Formation of methylphosphoryl inositol phosphates by extractions that employ methanol

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Fixatives that contain methanol extract an unknown compound from several tissues including the retinas of squid (*Loligo*). We have determined that the compound probably contains (1) a *myo*-inositol ring that is phosphorylated in more than one position (including at the 5-hydroxyl), (2) a charged moiety that is not susceptible to alkaline phosphatase, and (3) a methyl group. We have found that the compound can be made by treating either phosphatidylinositol bisphosphate or human red cell ghosts with acidic methanol. We have confirmed the observation of Lips, Bross & Majerus [Proc. Natl. Acad. Sci. U.S.A. **85**, 88–92] that the compound also can be made by methanolysis of inositol (cyclic 1:2,4,5)trisphosphate; however, we have not found inositol (cyclic 1:2,4,5)trisphosphate in either stimulated or unstimulated squid retinas. We tentatively identify the compound as (1-methylphosphoryl)inositol 4,5-bisphosphate formed by methanolysis of phosphatidylinositol 4,5-bisphosphate. By using this methanolysis to incorporate label from [¹⁴C]methanol, we have estimated the mass of inositol 1,4,5-trisphosphate in squid retinas to be approx. 30 μ mol/l of retinal volume.

INTRODUCTION

myo-Inositol phosphates have been implicated in a wide variety of physiological events. Studies have focused largely on Ins(1,4,5) P_3 and its hydrolytic degradation products (Berridge & Irvine, 1984), although Ins(1,3,4) P_3 (Irvine *et al.*, 1985, 1986), Ins(cyc 1:2,4,5) P_3 (Wilson *et al.*, 1985), and Ins(1,3,4,5) P_4 (Batty *et al.*, 1985; Irvine & Moor, 1987) have also been described as physiologically interesting compounds. In studies designed to reveal the presence of these isomers of inositol phosphates, unknown compounds that contain *myo*-inositol have been found [e.g. in rat parotid glands (Hawkins *et al.*, 1987); in mouse pancreas (Sekar *et al.*, 1987); in squid retinas (Brown *et al.*, 1987)].

Squid retinas incubated with [³H]inositol incorporate radiolabel into membrane phosphoinositides and water-soluble compounds (Szuts *et al.*, 1986; Brown *et al.*, 1987). The water-soluble compounds can be chromatographically resolved into several peaks, three of which co-elute with *bona fide* standards of Ins(1,4,5) P_3 , Ins(1,4) P_2 , and Ins(1) P_1 . By this chromatographic criterion, these peaks have been assigned to their respective co-eluant compounds. One unidentified peak, M3 (peak 5 in the numbering of Brown *et al.*, 1987), survives fixation in acidified chloroform/methanol and has a chromatographic retention between those of Ins(1,4,5) P_3 and Ins(1,4) P_2 . The chromatographic behaviour of the compound in this peak resembles that of the compounds extracted from rat parotid glands (Hawkins *et al.*, 1987) and from mouse pancreas (Sekar *et al.*, 1987). We have attempted to identify this compound extracted from squid retinas and to determine its origin and properties.

MATERIALS AND METHODS

Squids (*Loligo pealei* from Woods Hole, MA or *Loligo opalescens* from Santa Catalina, CA, U.S.A.) were dark-adapted for at least 2 h and injected intraocularly with 0.05 ml of artificial sea water containing 0.0625 or 0.125 mCi of [³H]*myo*-inositol. After injection, each squid was returned either to a large tank of running, aerated sea water or to a half-gallon tank containing sea water continuously equilibrated with O₂. After 2–4 h in darkness, each eye was dissected under i.r. illumination with the aid of image-converters; the front half of the eye was discarded, and the eye cup was rapidly immersed in either a fixative solution (at –70 °C) or liquid N₂ (and later stored at –70 °C). Eyecups, either fresh or previously frozen, were fixed in one of several different solutions and extracted so that the aqueous extracts could be analysed by h.p.l.c. according to the technique of Irvine *et al.* (1985). Typical chromatograms are shown in Fig. 1. Peak P3 co-chromatographed with *bona fide* Ins(1,4,5) P_3 [made from human red-cell ghosts (Downes & Michell, 1981)], Peak P2 co-chromatographed with *bona fide* Ins(1,4) P_2 , and Peak P1 co-chromatographed with *bona fide* Ins(1) P_1 . The material in peak M3 was collected and pooled from a large number of retinas for chemical analyses.

