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Phosphoinositide metabolism in cultured glioma and neuroblastoma cells: subcellular distribution of enzymes indicate incomplete turnover at the plasma membrane

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The hypothesis that the small portion of cellular phosphoinositide participating in signal transduction might be preferentially recycled within the plasma membrane was tested in rat glioma (C6) and murine neuroblastoma (N1E-115) cells. Percoll density gradient centrifugation was used to isolate a purified plasma membrane fraction and the subcellular distribution of all enzymes mediating phosphoinositide turnover was assessed. A small but signifiant proportion of PtdInsP$_2$-specific phosphodiesterase was located in the plasma membrane but only two of the five enzymes required to replace PtdInsP$_2$ (diacylglycerol kinase and PtdInsP kinase) also were present. CTP: phosphatidate cytidylyltransferase and CMP-phosphatidate: inositol phosphatidyltransferase were located exclusively in a microsomal fraction containing enriched levels of endoplasmic reticulum markers. Thus, diacylglycerol from agonist-stimulated cleavage of PtdInsP$_2$, or phosphatidic acid formed from it, must be transferred to the endoplasmic reticulum for conversion to PtdIns. Plasma membrane also lacked PtdIns kinase. If the soluble PtdIns kinase has access to membrane-bound substrate, PtdIns may be phosphorylated to PtdInsP before or during transport to the plasma membrane. Phosphorylation by the predominantly plasma membrane PtdInsP kinase to form PtdInsP$_2$ completes the cycle. PtdInsP phosphatase was present in all membrane fractions suggesting that PtdInsP can be returned to the PtdIns pool in plasma membrane and elsewhere. PtdInsP$_2$ phosphatase was almost exclusively in the cytosol suggesting that reversible interchange between PtdInsP and PtdInsP$_2$ in the plasma membrane may be modulated by the ability of this phosphatase to act on PtdInsP$_2$ in the membrane. Thus, PtdIns resynthesis in the plasma membrane of these cells does not occur and is not required for phosphoinositide-mediated signal transduction.

Introduction

Many cells in culture, including rat glioma and mouse neuroblastoma exhibit enhanced phosphoinositide turnover in response to external stimuli [1–3]. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP$_2$) in the plasma membrane by a specific, receptor-linked phosphodiesterase is the initial event [4]. The 1-stearoyl-2-arachidonoyl-diacylglycerol backbone of agonist-sensitive phosphoinositide is preferentially recycled back into phosphoinositide [5] but evidence for a functionally separate, agonist sensitive pool of phosphoinositide is contradictory. There seems to be a small, metabolically discrete, agonist-sensitive pool of phosphoinositide representing less than 10% of cellular phosphoinositide in many cells but the biochemical basis for this separation is not known [6,7]. The most convincing experiments show a restricted pool of PtdIns that is rapidly labeled from radioactive precursors only in the presence of agonist, is preferentially lost upon readdition of agonist [8,9], and equilibrates with a limited pool of agonist-sensitive PtdInsP$_2$ [10]. Evidence for preferential hydrolysis of a more metabolically active, recently synthesized pool of phosphoinositide in 5-hydroxytryptamine

Abbreviations: PtdIns, 1-(3-*sn*-phosphatidyl)D-*myo*-inositol; PtdInsP, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4-phosphate; PtdInsP$_2$, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4,5-bisphosphate; DMEM, Dulbecco's modified Eagle's medium; CTAB, cetyltrimethylammonium bromide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate; DAG, diacylglycerol; PtdOH, phosphatidic acid.

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stimulated salivary glands [11] has not been found in other cells [12,13].

The plasma membrane is clearly the site of agonist-stimulated phosphodiesterase cleavage of $PtdInsP_2$ [14–16]. Replacement of the $PtdInsP_2$ requires 5 steps: DAG → PtdOH → CMP-PtdOH → PtdIns → PtdInsP → $PtdInsP_2$. Diacylglycerol kinase also occurs in the plasma membrane [17–19]. Local phosphorylation of agonist-generated diacylglycerol at or near the site of origin is consistent with enhanced formation of PtdOH in plasma membrane following agonist-stimulated hydrolysis of $PtdInsP_2$ [14,20]. The CTP : phosphatidate cytidylyltransferase, and CMP-phosphatidate : inositol phosphatidyltransferase (PtdIns synthase) required for PtdIns resynthesis are generally believed to reside in the endoplasmic reticulum [21,22]. Specific mechanisms to transport PtdOH or DAG to the endoplasmic reticulum and to maintain a metabolically separate pool of phosphoinositide have not been identified. Localized, rapid resynthesis of agonist-sensitive PtdIns and its phosphorylated derivatives in the plasma membrane is an attractive hypothesis for which the evidence is contradictory. Detection of PtdIns synthase activity in the plasma membrane of rat liver [23] and pituitary tumor (GH_3) cells [24] suggests that resynthesis of stimulus-responsive phosphoinositide could occur within the plasma membrane of these cells. However, plasma membrane PtdIns synthase activity was not found in WRK-1 cells [25] and recent studies suggest that neither CTP : PtdOH cytidylyltransferase nor PtdIns synthase are present in hepatocyte plasma membrane [26]. PtdIns kinase and PtdInsP kinase activities are widely distributed among cellular compartments and have been reported in the plasma membrane [17,18,19,27]. It has been presumed that plasma membrane PtdIns, or a specific portion thereof, is phosphorylated to replace $PtdInsP_2$ used for signal transduction. However, the presence of PtdIns kinase in the plasma membrane has been challenged recently [26].

