Organization of the phosphoinositide cycle

Assessment of inositol transferase activity in purified plasma membranes

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Experiments were carried out to determine whether or not CDP-diacylglycerol:myo-inositol 3-phosphatidyltransferase (IT) activity (EC 2.7.8.11) could be detected in purified plasma-membrane fractions from WRK-1 rat mammary tumour cells. These cells have previously been shown to have a very active phosphoinositide cycle. Sucrose-density-gradient-purified plasma mem-

branes contained no IT activity that could not be accounted for by endoplasmic-reticulum contamination. However, we also determined that the relative amount of IT activity in endoplasmic reticulum and plasma-membrane fractions could be altered by changing the concentration of detergent in the assay system.

INTRODUCTION

There is evidence to suggest that the phosphoinositide cycle is localized to the plasma membrane of the cell, closely associated with the receptors that trigger phosphoinositide turnover [1–4]. Many of the enzymes required for functioning of the cycle, such as phospholipase C [5], diacylglycerol kinase [6] and phosphoinositide kinases [7], can be found in the plasma membrane, and it has been hypothesized that the entire cycle takes place there. However, the data describing the subcellular location of the resynthetic enzymes is equivocal. Phosphatidate cytidyltransferase activity has been found predominantly in the endoplasmic reticulum, with some activity localizing to the mitochondria and nucleus [8–10]; in one report [11], a small percentage (2.1 % of the total activity) was found in the plasma membrane. On the other hand, there have been several reports of plasma-membrane inositol transferase (IT) activity [12–15], although most investigators have localized the activity to the endoplasmic reticulum [9,11,16–18].

In the present study, we purify plasma membrane from WRK-1 cells and compare the levels of IT activity in this fraction with those for the known endoplasmic-reticulum marker enzymes, cholinephosphotransferase (CPT) and NADPH-cytochrome *c* reductase (NCR). In addition, we characterize the activity in the plasma membrane and compare it with that found in a crude microsomal pellet, with respect to kinetics, metal requirement and the effect of detergent. We conclude that in WRK-1 cells there is no evidence for a distinct plasma-membrane IT activity if the IT assays are carried out in the presence of detergent; however, in the absence of detergent the relative amounts of IT activity in various subcellular fractions are altered, resulting in what appears to be a plasma-membrane IT activity.

MATERIALS AND METHODS

Materials

Tissue-culture media, sera, trypsin and Dulbecco's phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+} were supplied by Grand Island Biological Co. ^{125}I -transferrin and ^{14}C CDP-choline were obtained from New England Nuclear. ^3H Inositol

(15 Ci/mmol) was from American Radiolabelled Chemicals. Aqasol II was from New England Nuclear.

Cells

WRK-1 cells were established from a dimethylbenz[*a*]anthracene-induced rat mammary tumour as previously described [19]. In brief, cells were grown in monolayer culture in minimal essential medium containing Earle's salts and supplemented with penicillin (100 units/ml), streptomycin (100 $\mu\text{g}/\text{ml}$), fungizone (0.25 $\mu\text{g}/\text{ml}$), calf serum (5 %) and rat serum (2 %). Cells were harvested with a solution of 1 mM EDTA in PBS. After harvesting, cells were collected by centrifugation, washed twice with PBS, and the resulting pellet was frozen at -135°C until used.

Preparation of subcellular fractions

Approx. 2×10^8 cells were used for each plasma-membrane preparation. The cell pellet was thawed and diluted in 1 vol. of lysis buffer (1 mM NaHCO_3 , 0.5 mM dithiothreitol and 1 mM EDTA, pH 7.9). The cells were then incubated at 37°C for 10 min in a shaker bath, followed by vortex-mixing for 1 min. A further 1 vol. of ice-cold lysis buffer was then added, and the cellular disruption was completed by a further vortex-mixing for 1 min. From this point on, all procedures were carried out at 4°C . The lysed cells were then centrifuged for 10 min at 400 *g* to remove whole cells and nuclei. The supernatant was designated the cellular homogenate and centrifuged at 8000 *g* for 30 min. The resulting supernatant was further centrifuged at 100000 *g* for 1 h, and the resulting pellet, diluted to 2 ml with lysis buffer, was utilized as a crude microsomal preparation ('100K') for determination of the endoplasmic-reticulum enzyme activity. The 8000 *g* pellet was diluted in 1 vol. of lysis buffer and placed at the bottom of a discontinuous sucrose-density gradient. The gradient consisted of 57 %, 34 % and 8.55 % (w/v) sucrose dissolved in 5 mM Tris buffer, pH 8.0, in the proportions 2.67:4:1 ml respectively. The gradient was centrifuged at 24000 *g* for 3 h in an SW40 swinging-bucket rotor. Two clear bands were obtained. Band 1 (B1) was at the 57 %/34 % interface, and band 2 (B2) was at the 34 %/8.55 % interface. Each band was aspirated with a 20-gauge needle, diluted to 10 ml with lysis buffer and

Abbreviations used: IT, inositol transferase (EC 2.7.8.11); CPT, cholinephosphotransferase (EC 2.7.8.2); NCR, NADPH-cytochrome *c* reductase; TB, transferrin binding.