Preparation of unknown compound for chemical analyses

Fractions that contained the unknown compound in peak M3 from h.p.l.c. (Brown *et al.*, 1987) were diluted and passed through Dowex anion-exchange columns to remove inorganic phosphate (as described in Irvine *et al.*,

Abbreviations used: PtdIns(4,5) P_2 , phosphatidylinositol 4,5-bisphosphate; Ins(1) P_1 , inositol 1-phosphate; Ins(4) P_1 , inositol 4-phosphate; Ins(1,4) P_2 , inositol 1,4-bisphosphate; Ins(1,4,5) P_3 , inositol 1,4,5-trisphosphate; Ins(1,3,4) P_3 , inositol 1,3,4-trisphosphate; Ins(cyc 1:2,4,5) P_3 , inositol cyclic (1:2),4,5-trisphosphate; Ins(1,3,4,5) P_4 , inositol 1,3,4,5-tetrakisphosphate; MeIns P_3 , (1-methylphosphoryl)-inositol-4,5-bisphosphate; GroPIns(4,5) P_2 , glycerophosphoinositol 4,5-bisphosphate.

1986). 200 nmol of unlabelled $\text{Ins}(1,4,5)\text{P}_3$ was added, and the samples were desalted by conversion to the lithium salts, with subsequent removal of LiCl with ethanol (Burgess *et al.*, 1984).

Ionophoretic analysis

Samples were subjected to ionophoresis on paper in either of two buffers, pyridine/acetic acid, pH 3.6 (Dawson & Clarke, 1972), or sodium oxalate, pH 1.5 (Seiffert & Agranoff, 1965). Internal standards of $\text{Ins}(1,4,5)\text{P}_3$ (Irvine *et al.*, 1986) and $\text{GroPIns}(4,5)\text{P}_2$ (Irvine *et al.*, 1984a) were included. Phosphate-containing compounds were detected by spraying the paper with molybdate-perchloric acid (Clarke & Dawson, 1981). As an additional internal standard, we prepared $[\text{^3H}]\text{GroPIns}(4,5)\text{P}_2$ by deacylation (Clarke & Dawson, 1981) of $[\text{^3H}]\text{PtdIns}(4,5)\text{P}_2$. The paper was then cut into 0.5 cm strips that were counted by liquid scintillation spectrometry.

Dephosphorylation

Samples were dephosphorylated either with alkaline phosphatase (Boehringer-Mannheim grade II; 0.4 mg/ml for 2 h) as in Shears *et al.* (1987), or with 6 M-HCl at 120 °C for 18 h. In the latter case, the products were analysed by ionophoresis on paper in 0.1 M-NaOH (Frahn & Mills, 1959; Irvine *et al.*, 1984b).

We also treated the compound in peak M3 with human red cell ghosts. ^{32}P -labelled $\text{Ins}(1,4,5)\text{P}_3$ was prepared from $\text{PtdIns}(4,5)\text{P}_2$ extracted from ^{32}P -labelled *Chlamydomonas eugametus* (R. F. Irvine & A. Musgrave, unpublished results). This $[\text{^32P}]\text{InsP}_3$ has equal amounts of radiolabel in the 4- and 5-phosphates, but little radiolabel (< 5%) in the 1-phosphate. We used *C. eugametus* $[\text{^32P}]\text{InsP}_3$ as an internal standard for the hydrolysis products of the compound in peak M3 because the retention of both $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ could be determined in the same chromatographic run. Aliquots of the compound in peak M3 (2000 d.p.m.) were mixed with 3000 d.p.m. of *C. eugametus* $[\text{^32P}]\text{InsP}_3$ and incubated for up to 60 min with human red cell membranes, as described in Irvine *et al.* (1984b); after quenching with perchloric acid and neutralizing (Wreggett & Irvine, 1987), the samples were analysed by h.p.l.c. (Irvine *et al.*, 1985).

