in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum for 4 days when confluence was reached. Cultures were then passaged and maintained for a further week on 35 mm-diam. dishes in a medium containing 70 μCi of $[^3H]Ins/ml$. On the day of the experiment cultures were washed three times with KRB and either incubated with KRB or stimulated for 60 s with 1 unit of thrombin/ml. After 60 s the incubation medium was discarded and 1 ml of ice-cold 10% (w/v) trichloroacetic acid was added to stop the reaction. Samples were then processed in the same way as the smooth muscle fragments. The mass determination was made on a stimulated sample using three 35 mm culture dishes, equivalent to 2.3 mg of protein.

H.p.l.c. analysis

H.p.l.c. analysis was performed as previously described (Irvine *et al.*, 1985; Batty *et al.*, 1985), as modified by Salmon & Bolton (1988). Eluate from the h.p.l.c. was collected in 120 0.5 ml fractions using an Anachem 232 fraction collector. The fractions were then divided as follows: 150 μl of each fraction was removed and 3 ml of Hisafe 3 scintillant (LKB) was added. Radioactivity was determined using a Beckman LS1701 scintillation counter and, after scaling according to their protein content, the elution profiles of the InsPs containing $[^3H]Ins$ were plotted. For mass determination, 300 μl of each fraction was removed and 3 ml of water was added. These samples were either processed immediately or stored at -20 °C ready for desalting.

Measurement of $InsP_1$ isomers

Preparation for the mass determination of $InsP_1$ isomers was different from that of the other InsPs, because Q-Sepharose does not adequately separate them from P_1; thus not allowing proper desalting with high recovery. Therefore 300 μl fractions containing $InsP_1$ isomers were collected, diluted to 3 ml with water and freeze-dried to remove ammonium formate. They were then reconstituted in 300 μl with water, and 100 μl aliquots were removed and incubated with 150 units of alk.P/ml at 37 °C for 48 h in 200 mM-Tris/HCl, pH 7.0 (these are higher concentrations of Tris and alk.P than required for the other InsPs because a full desalting protocol was not adopted here). Standards using $Ins1P_1$ (Sigma) were made in eluate from an h.p.l.c. run without loading a sample on to the column. After boiling (to destroy the alk.P) and centrifugation, the samples were freeze-dried, reconstituted in 130 μl of water and assayed in duplicate. If standard curves were constructed in 200 mM-Tris/HCl, pH 7.0, (alone with no blank h.p.l.c. eluate added) followed by freeze-drying and reconstitution, the monitoring reagent was considerably inhibited, lowering the sensitivity of the assay. The phosphate in the h.p.l.c. eluate served to buffer the Tris and prevent it quenching the sample, since the freeze-drying step removed the HCl but not the Tris.
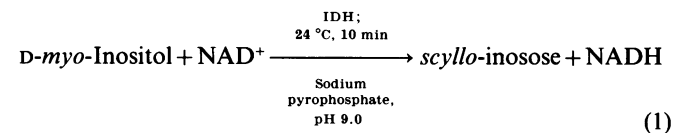
Measurement of other $InsP$ isomers

Desalting. $InsP_2$ or 'higher' InsPs were desalted as follows. Samples were loaded on to 10 ml Econocolumns (Bio-Rad, 10 cm) containing 0.2 ml of Q-Sepharose which had first been regenerated as follows: (1) 5 mls of 1 M-NaOH; (2) 2×10 ml of water; (3) 5 ml of 1 mM-EDTA; (4) 10 ml of water; (5) 4 ml of 20% (v/v) ethanol; (6) 10 ml of water; (7) 6 ml of 1 M-HCl; (8) 10 ml of water. All solutions, with the exception of NaOH and ethanol, were filtered through a non-sterile 0.2 μm Millipore filter before use. Steps 5–8 were carried out immediately before sample application in order to obtain reproducible high recoveries of the samples. After washing the columns with 2×10 ml of 3.75 mM-HCl for $InsP_2$, 5 mM-HCl for $InsP_3$ and 7.5 mM-HCl for $\geqslant InsP_4$, the InsPs were then eluted from the Sepharose Q with 4 ml of 150 mM-HCl. Samples were freeze-dried to remove the HCl, followed by transfer to 1.9 ml Microfuge tubes in a volume of 750 μl of water. After further freeze-drying they were ready for dephosphorylation using alk.P. The HCl concentration was low enough to prevent hydrolysis or acid migration and to allow its removal by freeze-drying (Mayr, 1988). Standard curves using Sigma $InsP_3$ in eluate from h.p.l.c. were processed at the same time as the samples. Blanks were also run at the same time from a run collected from the h.p.l.c. without a sample loaded on to the column.

Dephosphorylation. Samples were incubated in 70 μl of 50 mM-Tris/HCl, pH 9.0, containing 142 units of alk.P/ml for 24 h at 37 °C. Following this, the tubes were centrifuged, boiled for 5 min to destroy the enzyme activity and recentrifuged (to remove liquid condensed on the caps), followed by freeze-drying. Standards run under the same conditions as the samples were completely dephosphorylated under these conditions. The same standards were completely dephosphorylated in the absence of residual phosphate within 2 h of incubation using the above conditions (results not shown). The presence of Mg^{2+} in the incubation did not alter the time course for the alk.P reaction. When aliquots from the $InsP_1$ region of the h.p.l.c. profile were dephosphorylated with alk.P a precipitate was formed if Mg^{2+} was present, and Mg^{2+} was therefore not included.
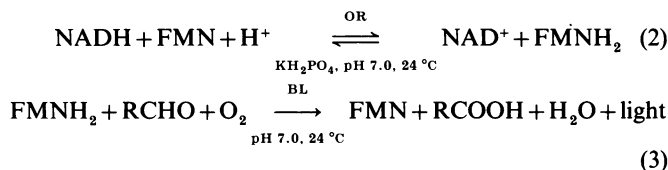
Coupled assays