To clarify the issue of plasma membrane participation in phosphoinositide recycling we have used a subcellular fractionation procedure, which gives a distinct separation of highly enriched plasma membrane from other cellular membranes, to assess the potential for phosphoinositide synthesis in the plasma membrane of cultured cells of glial (rat C6 glioma) and neuronal (murine N1E 115 neuroblastoma) origin. We show that only three of the six enzymes necessary for the phosphoinositide cycle are present in the plasma membrane. CTP : PtdOH cytidylyltransferase, PtdIns synthase, and PtdIns kinase are absent from the plasma membrane. We conclude that PtdIns synthesis in the plasma membrane of these agonist-responsive cells is not possible and therefore is not a necessary feature of the phosphoinositide-mediated signal transduction system.

Materials and Methods

Materials. Percoll was purchased from Pharmacia Fine Chemicals, Montreal, PQ, Canada. [2-^{3}H]Adenosine 5′-monophosphate (15 Ci/mmol), and *myo*-[2-^{3}H]inositol (16.2 Ci/mmol) were from Dupont (NEN Products) Canada (Mississauga, ON). [γ-^{32}P]ATP was prepared enzymically by Dr. Jason Hoffman, Department of Biochemistry, Dalhousie University [28]. [5-^{3}H]Cytidine triphosphate (20 Ci/mmol) was from Amersham Canada Ltd. (Oakville, ON). $PtdInsP_2$ was prepared from bovine brain [29] and used for the enzymic preparation of PtdInsP [30]. Phosphatidylinositol (yeast) and 1,2-diacylglycerol (from pig liver phosphatidylcholine) were from Serdary Research Laboratories (London, ON). Cytidine monophosphate-dipalmitoylphosphatidic acid, 1-stearoyl-2-arachidonoylphosphatidic acid, various buffers, and other substrates were from Sigma Chemical Co. (St. Louis, MO).

Cell culture. C6 rat glioma and N1E 115 murine neuroblastoma cell lines were grown in 150 cm^2 flasks in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% fetal bovine serum/5% newborn bovine serum, respectively [31]. Cells were removed from flasks by scraping and washed twice with ice-cold 0.25 M sucrose buffered at pH 7.0 with 40 mM Hepes.

Subcellular fractionation. Subcellular fractions of both glioma and neuroblastoma cells were prepared essentially as described for glioma cells [32] except that only a single gradient was used. The washed cell pellet ($(50–60) \cdot 10^6$ cells) was suspended in 5 ml of isotonic lysis buffer (25 mM Tricine, 100 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, pH 9.6). Suspended cells were disrupted by nitrogen cavitation (10 min at 600 lb/in^2 for glioma and 3 min at 100 lb/in^2 for neuroblastoma cells). An aliquot of the lysate was removed and the remainder was centrifuged at $900 \times g$ for 5 min. The supernatant was removed and the pellet resuspended in lysis buffer and sedimented as above. The resulting pellet (crude nuclear fraction) was resuspended in lysis buffer. A 2.5 ml aliquot of the combined supernatant was layered on top of 9.0 ml of a 40% Percoll solution prepared by diluting Percoll with buffer containing 400 mM KCl, 20 mM $MgCl_2$, and 400 mM Tricine and adjusting to pH 9.3 with 0.1 M NaOH. Fractions were separated in a self-forming gradient during centrifugation at $100\,000 \times g$ for 12 min ($8.4 \cdot 10^9$ $\omega^2 t$). Five fractions were collected from the top of the gradient. Volumes of fractions were 2.5, 1.9, 2.1, 2.9, and 1.5 ml for glioma cells and 3.0, 1.6, 3.0, 0.9, and 2.4 ml for neuroblastoma cells. All fractions were dialysed against 40 mM Hepes (pH 7.2) for 6–12 h and stored at 4 °C. The bicinchoninic acid reagent obtained from the Pierce Chemical Co. (Rockford, IL) was used for the de-

termination of protein [33]. Enzyme activities were assayed within 24 h.