Generation of compound in peak M3 in the absence of tissue

Aliquots (50000 d.p.m.) of $[\text{^3H}]\text{PtdIns}(4,5)\text{P}_2$ (Amersham, U.K.) whose identity and purity had been checked by t.l.c. were dried in glass tubes. To samples designated 'controls', 1.33 ml of chloroform was added and these were incubated for 1 h at 0 °C; then we added 1 ml of water, followed by 0.67 ml of methanol and then 20 μl of conc. HCl. After mixing and centrifuging, the upper phases were removed and dried. To each sample we added $[\text{^32P}]\text{Ins}(1,4,5)\text{P}_3$ (Amersham, U.K.) and $[\text{^32P}]\text{GroPIns}(4,5)\text{P}_2$ prepared by deacylation (Clarke & Dawson, 1981) of $\text{PtdIns}(4,5)\text{P}_2$ isolated from ^{32}P -labelled *C. eugametus* (R. F. Irvine, A. Musgrave, unpublished results). The samples were then analysed by h.p.l.c. To samples of $[\text{^3H}]\text{PtdIns}(4,5)\text{P}_2$ designated as 'incubated with acidic methanol', the methanol and conc. HCl were added first, and after incubation for 1 h at 0 °C, the chloroform and water were added, all in the

same quantities as above. The samples were then processed exactly as for the controls.

RESULTS AND DISCUSSION

Chromatographic behaviour and dephosphorylation

The behaviour of the compound in peak M3 on h.p.l.c. (Fig. 1) was similar to that of $\text{GroPIns}(4,5)\text{P}_2$ (Irvine *et al.*, 1985). However, when it was analysed by ionophoresis with internal standards of both $\text{Ins}(1,4,5)\text{P}_3$ and $\text{GroPIns}(4,5)\text{P}_2$, it ran as an inositol trisphosphate (Fig. 2).

Total dephosphorylation

The compound in peak M3 was totally dephosphorylated by strong acid and analysed by ionophoresis in 0.1 M-NaOH; in this procedure, we find that alditols migrate toward the cathode (Irvine *et al.*, 1984b), but many other sugars migrate towards the anode (Frahn & Mills, 1959; R. F. Irvine & A. J. Letcher, unpublished results). We therefore spotted the acid hydrolysate in the middle of the paper. After ionophoresis, the paper was sprayed with AgNO_3 to locate the internal standard of inositol (Irvine *et al.*, 1984b); then the paper was cut into strips and the radioactivity in each strip counted. The only radiolabel co-chromatographed with inositol. We conclude that the compound in peak M3 contains an inositol ring.

Alkaline phosphatase

The compound in peak M3 was treated extensively with alkaline phosphatase under conditions that completely dephosphorylate $\text{Ins}(1,4,5)\text{P}_3$ (Irvine *et al.*, 1984b). The product of this enzymic hydrolysis was found to have h.p.l.c. retention identical to that of the material in peak P1 (Fig. 1a). This observation was made four times with three different batches of alkaline phosphatase. In one experiment, we found that the product of extensive treatment with alkaline phosphatase was not retained by the h.p.l.c. column although the internal standard of $[\text{^32P}]\text{Ins}(1,4,5)\text{P}_3$ was normally retained; however, we have been unable to repeat this observation and suggest that this one batch of alkaline phosphatase was contaminated with other enzymic activities (e.g. phosphodiesterases). These experiments suggest that the compound in peak M3 contains phosphomonoester moieties, but that it is not a simple inositol phosphate because alkaline phosphatase does not convert it to inositol. We tentatively conclude that there is a compound 'M1' that co-elutes with peak P1 and is a dephosphorylated form of the compound in peak M3.

Periodate and nitrous oxide treatments

Mild periodate treatment (0.03 M-sodium periodate, pH 6.5 for 15 min) with or without subsequent treatment with dimethylhydrazine (Brown & Stewart, 1968; Irvine *et al.*, 1986) had no effect on the h.p.l.c. retention of the compound in peak M3. These treatments ought to remove any glycerol moieties or to oxidize any non-cyclic vicinal hydroxyl groups. In one experiment, prolonged treatment with periodate (4 days at pH 6.5 in the dark) caused a slight shift in the h.p.l.c. retention of the compound relative to the retention of the internal standard of $\text{Ins}(1,4,5)\text{P}_3$; that is, peak M3 eluted 0.5–1.0 min earlier than it did before periodate treatment. Although this change in retention is small, we believe