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centrifuged at 100 000 *g* for 1 h. The resulting pellets were diluted to 1 ml with lysis buffer.

Analysis of subcellular fractions

All protein determinations were done by the method of Lowry et al. [20]. The presence of plasma membrane was determined by measuring transferrin binding (TB) as follows: 6.55 pmol of ¹²⁵I-transferrin (0.72 µCi/µg) was added to a solution of 20 µg of protein in binding buffer (50 mM Hepes, 5 mM MgCl₂, 1 mM EDTA, 0.1% BSA and 0.5 mM phenylmethanesulphonyl fluoride) in a final volume of 500 µl. Non-specific binding was simultaneously measured by including 5 nmol of unlabelled transferrin to the reaction mixture. The samples were incubated for 30 min at room temperature and then centrifuged for 5 min in a microfuge. The pellets were immediately placed in ice and the supernatants aspirated. The pellets were washed three times with ice-cold PBS.

NCR activity was measured as follows: the reaction mixture, in a total volume of 1 ml, contained 20 µg of protein, NADPH (0.2 mg/ml), KCN (1 mM), cytochrome *c* (0.05%) and KH₂PO₄ buffer (50 mM, pH 7.4). The NADPH was added last to initiate the reaction, which was carried out at 37 °C for 10 min. The reaction tubes were then placed on ice. Reduced cytochrome *c* was monitored by measuring the *A*₅₅₀. To determine the presence of mitochondria, the identical assay utilizing NADH was employed.

CPT activity was measured as follows: the incorporation of [¹⁴C]CDP-choline into chloroform-soluble material was measured. The assay mixture consisted of diacylglycerol (100 µg/ml), CDP-choline (10 µg/ml), MgCl₂ (10 mM), Triton X-100 (0.1%), Tris/HCl buffer (25 mM, pH 7.5), [¹⁴C]CDP-choline (0.1 µCi) and protein in a final volume of 0.1 ml. The reaction mixture was incubated for 20 min at 37 °C and terminated by addition of 4 vol. of chloroform/methanol/HCl (200:100:1, by vol.). After mixing, the layers were separated by centrifugation at low speed, and a sample of the chloroform layer was transferred to a scintillation vial, evaporated and the radioactivity quantified.

IT activity was measured in a manner similar to that utilized for CPT. The reaction mixture contained inositol (1 mM), Triton X-100 (0.3%), MnCl₂ (2 mM), CDP-diacylglycerol (50 µM), Tris/HCl buffer (25 mM, pH 8.5), [³H]inositol (5 µCi) and protein in a final volume of 0.1 ml. The reaction mixture was incubated at 37 °C for 10 min. Termination and quantification were done as described above for CPT. Under the conditions of the assay there was no evidence for a CDP-diacylglycerol-independent inositol exchange activity. Incorporation of radioactive inositol into a chloroform-soluble compound was completely dependent on the presence of CDP-diacylglycerol.

RESULTS

Analysis of subcellular fractions

The purpose of these experiments was to determine whether or not purified plasma membranes contained any distinct IT activity that could not be accounted for by endoplasmic-reticulum contamination. For this reason, it was not deemed necessary to purify any fraction other than the plasma membrane. Results of sucrose-density-gradient fractionation of WRK-1 membranes are shown in Figure 1. Fraction B2 from the sucrose-density gradient comprises highly purified plasma membrane (19-fold purified); however, it is not devoid of endoplasmic reticulum, as evidenced by the presence of two markers, NCR and CPT, both of which are purified to some degree (3- and 2-fold respectively). When IT activity is measured, one finds a similar amount

Figure 1 Subcellular localization of IT activity in WRK-1 membranes

Methods were as described in the text. B1 and B2 refer to the heavier and lighter bands derived from the sucrose-density-gradient centrifugation of the 8000 *g* pellet; 100K refers to the 100 000 *g* pellet derived from the 8000 *g* supernatant. The results shown are derived from 3 (CPT), 10 (TB) and 15 (NCR and IT) separate experiments.

