Studies of inositol analogues as inhibitors of the phosphoinositide pathway, and incorporation of 2-deoxy-2-fluoro-*myo*-inositol to give analogues of phosphatidylinositol intermediates

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The incorporation of [^3H]Ins into PtdIns by exchange of free and lipid-bound inositol moieties occurs via the action of at least two types of $\text{Mg}^{2+}/\text{Mn}^{2+}$ -dependent enzymes in turkey erythrocytes. One is a nucleotide-independent PtdIns/Ins exchange enzyme and its function is, as yet, unknown, whereas the other is CMP-dependent and appears to be an exchange reaction catalysed by PtdIns synthase. The effects of analogues with modifications of the substituent at the 1-, 2-, 3-, 4- and 5-positions on the incorporation of [^3H]Ins into PtdIns under both synthase and exchange reaction conditions were investigated in turkey erythrocytes. Analogues causing substantial inhibition of [^3H]Ins incorporation were then used in kinetic experiments to determine the type of inhibition involved. The analogues 1-deoxy-1-fluoro-*scyllo*-inositol and 5-*O*-methyl-*myo*-inositol exhibited the greatest effects on the incorporation of [^3H]Ins via both the synthase and exchange reactions, and the kinetic analysis indicated that they were competitive inhibitors of Ins. K_i values of 0.37 mM and 2.87 mM were observed for 1-deoxy-1-fluoro-*scyllo*-inositol under exchange and synthase reaction conditions respectively; similar K_i values of 0.26 mM and 2.80 mM were observed for 5-*O*-methyl-*myo*-inositol in the exchange and synthase reactions respectively. The ability of 1-deoxy-1-fluoro-*scyllo*-inositol and its diastereoisomer, 2-deoxy-2-fluoro-*myo*-inositol, to act as substrates for the synthase and exchange reactions in turkey erythrocytes was investigated. The radiolabelled derivative of the former analogue was not incorporated into phospholipids, whereas the radiolabelled derivative of the latter analogue was a poor substrate for the synthase and exchange enzymes. In the presence of ATP, the labelled analogue of PtdIns, derived from 2-deoxy-2-fluoro-*myo*-[2- ^3H]inositol, appeared to be converted into phosphorylated PtdIns analogues, presumably by the enzymes of the phosphoinositide pathway.

INTRODUCTION

In recent years, turkey erythrocyte membranes have been used as a model system for studies of receptor and guanine-nucleotide-binding protein (G-protein) regulation of phosphoinositide-specific phospholipase C (PLC) (e.g. Boyer *et al.*, 1989). These membranes are also useful for the study of several aspects of inositol phospholipid metabolism. They possess PtdIns synthase and PtdIns/Ins exchange activities, which together account for the rapid incorporation of [^3H]Ins into membrane lipids (McPhee *et al.*, 1991), and they have PtdIns 4-kinase and PtdIns 4P 5-kinase activities that generate the respective polyphosphoinositides from the initially labelled PtdIns pool. PtdIns 3-kinase, however, which produces a novel series of 3-phosphorylated inositol phospholipids (Whitman *et al.*, 1988), has not been detected in turkey erythrocyte membranes.

The second messengers produced upon PLC-mediated hydrolysis of PtdIns(4,5) P_2 [inositol triphosphate and diacylglycerol (DG)] control a variety of important cellular functions, including secretion, smooth muscle contraction, inflammatory cell responses and cell division, and are implicated in disease states involving malfunctions of such cellular responses. Hence an inhibitor of inositol function would be an attractive target for pharmacological intervention. In this study, the inhibitory effects of a number of analogues of *myo*-inositol were investigated by analysing their ability to prevent the incorporation of [^3H]Ins into PtdIns via the synthase and head-group exchange reactions, respectively, of turkey erythrocytes. Potential substrate activities of two of these analogues were also investigated using radio-

labelled analogues. Turkey erythrocyte membranes were selected for these studies because the effects of inhibitors could be tested on a variety of aspects of inositol function without any of the constraints imposed by problems of cell penetration that would apply to similar experiments on intact cells.

MATERIALS AND METHODS

Chemicals

myo-[2- ^3H]inositol was obtained from New England Nuclear, Boston, MA, U.S.A. The inositol analogues 1-*O*-methyl-, ($\pm$)1-*O*-methyl- and 3-deoxy-*myo*-inositol were kindly given by Dr. L. R. Stephens, A.F.R.C., Babraham, Cambridge, U.K. Inositol analogues with modified substituents in the 3- and 4-positions were donated by Dr. M. Voyle, Smith Kline Beecham Research Ltd., Welwyn.