Fig. 1. Chromatography of $[^3\text{H}]$ inositol-containing compounds from squid retinas

Retinas from dark-adapted squids that were previously incubated intraocularly with $[^3\text{H}]$ inositol were extracted and the water-soluble fractions were separated, dried and analysed by h.p.l.c. Fractions were collected every 30 s. (a) Each retina was extracted with chloroform/methanol/HCl (80:40:1) (solid line and filled circles) at -70°C for 1 h or with chloroform/methanol/2 M-KCl+0.1 M-EDTA/ H_2O (4:10:1:2) at -20°C for 1 h (dashed line and open circles). The retentions of standards on the same

that our chromatographic procedure is reliable enough (when successive runs are analysed) to say that the change is real and probably reflects the opening of the inositol ring by periodate (Grado & Ballou, 1961).

In two experiments we treated the compound in peak M3 with nitrous oxide to see if it had an *N*-glucosamine link, as does the suggested insulin mediator that contains inositol (Saltiel *et al.*, 1986); this treatment had no effect on the chromatographic retention of the compound. Thus, from all these experiments, we conclude that the compound is an inositol phosphate with an additional charged moiety that is not *N*-glucosamine linked and does not contain glycerol or vicinal non-cyclic hydroxyls.

5-Phosphatase

We also investigated the ability of human red cell membranes to hydrolyse the compound in peak M3. These membranes contain principally a phosphatase which specifically removes the 5-phosphate from $\text{Ins}(1,4,5)P_3$ (Downes *et al.*, 1982) and under the conditions used the membranes have apparently negligible non-specific phosphatase activity (Irvine *et al.*, 1986; Batty *et al.*, 1985). The lack of non-specific activity was confirmed in these experiments by the lack of detectable formation of any $\text{Ins}(1)P$ or $\text{Ins}(4)P$ from the internal standard of $[^{32}\text{P}]\text{Ins}(1,4,5)P_3$. We did not have sufficient of the compound in peak M3 to do detailed kinetic studies, but we found that under conditions in which 80% of the $[^{32}\text{P}]\text{Ins}(1,4,5)P_3$ was hydrolysed to $[^{32}\text{P}]\text{Ins}(1,4)P_2$ and ^{32}P inorganic phosphate with no non-specific hydrolysis (see above), 7% of the compound in peak M3 was converted to a compound which eluted 6 min before $\text{Ins}(1,4)P_2$ and 4 min after $\text{Ins}(1)P$ (elution times of $\text{Ins}(1)P$, $\text{Ins}(1,4)P_2$, peak M3, and $\text{Ins}(1,4,5)P_3$, 17.5, 28, 33 and 38 min respectively). Thus, incubation with red cell membranes converts the compound in peak M3 to a compound that has a retention similar to peak M2 (Fig. 1).

From these data we conclude that the compound in peak M3 is probably a poor substrate for the $\text{Ins}(1,4,5)P_3$ phosphatase. This is consistent with the complex substitution being in the 1-position in that (a) $\text{GroPIns}(4,5)P_2$ is a substrate for the phosphatase (Downes *et al.*, 1982), and (b) $\text{Ins}(1,4,5)P_3$ 'caged' by 1-(2-nitrophenyl)ethanol in the 1-phosphate is a substrate for the same enzyme, but if 'caged' in the 4 or 5 phosphates it is not a substrate (Walker *et al.*, 1987). We also tentatively conclude that the compound in peak M2 is a dephosphorylated form of the compound in peak M3.

column during different runs are indicated by arrows. In other experiments, inclusion of pH buffer (Tris at pH 7.0) did not qualitatively change the chromatogram. The peak labelled 'M3' corresponds to 'Peak 5' in Brown *et al.* (1987). (b) Each retina was extracted first with 2 ml of methanol for 1 h at 20°C followed by 1 ml of 0.1 M-HCl for 1 h at 20°C followed by 2 ml of methanol (dashed line); the small peak preceding peak M3 had the same retention as $\text{GroPIns}(4,5)P_2$. No material was recovered in either peak M2 or M3 when the tissue was acidified prior to exposure to methanol. (c) Each retina was extracted with phenol/chloroform/isoamyl alcohol (38:24:1) at -20°C for 1 h either without (dashed line) or with prior fixation with chloroform/methanol (2:1) at -70°C for 1 h (followed by lyophilization) (solid line). Without prior exposure to methanol, no material was recovered in peaks M2 and M3.