Enzyme assays. All enzyme reactions were measured under conditions where activity was linear with time and protein concentration. Lactate dehydrogenase (EC 1.1.1.27), 5′-nucleotidase (EC 3.1.3.5), and NADPH: cytochrome-*c* reductase (EC 1.6.99.1) were measured as described previously [34]. CMP-phosphatidate: inositol phosphatidyltransferase (EC 2.7.8.11) was measured by incubating 10–50 μg protein of cell fraction in the presence of 100 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM [^{3}H]inositol, 0.1 mM CMP-phosphatidic acid, and 0.1% Triton X-100 at 37 °C in a total volume of 100 μl. Reactions were terminated by addition of 2 ml of chloroform/methanol/12 M HCl (100 : 100 : 1, by vol.) and the phases separated by addition of 0.5 ml of 2 M KCl. The lower phase was washed three times with pre-equilibrated upper phase and radioactivity incorporated into lower phase lipid was measured by scintillation counting. No incorporation of [^{3}H]inositol occurred in the absence of CMP-phosphatidate. CTP: phosphatidate cytidylyltransferase (EC 2.7.7.41) activity was measured by a modification of the method of Liteplo and Sribney [35]. Portions of the cell fractions (10–50 μg protein) were pre-incubated for 5 min at 37 °C in the presence of 100 mM Tris-HCl (pH 7.2), 4 mM dithiothreitol, 1 mM [^{3}H]CTP, 0.1% Triton X-100, and 1 mM phosphatidate (total volume of 90 μl). Reactions were initiated by addition of 10 μl of 200 mM $MgCl_2$. Reactions were stopped and samples washed as in the assay for CMP-phosphatidic acid: inositol phosphatidyltransferase.

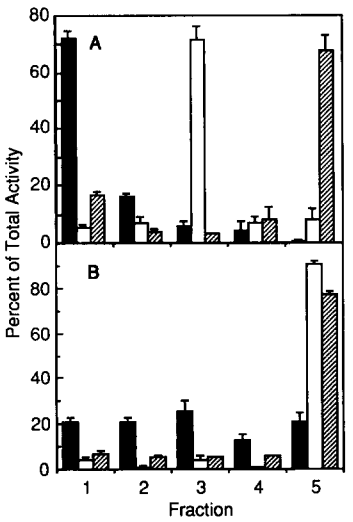

Fig. 1. Subcellular distribution of enzymes of phosphatidylinositol synthesis in rat glioma cells (C6). Results are expressed as percent of the initial activity in the post-nuclear supernatant applied to the gradient. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient is in Table I. Enzyme markers in panel A are lactate dehydrogenase (solid bars), 5′-nucleotidase (open bars), and NADPH: cytochrome-*c* reductase (hatched bars). Panel B is diacylglycerol kinase (solid bars), CTP: phosphatidic acid cytidylyltransferase, (open bars) and PtdIns synthase (hatched bars). Data are the means ± S.D. for three independent experiments in which all assays were done in triplicate.

DAG and phosphoinositide kinases were measured by following the incorporation of radioactivity from [γ-^{32}P]ATP into the appropriate product in the presence of exogenous substrate. Diacylglycerol kinase (EC 2.7.1.107) was measured by the method of Besterman et al. [36]. Since the only radioactive product formed was identified as phosphatidic acid by thin-layer chromatography, only the formation of radioactive lipid (lower phase) was routinely measured. Reaction conditions for PtdIns kinase (EC 2.7.1.67) and PtdInsP kinase (EC 2.7.1.68) were as described by MacDonald et al. [37] using purified PtdIns and PtdInsP, respectively, as substrates. Reaction products were isolated on oxalate-treated silica gel HL plates developed in chloroform/methanol/acetone/acetic acid/water (40 : 26 : 30 : 24 : 16, by vol.). Bands corresponding to PtdInsP and $PtdInsP_2$ were scraped off and radioactivity determined by scintillation counting.

$PtdInsP_2$ specific phosphodiesterase (EC 3.1.4.11) was measured by the method of Palmer [38]. The PtdInsP and $PtdInsP_2$ (EC 3.1.3.36) specific phosphatases were measured by the method of Mack and Palmer [39]. Measurement of inorganic phosphate in detergent-solubilized reaction mixtures with an autoanalyser is the final step in all three assays. Interference by Percoll particles from the gradients was avoided by including a continuous flow dialyser in the autoanalyser system. Retrieval of inorganic phosphate from Percoll-containing samples, blanks and standards was 37%.

Results

Subcellular fractionation of glioma cells

Based on initial studies of the distribution of marker enzymes, the gradients were divided into five fractions (Fig. 1A, Table I) that minimized cross contamination between the three major fractions representing cytosol (fraction 1), plasma membrane (fraction 3) and a heterogeneous 'microsomal' fraction (fraction 5). The greatest overlap of marker enzymes was found in intervening fractions (fractions 2 and 4). Fraction 1 exhibited characteristics similar to those observed before [32]. It contained 60% of the protein and 72% of the lactate dehydrogenase. A fraction roughly equivalent to fractions 2 and 3 had been taken previously as representative of plasma membrane and contained most (> 60%) of the 5′-nucleotidase activity. Loss of some plasma membrane into fraction 2 was sacrificed to ensure minimal contamination of the plasma membrane fraction with cytosol (lactate dehydrogenase activity) (Table I). Separation of fraction 4 minimized contamination of the plasma membrane fraction with endoplasmic reticulum (NADPH: cytochrome-*c* reductase activity).