Ins oxidation. Catalytic oxidation of Ins occurs in the presence of NAD^+ by the enzyme IDH (Weissbach, 1974) as follows:

$$\text{D-}myo\text{-Inositol} + NAD^+ \xrightarrow[\substack{\text{Sodium} \\ \text{pyrophosphate,} \\ \text{pH 9.0}}]{\substack{\text{IDH;} \\ \text{24 °C, 10 min}}} scyllo\text{-inosose} + NADH \quad (1)$$

Samples were reconstituted in 65 μl of water and vortex-mixed, and 60 μl was transferred to a bioluminometer tube (since there was enough Ins present from the original InsP, samples could be made up to 65μl; however, if the level of InsP is very low samples can be transferred to the bioluminometer tube in 150 μl of water, freeze-dried and reconstituted to 60 μl and assayed directly from the tube). The assay was carried out as follows: 25 μl of 100 mM-sodium pyrophosphate, pH 9.0, was added to each tube. The reaction was then initiated by the addition of 10 μl of 5 mM-NAD^+ and 5 munits of IDH in 10 μl (total sample volume 105 μl). Samples were incubated for 10 min at room temperature (24 ± 1 °C). Standards containing Ins (2.5–200 pmol) were assayed at the same time and compared with $InsP_3$ standards that had been batch-processed along with the samples. Standards were measured throughout the assay to monitor the activity of

the IDH. The NADH generated by the action of IDH was assayed as follows.

NADH-coupled bioluminescence assay. OR coupled to BL produces light during the catalytic oxidation of NADH in the presence of FMN, decanal and O_2 (Hastings & Nealson, 1977), according to the following reactions:

$$NADH + FMN + H^+ \underset{KH_2PO_4, \ pH \ 7.0, \ 24 \ ^\circ C}{\overset{OR}{\rightleftharpoons}} NAD^+ + FMNH_2 \quad (2)$$

$$FMNH_2 + RCHO + O_2 \overset{BL}{\underset{pH \ 7.0, \ 24 \ ^\circ C}{\longrightarrow}} FMN + RCOOH + H_2O + light \quad (3)$$

where RCHO is a long-chain aliphatic aldehyde (decanal) and RCOOH is the corresponding carboxylic acid (decanoic acid). The $FMNH_2$ produced from reaction (2) is utilized by BL in reaction (3) to produce light. At low concentrations of OR, the consumption of NADH is slow and consequently the $FMNH_2$ is supplied for light formation over a period of minutes (Lövgren et al., 1982; Lavi et al., 1983), so that the signal rises to a plateau which is well maintained without decay during the period of measurement. If the concentration of OR is too high, the signal reaches a peak and then decays. However, decay is not produced by high concentrations of BL (Lövgren et al., 1982). The light intensity is affected by the concentrations of both OR and BL. If reaction (3) is too slow, all of the free $FMNH_2$ will be oxidized by the O_2 dissolved in the monitoring reagent (dark reaction) and no photons will be produced (Hastings & Nealson, 1977). If contaminants of the sample cause quenching by a decrease in the photon output due to the slowing of the rate of reaction (3), then increasing the concentration of BL can overcome this. Eqns. (2) and (3) are simplified to illustrate the general reaction. A chain of intermediate reactions occurs which results in the formation of an enzyme–flavin–aldehyde complex which decays with the emission of light (Meighen & Mackenzie, 1973; Hastings & Nealson, 1977). The aldehyde is required for the light reaction to take place. Increasing the FMN concentration caused a decrease in light output due to the yellow colour of the FMN absorbing the increased amounts of emitted light. The reagents are not inactivated during the total measurement period, which allows the light signal produced by a NADH internal standard to be used to calibrate the first response. Repeated additions of NADH produce the same responses.

The sample was placed into the chamber of a Berthold Biolumat LB 9500 which was linked to a BBC microcomputer. A simple program was written for automatic calculation of the results. At 10 min after beginning the IDH reaction, 100 μl of monitoring reagent was added to initiate the final bioluminescence assay (Fig. 1). A 5 s delay in measurement of photons was introduced to allow for mixing, a pH change from 9.0 to 7.0, and a temperature change, since the 105 μl from the IDH reaction was at 24 °C and 100 μl of monitoring reagent was at 0 °C. The intensity of light emission was then integrated over a 40 s period, at which time 150 pmol of NADH was added in 20 μl to the assay tube and a further 30 s integration of photons was recorded. Using this protocol it was possible to assay 36 samples/h.

The monitoring reagent solution contained the following (final concentrations in 100 μl, therefore 2.05 × final assay concentrations): 4 μM-FMN, 40 μM-decanal, 6.25 mg of BL/ml, 10 units of OR/litre, 0.212 M-KH_2PO_4 and 0.1 % fatty-acid free BSA. Once made up, the reagent was kept on ice, covered in foil and left for 30 min before use. It was stable for at least 1 month at 4 °C. Since the reagent was not sterile at this stage, growth of
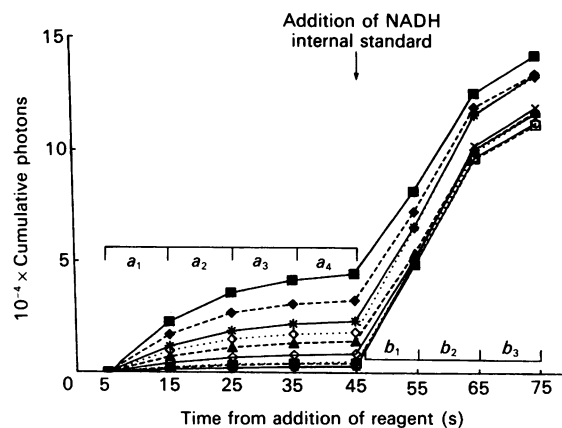

Fig. 1. **Measurement of photons generated from standards in the presence and absence of Ins using the bioluminescence assay**

Photon emission is shown after addition of monitoring reagent (at zero time) to samples containing either no Ins or 2.5–200 pmol of Ins. After a 10 min incubation at 24 ± 1 °C in the presence of NAD^+, sodium pyrophosphate and IDH, the sample was placed into the chamber of a bioluminometer and monitoring reagent was added. After a 5 s delay to allow for mixing, temperature change and pH change, photon generation was monitored and counted over four successive periods. At 45 s after the addition of the reagent, the NADH internal standard was added and a further three periods of 10 s were recorded. [Ins] (pmol): ×, 0; ○, 2.5; □, 5; ●, 10; △, 25; ▲, 50; ◇, 75; ✳, 100; ◆, 150; ■, 200. a_1–a_4 and b_1–b_3 are defined in the text.

bacteria probably determined its stability. The presence of bacteria caused the background photon levels to increase dramatically and the monitoring reagent could not be used. At room temperature the reagent lost at least 40 % of its activity in 4 h. This was seen as a lowered photon output due to the instability of the reagents at room temperature.