Fig. 2. Ionophoresis of the material in peak M3 from squid retinas

(a) Sodium oxalate buffer, pH 1.5; (b) acetic acid, pyridine buffer, pH 3.6. Recovery of radioactivity spotted onto the paper was < 90 % in each instance; results are typical of two experiments for each buffer. Ionophoretic mobilities of internal standards of GroPIns(4,5) P_2 and Ins(1,4,5) P_3 are indicated by bars with arrowheads.

Extraction methods

The material in peak M3 was not reported by Szuts *et al.* (1986), who fixed and extracted squid retinas in cold 10 % trichloroacetic acid. We confirmed that the material in peak M3 did not appear in our h.p.l.c. chromatograms after fixation in 10 % trichloroacetic acid in chloroform. The material also was absent when the tissue was fixed in chloroform/butanol/HCl (80:40:1). The material was present when the tissue was initially fixed in methanol alone (20 °C for 60 min) and later acidified with 0.1 M-HCl (20 °C for 60 min), whereas the material was absent if the methanol was added after the tissue was treated with 0.1 M-HCl (Fig. 1b).

The material in peak M3 could also be extracted at neutral pH by fixing the tissue in chloroform/methanol/KCl (Ishii *et al.*, 1986) (Fig. 1a) or by treating tissue previously fixed in chloroform/methanol (2:1) with phenol/chloroform/isoamyl alcohol (38:24:1) (Slater *et al.*, 1973). However, treatment by phenol/chloroform/isoamyl alcohol alone without prior exposure to methanol did not extract any radiolabelled material that chromatographed with the retention of peak M3 (Fig. 1c). The compound found by Hawkins *et al.* (1987) was extracted in the same manner from rat parotid glands

Fig. 3. Chromatogram of water-soluble compounds extracted with [${}^{14}\text{C}$]methanol

Two dark-adapted squid retinas that had been incubated intraocularly with [${}^3\text{H}$]inositol were fixed in chloroform/methanol/HCl (80:40:1) at -70 °C for 1 h then warmed to 20 °C for 30 min. The methanol included [${}^{14}\text{C}$]methanol (3 mCi/100 μl). The aqueous phase was separated, dried and chromatographed by h.p.l.c. ${}^3\text{H}$ c.p.m., solid line. ${}^{14}\text{C}$ c.p.m., dashed line.

after the tissue was initially fixed in chloroform/methanol and dried. This compound appears to have the same chromatographic properties as does the compound in peak M3 extracted from squid retinas. Similarly, the compound from mouse pancreas (Sekar *et al.*, 1987) was extracted by neutral chloroform/methanol and had less retention time on Partisil 10 SAX with a gradient of ammonium formate plus phosphoric acid (pH 5.3) than did Ins(1,4,5) P_3 .

The conditions for the formation of the material in peak M3 all include methanol in the extraction protocol. These findings suggest that a methanolysis reaction may occur during fixation and extraction. Other non-enzymic methanolysis reactions have been reported. Eichberg & Dawson (1964) found that treating ATP with methanol and HCl produced methyl-phosphate; trichloroacetic acid and methanol did not support this reaction. To examine the possibility that methanolysis occurred during fixation of the tissue, living squid retinas that had been radiolabelled intraocularly with [${}^3\text{H}$]inositol (for 3 h in darkness) were fixed in chloroform/[${}^{14}\text{C}$]methanol/HCl (at specific activity 30 mCi [${}^{14}\text{C}$]/ml methanol) at -70 °C. The retinas were extracted and analysed by h.p.l.c., as before. A chromatogram of the aqueous extract from one of two experiments is shown in Fig. 3. ${}^{14}\text{C}$ radioactivity was recovered in three of the peaks including peak M3. This finding supports the idea that methanolysis during fixation is involved in the formation of the material in peak M3. The material in the other two peaks that were labelled by ${}^{14}\text{C}$ may be made either (most likely) by methanolysis of two additional compounds (e.g. two analogues of the precursor of the material in peak M3 with fewer phosphates, probably PtdIns and PtdIns(4) P , see below) or possibly by some form of non-enzymic degradation of the material in peak M3.

