Phosphatidylinositol synthase and phosphatidylinositol/inositol exchange reactions in turkey erythrocyte membranes

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Unlike human erythrocytes, those from avian species, such as turkeys and chicks, rapidly incorporate *myo*-[³H]inositol into membrane phospholipids. The mechanisms regulating [³H]Ins labelling of phosphatidylinositol have been investigated using turkey erythrocyte membranes. In the absence of added nucleotides, [³H]inositol incorporation appears to proceed via phosphatidylinositol/inositol exchange, with a K_m for inositol of 0.01 mM. The reaction was dependent upon divalent cations, either Mg²⁺ or Mn²⁺, with the latter metal ion being the more effective. [³H]inositol incorporation was accelerated by CMP, especially when the concentration of Ins was greater than the K_m for the exchange reaction. CMP-dependent labelling of PtdIns had a K_m for inositol of 0.3 mM and for CMP of 0.015 mM. Divalent cations were also required for this reaction: activity peaked at 0.5 mM-Mn²⁺ and declined at higher concentrations. At relatively high concentrations, Mg²⁺ was more effective than Mn²⁺, with peak activity being achieved above 10 mM. CMP-dependent incorporation of [³H]inositol appears to reflect an exchange reaction catalysed by PtdIns synthase. Definitive evidence for the occurrence of PtdIns synthase in turkey erythrocyte membranes was obtained by demonstrating the formation of [¹⁴C]CMP-phosphatidate from [¹⁴C]CMP. The radioactivity could be efficiently chased from [¹⁴C]CMP-phosphatidate in the presence of unlabelled inositol. The detection of PtdIns synthase activity in morphologically simple turkey erythrocytes should help to clarify the subcellular distribution of this important component of the phosphatidylinositol cycle.

INTRODUCTION

Receptor-stimulated PtdInsP₂ hydrolysis is a ubiquitous signal transduction mechanism which generates the intracellular second messengers diacylglycerol and Ins(1,4,5)P₃ (Hirasawa & Nishizuka, 1985; Berridge & Irvine, 1989). Cells appear to contain a finite hormone-sensitive pool of inositol phospholipids (see below), the replenishment of which during continued hormonal stimulation requires efficient recycling of diacylglycerol and the inositol moiety of Ins(1,4,5)P₃. These recycling reactions culminate in the combination of CMP-phosphatidate and inositol to give PtdIns, a reaction catalysed by CMP-phosphatidate: inositol phosphatidyltransferase (PtdIns synthase; reviewed by Downes & Michell, 1985). Recycling of the inositol moiety of Ins(1,4,5)P₃ is blocked by lithium ions at the level of inositol polyphosphate 1-phosphomonoesterase and inositol monophosphate phosphomonoesterase (Hallcher & Sherman, 1980; Majerus *et al.*, 1988), an effect which may underlie the drug's therapeutic utility in the treatment of manic depressive psychoses (Berridge *et al.*, 1989). Moreover, since agonists which stimulate PtdInsP₂ hydrolysis enhance the rate of incorporation of [³²P]P_i or [³H]inositol into cellular PtdIns (even when the specific radioactivity of phosphatidate, from which PtdIns obtains its phosphodiester phosphate, is not changing; Weiss *et al.*, 1982), it is evident that PtdIns synthase activity is increased during stimulation (Downes & Michell, 1985). Neither the mechanism nor the function of the receptor-mediated stimulation of PtdIns synthase is currently understood, although Imai & Gershengorn (1989) suggested that it may be due to relief of product inhibition by PtdIns.

Although it is self-evident that hormone-stimulated PtdInsP₂ hydrolysis is initiated in the plasma membranes of cells, whether the complete inositol phospholipid metabolic cycle takes place in a single membrane compartment is currently a matter of debate. Metabolic turnover studies providing evidence both for and

against the existence of a discrete hormone-sensitive pool of inositol phospholipids have been reported (Monaco, 1982, 1987; Michell *et al.*, 1988), and the well-documented occurrence of a PtdIns transfer protein could facilitate rapid equilibration of this lipid species between subcellular compartments (Van Paridon *et al.*, 1987). In an attempt to resolve these issues, Gershengorn and his colleagues have sought and characterized a PtdIns syn-