Calculation of results

Total photons (A) emitted over four successive 10 s periods (a_1, a_2, a_3 and a_4) were recorded and summed (i.e. $A = a_1 + a_2 + a_3 + a_4$). The total photons ($B$) emitted over the three successive 10 s periods in the presence of 150 pmol of NADH internal standard were recorded and summed. The final 10 s period before the addition of NADH was multiplied by three and subtracted from B; this gave the photons due to the presence of NADH only (C) (see Fig. 1), i.e. $C = B - 3a_4$. The photon emission due to the sample (V) was expressed in pmol equivalents of NADH, i.e. $V = 150A/C$. This quantity, V, was plotted against the concentration of added Ins in the standards (either from Ins or from InsP-derived Ins; see Fig. 2). A linear relationship was obtained under the above conditions from 2.5 to 200 pmol of Ins (Fig. 2). Samples were diluted to fall within this range and values for Ins were read off the linear regression curve fitted by the method of least squares of deviations about the line.

Losses from the h.p.l.c. step to the end of the assay were monitored by scintillation counting of labelled samples after the addition of 3 ml of Hisafe 3 scintillant to the sample at the end of the bioluminescence assay. Any residual bioluminescence did not interfere with the counting. The label used was [³H]Ins so that the measurement of d.p.m. would at least reflect InsP-containing compounds, and co-eluting compounds would not be detected in the d.p.m. profile. However, using [³H]Ins label which has not been purified, or storing labelled samples for a long period of time before analysis, is known to cause the appearance of radiolysis products which co-elute with InsPs (Bielkiewicz-Vollrath et al., 1986). Background radioactivity was determined
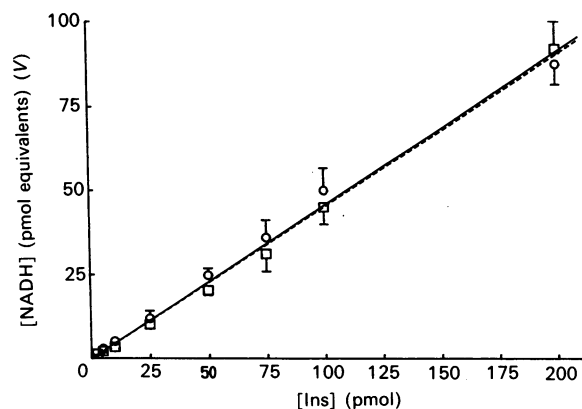
Fig. 2. Standard curve obtained using the bioluminescence assay for Ins

Standards of Ins(1,4,5)P_3 (5–200 pmol) were prepared in exactly the same way as the samples (see the Materials and methods section). The Ins from these samples was measured in the IDH:NADH-coupled bioluminescence assay after alk.P treatment (○, solid line) ($n = 5$–15). Standards curves with Ins were assayed at the same time for comparison (□, broken line) ($n = 7$–17). The photons produced were expressed as [NADH] (pmol equivalent), and Ins(1,4,5)P_3 was expressed as Ins added. The method corrects for any quenching of the signal by the addition of a known amount of NADH to act as an internal standard. The standard curve was generated after background photon subtraction. A linear relationship between Ins concentration (2.5–200 pmol) and the number of emitted photons was seen.

for each experiment and subtracted. Also, the h.p.l.c. column was washed at the beginning of each run, ensuring the removal of any residual [³H]Ins which might contribute to background radioactivity. Such [³H]Ins, and any unlabelled Ins, will not interfere with the mass determination, as it will not be retained on a Q-Sepharose column when the samples are desalted. However, if present, residual [³H]Ins will be washed off, resulting in an apparent lowering of the recovery of the sample as estimated from radioactivity measurements. For experiments where tissue was not incubated with label, a known quantity of a [³H]Ins$P$ standard was added to the samples for recovery determinations. Such [³H]Ins$P$ standards (Amersham) are of a high enough SA to allow sufficient radioactivity to be added to each sample in less than 1 pmol of unlabelled Ins$P$. Standards prepared from the human erythrocytes are not of high enough SA for use here (see Table 1a).

Since this assay measures single photon events, other sources of light can interfere and lead to high and variable background counts (Stanley, 1974). All tubes and solutions were kept away from direct sunlight and fluorescent lights to minimize phosphorescence. The assay was performed in a darkened room lit only by tungsten lights.

Statistics

Differences in mass, d.p.m. or SA for the three Ins$P$ isomers from erythrocyte membranes were tested for significance using an unpaired Student's t test. To test for significance, a paired t test was applied to pair differences between control and stimulated d.p.m., mass values and SAs obtained from smooth muscle fragments.

RESULTS AND DISCUSSION

Three different cell systems were used: (1) human erythrocyte membranes, (2) cultured rat fibroblasts, and (3) fragments of longitudinal muscle from the guinea pig small intestine.

[³²P]P$_i$-labelled human erythrocyte membranes

Two [³²P]P$_i$-labelled Ins$P$ species, namely Ins(1,4)P_2 and Ins(1,4,5)P_3, which can be clearly resolved without significant contamination by other compounds, can be generated from [³²P]P$_i$-labelled human erythrocytes. In addition to these two standards, a further standard can be obtained indirectly from the erythrocytes. Ins(1,3,4,5)P_4 was produced from Ins(1,4,5)P_3 after incubation with a rat brain homogenate as the source of the 3-kinase enzyme (Irvine et al., 1986). These relatively pure standards were therefore suitable for comparisons between the measurements of labelled and unlabelled Ins$P$s.