Concentration of Ins(1,4,5) P_3 in the retina

Because we know the specific activity of the [${}^{14}\text{C}$]methanol, these experiments afford an opportunity to

Fig. 4. Chromatograms of water-soluble products from human red cell ghosts

(a) Human red cell ghosts were challenged with 1 mM- Ca^{2+} at pH 5.5. A portion of the aqueous supernatant was dried and analysed by h.p.l.c. (solid line). Another dried portion of the supernatant was treated with chloroform/methanol/HCl (80:40:1) and the aqueous extract analysed by h.p.l.c. (dashed line). The material co-eluting with the material in peak M3 from squid retinas appeared after chloroform/methanol/HCl treatment. Note that the calcium-challenged ghost membranes produce $[\text{32P}]\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_2$ (Hawkins *et al.*, 1987), but only as a small fraction of the $[\text{32P}]\text{Ins}(1,4,5)\text{P}_3$ produced. (b) Ghosts, not challenged with Ca^{2+} , were lyophilized and fixed with chloroform/methanol/HCl (80:40:1) at -70°C for 1 h, and the aqueous extract analysed by h.p.l.c. Note that there is more radiolabelled material in peak M3 than in $\text{Ins}(1,4,5)\text{P}_3$.

estimate the mass of $\text{Ins}(1,4,5)\text{P}_3$ in the retina. The reasoning is that, assuming that there is only one compound in peak M3, we can calculate the mass of the material in that peak to be approx. 4.5×10^{-10} mol/retina. Consequently, the specific activity of $[\text{3H}]\text{inositol}$ in that material is approx. 4.4×10^{12} c.p.m./mol. Assuming that the specific activity of $[\text{3H}]\text{inositol}$ has approximately reached steady-state in the labelled $\text{PtdIns}(4,5)\text{P}_3$ and its degradation products $[\text{Ins}(1,4,5)\text{P}_3]$

and $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3]$ during our prolonged (3 h) incubation, then we can calculate that the mass of $\text{Ins}(1,4,5)\text{P}_3$ is approx. 1.6×10^{-9} mol/retina.

The bulk of the volume of the squid retina is comprised of photoreceptor cells (Cajal, 1917; Cohen, 1973). The retina was about 500 μm thick and each retina extracted was about 0.8 cm in diameter. Thus, the total content of $\text{Ins}(1,4,5)\text{P}_3$ was approx. 32 $\mu\text{mol/l}$ of whole (unstimulated) retinal volume. This estimate can only be approximate in that several assumptions have been made during the evaluation. A resting concentration for a proposed second messenger of 32 μM may seem very high (cf. Berridge & Irvine, 1984), but the resting levels of radiolabelled $\text{Ins}(1,4,5)\text{P}_3$ in these experiments are also high (e.g. Brown *et al.*, 1987), and it may be that not all of this $\text{Ins}(1,4,5)\text{P}_3$ has access to its site of action. We cannot at present offer any firm conclusion about the calculations above with regard to resting levels of cytoplasmic $\text{Ins}(1,4,5)\text{P}_3$.

Generation of M3 from $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$

Lips *et al.* (1988) have found that treating $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ with methanol synthesizes a compound that they identify as MeInsP_3 that has chromatographic properties similar to the compound in peak M3. Furthermore, they report that fixation of 3T3 cells that contain $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ in chloroform/methanol (either acidified with HCl or not) causes the formation of the same MeInsP_3 . Also, significant amounts of $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ are found in the supernatant after calcium-elicited production of inositol phosphates by red cell ghosts (Hawkins *et al.*, 1987). Therefore, $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ is a likely precursor for the compound in peak M3 formed after addition of methanol to that preparation (Fig. 4a). We found that prior acidification destroys the ability of squid retinas to form the compound in peak M3 (Fig. 1b). Thus, $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ is a logically possible precursor of the compound in peak M3 extracted from squid retinas.