Table 1 Plasma-membrane IT activity in rat liver

Methods were as described for Figure 1. Abbreviations: T, total; FP, fold purification.

Fraction	Protein (% of T)	TB		NCR activity		IT activity	
		(% of T)	(FP)	(% of T)	(FP)	(% of T)	(FP)
B2	0.05	3.70	79	0.14	3	0.04	1
B1	0.71	16.67	22	1.22	2	2.53	3
100K	5.61	60.85	11	8.96	2	18.61	3

Table 2 Effect of Triton X-100 on IT activity in individual subcellular fractions

The total activity was defined in each case as that present in the CH (cellular homogenate) before fractionation. In reality, the activity measured without Triton X-100 in the CH was 38.57% of that observed with detergent, but was normalized to 100% for calculating the relative amounts in the subcellular fractions.

Fraction	Activity (% of total)		Stimulation by Triton (fold)
	+ Triton	No Triton	
CH	100	100	2.59
B2	0.90	1.56	1.64
B1	8.49	13.40	1.50
100K	10.75	7.31	3.81

Figure 2 Hypothetical replot of Figure 1 using the data shown in Table 2**Table 3** Substrate K_m values for the plasma-membrane and endoplasmic-reticulum IT activity in WRK-1 cells

Subcellular fractionation and enzyme assays were carried out as described in the text.

Substrate	Fraction ... B2	100K
CDP-diacylglycerol	4.13 μ M	6.03 μ M
Inositol	0.91 mM	0.90 mM

(determined as percentage of total activity) to that observed for the endoplasmic-reticulum markers. In addition, the fold purification of IT in this fraction is identical with that for NCR. The heavier sucrose-density-purified fraction, B1, also contains

Figure 3 Metal-dependence of IT activity in the plasma-membrane-enriched fraction (B2) and the crude microsomal pellet (100K)

Assays were carried out as described in the text.

purified plasma membrane; however, the contamination with endoplasmic reticulum is much higher. In fact, the CPT and NCR markers are purified 4- and 3-fold in this fraction. Again, the level of IT and its fold purification are similar to those seen for the endoplasmic-reticulum marker enzymes. Mitochondria were present in all three fractions: 11.5% of the total in B1, 3.3% in B2 and 14.6% in 100K (combined results of three separate experiments; results not shown). Thus, as measured by the assay systems described in the Materials and methods section, there was no IT activity present in the plasma membranes of WRK-1 cells that could not be explained by contamination with endoplasmic reticulum.

Subcellular fractionation of cultured cells is particularly difficult, and, in addition, cultured tumour cells do not always reflect the normal situation. We therefore carried out the identical experiment using a rat liver preparation. Results are given in Table 1. With liver we were able to achieve a 79-fold purification of plasma membrane, although the recovery in terms of total TB was small. In this very highly purified preparation there was negligible IT activity: 3-fold less in terms of percentage of total than NCR activity.

Effect of detergent on subcellular fractions

The results reported by Imai and Gershengorn [13] demonstrating substantial IT activity present in pituitary plasma membrane were derived from enzyme assays done in the absence of detergent.

In order to determine whether relative levels of IT activity could be altered depending on the absence or presence of Triton X-100 in the assay mixture, we compared the IT activity in four fractions measured with or without the detergent. Results are shown in Table 2. The amount of latent IT activity (that absent in the absence of Triton X-100, 0.3 %) was related to the density of the fraction, with the least dense, B2 fraction having the least latent activity. The densest (100K) fraction had the most latent activity, whereas the cellular homogenate had an intermediate level. When results are expressed as a percentage of total activity, the relative amount of IT in the absence of detergent increases in fractions B1 and B2, whereas that in the 100K fraction decreases. Figure 2 is a hypothetical replot of Figure 1 in the absence of detergent. Under this condition, IT appears to purify with both the plasma-membrane and endoplasmic-reticulum fractions.

Comparison of kinetic parameters

When substrate K_m values and metal-dependence were determined for the B2 fraction and the 100K fraction, no differences were observed. Table 3 compares K_m values for inositol and CDP-diacylglycerol; Figure 3 compares dependence on Mn^{2+} and Mg^{2+} .