Profiles of radioactivity from the h.p.l.c. analysis for the [³²P]P$_i$-labelled standards revealed three well-resolved peaks (Fig. 3). When aliquots of these fractions from h.p.l.c. were taken and analysed using the procedure for mass determination, peaks of InsP_2, InsP_3 and InsP_4 were obtained, which corresponded very well with the [³²P]P$_i$ d.p.m. profile from the same membranes. Between peaks, radioactivity was at background levels and Ins assay measurements were close to blank values. The results shown in Fig. 3 represent the means ± s.e.m. of individual fractions for each Ins$P$ isomer determined on three to five separate occasions. In additional experiments, however, peaks from h.p.l.c. were pooled after Čerenkov counting and one radioactivity and one mass value was calculated for each isomer. Thus the values in Table 1(a) represent the means ± s.e.m. for five to seven determinations. Under conditions of isotopic equilibrium it would be expected that the SA of InsP_3 would be twice that of InsP_2. Under similar conditions to ours, but after labelling for 2 h, Downes et al. (1982) calculated an SA for InsP_3 of 1.37 times that of InsP_2, whereas we obtained a value of 1.54. This result compared well, as we labelled for 3 h. It can be seen from the values obtained for the SA (Table 1a) that these Ins$P$ isomers are not of high enough SA for use as standards for spiking samples for recovery determinations in the bioluminescence assay, but higher-SA Amersham standards can be used.

Fibroblast cultures

Measurements of Ins$P$ isomer masses were made in a stimulated sample in one experiment only so that a comparison could be made of the mass and d.p.m. measurements in a cultured cell system.
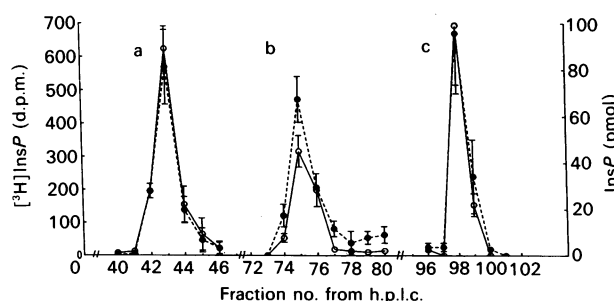

Fig. 3. Comparison between the profiles of mass and radioactivity obtained for Ins(1,4)P_2 (peak a), Ins(1,4,5)P_3 (peak b) and Ins(1,3,4,5)P_4 (peak c) from human erythrocytes after h.p.l.c. analysis

Human erythrocyte membranes were labelled with ³²P using the protocol of Downes & Michell (1981). After stimulation with Ca²⁺, Ins(1,4)P_2 and Ins(1,4,5)P_3 were obtained using an AG1X8 column. Ins(1,4,5)P_3 was converted to Ins(1,3,4,5)P_4 by incubation with rat brain homogenate (Irvine et al., 1986). A mixture of these isomers was applied to an h.p.l.c. column. Aliquots from the fractions collected were counted for radioactivity (●). A second series of aliquots from these samples was analysed for mass determination (○). The results are expressed as the means ± s.e.m. for 3–5 determinations.

Table 1. Mass, d.p.m. and SA values obtained from erythrocytes, cultured fibroblasts and smooth muscle tissue

(a) Mass, d.p.m. and SA values for three standards generated from human erythrocyte membranes. Results are expressed as means ± S.E.M. for 3–7 determinations. There were no significant differences ($P > 0.05$) in the mass, d.p.m. or SA between any of the three isomers analysed (by unpaired Student's t test). (b) Mass, d.p.m. and SA values from a single experiment using cultured rat fibroblasts. Fibroblasts were stimulated with 1 unit of thrombin/ml for 60 s at 37 °C (see the Materials and methods section for details). Mass determinations were made only on the stimulated samples for comparison with other types of cell systems. The control d.p.m. values (but not mass values) were also measured (results not shown). Radioactivity was increased for the four isomers measured upon stimulation with thrombin. (c) Mass, d.p.m. and apparent SA values for InsP from control and stimulated guinea pig smooth muscle fragments. Results are expressed as the means ± S.E.M. for 5–6 determinations of the amounts of various InsP isomers/mg of protein: this corresponds to about 40 mg wet wt. of tissue. C, control; S, stimulated for 5 s with 100 μM-carbachol at 37 °C. Tests of significance were made on mean pair differences (stimulated − control) of results on each guinea pig: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Other results were not significant.

		Mass (pmol)	Radioactivity (d.p.m.)	SA (mCi/mmol)
(a) Human erythrocyte membranes				
Ins(1,4)P_2		179 ± 20	1000	2.8 ± 0.29
Ins(1,4,5)P_3		125 ± 21	1000	4.3 ± 0.75
Ins(1,3,4,5)P_4		135 ± 21	1000	3.6 ± 0.58

