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Molecular Species of Phosphatidylinositol-Cycle Intermediates in the Endoplasmic Reticulum and Plasma Membrane†

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Abstract

Phosphatidylinositol (PI) turnover is a process requiring both the plasma and ER membranes. We have determined the distribution of phosphatidic acid (PA) and PI and their acyl chain compositions in these two subcellular membranes using mass spectrometry. We assessed the role of PI cycling in determining the molecular species and quantity of these lipids by comparing the compositions of the two membranes isolated from embryonic fibroblasts obtained from diacylglycerol kinase epsilon (DGKε) knock out (KO) and wild type (WT) mice. In the KO cells the conversion of arachidonoyl-rich DAG to PA is blocked by the absence of DGKε, resulting in reducing the rate of PI-cycling. The acyl chain composition is very similar for PI or PA in the endoplasmic reticulum (ER) vs. plasma membrane (PM) and for WT vs. KO. However, the acyl chain profile for PI is very different from that for PA. This indicates that DGKε is not facilitating the direct transfer of a specific species of PA between the PM and the ER. About 20% of the PA in the ER membrane has one short acyl chain of 14 carbons or less. These species of PA are not converted into PI but may play a role in stabilizing regions of high positive curvature in the ER. There are also PI species in both the ER and PM for which there is no detectable PA precursor, indicating that these species of PI are unlikely to arise via the PI-cycle. We find that in the PM of KO cells the levels of PI and of PA are decreased about three-fold in comparison with either the PM of WT cells or in comparison with the ER of KO cells. The PI-cycle is slowed in the KO cells, hence the lipid intermediates of the PI-cycle can no longer be interconverted and are depleted from the PI-cycle by conversion to other species. There is less of an effect of the depletion in the ER where *de novo* synthesis of PA occurs in comparison with the PM.

A major pathway for hormonal stimulation of cells is through the activation of PI(4,5)P₂-specific isoforms of phospholipase C that catalyzes the hydrolysis of PI(4,5)P₂¹ to the two signaling molecules, diacylglycerol (DAG) and inositol triphosphate. The efficiency of this system is due in part to the fact that the initial substrate, PI(4,5)P₂, is regenerated from DAG

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Supporting Information Available: Results of an analysis giving the relative quantitation based on the percent composition of the PA and PI molecular species, normalized by total phospholipid are presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

through a biochemical cycle referred to as the PI-cycle. The hormone-stimulated initial cleavage of PI(4,5)P₂ occurs in the PM but the regeneration of PI(4,5)P₂ requires participation of enzymes found only in the ER (Fig. 1). Thus, the functioning of the PI-cycle requires transfer of lipids between these two membranes.

Several of the lipid intermediates of the PI-cycle have important signaling properties, yet little is known about how they are distributed between the two membranes involved in the PI-cycle, nor is the acyl chain composition for these lipids known in these two membranes. Among the lipid intermediates of the PI-cycle with important signaling properties are the various species of phosphorylated PI, PIP_n. This lipid class, of PI plus PIP_n, comprises only 5–8% of total lipids in mammalian cells (1). However these lipids regulate fundamental cell processes, including cell growth, cytoskeleton dynamics, membrane trafficking and nuclear events (2). PI(4,5)P₂ is not the only form of PIP_n with important cellular functions, rather PIP_n species undergo rapid interconversion through cycles of phosphorylation/dephosphorylation, tightly regulated by numerous PI and PIP_n kinases and phosphatases to form PI and various species of PIP_n with varying number and positions of phosphorylation in the inositol moiety. All of the different forms of PIP_n serve as individual signaling molecules. Another important signaling lipid of the PI-cycle is phosphatidic acid (PA). PA is essential in controlling cell processes such as cytoskeletal rearrangement, proliferation and cell survival (3). PA is required for vesicular trafficking. Decreasing PA production results in reduced exocytosis (4). PA regulates fusion through promotion of the negative membrane curvature (3). Another lipid intermediate of the PI-cycle, diacylglycerol (DAG), is a lipid second messenger whose importance in cell signaling is well established (5). DAG's diverse range of effectors allows it to modulate a large variety of cellular events, resulting in its broad effects on the cell (6).