Assay of PtdIns synthase and PtdIns/Ins exchange activities

Turkey erythrocytes were prepared by hypo-osmotic lysis and washing, exactly as described previously (Harden *et al.*, 1988). The ghost pellet was finally resuspended in an equal volume of 10 mM-Hepes, pH 7.0, and stored on ice before use. The protein content of each ghost preparation was assessed by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

PtdIns synthase was assayed by exploiting its ability to catalyse CMP-dependent exchange of inositol into PtdIns in the presence of [^3H]inositol with no added CMP-phosphatidate. The rationale for this approach has been discussed previously (McPhee *et al.*, 1991). The assays contained approx. 50 μg of ghost protein in a

Abbreviations used: G-protein, guanine-nucleotide-binding protein; GroPIns, glycerophospho-*myo*-inositol; Ins P_2 , Ins P_3 , inositol polyphosphates of unknown isomerism; DG, 1,2-diacylglycerol; GTP[S], guanosine 5'-[γ -thio]triphosphate; PLC, phospholipase C.

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buffer (100 μ l) of the following composition: MgCl_2 , 10 mM; Hepes, 40 mM (pH 7.0); inositol, 0.3 mM; CMP, 1 mM; [^3H]inositol (20 Ci $\cdot$ mmol $^{-1}$), 0.5 μ Ci; plus the indicated concentration of an inositol analogue. The assay mixture was incubated at 37 °C for 2 min. The assays were then started by addition of membranes and the incubations were continued for a further 5 min. Under these conditions, more than 95% of [^3H]inositol incorporation into PtdIns occurs via the synthase-catalysed reaction (McPhee *et al.*, 1991).

PtdIns/inositol head-group exchange (the CMP-independent reaction) was assayed using the same amount of ghost protein as above, in 100 μ l of a medium comprising MnCl_2 , 10 mM; Hepes, 10 mM, pH 7.0; inositol, 0.01 mM; [^3H]inositol, 0.5 μ Ci. Incubations were started by addition of membranes after a 2 min preincubation at 37 °C, and terminated after 10 min.

PtdIns synthase and head-group exchange reactions were stopped by the addition of 750 μ l of chloroform/methanol/0.1 M-HCl (80:40:1, by vol.) and a phospholipid fraction obtained as described previously (McPhee *et al.*, 1991). A 500 μ l portion of the final organic phase, containing radioactive phospholipids, was mixed with scintillant, and radioactivity was determined by liquid scintillation counting.

For the kinetic analysis of inhibition of these activities by inositol analogues, the experimental results were fitted to the corresponding kinetic equations by the non-linear regression facility of the statistical software package KINPAC.

Phosphorylation of PtdIns and PtdIns analogues by endogenous kinases of turkey erythrocyte membranes

Erythrocyte ghosts (0.1–0.5 mg of protein/ml) were labelled with [^3H]inositol or a ^3H -labelled inositol analogue (details are given in appropriate Figure legends) in 2 ml of a medium of the following composition: MgCl_2 , 10 mM; Hepes, 40 mM, pH 7.0; CMP, 1 mM; and the indicated amount of radiolabelled inositol or an analogue. Labelling periods ranging from 1 to 24 h were employed (see the Results section). For incubations longer than 1 h, the above medium was supplemented with penicillin (5 mg/ml) and streptomycin (5000 units/ml). After the prelabelling period, the incubation was terminated by the addition of 15 vol. of ice-cold 10 mM-Hepes, pH 7.0. The labelled ghosts were collected by centrifugation at 12000 *g* for 10 min. The pellet was washed once with Hepes buffer to remove excess radiolabel and CMP, and finally resuspended in 1 vol. of 10 mM-Hepes, pH 7.0.