		Mass (pmol/mg of protein)	Radioactivity (d.p.m./mg of protein)	Apparent SA (ml/mmol)
(b) Cultured fibroblasts – stimulated with thrombin				
Ins(1,4)P_2		284	1075	1.72
Ins(1,3,4)P_3		95	111	0.53
Ins(1,4,5)P_3		38	325	3.95
InsP_4		54	57	0.48
InsP_5		36	235	2.93
(c) Guinea pig small intestine fragments				
Ins1P_1	C	79 ± 17	1005 ± 215	7.2 ± 1.4
	S	62 ± 14	1139 ± 217**	9.5 ± 1.7
Ins4P_1	C	40 ± 9	483 ± 56	9.8 ± 2.6
	S	29 ± 4	992 ± 198*	18.0 ± 2.3*
Ins(1,4)P_2	C	31 ± 7	869 ± 201	15.0 ± 3.2
	S	164 ± 17***	3104 ± 524**	9.0 ± 1.2
InsP_2	C	13 ± 2	133 ± 24	6.0 ± 0.6
	S	12 ± 2	160 ± 29	7.0 ± 1.0
Ins(1,3,4)P_3	C	5.2 ± 1.5	30 ± 6	3.7 ± 0.7
	S	6.9 ± 1.1	116 ± 20**	8.0 ± 1.4*
Ins(1,4,5)P_3	C	7.4 ± 0.8	212 ± 32	13.0 ± 2.1
	S	14.0 ± 1.6**	785 ± 168**	27.0 ± 4.4**
InsP_3	C	2.4 ± 0.6	22 ± 5	6.1 ± 1.8
	S	1.5 ± 0.2	39 ± 6*	11.4 ± 1.6
Ins(1,3,4,5)P_4	C	13.0 ± 1.7	141 ± 25	7.7 ± 3.5
	S	14.0 ± 0.7	196 ± 39*	6.0 ± 1.5

Experiments on cultured cells allow longer incubation periods with the isotope and therefore provide a better chance of obtaining isotopic equilibrium. However, if the cells are incubated in a culture medium such as Dulbecco's modified Eagle's medium which contains approx. 50 μM-Ins, then inadequate labelling of the Ins pools may result, despite several days' incubation with label, and for this reason cultured cells which have been depleted of Ins have recently been used in an attempt to label to equilibrium all of the Ins lipid pools (Stephens et al., 1989a). The values of pmol/mg of protein and d.p.m./mg of protein for five InsP

isomers from a thrombin-stimulated fibroblast culture experiment are shown in Table 1(b).

Peaks of ^{3}H radioactivity which co-eluted with Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 standards, and peaks assumed to be Ins(1,3,4)P_3 and InsP_5, were studied. The 'apparent' SAs of the various InsP peaks varied widely (Table 1b), probably because each contained several isomers. The apparent SAs of Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 were much lower than those of other InsPs and quite similar to each other. The apparent SA of InsP_5 was surprisingly high and approached that of Ins(1,4,5)P_3. The differences in apparent SAs were not investigated further. More than one peak was detected by mass measurement in the InsP_4 and InsP_5 regions, but only one peak in each region was [^{3}H]Ins-labelled; only labelled peaks were used for calculation of SAs. The labelled InsP_4 peak was co-eluted with the Ins(1,3,4,5)P_4 standard.

Small intestine smooth muscle fragments

The main thrust of these experiments was to measure the mass of Ins-containing compounds and to show that the elution profile of these was close to the profile of ^{3}H-labelled Ins-containing compounds. Some discrepancies were apparent, some of which seemed to arise due to combination of several h.p.l.c. runs which showed some variations in peak shape. At this stage minor apparent anomalies were not pursued in detail.

Peaks of ^{3}H radioactivity which were co-eluted with Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 standards were studied in fragments after 5 s of stimulation with carbachol (0.1 mM) and without such stimulation (control) (Fig. 4, Table 1c). A peak of ^{3}H radioactivity eluting just before Ins(1.4,5)P_3 and usually assumed to be Ins(1,3,4)P_3 was also studied. Fractions containing [^{3}H]InsP_5 and [^{3}H]InsP_6 isomers were not collected in these experiments. The h.p.l.c. eluate was collected in 120 0.5 ml samples. Two aliquots were taken from each sample: 150 μl was
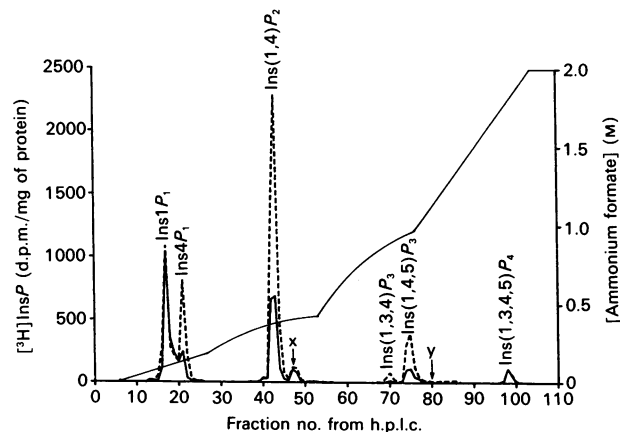

Fig. 4. A typical h.p.l.c. separation of [^{3}H]Ins-containing InsPs from control and carbachol-stimulated smooth muscle fragments