There is interconversion among the three types of lipid signaling molecules, PI/PIP_n, PA and DAG in the PI-cycle. One important step in PI turnover is the conversion of DAG to PA, the first step in the resynthesis of PI, catalyzed by diacylglycerol kinases (DGK), a family of lipid signaling enzymes (7–10). Among all the isoforms of DGK, DGK ϵ appears to be most important for catalyzing this step in the PI-cycle (11;12). DGK ϵ is located in both plasma and ER membranes (13), and it has specificity for 1-stearoyl-2-arachidonoyl glycerol (14) and through the PI-cycle DGK ϵ contributes to enriching the PI with these acyl chains (14). In the present work we assess the role of the PI-cycle in determining the location and acyl chain composition of the lipid intermediates of the cycle by affecting the cycle with the deletion of DGK ϵ . For this purpose we have compared the PA and PI of the ER and PM isolated from embryonic fibroblasts derived from DGK ϵ KO and WT mice using mass spectrometry. This is a reliable method of detecting PI and PA and also allows determination of the acyl chain composition of these lipids. There have been studies using fluorescent protein-tagged protein domains that specifically recognize PI lipids to determine their cellular localization, but these methods have their own limitations (15). In addition, there is no acceptable fluorescent probe, specific for non-PI lipids, such as PA.

Experimental Procedures

Tissue Culture

Mouse fibroblasts were obtained from embryos of mice that were made deficient in DGK ϵ and are designated as DGK ϵ KO mouse embryonic fibroblasts (MEF) (11). In each experiment, these cells were compared with wild-type embryonic fibroblasts obtained from siblings of the

¹Abbreviations: DGK, diacylglycerol kinase; DAG, diacylglycerol; PA, phosphatidic acid; PI, phosphatidylinositol; PIP_n, all phosphorylated forms of PI; PLC, phospholipase C; PI(4,5)P₂, phosphatidylinositol (4,5) bisphosphate; WT, wild type; KO, DGK ϵ -knockout; ER, endoplasmic reticulum; PM, plasma membrane; SAG, 1-stearoyl-2-arachidonoyl glycerol; SAPA, 1-stearoyl-2-arachidonoyl phosphatidic acid; DMEM, Dulbecco's modified Eagle's medium; PNS, post-nuclear supernatant; FBS, fetal bovine serum.

($-/-$) mice. These cells, derived from DGK ϵ ($+/+$) embryos, are designated as DGK ϵ WT MEFs. All cells were immortalized by transfection with the SV40 large T antigen. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 25 mM HEPES, at 37 °C in a humidified atmosphere with 5% CO₂.

Subcellular Fractionation

Subcellular fractionation was adapted from a previously described procedure (16). The fractionation was performed using the OptiPrep gradient (Sigma-Aldrich), according to the manufacturer's instructions. The method has been shown to give good separation of ER and PM despite the fact that these two organelles have very similar densities of 1.16 g/cm³ (17). Briefly, DGK ϵ KO and WT MEF cells were grown at 37 °C in DMEM medium with 10% FBS until approaching confluency (80%). 32 dishes (10 cm) of each cell line were washed two times with ice-cold PBS and scraped into ice-cold PBS containing 1 × protease inhibitor cocktail for use with mammalian cell and tissue extracts (Sigma-Aldrich). The cells were collected by centrifugation at 1000g for 5 min, 4 °C and resuspended in 850 μ L of ice-cold homogenization buffer (0.25 M sucrose, 10 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM KCl, 20 mM NaCl, 1 × protease inhibitor cocktail). The cells were broken by 20 passages through a 25-gauge needle syringe. Unbroken cells and nuclei were removed from the cell homogenate by centrifugation at 1000g for 10 min at 4 °C to generate a postnuclear supernatant (PNS). The crude microsomal sample was diluted with the 50% Optiprep Density Gradient Medium (Iodixanol, from Sigma) to a final concentration of 25% Optiprep. The vesicle suspension was layered underneath an OptiPrep gradient consisting of 3%, 6.5%, 10%, 13.5%, 17% and 20.5% (w/v) iodixanol solutions. Gradients were centrifuged using a SW41Ti rotor in a Beckman Optima L-100 XP ultracentrifuge at 50,000g for 18 h, 4 °C. Eighteen fractions were collected and concentrated using Vivaspinn-500 columns (30-kDa cut-off, GE Healthcare). The presence of organelle-specific proteins was detected by SDS-PAGE and Western blotting using rabbit anti-Na/K ATPase α polyclonal antibody (Santa Cruz Biotech) and anti-GRP-94 polyclonal antibody (Santa Cruz Biotech). Marker enzymes indicate an excellent separation of PM and ER. It is not likely that there would be much contamination with other organelles that have an even greater difference in density. In addition, we are measuring the total PA and PI species in these membranes, so that a minor contamination with another organelle would not greatly affect the results. This is different, for example, from a common use of subcellular fractionation to determine the location of an enzyme, where a small contamination can falsely identify a fraction as being the one where the enzyme is located. Nevertheless, we recognize that there is likely some overlap in the distribution of subcellular organelles that is in part unavoidable because several of these membranes undergo exchange of materials and cycling and there are probably membrane particles of intermediate density. The ER contains the largest amount of membrane material in the cell, so any contamination of this fraction would be a small percent of the total. Because of its similar density, the ER would be the most likely contaminant of the PM. However, there is little overlap of the two peaks for the marker enzymes and the lipid composition is distinctly different between the PM and ER fractions. Furthermore, there is not likely to be a major difference in contamination of the organelles between the two cell lines since the acyl chain compositions in the PM and ER, although different from each other, are the same for WT and KO.