Fig. 1. Time course of incorporation of [³H]inositol into turkey erythrocyte PtdIns

Turkey erythrocyte membranes were incubated with [³H]inositol in basic incubation buffer containing 1 mM-MgCl₂ for the indicated times. Radiolabelled PtdIns was extracted and quantified as described in the Materials and methods section. The results are expressed as [³H]inositol incorporated into membrane PtdIns in the presence (●) or the absence (○) of 1 mM-CMP, and each data point is the mean of duplicate incubations which generally differed by no more than 5%. The Figure shows a single experiment which was reproduced several times.

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thase activity in membrane fractions enriched in plasma membranes of GH₃ pituitary cells (Imai & Gershengorn, 1987; Cubitt & Gershengorn, 1989). The putative plasma membrane PtdIns synthase could be distinguished on kinetic grounds from the endoplasmic reticulum enzyme, having a lower K_m for inositol. If substantiated in a variety of cell types, this observation would provide compelling evidence in favour of concerted recycling of a hormone-sensitive pool of inositol phospholipids within the plasma membrane. Such a possibility is also suggested by the accumulation of both CMP-phosphatidate synthase and PtdIns synthase in post-Golgi apparatus secretory vesicles destined for the plasma membranes of the yeast *Saccharomyces cerevisiae* (Kinney & Carman, 1990).

Our observations that turkey erythrocytes, by contrast with erythrocytes obtained from mammalian species, incorporate large amounts of [³H]inositol into PtdIns (Harden *et al.*, 1988) prompted the present study to determine the characteristics of the enzymes involved. The results suggest that turkey erythrocyte membranes possess both PtdIns synthase and PtdIns/inositol exchange activities. The occurrence of PtdIns synthase in anatomically simple avian erythrocytes, which are nucleated but contain very little endoplasmic reticulum, may be a further pointer to the existence of a plasma membrane isoform of this enzyme.

MATERIALS AND METHODS

Assays of PtdIns synthase and PtdIns/inositol exchange reactions in turkey erythrocyte membranes

Turkey erythrocyte ghosts were prepared by hypo-osmotic lysis and washing exactly as described previously (Harden *et al.*, 1988). Incubations were carried out at 37 °C in 200 µl of 20 mM-Hepes, pH 7.0, containing 0.1–0.25 mg of membrane protein, [³H]inositol (0.5–1 µCi) or [¹⁴C]CMP (0.1 µCi) and various additions as indicated in the legends to Figures and Tables. The reactions were stopped by the addition of 750 µl of chloroform/methanol/0.1 M-HCl (80:40:1, by vol.). The contents were mixed vigorously and a further 500 µl of chloroform, 250 µl of methanol and 250 µl of 0.1 M-HCl were added. After vortex-mixing, the phases were separated by a brief centrifugation. The resulting upper phase was aspirated and the lower phase was then washed with 2 × 1 ml portions of methanol/0.1 M-HCl/chloroform (48:47:3, by vol.). A 500 µl portion of the final lower phase was then mixed with 10 ml of Packard Instagel and radioactivity was determined by scintillation counting. Zero-time controls confirmed that the wash procedure removed essentially all of the unincorporated [³H]inositol.

T.l.c.

PtdIns and CMP-phosphatidate were identified using oxalate-impregnated silica-gel 60 plates (Merck) prepared by dipping the plates in a solution of 1% potassium oxalate. The latter were then air-dried and pre-activated by heating at 100 °C for 15 min. Two different solvent systems were employed: (1) chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, by vol.) according to Jolles *et al.* (1981), or (2) a double solvent system developed in a single direction starting with chloroform/methanol/acetic acid/water (40:10:10:1, by vol.) followed by the same combination of solvents in the proportions 120:46:19:3 (by vol.) (Godfrey, 1989). Phospholipids, including standards, were visualized by iodine staining and the positions of radiolabelled phospholipids were verified using a radio t.l.c. scanner or, in the case of [¹⁴C]CMP-phosphatidate, by autoradiography.