After a 3 h incubation with [^{3}H]Ins followed by washing to remove the excess label, the fragments were divided into two equal portions; one portion was stimulated for 5 s at 37 °C with 100 μM-carbachol (broken line), and the other portion acted as a control (solid line). The reaction was stopped with trichloroacetic acid and, after four washes with diethyl ether to remove the acid, samples were neutralized and stored ready for h.p.l.c. analysis. H.p.l.c. analysis was performed on a Partisal SAX anion-exchange column using an ammonium formate buffer, pH 3.7, containing phosphoric acid (see the text for full details). The positions at which various isomers are assumed to elute, determined by the use of standards in most cases, are shown. For x and y, see the text.
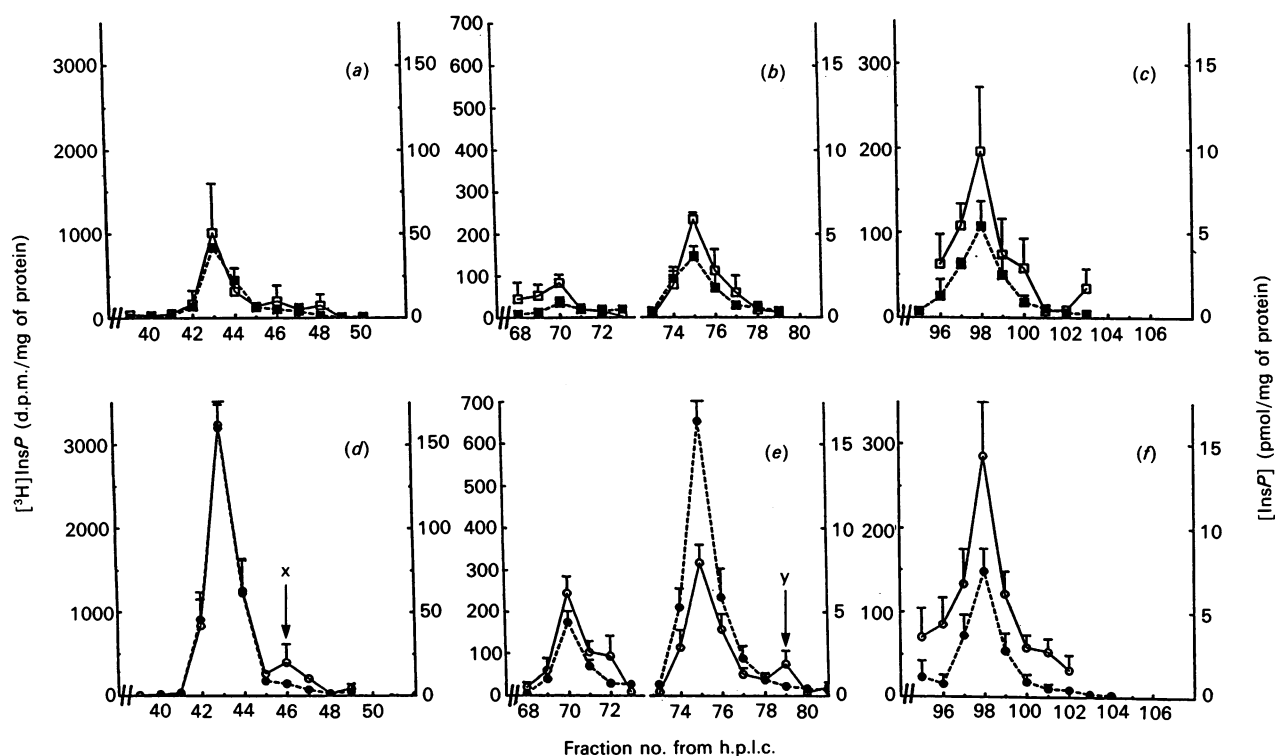
Fig. 5. Comparisons between mass and radioactivity of InsPs in control and stimulated smooth muscle fragments after h.p.l.c. analysis

Aqueous extracts of InsPs were generated from smooth muscle fragments which had been incubated with [³H]Ins, followed by a 5 s incubation without drug or a 5 s stimulation with carbachol (see the text for details of tissue preparation and assay conditions). The eluate from h.p.l.c. was collected and the samples were divided and assayed for both their d.p.m. profile and their mass profile. (a)–(c) show the profiles per mg of protein without stimulation; □, mass; ■, d.p.m. (d)–(f) show the profiles after a 5 s stimulation with 100 μM-carbachol; O, mass; ●, d.p.m. Shown are the profiles for Ins(1,4)P_2, (a, d), Ins(1,3,4)P_3 and Ins(1,4,5)P_3 (b, e) and Ins(1,3,4,5)P_4 (c, f). The peaks marked x and y are referred to in the text. Results are expressed as the means ± S.E.M. for 3–6 determinations. These experiments were done not to demonstrate the sensitivity of the method but to compare the elution profiles of the mass and radioactivity of InsPs.

used to determine the profile of d.p.m. in [³H]Ins-containing compounds, and 300 μl was used to determine the profile of the mass of Ins in Ins-containing compounds. In both control (Figs. 5a–5c) and stimulated (Figs. 5d–5f) smooth muscle fragments it was found that the profiles of d.p.m. and mass were similar. Between the peaks of radioactivity or of Ins-containing compounds measurements were generally at background levels, although some unlabelled InsP peaks were noted but not investigated.

By the use of standards, peaks eluting with Ins1P_1, Ins(1,4)P_2, Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were identified. However, it is uncertain to what extent these peaks are contaminated with other InsP isomers; for convenience these peaks were designated by the names of the InsP isomer which was co-eluted with them. Experiments were not designed to reveal changes in SA, which would require that h.p.l.c. peaks were pure single InsP isomers.

For further experiments, fractions containing radioactivity corresponding to Ins1P_1, Ins4P_1, Ins(1,4)P_2, an unidentified InsP$_2$ (marked x in Figs. 4 and 5d), Ins(1,3,4)P_3, Ins(1,4,5)P_3, an InsP$_3$ eluting immediately after the Ins(1,4,5)P_3 peak (marked y in Figs. 4 and 5e) and Ins(1,3,4,5)P_4 were collected after elution from h.p.l.c. After removal of an aliquot for radioactivity determination, fractions corresponding to each peak were then pooled and further analysis performed for mass determination (see Table 1c). Blank (containing no InsPs) h.p.l.c. runs, collected at the same time as samples from a tissue, were analysed and fractions corresponding to the positions of InsPs were also pooled and analysed along with the samples for background determinations.

There was an overall increase of 1.6-fold in the total mass of

all InsPs upon stimulation [control, 193 ± 0.4 pmol/mg of protein (means ± S.E.M., n = 6); carbachol, 301 ± 32 pmol/mg of protein] and a 2.7-fold increase in the total InsP d.p.m. (control 1978 ± 292 d.p.m./mg of protein; carbachol 5417 ± 969 d.p.m./mg of protein). For the masses the ranges of values were 107–278 pmol/mg of protein (control) and 200–431 pmol/mg of protein (carbachol). For d.p.m. the ranges of values were 1239–2900 d.p.m./mg of protein (control) and 2679–9200 d.p.m./mg (carbachol). The range of variation of values for replicates within a guinea pig experiment was very small for both the control and stimulated fragments, and thus significant differences between control and stimulated values were apparent using paired t test analysis. Thus statistical variation between guinea pigs was greater than that within guinea pigs.