Formation of polyphosphoinositides from prelabelled PtdIns was then measured in incubations containing a portion of the resuspended pellet from above in 0.2 ml of an assay buffer of the following composition: KCl, 115 mM; CaCl_2 , 0.46 mM/EGTA, 2 mM (pCa $^{2+}$ approx. 7); MgSO_4 , 0.9 mM; KH_2PO_4 , 5 mM; ouabain, 0.1 mM; Hepes, 10 mM, pH 7.0; and an ATP-regenerating mixture comprising 5 mM-MgATP, 20 mM-phosphocreatine and creatine kinase (1–3 units per assay tube). The incubations were terminated by the addition of 750 μ l of HClO_4 (6%, v/v). The samples were kept on ice for 10 min and then centrifuged before removal of the supernatant. Lipids were extracted from the resulting pellets and analysed as described below. After 15 min at 37 °C in a typical experiment, approximate values for the proportion of label (out of a total of 1.0) in each of the inositol lipids were: PtdIns, 0.70; PtdIns4P, 0.24; PtdIns(4,5) P_2 , 0.057.

Extraction and analysis of phospholipids

Phospholipids were extracted exactly as described previously (Harden *et al.*, 1988) and deacylated using methylamine according to the method described in Hawkins *et al.* (1986). The deacylated phospholipids were separated by ion-exchange

chromatography on small columns of Bio-Rad AG 1 ($\times 8$; formate form; 200–400 mesh) as described previously (Harden *et al.*, 1988) or subjected to h.p.l.c. on a Whatman Partisphere SAX anion-exchange column (5 μ m particle size; 12.5 cm cartridge) eluted with a gradient of water (A) and 1.25 M- $(\text{NH}_4)_2\text{HPO}_4$, pH 3.8, with H_3PO_4 (B) as follows: 0 min, 0% B; 5 min, 0% B; 45 min, 12% B; 60 min, 30% B; 61 min, 100% B; 65 min 100% B; 66 min, 0% B. The flow rate was 1 ml/min, and 143 fractions of 0.43 ml were collected. The fractions were mixed with 1 ml of 50% (v/v) methanol and 5 ml of Instagel liquid scintillation cocktail, and radioactivity was measured by liquid scintillation counting.

Identification of inositol analogues incorporated into membrane lipids

The purity of the labelled compounds has been established as 97.5% for 1-deoxy-1-fluoro-*scyllo*-[1- ^3H]inositol and 98.4% for 2-deoxy-2-fluoro-*myo*-[2- ^3H]inositol (Lowe & McPhee, 1991). In experiments employing ^3H -labelled inositol analogues, only a small proportion of the label (less than 1%) was incorporated, even during prolonged incubations (see the Results section), so it was essential to show that the incorporated label was not due to a minor contaminant or to a metabolite of the radiolabelled material. This was achieved by extraction and deacylation of the labelled PtdIns analogue as described above, followed by deglyceration (Brown & Stewart, 1966) and dephosphorylation using alkaline phosphatase as described previously (Stephens *et al.*, 1988). The resulting [^3H]inositol analogue should be the same as the starting material. This was verified by h.p.l.c. on a Technical Cyclobond 111 (α) analytical column eluted with a gradient of water (A) and acetonitrile (B) as follows: 0 min, 95% B; 20 min, 85% B; 25 min, 50% B; 35 min, 50% B; 36 min, 95% B. The flow rate was 1 ml/min and 100 fractions (0.3 ml each) were collected. Instagel (3 ml) was added to each fraction and radioactivity was determined by liquid scintillation counting.

RESULTS

Effect of analogues of *myo*-inositol on [^3H]Ins incorporation

The influence of a number of analogues of *myo*-inositol on the incorporation of [^3H]Ins into PtdIns of turkey erythrocyte membranes was examined. The assay conditions used allowed for the incorporation of [^3H]Ins into PtdIns via either the PtdIns synthase or exchange activities, with the inositol concentration for each type of assay being close to the K_m for the synthase- and exchange-catalysed reactions respectively (McPhee *et al.*, 1991).

A series of analogues of *myo*-inositol, modified at the 1-, 2-, 3-, 4- and 5-positions, was employed to evaluate the inhibitory effects of changes at each position on the ring on the incorporation of [^3H]Ins via both the synthase and exchange processes. The assay conditions used are described in Table 1; the inhibitory effects of each analogue on the incorporation of the tritiated substrate are also given for both processes. As shown in Table 1, 5-*O*-methyl-*myo*-inositol and 1-deoxy-1-fluoro-*scyllo*-inositol exhibited the largest inhibitory effects, followed by 3-deoxy-3-fluoro-*myo*-inositol, with 4-deoxy-4-fluoro-, 1-*O*-methyl-, 3-deoxy- and 2-*O*-methyl-*myo*-inositol all demonstrating inhibitory effects greater than 50% on the exchange activity. 1-Deoxy-1-fluoro-*myo*-inositol and 4-amino-4-deoxy-*myo*-inositol were poor inhibitors by comparison, while the racemic 1-*O*-methyl-*myo*-inositol showed a lower inhibition of the exchange reaction compared with the enantiomerically pure compound. By contrast, the racemic compound showed a greater inhibitory effect on the synthase reaction, implying that 3-*O*-methyl-*myo*-inositol is a better inhibitor of the synthase than is its enantiomer. The majority of analogues tested showed greater inhibitory effects on