Materials

[5-¹⁴C]CMP and *myo*-[2-³H]inositol were obtained from New England Nuclear, Boston, MA, U.S.A. CDP-dipalmitoylglycerol (commercial CMP-phosphatidate), potassium salt, was obtained from Sigma Chemical Company.

RESULTS

When turkey erythrocyte ghosts were incubated with 0.1 mM-[³H]inositol and 1 mM-MgCl₂ under the conditions defined above, the radiolabel was rapidly incorporated into membrane phospholipid. T.l.c. analysis indicated that the label was incorporated specifically into PtdIns. As shown in Fig. 1, the rate of incorporation of [³H]inositol into membrane PtdIns was greatly increased in the presence of 1 mM-CMP. In the presence of CMP, incorporation of radiolabel reached a plateau within about 30 min and was linear for no more than the first 5 min of the incubation at 37 °C (Fig. 1). In the absence of CMP the incorporation of [³H]inositol was approximately linear for at least 15 min. Subsequent experiments to determine the divalent cation, inositol and CMP requirements of these reactions used an incubation time of 5 min to obtain approximate initial incorporation rates for the CMP-dependent and -independent processes.

The divalent cation requirements of the CMP-dependent and -independent reactions were assessed (Fig. 2). CMP-independent incorporation of 0.01 mM-[³H]inositol was absolutely dependent on divalent cations. Both Mg²⁺ and Mn²⁺ were effective over the same concentration range, with the latter giving incorporation rates approximately twice that obtained using the same concentration of Mg²⁺ (each as their chloride salts). The maximum

Fig. 2. Effects of divalent cations on [³H]inositol labelling of membrane PtdIns

Turkey erythrocyte membranes were incubated with [³H]inositol in basic incubation buffer containing the indicated concentrations of MgCl₂ (●) or MnCl₂ (○) and either without (a) or with (b) 1 mM-CMP for 5 min. Data points are the means of duplicate incubations which differed by no more than 5%, and the results are from one of three essentially identical experiments.

rates could not be properly assessed, as the incorporation rate was still increasing at concentrations of greater than 10 mM for both cations. CMP-dependent incorporation of [^3H]inositol was defined as the difference in radiolabel incorporated between parallel CMP-containing and CMP-free incubations at each divalent cation concentration and was maximized by utilizing a relatively high concentration of [^3H]inositol (1 mM; see below). This process was also dependent upon divalent cations, but the effects of Mn^{2+} and Mg^{2+} differed markedly from those described above. Mn^{2+} was a relatively potent cofactor, but its effects were biphasic, with peak activity observed at about 0.5 mM- Mn^{2+} . Further increasing [Mn^{2+}] only caused the CMP-dependent incorporation of [^3H]inositol to decline. By contrast, the effects of Mg^{2+} appeared to be monophasic, with half-maximal activity occurring at about 2 mM. At concentrations above 2 mM, Mg^{2+} was markedly more effective than Mn^{2+} .

The different divalent cation requirements for the CMP-dependent and -independent reactions noted above were the first indication that these processes may be catalysed by distinct enzymes in turkey erythrocyte ghosts, rather than simply reflecting the enhancement by CMP of a single enzyme activity. Preliminary experiments also revealed markedly different inositol requirements for the two processes when the assays were carried out under identical conditions. Evidence will be presented below which indicates that the CMP-dependent incorporation of [^3H]inositol represents an exchange reaction catalysed by PtdIns synthase, whereas the nucleotide-independent reaction appears to be catalysed by a PtdIns/inositol exchange activity. Henceforth these processes will be referred to as PtdIns synthase and exchange reactions respectively.

The inositol requirements of the synthase and exchange reactions are shown in Figs. 3(a) and 3(b). In order to optimize their respective rates the synthase assay contained 5 mM- MgCl_2 and 1 mM-CMP, whereas the exchange assay contained 5 mM- MnCl_2 . Both reactions showed typical saturation kinetics, thus allowing V_{max} and K_m values to be determined from the Eadie-Hofstee plots (Fig. 3). The synthase, with a V_{max} of $371 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ and a K_m of $0.30 \pm 0.05 \text{ mM}$ ($n = 5$), is a relatively low-affinity high-capacity system, whereas the exchange mechanism has a high affinity for inositol ($K_m = 0.012 \pm 0.0015 \text{ mM}$) but a low capacity ($V_{\text{max}} = 6.5 \text{ pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$).