TABLE I

Relative specific activity and recovery of enzyme activities in subcellular fractions of rat glioma (C6) cells

Cells from four flasks were used for each fractionation (65.8 ± 19.3 mg protein, mean ± S.D., 13 experiments). After fractionation, 32.4 ± 5.9 mg, 4.1 ± 0.8 mg, 3.3 ± 1.0 mg, 2.2 ± 0.9 mg, and 4.4 ± 1.9 mg was recovered in gradient fractions 1 to 5, respectively, and the remainder in the initial 900 × *g* 'nuclear pellet'. Enzyme activities in the total cell lysate are expressed as nmol/min per mg protein except for CTP:PtdOH cytidylyltransferase and PtdIns synthase which are pmol/min per mg protein. Relative specific activities were obtained by dividing the specific activity in each fraction by the specific activity of the initial lysate. Activity data is reported as the mean ± S.D. for three experiments in which each determination was in triplicate.

	Specific activity (lysate)	Relative specific activity in gradient fractions					Recovery (%)
		1	2	3	4	5	
Lactate dehydrogenase	50.7 ± 7.4	1.1 ± 0.03	1.3 ± 0.2	0.8 ± 0.03	0.5 ± 0.01	0.1 ± 0.02	94.3 ± 7.6
5′-Nucleotidase	159 ± 4.7	0.1 ± 0.03	1.9 ± 0.7	8.3 ± 2.2	1.7 ± 0.5	0.7 ± 0.1	99.5 ± 13.0
NADPH:cyt.-*c* reductase	7.0 ± 0.4	0.1 ± 0.02	0.3 ± 0.03	0.3 ± 0.05	1.1 ± 0.4	8.6 ± 0.4	86.0 ± 19.9
DAG kinase	0.5 ± 0.1	0.3 ± 0.1	1.9 ± 0.8	3.3 ± 1.7	2.5 ± 0.7	1.0 ± 0.2	101 ± 7.5
CTP:PtdOH cytidylyltransferase	261 ± 24	< 0.1	0.1 ± 0.01	0.4 ± 0.1	0.1 ± 0.04	3.8 ± 0.06	70.0 ± 3.5
PtdIns synthase	79.8 ± 2.7	0.1 ± 0.1	2.1 ± 2.2	3.3 ± 2.3	9.3 ± 3.9	3.5 ± 0.4	110 ± 10.9
PtdIns kinase	31.5 ± 1.4	0.8 ± 0.004	1.1 ± 0.01	1.2 ± 0.2	1.9 ± 0.02	2.9 ± 0.1	81.7 ± 2.5
PtdInsP kinase	11.7 ± 0.7	0.2 ± 0.03	0.8 ± 0.03	5.7 ± 0.3	6.2 ± 0.1	3.8 ± 0.03	104 ± 9.7
$PtdInsP_2$ phosphodiesterase	140 ± 31.5	1.7 ± 0.2	1.0 ± 1.0	1.2 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	78.9 ± 7.4
$PtdInsP_2$ phosphatase	39.7 ± 2.1	0.3 ± 0.02	0.8 ± 0.2	1.9 ± 0.8	3.8 ± 1.3	0.7 ± 0.07	85.7 ± 4.7
PtdInsP phosphatase	47.8 ± 1.1	1.8 ± 0.5	2.0 ± 0.03	6.0 ± 0.06	4.4 ± 0.02	0.8 ± 0.2	64.1 ± 3.5

Subcellular distribution of PtdIns synthesis in glioma cells

PtdIns resynthesis following phospholipase C degradation of $PtdInsP_2$ requires three enzymes. The first of these, diacylglycerol kinase, was distributed throughout the gradient (Fig. 1B). CTP:PtdOH cytidylyltransferase and CMP-PtdOH:inositol phosphatidyltransferase (PtdIns synthase) activities were recovered primarily in the microsomal fraction. However, small amounts of both activities (4 and 7%, respectively) were found in the plasma membrane fraction. Since agonist-sensitive phosphoinositide represents only a small portion of the total cellular phosphoinositide (variously estimated at 5–15%), resynthesis of this pool of lipid in the plasma membrane might require only a small portion of the synthetic enzymes to be located there. To assess whether cytidylyltransferase and PtdIns synthase activities observed in the plasma membrane fraction represent contamination with endoplasmic reticulum or limited capacity of the plasma membrane for PtdIns synthesis, increasing amounts of the microsomal fraction were added to a constant amount of plasma membrane fraction and the synthetic activities compared to the activity of the endoplasmic reticulum

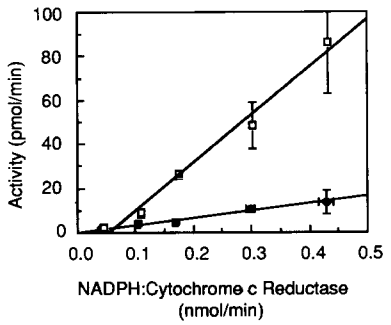

Fig. 2. Estimation of contamination-dependent CTP:PtdOH cytidylyltransferase (□) and PtdIns synthase (●) activities in rat glioma plasma membranes. Increasing aliquots of microsomes (fraction 5) were added to a fixed aliquot of plasma membrane (fraction 3). Data are for one experiment in which each point represents the mean ± S.D. for three mixtures of the two fractions from a single gradient. Results are typical of three experiments in which only the slope of the regression line varied due to the gradient fractions having different specific activities of the enzymes.