Table 1. Effects of analogues of *myo*-inositol on the incorporation of [³H]Ins into PtdIns via the PtdIns synthase or PtdIns exchange reactions

In general, the final concentration of the analogue was 10 mM; *represents a 5 mM concentration of analogue in the assay, and † represents a 1 mM concentration of analogue in the assay. For the synthase assay, turkey erythrocytes (1 mg) were incubated with 5 mM-MgCl₂, 0.2 mM-[³H]Ins, 1 mM-CMP and the analogue for 5 min at 37 °C. For the exchange assay, turkey erythrocytes (1 mg) were incubated with 5 mM-MnCl₂, 0.01 mM-[³H]Ins and the analogue for 10 min at 37 °C. The results are expressed as means±S.E.M. for two experiments, each carried out in duplicate. Analogues are numbered according to the D-configuration of *myo*-inositol.

Analogue structure and name	Inhibition of the exchange reaction (%)	Inhibition of the synthase reaction (%)	
	1- <i>O</i> -Methyl-*	52.5±3.5	6
	1-Deoxy-1-fluoro-*	26.5±4.5	11.5±1.5
	1-Deoxy-1-fluoro- <i>scyllo</i> -	93±3	61±5
	1- <i>O</i> -Methyl- and 3- <i>O</i> -methyl-*	28±2	14.4±4.5
	2- <i>O</i> -Methyl-	58±6	6.5±3.5
	2-Deoxy-2-fluoro-	10.5±3.5†	23*
	3-Deoxy-*	58±3	6.5±0.5
	3-Deoxy-3-fluoro-*	82±1	26.5±7.5
	4-Amino-4-deoxy-	13±7	7
	4-Deoxy-	59.5±6.5	20±2
	4-Deoxy-4-fluoro-	77.5±2.5	40±1
	5- <i>O</i> -Methyl-	84±5*	65±1†

Fig. 1. Kinetic studies for the elucidation of the type of inhibition induced by 1-deoxy-1-fluoro-scylo-inositol in the synthase and exchange assays

Lysed turkey erythrocyte membranes were incubated under similar assay conditions to those stated in the legend to Table 1. The modifications to the assays are described below. The inositol concentrations used in these studies ranged from $0.5 \times K_m$ to $5 \times K_m$ for the synthase and exchange reactions respectively. The effects of a series of analogue concentrations on the incorporation of [^{3} H]Ins were measured in assays containing the above-mentioned range of inositol concentrations. For each experiment four graphs were plotted, where one graph represented the set of control assays in which the analogue was absent, while the other three contained the respective analogue concentrations. (a) and (b) show the Lineweaver-Burk plots obtained for the synthase and exchange reactions respectively when 1-deoxy-1-fluoro-scylo-inositol was used. Each assay was carried out in duplicate. A computer program was used to fit the best lines through the data. Concentrations used: (a) $\triangle$, 0 mM; $\blacksquare$, 0.55 mM; $\circ$, 2 mM; $\bullet$, 5 mM; (b) $\triangle$, 0 mM; $\blacksquare$, 0.4 mM; $\circ$, 1.2 mM; $\bullet$, 4 mM.

the exchange activity compared with the synthase activity, except for 2-deoxy-2-fluoro-*myo*-inositol where, interestingly, the effects were reversed. An explanation for this observation must lie in the different requirements of the active sites of the PtdIns synthase and exchange enzymes.