Table 1. Properties of turkey erythrocyte membrane PtdIns synthase and headgroup exchange enzymes

PtdIns synthase and headgroup exchange activities were measured under a variety of conditions as described in the Materials and methods section and the legends to Figs. 2, 3 and 4. V_{max} and K_m values were determined from the ordinate intercepts and negative slopes respectively of the Eadie-Hofstee plots, typical data being shown in Figs. 3 and 4. Numbers in parentheses are the numbers of independent experiments from which the mean value of each parameter was calculated.

List of parameters	PtdIns synthase	Headgroup exchange
K_m (mM)		
Inositol	0.303 (7)	0.012 (7)
CMP	0.015 (4)	—
V_{max} ($\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)		
Inositol	371 (7)	6.5 (7)
CMP	500 (5)	—
Divalent cation optimum (mM)		
Mg^{2+}	10 (3)	> 10 (2)
Mn^{2+}	0.5 (2)	> 10 (3)

Fig. 3. Inositol-dependence of PtdIns synthase and headgroup exchange activities of turkey erythrocyte membranes

Turkey erythrocyte membranes were labelled under 'synthase' (5 mM- MgCl_2 , 1 mM-CMP; a) or 'headgroup exchange' (5 mM- MnCl_2 ; b) conditions in the presence of various concentrations of unlabelled inositol for 5 min. Incorporation rates were calculated from the radioactivity incorporated into PtdIns and the known specific radioactivity of inositol in each incubation. The data were plotted according to Eadie & Hofstee. Each point is the mean of duplicates and the results from several individual experiments were used to compile Table 1.

Fig. 4. CMP-dependence of turkey erythrocyte PtdIns synthase

Turkey erythrocyte membranes were labelled under 'synthase' conditions in the presence of the indicated concentrations of CMP for 5 min. Incorporation rates were calculated as for Fig. 3. The Figure shows the Eadie-Hofstee transformation of the raw data. Data points are the means of duplicates and the results from several experiments were used to compile Table 1.

The CMP requirement of the synthase was also determined at an inositol concentration close to the K_m (0.2 mM). The results are shown in Fig. 4 and the K_m , determined from the Eadie-Hofstee plot, was found to be 0.015 mM ($n = 4$). The

Fig. 5. Incorporation of [^{14}C]CMP into turkey erythrocyte membrane CMP-phosphatidate

Turkey erythrocyte membranes were labelled in standard incubation buffer containing 5 mM-MgCl₂ and 0.1 μCi of [^{14}C]CMP, giving a final concentration of the latter of approx. 10 μM , for the times indicated in the presence (○) or the absence (●) of 1 mM unlabelled inositol. Data points are the means of duplicates, and the results are from a single experiment which is typical of three giving essentially identical results.

Fig. 6. Time-dependent chase of radioactivity from prelabelled PtdIns

Turkey erythrocyte membranes were labelled with [^3H]inositol under 'exchange' conditions for 60 min. The membranes were washed free of labelled inositol and resuspended in basic incubation buffer containing 5 mM-MgCl₂ and either 1 mM (■) or 10 mM (◆, ◇) unlabelled inositol in the presence (◇) or absence (◆, ■) of 1 mM-CMP. Controls (□) contained no unlabelled inositol or CMP. The incubations were continued at 37 °C for the indicated times. The data points are means of duplicates and the Figure shows one of two experiments giving essentially similar results.

respective properties of the synthase and exchange reactions are summarized in Table 1.

PtdIns synthase catalyses the reversible combination of CMP-phosphatidate and inositol to yield PtdIns and CMP. The reversible nature of this enzymic process was exploited to unequivocally establish the presence of PtdIns synthase activity in turkey erythrocyte ghosts. As shown in Fig. 5, the ghosts catalysed the incorporation of [^{14}C]CMP into a lipid which was

identified as CMP-phosphatidate by co-chromatography with a known standard on t.l.c. using two different solvent systems (results not shown). Moreover, the radiolabel incorporated into CMP-phosphatidate in this way could be effectively and rapidly chased by adding inositol to the incubations (Fig. 5).