DISCUSSION

It has been proposed that the phosphoinositide cycle might take place entirely at the plasma membrane, thus obviating the need for a shuttle to transfer the diacylglycerol and inositol generated by phospholipase C activity to the endoplasmic reticulum, and subsequently to transfer phosphatidylinositol back to the plasma membrane. Furthermore, data supporting the existence of individual hormone-sensitive lipid pools and suggesting that the cycle is closed [21–23] make the notion of a plasma-membrane IT activity attractive. Most investigators have failed to find a plasma-membrane IT activity that could not be accounted for by endoplasmic-reticulum contamination [9,15–18]; however, Takenawa et al. [12] reported an IT activity that could not be accounted for by endoplasmic-reticulum contamination in a 5'-nucleotidase-rich fraction of rat liver purified by sucrose-density-gradient centrifugation. The IT activity of this fraction had similar pH, Mn^{2+} and Mg^{2+} requirements to the activity recovered from a glucose-6-phosphatase-rich fraction. Imai and Gershengorn [13] reported an IT activity in the plasma membranes of pituitary tumour cells purified by sucrose-density-gradient centrifugation. This plasma-membrane fraction was devoid of glucose-6-phosphatase activity, but contained 5'-nucleotidase activity. The specific activity of the 5'-nucleotidase-associated IT was twice that of the glucose-6-phosphatase-associated activity. Since the total protein recovered in the two fractions was equal, the plasma-membrane IT accounted for the bulk of the IT activity. The kinetic parameters of the IT in the plasma-membrane fraction differed significantly from those in the endoplasmic reticulum. However, it must be kept in mind that the IT assays were done in the absence of detergent, which in WRK-1 cells changes the relative amounts of plasma-membrane and endoplasmic-reticulum activity. In addition, assays were carried out at pH 7.4, which may also alter the results.

Recently, McPhee et al. [24] reported the occurrence of IT activity in turkey erythrocyte membranes, as measured by CMP-dependent incorporation of radiolabelled inositol; that is, they measured IT-catalysed exchange of inositol. Since avian erythrocyte membranes comprise predominantly nuclear and plasma membrane, any non-nuclear localization can generally be considered to be in the plasma membrane, and those authors report

that they have preliminary data suggesting that this is indeed the case. A study in rat pancreatic islets localized the bulk of the IT activity to secretory granules (46.8 %) and a combined nuclei/plasma-membrane fraction (28 %) [25].

An additional enzyme required for resynthesis of phosphatidylinositol is phosphatidate cytidylyltransferase. There is only one report of minimal plasma-membrane activity in rat liver [11], whereas several studies have failed to find evidence for a plasma-membrane activity, including one done in rat liver [8–10]. In a recent study by Morris et al. [17], neither the IT nor the cytidylyltransferase activity could be found in plasma membrane isolated from glioma and neuroblastoma cells.

In the present study, no evidence for a plasma-membrane IT activity could be found when assays were carried out in the presence of detergent. In terms of the percentages of both total and specific activities, the values obtained for IT closely paralleled those obtained for two endoplasmic-reticulum markers, CPT and NCR. A 19-fold purification of TB activity, which is a marker for plasma membrane, resulted in only a 2-fold increase in IT specific activity (CPT increased 3-fold, and NCR < 2-fold). Similar experiments in rat liver resulted in a 79-fold increase in TB specific activity and no increase in IT specific activity. These results are in agreement with previous findings in rat liver [9,11,16].

The absence of Triton X-100 from the assay medium drastically decreased the amount of IT activity observed in the dense 100K fraction, but had a much lesser effect on plasma-membrane activity. This latency of enzyme activity probably reflects an inaccessibility of the CDP-diacylglycerol substrate to the enzyme, which is more pronounced in the denser microsomal fraction, perhaps due to a greater proportion of closed vesicles in this fraction. Thus it is clear that the relative amount of IT, and therefore the subcellular localization, can be altered, depending upon the presence of detergent in the assay medium. Since the determination of the subcellular location of a given enzyme is considered dependent on the measurement of maximal rates of that enzyme's activity [26], it is our bias that IT assays done in the absence of detergent are not indicative of total activity and therefore do not yield an accurate assessment of subcellular location. However, as has recently been demonstrated for the enzyme CTP:phosphocholine cytidylyltransferase, subcellular-fractionation studies may not reflect the situation in whole cells [27]. In this case, an enzyme which had been localized to the endoplasmic reticulum by subcellular-fractionation techniques was found to reside on the nuclear envelope when immunocytochemical methods were employed.

In summary, the question of whether or not the plasma membrane contains IT activity is still unanswered, and it appears that assay conditions and/or tissue type can significantly alter the outcome of localization experiments. We are currently purifying IT in order to localize it immunocytochemically, and thus to avoid having to measure activity or disrupt the integrity of the membrane structure of the cell.

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