Analogues displaying significant inhibition (> 50%) of the synthase and exchange mechanisms were analysed further, although the available quantity of inhibitor was a limiting factor in certain cases. Kinetic studies were undertaken to elucidate the type of inhibition occurring in the presence of these analogues. Assay conditions used are described in the appropriate legends to Figures and Tables. K_i values were determined from the double-reciprocal ($1/v$ versus $1/[S]$) kinetic plots in the absence or in the presence of three concentrations of each analogue (selected following inspection of the preliminary data displayed in Table 1). Kinetic parameters and the type of inhibition were computed using KINPAC. In general, the analogues exhibited competitive inhibition. An example of the plots is shown for 1-deoxy-1-fluoro-scylo-inositol in Fig. 1. The calculated K_i values for all analogues tested are shown in Table 2.

Investigations analysing the inhibitory effects of a number of analogues on the incorporation of [^{3} H]Ins into PtdIns via both the PtdIns synthase and exchange activities indicated that all analogues inhibited incorporation to some extent, although the degree of inhibition varied greatly. Kinetic studies confirmed that 1-deoxy-1-fluoro-scylo-inositol and 5-*O*-methyl-*myo*-inosi-

Table 2. Results of the kinetic studies carried out on analogues of *myo*-inositol

myo-Inositol analogue K_i values were elucidated using computer software designed for analysing kinetic data (KINPAC software, designed by Walter E. Dewolf, Jr.). The two analogues which did not exhibit competitive inhibition were 2-methoxy-*myo*-inositol and 4-deoxy-4-fluoro-*myo*-inositol. A series of plots was obtained for 2-methoxy-*myo*-inositol, but the Lineweaver-Burk plots did not intercept at the y -axis. A computer plot causing the data to intercept at a common point on the y -axis was inaccurate; thus a credible K_i value could not be determined for this analogue. Uninterpretable kinetic data were also obtained for 4-deoxy-4-fluoro-*myo*-inositol, since an increase in the analogue concentration (from 6 mM to 12 mM) resulted in a decrease in inhibition.

Analogue	Type of reaction	Type of inhibition	K_i (mM)
1- <i>O</i> -Methyl- <i>myo</i> -inositol	Exchange	Competitive	1.56 ± 0.16
1-Deoxy-1-fluoro- <i>myo</i> -inositol	Synthase	Competitive	25.07 ± 2.37
	Exchange	Competitive	19.46 ± 0.18
1-Deoxy-1-fluoro- <i>scyllo</i> -inositol	Synthase	Competitive	2.87 ± 0.18
	Exchange	Competitive	0.37 ± 0.06
2- <i>O</i> -Methyl- <i>myo</i> -inositol	Exchange	—	—
3-Deoxy- <i>myo</i> -inositol	Exchange	Competitive	2.96 ± 0.10
3-Deoxy-3-fluoro- <i>myo</i> -inositol	Synthase	Competitive	5.46 ± 1.7
	Exchange	Competitive	0.69 ± 0.17
4-Deoxy- <i>myo</i> -inositol	Exchange	Competitive	7.8 ± 1.2
4-Deoxy-4-fluoro- <i>myo</i> -inositol	Exchange	—	—
5- <i>O</i> -Methyl- <i>myo</i> -inositol	Synthase	Competitive	2.8 ± 0.4
	Exchange	Competitive	0.26 ± 0.02

tol exhibited the greatest competitive effects on the substrate for both enzyme activities.

The syntheses of 1*D*-1-deoxy-1-fluoro-*myo*-inositol and 1*D*-3-deoxy-3-fluoro-*myo*-inositol have been reported (Kozikowski *et al.*, 1990), and the ability of these inositol analogues to affect cell growth of NIH 3T3 (normal fibroblasts) and v-*sis*-transformed NIH 3T3 cells has been investigated. The latter analogue, at a concentration of 5 mM, was capable of inhibiting v-*sis*-transformed NIH 3T3 cell growth by approx. 90% when the medium utilized was free of inositol, but when 0.2 mM-inositol was present in the medium, inhibition of growth was minimal. These results are interesting in comparison with the effects of the same analogues on PtdIns synthase in turkey erythrocytes. In the lysed turkey erythrocyte system, the analogues appear to exert a greater competitive effect on the incorporation of [^{3} H]Ins into PtdIns than their inhibitory effects on cell growth in mutant fibroblasts (Kozikowski *et al.*, 1990), but this may be explained by poor penetration of the compound in the intact cell system. Studies of the cellular uptake of 3-deoxy-3-fluoro-*myo*-inositol will help to resolve this issue.

Are inositol analogues substrates or inhibitors of the *myo*-inositol incorporation enzymes?