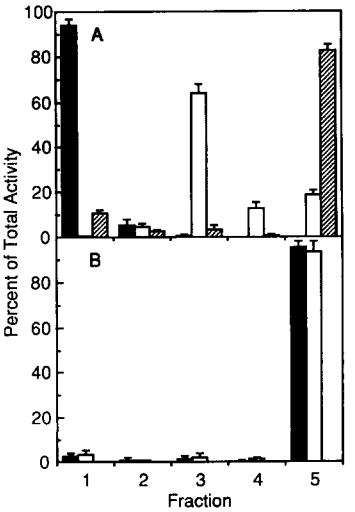

Fig. 3. Subcellular distribution of enzymes of phosphatidylinositol synthesis in murine neuroblastoma cells (N1E-115). Enzymes in panel A are lactate dehydrogenase (solid bars), 5′-nucleotidase (open bars), and NADPH:cytochrome *c* reductase (hatched bars). Panel B is CTP:phosphatidic acid cytidylyltransferase, (solid bars) and PtdIns synthase (open bars). Results are expressed as described for Fig. 1. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient is in Table II.

TABLE II

Relative specific activity and recovery of enzyme activities in subcellular fractions of murine neuroblastoma (N1E-115) cells

Data presented as described for Table I. Cell lysates used for each gradient contained 79.5 ± 6.5 mg protein (mean ± S.D., 13 experiments). After fractionation, 43.4 ± 4.1 mg, 2.7 ± 0.1 mg, 2.1 ± 0.2 mg, 0.4 ± 0.004 mg, and 10.5 ± 0.4 mg was recovered in gradient fractions 1 to 5, respectively, with the remainder in the 900 × *g* 'nuclear pellet'.

	Specific activity (lysate)	Relative specific activity in gradient fractions					Recovery (%)
		1	2	3	4	5	
Lactate dehydrogenase	1.5 ± 0.04	1.3 ± 0.01	1.2 ± 0.4	0.3 ± 0.6	< 0.1	< 0.1	99.6 ± 3.8
5′-Nucleotidase	2.4 ± 0.6	< 0.1	1.1 ± 0.2	8.6 ± 1.2	2.0 ± 0.2	1.4 ± 0.3	79.4 ± 19.6
NADPH : cyt.-*c* reductase	13.6 ± 0.6	0.1 ± 0.03	0.5 ± 0.3	0.9 ± 0.4	1.5 ± 0.3	4.3 ± 0.1	77.5 ± 12.0
CTP : PtdOH cytidylyltansferase	22.0 ± 0.2	< 0.1	< 0.1	0.3 ± 0.1	1.1 ± 0.3	3.7 ± 1.2	75.8 ± 8.1
PtdIns synthase	3.1 ± 0.4	< 0.1	< 0.1	0.7 ± 0.1	4.3 ± 0.3	2.1 ± 0.1	68.7 ± 15.2

marker, NADPH : cytochrome-*c* reductase (Fig. 2). When extrapolated to zero reductase activity, regression lines for both cytidylyltransferase and PtdIns synthase activities passed the vertical axis at or slightly below zero. In these experiments, the plasma membrane marker (5′-nucleotidase) remained constant. Assuming NADPH : cytochrome-*c* reductase to be a valid endoplasmic reticulum marker that is absent from plasma membrane, the results indicate that glioma plasma membrane does not have either of the two enzyme activities necessary to synthesis PtdIns from phosphatidic acid.

Subcellular distribution of PtdIns synthesis in neuroblastoma cells

Since observations for glioma cells conflicted with published reports of substantial PtdIns synthase activity in the plasma membrane of GH_3 cells [24], a second cell line (N1E-115 neuroblastoma) was investigated. Subcellular distribution of marker enzymes in the continuous gradient (Fig. 3) was similar to that observed before for this cell line using a discontinuous Percoll gradient and dividing the gradient into five fractions of equal volume [34]. Fraction sizes were modified to optimize the purity of the three major fractions (Table II). Distribution of marker enzyme activities was similar to that seen with glioma cells except that 5′-nucleotidase was distributed lower in the gradient. The cytosol fraction had no nucleotidase activity while fraction 5 at the bottom of the gradient (microsomes) was slightly contaminated with the plasma membrane marker (Fig. 3A). However, the plasma membrane fraction was almost devoid of contamination with either cytosol (lactate dehydrogenase) or endoplasmic reticulum (NADPH : cytochrome-*c* reductase). Virtually all CTP : PtdOH cytidylyltransferase and PtdIns synthase activities were recovered in the microsomal fraction (Fig. 3B). The activities of these two enzymes in plasma membrane were lower in neuroblastoma cells than in glioma cells and could be attributed entirely to microsomal contamination on the basis of mixing experiments similar to those described above and extrapolation of the measured activities to zero NADPH : cytochrome-*c* reductase activity (data not shown).