Stimulation with carbachol resulted in significant (P < 0.05) increases in the mass and d.p.m. values for Ins(1,4)P_2 and Ins(1,4,5)P_3 (Table 1c). However, Ins1P_1, Ins4P_1, Ins(1,3,4)P_3, InsP$_3$ and Ins(1,3,4,5)P_4 all increased significantly in their d.p.m. values upon stimulation, without significant changes in their masses (Table 1c). There was a large rise in the mass of Ins(1,4)P_2 with little change in its apparent SA; however, the apparent SA of Ins(1,4,5)P_3 approximately doubled, which does not support the idea that Ins(1,4,5)P_3 is the sole precursor (not necessarily direct) of Ins(1,4)P_2. The apparent SAs of Ins(1,3,4,5)P_4 and Ins(1,3,4)P_3 were less than that of Ins(1,4,5)P_3, and the apparent SA of Ins(1,3,4,5)P_4 did not increase upon stimulation.

The variation in SAs indicates that the tissues had not been incubated with the label to isotopic equilibrium. Since our primary intention was to develop a versatile method to measure any InsP isomer, further investigation into the changes in SA was

not attempted. The results are open to several interpretations. It is likely that pools exist which have not all achieved the same degree of labelling and which have different turnover rates (King *et al.*, 1987). It is also possible that other isomers are present in some of the Ins*P* peaks and these do not change upon stimulation (Stephens *et al.*, 1989*b*). The latter would account for the alteration in the SA upon stimulation of some but not other Ins*P*s. Discrepancies between the increase in mass and increase in d.p.m. content upon stimulation have been noticed by several other workers in different tissues, e.g., bradykinin on renal papillary collecting tubule fragments, (Shayman & Kirkwood, 1987) and carbachol on rat cortical slices (Challiss *et al.*, 1988).

Measurement of mass

Accurate measurement of mass and radioactivity depends on a number of factors in a multistep assay, and failure to take any one of these into account will lead to an inaccurate estimation of either or both the mass and the radioactivity, leading to an error in the SA measurement. Purity of peaks is essential for SA measurement, and this will depend on the h.p.l.c. system used. Numerous other checks on the methods used to determine the d.p.m. and mass were also made. These checks included proper background determination, measurement of recoveries at each step and checking for the absence of any interfering compounds.

Absence of interfering compounds. Several tests were performed which showed that compounds co-eluting with the Ins*P* isomers do not interfere with the mass determination. Samples from two experiments in which smooth muscle fragments were stimulated with carbachol for 5 s were analysed by h.p.l.c. and the corresponding fractions containing Ins*P* isomers were pooled and divided (to achieve a normal but equal concentrations of Ins*P* isomers in each pair of samples). To one sample of each pair, 25 pmol of Ins was added just before the bioluminescence assay. Measured Ins was 92.4% of that added. In separate experiments performed as described above, 25 pmol of Ins*P*$_3$ was added just before the alk.P step and 93.3% of this could be detected in the bioluminescence assay. A further experiment was performed in which the tissue samples, after trichloroacetic acid treatment, were pooled and divided into two equal portions; 100 pmol of Ins(1,4,5)*P*$_3$ (Sigma) was added to one portion. In this case 92% was recovered as mass of Ins*P*$_3$ in the bioluminescence assay vials. Therefore there was no significant interference with the measuremer t of the mass by compounds co-eluting with the Ins*P* isomers. Furthermore, recovery of Ins*P* through the entire assay system was good.

Recoveries. For recovery experiments, [^{32}P]Ins(1,4,5)*P*$_3$ was added with trichloroacetic acid to unlabelled tissue fragments, and $97.5 \pm 0.6\%$ ($n = 3$) of the radioactivity was recovered from the h.p.l.c. eluate. In other experiments, [^{3}H]Ins(1,4,5)*P*$_3$ standard (Amersham) was added to a solution made to the same composition as an h.p.l.c. sample, i.e. it contained ammonium formate at 0.3, 1.0 or 1.73 M buffered with phosphoric acid to pH 3.7. The ammonium formate concentrations corresponded to those at which Ins(1,4)*P*$_2$, Ins(1,4,5)*P*$_3$ and Ins(1,3,4,5)*P*$_4$ respectively were eluted by h.p.l.c. The recovery of these standards after dilution, application and subsequent elution from the Q-Sepharose was $85.7 \pm 0.5\%$ ($n = 10$). The d.p.m. in each isomer was measured in samples coming off the h.p.l.c. obtained from [^{3}H]Ins-labelled smooth muscle fragments. These values were compared with the d.p.m. values measured in the bioluminescence assay pot after the assay measurement. For [^{3}H]Ins(1,4,5)*P*$_3$, $75.4 \pm 0.3\%$ ($n = 32$) of the radioactivity could be recovered in the bioluminescence assay vials. The corresponding values for [^{3}H]Ins*P*$_2$ and [^{3}H]Ins*P*$_4$ were $78.1 \pm 0.1\%$ ($n = 80$) and

$73.2 \pm 0.4\%$ ($n = 21$) respectively. These lower recoveries (around 75% of radioactivity from tissue fragments labelled with [^{3}H]Ins were obtained if h.p.l.c. eluate and bioluminescence assay pots were compared. One explanation is the presence of ^{3}H-labelled compounds which co-elute on h.p.l.c. and contribute to the radioactivity measured at various points in the gradient. Further purification using the Q-Sepharose step presumably removed a substantial proportion of these. This conclusion is supported by the higher recovery (86%) of authentic [^{3}H]Ins(1,4,5)*P*$_3$ through the Q-Sepharose step, and the 92% recovery in the bioluminescence assay of authentic Ins(1,4,5)*P*$_3$ added to trichloroacetic acid-treated tissue.