In turkey erythrocytes, the ability of an analogue to act as a substrate in the PtdIns pathway can be explored by using radiolabelled derivatives of the analogue. Radiolabelled derivatives of 1-deoxy-1-fluoro-scylo-inositol and 2-deoxy-2-fluoro-*myo*-inositol were therefore synthesized (Lowe & McPhee, 1991). The former was chosen since it was a relatively potent competitive inhibitor of both the synthase and exchange reactions, and the synthetic route to incorporate radiolabel into the analogue was straightforward. The radiolabelled derivative of 2-deoxy-2-fluoro-*myo*-inositol was of considerable interest, since this analogue exerted relatively greater inhibitory effects on the synthase, whereas all other analogues exerted greater inhibitory effects on the exchange enzyme (Table 1).

Table 3. Incorporation of the tritiated analogues into PtdIns over 24 h

Basic system: lysed turkey erythrocyte membranes (0.321 mg) were incubated with assay buffer, 1 mM-CMP, penicillin/streptomycin (50 µg/ml, 1.6 µl) and 1 µCi of the appropriate tritiated analogue, in a total assay volume of 200 µl for 24 h at 37 °C. Ins (0.2 mM) was present in assays comparing the effects of CMP in order to determine the incorporation of the tritiated analogue via the PtdIns synthase reaction. To obtain an optimum response for incorporation by the PtdIns exchange mechanism, a Mn²⁺ assay buffer was utilized in the absence of CMP. Control assays containing [³H]Ins were run for 5 min, 1 h and 24 h respectively. Each result represents the mean ± S.E.M. from one experiment, in which each assay was carried out in duplicate.

Assay conditions	Incorporation of 2-deoxy-2- fluoro- <i>myo</i> - [2- ³ H]inositol (d.p.m.)	Incorporation of 1-deoxy-1- fluoro- <i>scyllo</i> - [1- ³ H]inositol (d.p.m.)	Incorporation of [³ H]Ins (d.p.m.)
Basic	568 ± 1	305 ± 25	391390 ± 820
+ CMP	504 ± 7	283 ± 19	167400 ± 110
- CMP	56 ± 0.5	45 ± 2	314800 ± 210
Mn ²⁺ assay buffer	685 ± 31	245 ± 10	1574900 ± 2510
Zero control	23	28 ± 1	28 ± 6

Fig. 2. Determination of whether 1-deoxy-1-fluoro-*scyllo*-[1-³H]inositol and 2-deoxy-2-fluoro-*myo*-[2-³H]inositol were incorporated into PtdIns

(a) Superimposed h.p.l.c. runs for the non-incubated analogue samples 1-deoxy-1-fluoro-*scyllo*-[1-³H]inositol (●) and 2-deoxy-2-fluoro-*myo*-[2-³H]inositol (2-F; ■), and [³H]Ins (▲), with respective retention times of approx. 20, 24 and 33 min. (b) Radioactive material retrieved from the PtdIns samples after the incubation with lysed turkey erythrocytes and the tritiated analogue 2-F. The retention time of the peak is 24 min, and is equivalent to that obtained for the non-incubated sample 2-F.

Fig. 3. H.p.l.c. data showing the respective incorporation of both 2-deoxy-2-fluoro-*myo*-[2-³H]inositol and [³H]Ins into the PtdIns pathway

Lysed turkey erythrocyte membranes (0.631 mg) were incubated with 2 mM-CMP, penicillin/streptomycin (50 µg/ml; 1.6 µl), a Mg²⁺ assay buffer (100 µl) and either (a) 25 pmol of [³H]Ins or (b) 2-deoxy-2-fluoro-*myo*-[2-³H]inositol (< 1 µCi) for 14 h at 37 °C. The pre-labelled membrane PtdIns had three fates: (1) in a control assay incubated with ATP for zero min (---), (2) in an assay incubated with ATP for 10 min, then with Hepes buffer (15 µl) for a further 10 min (·····), and (3) in an assay incubated with ATP for 10 min, then with GTP[S] (15 µM) for a further 10 min (—). The components present were analysed by h.p.l.c. and the results are shown in (a). The [³H]PtdIns analogue, derived from the tritiated analogue, had two fates which corresponded to the conditions outlined in (1) and (3). The components produced in this experiment were analysed by h.p.l.c. and the results are shown in (b). The assays in (a) were carried out in triplicate and the assays in (b) were carried out in duplicate. The limited amount of inositol analogue precluded examination of all the conditions described in (2).