In view of the occurrence of multiple metabolically segregated pools of inositol phospholipids, even in anatomically simple human erythrocyte membranes (King *et al.*, 1987), it was important to investigate the relationship between the lipid pools labelled by the synthase and exchange reactions. Time courses similar to those illustrated in Fig. 1, but carried out under approximately optimal conditions for the synthase and exchange reactions respectively (results not shown), revealed that the synthase reaction appears to equilibrate after about 10 min, when approx. 5 nmol of inositol had been incorporated. The exchange reaction is much slower and does not equilibrate, even after 60 min, after which time only about 0.8 nmol of inositol had been incorporated. The data are compatible with the possibility that each enzyme labels the same pool of inositol lipids, but at very different rates. This was confirmed by first labelling membrane PtdIns under exchange conditions followed by the addition of an excess of unlabelled inositol to act as a chase. The radiolabel was chased from endogenous membrane PtdIns only slowly if exchange conditions were maintained, but the rate of chase was dramatically increased if 1 mM-CMP was added together with the unlabelled inositol (Fig. 6). As the combination of CMP and unlabelled inositol rapidly chased > 90 % of the label from PtdIns, it follows that essentially all of the PtdIns labelled by the exchange reaction is accessed by PtdIns synthase. Whether PtdIns synthase has access to a larger pool of PtdIns than the exchange enzyme cannot be determined from this experiment.

DISCUSSION

The incorporation of inositol into PtdIns by exchange of free and lipid-bound inositol moieties has been described for a number of tissues (Berry *et al.*, 1983). At least two types of Mg²⁺/Mn²⁺-dependent enzymes appear to be responsible for catalysing these exchange reactions. One is nucleotide-independent and its function is unknown (Takenawa *et al.*, 1987). The other is CMP-dependent and appears to be an exchange reaction catalysed by PtdIns synthase (Berry *et al.*, 1983; Fischl *et al.*, 1986). PtdIns synthase catalyses the reversible combination of CMP-phosphatidate and inositol to give PtdIns and CMP, as shown below:

The yeast enzyme has been purified to homogeneity and shown to function by a sequential mechanism (Fischl *et al.*, 1986; Nikawa *et al.*, 1987). It efficiently catalyses both the forward and reverse reactions depicted above and will also catalyse exchange between inositol and PtdIns in the presence of CMP. By contrast, the yeast enzyme does not enhance exchange between CMP and CMP-phosphatidate in the presence of inositol, suggesting that the enzyme binds CMP-phosphatidate before inositol, and releases first PtdIns and then CMP (Nikawa *et al.*, 1987).

If, as seems likely, the above mechanism applies to other forms of PtdIns synthase, then the ability of CMP to enhance the incorporation of [^3H]inositol into endogenous membrane PtdIns may represent a convenient means of detecting PtdIns synthase activity in subcellular fractions. In turkey erythrocyte membranes the CMP-dependent component of [^3H]inositol incorporation is clearly an exchange process, because CMP also accelerates the rate at which unlabelled inositol chases radioactivity from prelabelled PtdIns (Fig. 6). Moreover, the CMP-dependent

component can be distinguished from nucleotide-independent labelling, on the basis of their distinct divalent cation and inositol requirements. Direct confirmation of the occurrence of PtdIns synthase in turkey erythrocyte membranes was provided by exploiting the reverse reaction (Hokin-Neaverson *et al.*, 1977), which was able to incorporate [³H]CMP into a lipid that was identified as CMP-phosphatidate.

Cubitt *et al.* (1990) recently showed that CMP greatly enhanced the incorporation of [³H]inositol into a hormone-insensitive pool of PtdIns in permeabilized GH₃ pituitary cells. However, they interpreted their results by considering that CMP was acting by enhancing the headgroup exchange reaction, and did not consider that they were probably exploiting a mechanistic feature of PtdIns synthase. The latter possibility would require a completely different interpretation of their results, i.e. that much of the PtdIns synthase of GH₃ cells is associated with a hormone-insensitive pool of PtdIns.