Baseline values. The background values in the bioluminescence assay were usually equivalent to 6–8 pmol of NADH. This value was measured by adding monitoring reagent followed by the NADH internal standard to samples consisting of sodium pyrophosphate buffer, IDH, NAD$^+$ and water and which had been incubated for 10 min at 24 °C. When samples analysed by h.p.l.c. were intended for subsequent mass analysis, an h.p.l.c. run without an added sample was also collected. Fractions from this run were analysed along with the tissue samples to determine 'blank' values in the bioluminescence assay. Such values obtained in the assay corresponded to between 8 and 12 pmol equivalents of NADH; this value was then subtracted from the tissue sample values. When determining the bioluminescence assay measurement (as pmol equivalents of NADH) for erythrocyte samples, values either side of the main peak were close to the basal values from a blank run. For the measurements made on the smooth muscle fragments or the fibroblast cultures, some increases above these basal values were seen in tubes other than those which corresponded to the labelled peaks. These were thought to be due to the presence of other Ins*P* isomers not identified here.

Samples from a smooth muscle experiment were analysed by h.p.l.c. and collected for mass determination. Immediately before the alk.P step, two samples were pooled and divided to give the same concentrations of compounds in each. To one half was added alk.P, as described in the Materials and methods section. To the other half was added boiled inactivated alk.P. When these samples were analysed for mass, 'blank' values were obtained for the samples which had been treated with boiled alk.P, whereas peaks which corresponded to the Ins*P* isomers were detected in the other halves of the samples, and these compared closely with the d.p.m. profile. This result indicates that IDH does not react significantly with Ins*P*s.

Quenching. Quenching of the bioluminescence reaction occurred when samples from h.p.l.c. eluates were analysed and compared with standards in water; correction was always made for this by the addition of NADH as an internal standard. This was very consistent quenching, mainly due to the presence of Tris, which is introduced at the alk.P step. Residual buffer ions from the h.p.l.c. eluate, residual ammonium sulphate from the alk.P, sodium pyrophosphate, NAD$^+$ and IDH also contributed to this quenching. The residual phosphate from the h.p.l.c. eluate lowered the quenching effect of Tris on the bioluminescence assay. Phosphate ions bind to a subsite on BL, stabilizing the enzyme–flavin intermediates (Meighen & Mackenzie, 1973). Variable quenching, however, could occur due to dirt, dust and bacteria on tubes and in solutions. Therefore, for reproducibility, great care was taken to minimize contamination.

Comments on the bioluminescence assay

The BL bioluminescent system has been coupled with the analysis of several substrates (LKB application sheet 506). Although both LKB and Boehringer produce an NADH-linked

bioluminescence assay kit, the Boehringer kit was not available to us at the time of setting up the assay and the LKB kit proved to be unsuitable for our samples. Guderman & Cooper (1986) measured Ins by coupling the IDH assay to the LKB kit.

When we used the LKB kit we found that some samples inhibited some batches of the monitoring reagent more than others, decreasing the light signal. Also, the reagent was not stable, since a decline in activity was observed in several batches between 2 days to 1 week at 4 °C; up to 80% of activity was lost when the monitoring reagent was kept at room temperature for 4 h. With the kit the proportions of BL and OR are fixed and so could not be adjusted to optimize the assay for our samples. The use of an NADH internal standard is essential for decreasing variability and correcting for quenching. An internal standard was not used by Guderman & Cooper (1986). When using the LKB kit we could not achieve a stable light signal for long enough in the presence of our sample to accurately measure the light produced after further addition of the NADH. The assay was, however, excellent for the measurement of NADH itself, i.e. when not coupled with the IDH assay.

We found that it was necessary to purchase the individual components for the bioluminescence assay rather than use the LKB kit, and to adjust the BL or OR concentrations so that a stable light signal could be generated with our samples (Lövgren et al., 1982; Lavi et al., 1983). Purchasing the components for the assay proved to be much cheaper and allowed multi-sample analysis using the same batches of enzymes. Note that crude luciferase (Sigma) cannot be used, since one of the impurities is OR.

The activity of the BL decreased slightly during a 12 month period when stored as aliquots at −20 °C. This was indicated by a decrease in light intensity with no change in the rate of decay of the signal. The slope for the standard curve was reduced from 1.00 ± 0.11 ($n = 10$) to 0.46 ± 0.03 ($n = 10$). When fresh aliquots of BL was used, the slope for the standard curve increased. Since the BL does not affect the rate of decay of the signal (Lövgren et al., 1982), the concentration could have been increased to compensate for this loss in light intensity if desired. There was, therefore, a large variation between assays done over a period of 12 months due to the change in the slope, but a very small intra-assay variability.

We have used a bioluminometer to measure the photons produced in the final assay. A scintillation counter in the single photon monitor mode, i.e. out of coincidence (Cantarow & Stollar, 1976), can be used for the assay. Photons per unit time will be directly proportional to the NADH concentration in the sample. In the coincidence mode a squared proportionality is observed, and so the useful range is smaller. We found it impossible to measure the photons produced by this method with sensitivity and accuracy when the coincidence circuit was switched on.

Conclusion

Until now there has not been a technique which could be routinely applied to the mass measurement of all of the Ins$P$ isomers. We have succeeded in developing a method which can be applied to multi-sample analyses and which allows the measurement of picomole amounts of any Ins$P$ separable by h.p.l.c. It is an extension of the h.p.l.c. method most commonly used for the analysis of radiolabelled Ins$P$s, using an ammonium formate buffer made to pH 3.7 with phosphoric acid (Irvine et al., 1985), but it is equally applicable to other h.p.l.c. methods which use ammonium phosphate buffers to obtain better separation of Ins$P$ isomers (e.g. Dean & Moyer, 1987). Extensive treatments to remove contaminating compounds are not required, as interference or masking by co-eluting compounds is not

a problem. The specificity of the technique for Ins$P$ arises largely from the use of IDH and alk.P enzymes. Complete conversion of Ins(1,4,5)P_3 to Ins was obtained (Fig. 2). If inactivated alk.P was used no Ins was detected, indicating the absence of significant amounts of other compounds which could give rise to a bioluminescence assay signal. Recovery at the bioluminescence assay of authentic Ins(1,4,5)P_3 added to unlabelled tissue fragments was good (92%).

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