We attempted to extract $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ from squid retinas by two techniques (neutral chloroform/methanol/KCl, Ishii *et al.*, 1986; phenol/chloroform/isoamyl alcohol, Hawkins *et al.*, 1987). We were unable to find any $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ in squid retinas with either technique. Fig. 5(a) shows the results of one such experiment using the phenol extraction technique. We were able to recover $[\text{32P}]\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ that had been added to the frozen tissue sample prior to extraction; the $[\text{32P}]\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ was acid labile as shown in Fig. 5(b). If $[\text{32P}]\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ was added to a frozen tissue sample prior to extraction with chloroform/methanol/HCl (80:40:1), the ^{32}P marker was recovered in $\text{Ins}(1,4,5)\text{P}_3$ and no ^{32}P was detected in the material in peak M3 (Fig. 5c). We assume this is because the water content of the sample was high enough so that hydrolysis overwhelmed methanolysis of the $[\text{32P}]\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ (see Eichberg & Dawson, 1964) and an undetectable amount of ^{32}P radiolabel appeared in peak M3. We should also note at this point that Hawkins *et al.* (1987) achieved a high recovery of added $[\text{32P}]\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ from parotid glands, and at the same time extracted a tritium-labelled compound which had chromatographic properties very similar to M3, but no ^{32}P radiolabel. Thus, it is very unlikely that this MeInsP_3 -like compound was generated from $\text{Ins}(\text{cyc } 1:2,4,5)\text{P}_3$ in their experiments.

Fig. 5. Cyclic inositol trisphosphate in squid retinas

A squid retina stimulated by bright illumination was extracted with phenol/chloroform/isoamyl alcohol (38:24:1) (Hawkins *et al.*, 1987). The retina had previously been labelled with [${}^3\text{H}$]inositol and was quickly frozen in liquid N_2 during illumination. [${}^{32}\text{P}$]Ins(cyc 1:2,4,5) P_3 was added to the frozen tissue prior to extraction. ${}^{32}\text{P}$ c.p.m., dashed lines. ${}^3\text{H}$ c.p.m., solid lines. (a) A portion of the aqueous extract was chromatographed. (b) Another portion was acidified (with HCl at 80°C) and chromatographed. After acidification, the ${}^{32}\text{P}$ label co-eluted with Ins(1,4,5) P_3 . Thus, the extraction procedure could

If the compound in peak M3 is not predominately formed from Ins(cyc 1:2,4,5) P_3 accumulated in the tissue, then the prevention of formation of the compound by prior exposure to dilute acid at 20°C requires explanation. We believe that the formation of the compound depends on the competition of hydrolysis and methanolysis reactions, and the latter competes favourably at -70°C because the methanol can dehydrate (at least partially) the frozen tissue.

Generation of M3 from PtdIns(4,5) P_2

The material in peak M3 was reported (Brown *et al.*, 1987) to decline with illumination of squid retinas. In tissue fixed in chloroform/methanol/HCl, the only other compound that contains *myo*-inositol and reported to decline with illumination was PtdIns(4,5) P_2 . We have examined if PtdIns(4,5) P_2 can be a precursor for the compound in peak M3 in the absence of tissue. We incubated aliquots of [${}^3\text{H}$]PtdIns(4,5) P_2 in acidic methanol (see Materials and methods) and analysed the samples by h.p.l.c. with internal standards of [${}^{32}\text{P}$]GroPIns(4,5) P_2 and [${}^{32}\text{P}$]Ins(1,4,5) P_3 . We found that a compound with h.p.l.c. retention identical to peak M3 was formed (Fig. 6). Controls in which the methanol and acid were added after the water did not contain this compound. These results strongly support the suggestion that the compound in peak M3 is methylphosphoryl-Ins(4,5) P_2 , and that it is formed by methanolysis of PtdIns(4,5) P_2 in the presence of acid.