From the results presented in this paper, it appears that anatomically simple turkey erythrocytes possess both a PtdIns synthase and a nucleotide-independent PtdIns/inositol base exchange enzyme. The former may be required in avian erythrocytes because they possess a purinoreceptor-stimulated phospholipase C which cleaves the phosphodiester bond in PtdIns4P and PtdIns(4,5)P₂ (Harden *et al.*, 1988; Boyer *et al.*, 1989). As this receptor is likely to be stimulated by circulating ATP and/or ADP during the lifetime of the erythrocyte, it may be that the PtdIns synthase is responsible for maintaining the cellular content of the phosphoinositides. Alternatively, the receptor-stimulated phospholipase C and other enzymes of phosphoinositide metabolism may be relics of erythroid precursor cells and may serve no useful purpose in mature erythrocytes. Whatever the explanation, the occurrence of PtdIns synthase in these cells offers an ideal opportunity to extend the observations of Gershengorn and his colleagues which suggested the existence of multiple forms of PtdIns synthase, one of which appears to be present in plasma membranes (Imai & Gershengorn, 1987; Cubitt & Gershengorn, 1989). Avian erythrocytes consist of a plasma membrane and a nucleus, with other intracellular membranes almost absent (Beam *et al.*, 1979), so that subcellular fractionation is relatively simple. Preliminary studies indicate that the PtdIns synthase activity in these cells is not in the nucleus and that PtdIns labelled exclusively via the synthase mechanism can be converted into polyphosphoinositides, which are substrates for the purinoreceptor-stimulated phospholipase C. These data, which will be presented in detail elsewhere, strongly suggest that the turkey erythrocyte PtdIns synthase is located in plasma membranes.

An important distinguishing feature of the plasma membrane PtdIns synthase of GH₃ pituitary cells is its relatively high affinity for inositol (K_m 0.06 mM) compared with its endoplasmic-reticulum-located counterpart in the same cells (0.26 mM) or the enzyme from rat liver (2.5 mM), rat brain (4.6 mM) or dog pancreas (0.76 mM; Takenawa & Egawa, 1977; Parries & Hokin-Neaverson, 1984; Ghalayini & Eichberg, 1985; Imai & Gershengorn, 1987). The turkey erythrocyte PtdIns synthase appears to have an intermediate affinity for inositol with a K_m of approx. 0.3 mM. The variation in this parameter alone is sufficient to suggest that there may be considerable heterogeneity amongst PtdIns synthases in different tissues or located in different cellular compartments. This is particularly important with regard to the proposal that the therapeutic activity of the anti-manic drug, lithium, results from its uncompetitive inhibition of inositol monophosphate phosphomonoesterase, which causes inositol depletion of cells undergoing active stimulation of phospholipase C-coupled receptors (Berridge *et al.*, 1989). This in turn may lead to inhibition of PtdIns synthase by substrate limitation. Cells in

which the hormone-sensitive pool of inositol phospholipids is generated by a PtdIns synthase with high affinity for inositol will be relatively protected from the inhibitory effects of the drug. By contrast, the enzyme described in rat brain, with an affinity almost 100 times lower than that of the GH₃ cell plasma membrane enzyme, may be especially susceptible to inhibitory mechanisms based on inositol depletion.

The occurrence of the exchange reactions documented in this and other publications raises important questions about experimental strategies which utilize radioactive inositol labelling to study hormone-sensitive and -insensitive pools of inositol phospholipids (Michell *et al.*, 1988). Its relatively high affinity for CMP suggests that the exchange reaction catalysed by PtdIns synthase may operate at a significant rate in intact cells. Exchange reactions provide a means of labelling the inositol moiety of PtdIns independently of the turnover of the phosphodiester phosphorus. Since it is the rapid turnover of the phosphodiester phosphorus that most clearly characterizes the hormone-sensitive inositol phospholipid pool, for example in WRK-1 cells (Monaco, 1982, 1987), it follows that the ability to detect such a pool by radioactive inositol labelling will depend upon the relative rates of the combined exchange processes versus the metabolic flux from diacylglycerol to PtdIns. It may be best to defer interpretation of radiotracer inositol labelling experiments until more is known of the rates of the exchange reactions in intact cells.

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