Subcellular distribution of polyphosphoinositide metabolism in glioma cells

When assayed in the presence of exogenous substrate, the PtdIns and PtdInsP kinases exhibited different bimodal distributions (Fig. 4A). PtdIns kinase was recovered mainly in the cytosol (37%) and microsomal (41%) fractions but not in the plasma membrane. From the ratio of PtdIns kinase to lactate dehydrogenase activity in fraction 1 and the ratio of PtdIns kinase to reductase activity in fraction 5, it can be calculated that contamination of fraction 3 with cytosol and endoplasmic reticulum accounts for all PtdIns kinase activity

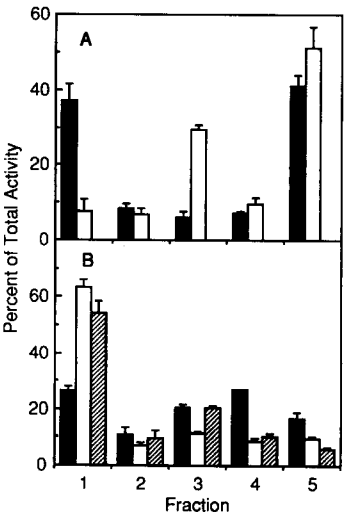

Fig. 4. Subcellular distribution of enzymes of polyphosphoinositide metabolism in rat glioma cells (C6). Enzymes in panel A are PtdIns kinase (solid bars), and PtdInsP kinase (open bars). Panel B is PtdInsP phosphatase (solid bars), $PtdInsP_2$ phosphatase (open bars) and $PtdInsP_2$ phosphodiesterase (hatched bars). Results are expressed as described for Fig. 1. Specific activities of the enzymes in the post-nuclear supernatant and their total recovery from the gradient is in Table I.

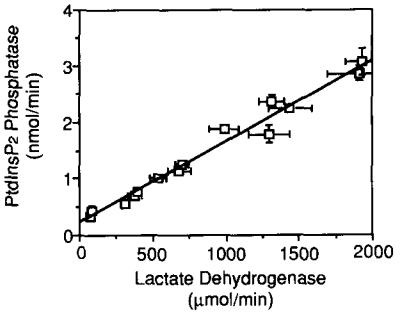

Fig. 5. Estimation of contamination-dependent $PtdInsP_2$ phosphatase activity in rat glioma plasma membranes. Increasing aliquots of cytosol (fraction 1) were added to a fixed aliquot of plasma membrane (fraction 3). Data are for three experiments in which each point represents the mean ± S.D. for three mixtures of the two fractions from a single gradient.

found in the plasma membrane fraction. PtdInsP kinase was found mainly in the membranous fractions 3 (29%) and 5 (51%).

The two phosphatases that sequentially dephosphorylate $PtdInsP_2$ also showed different distributions in the Percoll gradient (Fig. 4B). PtdInsP phosphatase was distributed throughout the gradient whereas $PtdInsP_2$ phosphatase was recovered mainly (63%) in the cytosol. The distribution of $PtdInsP_2$ phosphatase was further investigated to determine if the activity measured in the plasma membrane fraction could be attributed to contamination with cytosol. Increasing volumes of cytosol (fraction 1) were added to a constant amount of fraction 3. $PtdInsP_2$ phosphatase activity in the mixtures was plotted against lactate dehydrogenase activity (Fig. 5). Extrapolation to zero lactate dehydrogenase activity indicated that, in the absence of cross-contamination with cytosol, there was little or no $PtdInsP_2$ phosphatase activity in the plasma membrane fraction. The $PtdInsP_2$ specific phosphodiesterase was also recovered mostly in the cytosol (54%), but there was a significant peak of activity in fraction 3 (21%) indicating that a small portion of the diesterase was associated with the plasma membrane.

Discussion

Although turnover of inositol phospholipids following stimulation by a variety of extracellular agonists can be demonstrated at the plasma membrane [14,15], it is not clear which of the complete sequence of metabolic events take place in the plasma membrane. In view of evidence in a variety of tissues for a metabolically distinct pool of agonist-sensitive phosphoinositide, it seemed possible that recycling of this fraction of the total cellular phosphoinositide complement might occur at the site of agonist-induced polyphosphoinositide hydrolysis in the plasma membrane. We tested this hypothesis by determining the subcellular distribution of all participating enzymes in cultured neural cells using a fractionation procedure originally developed for the isolation of high purity plasma membranes [32].