to act as substrates for the PtdIns synthase and PtdIns exchange reactions. There was no detectable incorporation of the tritiated analogues into phospholipids after an incubation time of 1 h, indicating that these compounds are, at best, very poor substrates for the PtdIns synthase and exchange enzymes. When the incubation period was increased to 24 h (in the presence of penicillin and streptomycin to prevent bacterial growth), low levels of incorporation were detected (Table 3). However, the percentage of radioactivity incorporated was small with respect to the amount of radiolabel present, and the rate of incorporation compared with that of [³H]Ins was very low. These analogues thus appear to be very poor substrates.

Before investigating whether the [³H]PtdIns analogues could serve as substrates for the endogenous lipid kinases in turkey erythrocyte ghosts, it was necessary to know whether the low levels of labelling reflected the incorporation of the analogues themselves or whether minor contaminants were responsible. This was achieved by deacylation, deglyceration and dephosphorylation of the lipid analogue (see the Materials and methods section) and h.p.l.c. analysis of the ³H-labelled head-group so obtained. This procedure revealed that the compound derived from [³H]PtdIns that had been labelled with 1-deoxy-1-

Preliminary experiments employing 1-deoxy-1-fluoro-*scyllo*-[1-³H]inositol and 2-deoxy-2-fluoro-*myo*-[2-³H]inositol were undertaken to establish the capability of these tritiated analogues

fluoro-*scyllo*-[1-³H]inositol did not co-chromatograph with the starting material (results not shown) and, therefore, reflected the incorporation of an unknown contaminant. The same procedure applied to 2-deoxy-2-fluoro-*myo*-[2-³H]inositol revealed that the labelled PtdIns analogue indeed contained the authentic precursor (Fig. 2).

In the experiment depicted in Fig. 3, 2-deoxy-2-fluoro-*myo*-[2-³H]inositol was first incubated with erythrocyte ghosts for 14 h at 30 °C (conditions which were found to maintain the activity of PtdIns and PtdIns4P kinases in the preparation). Following this prelabelling period, the 2-deoxy-2-fluoro-*myo*-[2-³H]inositol was washed out and ATP was added to some of the samples. Before the addition of ATP these samples contained a single radioactive lipid which upon deacylation gave a compound with chromatographic properties similar to those of glycerophosphoinositol (GroPIIns). After addition of ATP several more polar compounds were present whose deacylation products resembled GroPIInsP and GroPIInsP₂. However, the proportion of the PtdIns analogue that was phosphorylated under these conditions was very much greater than in the corresponding experiment using *myo*-[³H]inositol. Moreover, the h.p.l.c. trace in Fig. 3(b) is much more complex than that in Fig. 3(a), in which erythrocyte lipids were labelled with *myo*-[³H]inositol. Unfortunately, there was insufficient of the labelled analogue to further characterize the components of the complex peaks in Fig. 3(b) or to convincingly determine whether the lipid analogues generated were PLC substrates. Guanosine 5'-[γ-thio]triphosphate (GTP[S]) was included in the incubations in an attempt to test the latter, but there was no detectable increase in the water-soluble [³H]labelled components resulting from this treatment. A possible interpretation of these results which, although unlikely, cannot be discounted, is that the PtdIns analogue provides an efficiently used substrate for inositol lipid kinases, but that the normal specificity of the enzymes is compromised by the substitution of a fluorine atom in place of the 2-OH group of *myo*-inositol.

DISCUSSION

1-Deoxy-1-fluoro-*scyllo*-inositol and 2-deoxy-2-fluoro-*myo*-inositol have the potential to be very useful reagents for studying the phosphoinositide pathway. The former analogue is of considerable interest, since it is a relatively good competitive inhibitor of the synthase and exchange enzymes, and has the potential to diminish the production of PtdIns, which, in turn, decreases the conversion of PtdIns into the polyphosphoinositides, hence decreasing the level of PtdInsP₂ for PLC-mediated second messenger production. 2-Deoxy-2-fluoro-*myo*-inositol is a very poor substrate for PtdIns synthase and the exchange enzyme. The PtdIns analogue produced appears to be readily recognized by the kinase enzymes to yield analogues of PtdInsP and PtdInsP₂.