Generation of M3 from human red blood cell ghosts

We found peak M3 in chromatograms of materials prepared from human red cell ghosts. We labelled ghost membranes with ${}^{32}\text{P}$ (Downes & Michell, 1981), elicited production of inositol phosphates with 1 mM-Ca^{2+} at pH 5.5 and formed a pellet of the membranes in an ultracentrifuge. The material in peak M3 appeared after addition of acidified methanol to dried samples of the aqueous supernatant (in the absence of ghost membranes) (Fig. 4a) or after fixation of the pellet of ghost membranes with chloroform/methanol/HCl (80:40:1) (results not shown). Red cell ghost membranes that have been challenged with Ca^{2+} (at pH 5.5) are known to produce Ins(cyc 1:2,4,5) P_3 [in amounts up to 10% of the amount of Ins(1,4,5) P_3] (Hawkins *et al.*, 1987). Thus, the material found in peak M3 from the pellet of ghost membranes might have come from methanolysis of the cyclic compound as discussed above. However, ghost membranes (labelled with ${}^{32}\text{P}$) that had not been challenged with Ca^{2+} were lyophilized and then fixed with chloroform/methanol/HCl (80:40:1) at -70°C . The chromatogram from such an experiment is shown in Fig. 4(b); there is more radiolabel in peak M3 than in the Ins(1,4,5) P_3 peak. This finding indicates that the material in peak M3 was formed without accumulation of Ins(cyc 1:2,4,5) P_3 ; either a transiently formed cyclic com-

recover the ${}^{32}\text{P}$ -labelled cyclic compound, but there was no detectable [${}^3\text{H}$]Ins(cyc 1:2,4,5) P_3 extracted from the tissue. (c) [${}^{32}\text{P}$]Ins(cyc 1:2,4,5) P_3 was added to a frozen squid retina prior to extraction with chloroform/methanol/HCl (80:40:1). The ${}^{32}\text{P}$ marker co-eluted with Ins(1,4,5) P_3 or eluted with the expected retention of Ins(2,4,5) P_3 but did not co-elute with the material in peak M3. The chromatography in (c) was done on a Partisil 10 SAX column different from that used for (a) and (b).

Fig. 6. Generation of compound 5 in the absence of tissue

Aliquots of [^3H]PtdIns P_2 were incubated with or without acidic methanol (see Materials and methods) and analysed by h.p.l.c. Fractions were collected every 20 s. Open circles, controls (no acidic methanol incubation); ●, incubated with acid methanol; △, a sample of the compound in peak M3 (from squid retinas) run separately, but in the same series of h.p.l.c. runs in which the retention of the internal [^{32}P]Ins(1,4,5) P_3 marker remained identical (retentions of the internal markers are indicated by arrows). Similar results were obtained in two other experiments.

pound was attacked by the methanol in the presence of minimal water (accounting for the minimal production of the non-cyclic compound), or the material in peak M3 was formed without a cyclic intermediate and there was a small amount of Ins(1,4,5) P_3 trapped in the red cell ghost membranes.

SUMMARY

The material in peak M3 is probably formed by methanolysis of PtdIns(4,5) P_2 independent of enzyme action, because it can be formed from PtdIns(4,5) P_2 in the absence of tissue and also because it is unlikely that enzymic activity in tissue would survive in acidified methanol at -70°C . The ability of tissue to form this compound is destroyed by acidification prior to addition of methanol, however, so we cannot rule out some enzymic methanolysis having occurred. Because the compound in peak M3 (1) contains *myo*-inositol, (2) contains one or more phosphates susceptible to alkaline phosphatase and a charged moiety not susceptible to that enzyme, (3) contains a phosphate hydrolysed by a phosphatase in red cell membranes, (4) requires methanol for its production, and (5) has similar chromatographic properties to the compound described by Lips *et al.* (1988), we suggest that the compound is 1-methylphosphoryl-Ins(4,5) P_2 . This compound can be formed both by methanolysis of free Ins(cyc 1:2,4,5) P_3 and of PtdIns(4,5) P_2 . In tissue, either or both of these pathways may contribute to the formation of the compound.

Finally, we tentatively suggest that the compounds in peaks M1 and M2 are (1-methylphosphoryl)inositol and (1-methylphosphoryl)inositol(4)phosphate respectively and that these compounds can be derived from the corresponding lipids PtdIns and PtdIns(4) P . The presence of these methylphosphoryl-inositol phosphates in tissue extracts may interfere with interpretation of chromatographic analyses of inositol phosphates from tissues extracted with methanol.

We thank Drs. R. M. C. Dawson and P. W. Majerus for several helpful discussions and Dr. W. L. Hubbell and K. R. Oldenburg for their assistance in obtaining squid from Santa Catalina Island. This work was supported in part by NIH grants EY-05166 and EY-05168.

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Received 21 December 1987/22 February 1988; accepted 20 April 1988