Our results for the $PtdInsP_2$ specific phosphodiesterase agree with other studies [26,40–42] showing most of this activity to be localized in the cytosol with a lesser amount in the plasma membrane. In contrast to one report [42], we did not detect $PtdInsP_2$ specific phosphodiesterase in the microsomal fraction above that which could be attributed to contamination with the plasma membrane fraction. It is presumed that the diesterase in the plasma membrane is responsible for agonist-dependent hydrolysis of $PtdInsP_2$. The relationship between soluble and plasma membrane forms of the enzyme is not clear. They may be the same since highly purified preparations of each have similar kinetic properties and at least one of the soluble diesterase isozymes has been detected immunologically in membranes [43,44]. This raises the possibility of translocation between the two compartments. However, we did not detect any agonist-dependent translocation of the $PtdInsP_2$ phosphodiesterase to membranes in experiments with digitonin-permeabilized glioma cells (unpublished results).

Diacylglycerol kinase has been found in the cytosol, plasma membrane, and endoplasmic reticulum fractions of brain and other tissues [19,45,46,47]. Our results extend these observations to cultured cells of neural origin and show diacylglycerol kinase activity distributed in approximately equal amounts throughout the cytosol, plasma membrane and microsomal membranes of glioma cells. Fractionation of hepatocytes in Nycodenz gradients showed the membrane-bound diacylglycerol kinase activity to be associated with the Golgi marker (galactosyltransferase) rather than with the endoplasmic reticulum [26]. In our Percoll gradients, galactosyltransferase is localized in the heterogeneous microsomal membrane fraction at the bottom of the gradients [32] so we could not distinguish between endoplasmic reticulum and Golgi-associated membranes. While Lundberg and Jergil [26] found no diacylglycerol kinase associated with the plasma membrane of hepatocytes, our data for glioma cells clearly support a plasma membrane location for a significant amount of the cellular diacylglycerol kinase activity. Since enhanced phosphatidic acid synthesis in the plasma membrane follows agonist-stimulated hydrolysis of $PtdInsP_2$ [14], it seems likely that the diacylglycerol is phosphorylated at or near its site of formation in the plasma membrane. A membrane-bound diacylglycerol kinase in 3T3 cells selectively phosphorylates arachidonoyl-DAG that would be produced by phosphoinositide hydrolysis [48]. This activity is distinct from the cytosolic activity which exhibits no acyl chain specificity, and has been implicated in the platelet-derived growth factor-stimulated formation of phospha-

tidic acid in these cells [20]. Furthermore, diacylglycerol kinase activity may translocate to the plasma membrane in response to the appearance of diacylglycerol [36] and be part of a mechanism for limiting the diacylglycerol-mediated signal that activates protein kinase C.

It has been believed that the CTP : PtdOH cytidylyltransferase and PtdIns synthase required for PtdIns synthesis from PtdOH via CMP-PtdOH occur only in the endoplasmic reticulum although most studies could not exclude the possibility that a small portion of the total cellular activity might reside in the plasma membrane [22]. PtdIns synthase activity has been reported in liver plasma membrane [23]. More recently, a substantial proportion of the cellular PtdIns synthase was found in the plasma membrane of rat pituitary (GH_3) cells [24]. It was suggested that PtdIns synthesis could occur in the plasma membranes of these cells although no source of CMP-PtdOH in the plasma membrane was identified. In contrast, we found no cytidylyltransferase or PtdIns synthase activity in purified plasma membranes of either glioma or neuroblastoma cells other than could be accounted for by the very low level of endoplasmic reticulum contamination. PtdIns synthase can be partially solubilized from GH_3 cell membranes (both plasma membrane and endoplasmic reticulum) by salt extraction [49]. The high ionic strength in our Percoll gradients raises the possibility that PtdIns synthase and possibly CTP : PtdOH cytidylyltransferase might have been extracted from the membranes during the fractionation. This is not the explanation for our results since (i) the salt concentration in the Percoll gradients was well below the optimal concentration (3 M) for solubilization of PtdIns synthase, and (ii) all the cytidylyltransferase and PtdIns synthase activities were in the microsomal fraction at the bottom of the gradient with no solubilized activities present higher in the gradient. Our results agree with those of Monaco [25] who could not detect PtdIns synthase activity in the plasma membrane fraction of WRK-1 cells and Lundberg and Jergil [26] who obtained similar results with hepatocytes. In the latter study, the fractionation method did not clearly separate plasma membrane from endoplasmic reticulum. However, profiles of CTP : PtdOH cytidylyltransferase and PtdIns synthase activities throughout the gradient followed the profile of the endoplasmic reticulum markers closely and they concluded that neither synthetic enzyme was present in plasma membrane.