The conversion of the PtdIns analogue to the polyphosphoinositide analogues observed in assays incubated with 2-deoxy-2-fluoro-*myo*-[2-³H]inositol was much greater than that observed under assay conditions incubated with [³H]Ins (Fig. 3), which suggests that the kinase enzymes catalysing these reactions were not as specific for their substrate as the PtdIns synthase or exchange enzymes. Studies undertaken by other groups have shown that the synthase enzyme has stringent requirements for

the *myo*-inositol isomer (Benjamins & Agranoff, 1969), although *scyllo*-inosose has been shown to compete with *myo*-inositol for PtdIns synthase in rat liver (Takenawa & Egawa, 1977).

Moyer *et al.* (1988) carried out inhibitory and substrate studies on PtdIns synthase from rat brain and uptake experiments utilizing L1210 cells. They concluded that, of the inositol analogues studied (modifications in the 2-, 3-, 4- and 5-positions; Jiang *et al.*, 1987), analogues with modifications in the 5-position were taken up into the cell, but only 5-deoxy-5-fluoro-*myo*-inositol was accepted as a substrate for PtdIns synthase. Moyer *et al.* (1988) also studied the inhibitor and substrate capabilities of 1-deoxy-1-fluoro-*scyllo*-inositol. This analogue appeared to be a better inhibitor under conditions utilized in our system.

It would be interesting to study the effects of the analogues synthesized with modifications in the 5-position (Moyer *et al.*, 1988) to see the extent of their inhibition in the turkey erythrocyte system. We analysed the kinetics of 5-*O*-methyl-*myo*-inositol, which yielded the greatest inhibitory effects on both the synthase and exchange reactions in turkey erythrocytes, and the *K_i* values were of the same order as those for 1-deoxy-1-fluoro-*scyllo*-inositol. Thus an investigation of the substrate capabilities of radiolabelled 5-*O*-methyl-*myo*-inositol would be useful. If incorporation of the radiolabelled analogue into the PtdIns pathway occurred, a comparative study with the inhibition induced by 5-deoxy-5-fluoro-*myo*-inositol (Moyer *et al.*, 1988) would be interesting.

Moyer *et al.* (1988) and Kozikowski *et al.* (1990) have shown that certain *myo*-inositol analogues can permeate into their cell systems. If analogues are to be used in studies with intact turkey erythrocytes, then it is essential that they can be taken up into the cell to act as substrates/inhibitors of PtdIns synthase and of the exchange activity. Hence the uptake of radiolabelled analogues into intact turkey erythrocytes, and perhaps into other cells, has to be investigated if the tritiated analogues synthesized are to be used in further studies of the phosphoinositide pathway.

REFERENCES

- Benjamins, J. A. & Agranoff, B. W. (1969) *J. Neurochem.* **16**, 513–527
- Boyer, J. L., Downes, C. P. & Harden, T. K. (1989) *J. Biol. Chem.* **264**, 884–890
- Brown, D. M. & Stewart, J. C. (1966) *Biochim. Biophys. Acta* **125**, 413–421
- Harden, T. K., Hawkins, P. T., Stephens, L., Boyer, J. L. & Downes, C. P. (1988) *Biochem. J.* **252**, 583–593
- Hawkins, P. T., Stephens, L. & Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
- Jiang, C., Moyer, J. D. & Baker, D. C. (1987) *J. Carbohydr. Chem.* **6**, 319–355
- Kozikowski, A. P., Fauq, A. H., Powis, G. & Melder, D. C. (1990) *J. Am. Chem. Soc.* **112**, 4528–4531
- Lowe, G. & McPhee, F. (1991) *J. Chem. Soc. Perkin Trans. 1*, 1249
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- McPhee, F., Lowe, G., Vaziri, C. & Downes, C. P. (1991) *Biochem. J.* **275**, 187–192
- Moyer, J. D., Reizes, O., Surender, A., Jiang, C., Malinowski, N. & Baker, D. C. (1988) *Mol. Pharmacol.* **33**, 683–689
- Stephens, L. R., Hawkins, P. T., Carter, N., Chatwala, S. B., Morris, A. J., Whetton, A. D. & Downes, C. P. (1988) *Biochem. J.* **249**, 271–282
- Takenawa, T. & Egawa, K. (1977) *J. Biol. Chem.* **252**, 5419–5423
- Whitman, M., Downes, C. P., Keeler, M., Keller, T. & Cantley, L. (1988) *Nature (London)* **332**, 644–646