Since the step-wise phosphorylation of PtdIns to $PtdInsP_2$ was shown to be catalyzed by two kinases located on the cytoplasmic side of human erythrocyte membranes [27], it has been presumed, with some experimental support, that PtdIns synthesized in the endoplasmic reticulum is phosphorylated after translocation to the plasma membrane. This is consistent with the view that at least a portion of plasma membrane PtdIns acts as a reservoir to replenish $PtdInsP_2$ used in signal transduction. Prior studies of the distribution of these two kinases in other mammalian tissues have produced conflicting results. PtdIns kinase has been found in the cytosol and in virtually every intracellular membrane from the nuclear envelope to the plasma membrane [17,19,50]. We found this enzyme activity localized in the cytosol and the microsomal fraction of glioma cells. The absence of PtdIns kinase from glioma plasma membrane, though surprising, is consistent with recent studies of hepatocytes [26] in which no appreciable activity was found in either the plasma membrane or the endoplasmic reticulum. Membrane-bound PtdIns kinase originally assigned to Golgi, has been associated with a slightly lighter particle (possibly a lipid transport vesicle). As noted earlier, Golgi membranes migrate to the bottom of our gradient and the PtdIns kinase activity we observed in the microsomal fraction may represent Golgi-associated particles rather than a true endoplasmic reticulum location for this enzyme. Our results indicate that the cytosolic PtdIns kinase may be active with PtdIns on the surface of the plasma membrane in vivo or, alternatively, PtdIns may be phosphorylated in transit to the plasma membrane as proposed by Lundberg & Jergil [26]. PtdInsP kinase is almost exclusively localized in the plasma membrane of hepatocytes [17,26]. It is more widely distributed among cell mebranes and the cytosol in whole brain [19]. While we found substantial PtdInsP kinase activity in the plasma membrane as well as the microsomal membranes of glioma cells, all activity found in the cytosol could be attributed to cross-contamination.

Steady-state levels of the polyphosphoinositides are maintained in erythrocyte membranes and other tissues by an energy-dependent cycle in which the action of PtdIns and PtdInsP kinases are reversed by the sequential action of a specific Mg^{2+}-dependent $PtdInsP_2$ phosphatase [51,52], and a cation-independent PtdInsP phosphatase [53]. Consistent with earlier studies of whole tissues [52], $PtdInsP_2$ phosphatase is almost entirely a soluble enzyme in glioma although there is very low activity in plasma membrane which is not attributable completely to cross-contamination. On the other hand, PtdInsP phosphatase is predominantly a membrane-bound activity present in the plasma membrane as well as intracellular membranes.

Our results with glioma and neuroblastoma cells and the data for hepatocytes [26] clearly demonstrate that the plasma membranes of these cells lack the ability to completely recycle phosphoinositides in unstimulated cells. This is probably also true for even a small pool of agonist-sensitive phosphoinositide in stimulated cells although the data do not specifically exclude the possibility of agonist-induced redistribution of these enzymes to the plasma membrane or activation of unmeasured cryptic activities in the plasma membrane. However,

neither possiiblity seems very likely. Since neither enzyme was present in the cytosol, translocation of membrane-bound enzyme from endoplasmic reticulum, rather than soluble enzyme, to the plasma membrane would be necessary. Further, use of saturating concentrations of exogenous substrates in optimized assays containing membrane-solubilizing detergent reduces the probability of undetected cryptic activities in the plasma membrane fraction. Although PtdIns resynthesis may occur in the plasma membranes of GH_3 cells (if CMP-PtdOH is available at that site), this is not a general phenomenon and, therefore, is not an obligatory requirement to sustain the inositide-linked signaling system. Diacylglycerol derived from agonist-induced hydrolysis of $PtdInsP_2$ by the receptor-coupled diesterase can, and probably is, phosphorylated in the plasma membrane. However, the PtdOH (possibly some of the diacylglycerol as well) must be transported to the endoplasmic reticulum for PtdIns resynthesis by the CTP : PtdOH cytidylyltransferase and PtdIns synthase enzymes that are exclusively located there. A mechanism for rapid PtdOH transport other than by non-specific phospholipid transfer proteins has not been identified. Furthermore, a mechanism for segregating this PtdOH from PtdOH arising from de novo synthesis is difficult to imagine in the absence of evidence for topographically isolated species of cytidylyltransferase and PtdIns synthase with specificity for stearoyl-arachidonoyl-DAG in the endoplasmic reticulum. Recent studies and associated discussion have developed largely hypothetical schemes to explain the roles of inositol 1,4,5-trisphosphate and inositol 1,3,4,5-tetrakisphosphate in calcium homeostasis. These have invoked various endoplasmic reticulum-like organelles such as 'calciosomes' and endoplasmic reticulum vesicles adjacent to the plasma membrane [54]. If isolated, such endoplasmic reticulum subfractions may provide a physical location for the required selectivity. Newly synthesized PtdIns is readily moved between cellular membranes by transport proteins but selective transport has not been demonstrated. Lundberg and Jergil [26] have proposed that PtdIns kinase activity is present in transport vesicles and that PtdIns is phosphorylated during transit to the plasma membrane. Since PtdIns kinase is absent from the plasma membrane, this may be a potential means of targeting PtdIns into the polyphosphoinositide signal transduction system as only PtdInsP delivered to the plasma membrane could be phosphorylated by the PtdInsP kinase in situ.

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