Determination of total protein concentration

Total protein concentration in the samples was measured using BCA Protein Assay Kit (Thermo Scientific) according to the product manual.

Glycerophospholipid Analysis

Phospholipids were extracted from the cellular fractions by a modified Bligh and Dyer extraction using acidified methanol. Briefly, an equal volume of ice cold 0.1 N methanolic HCl and ice cold CHCl_3 was added to each of the fractions. Following 1 min vortex at 4°C layers were separated by centrifugation (18,000g for 5 min, 4°C). After the extraction and standards addition, solvent was evaporated. The resulting lipid film was dissolved in 100 μl of Isopropanol (IPA):Hexane:100 mM $\text{NH}_4\text{COOH}_{(\text{aq})}$ 58:40:2 (mobile phase A). The mass spectrometric analysis and quantitation were performed essentially as described in (18). LC-MS technique was used with the utilization of synthetic odd-carbon phospholipid standards (four per each class). MDS SCIEX 4000QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA) was used for the analyses. Coupled to it was a Shimadzu HPLC system (Shimadzu Scientific Instruments, Inc., Columbia, MD) consisting of a SCL 10 APV controller, two LC 10 ADVP pumps and a CTC HTC PAL autosampler (Leap Technologies, Carrboro, NC). Phospholipids were separated on a Phenomenex Luna Silica column (Phenomenex, Torrance, CA) (2×250 mm, 5 μ particle size) using a 20 μL sample injection. A binary gradient consisting of IPA:Hexane: 100 mM $\text{NH}_4\text{COOH}_{(\text{aq})}$ 58:40:2 (mobile phase A) and IPA:Hexane: 100 mM $\text{NH}_4\text{COOH}_{(\text{aq})}$ 50:40:10 (mobile phase B) was used for the separation. The parameters of the mass spectrometer instrument and solvent gradient were as described in (18).

Statistical analysis

Experiments were performed in 5 independent repeats of each subcellular fraction/condition (ER/WT, ER/KO, PM/WT, PM/KO). The concentration of total protein was measured in each sample, and the amount of each lipid was normalized for the amount of the corresponding marker protein, relative to the total protein in the PNS. Results are presented as the mean \pm SEM. Data are analyzed by paired t-tests across either fractions (ER, PM) or genotypes (WT, KO) from the repeated experiments. Association of enrichment levels of PI in one fraction versus the other (the PM/ER ratio) with acyl chain length and fatty acid unsaturation is assessed by Spearman rank correlation (19).

Results

Subcellular Fractionation of DGK ϵ KO and WT MEF cells

We tested the role of PI-cycling in determining the relative amounts of specific species of PA and PI, as well as their partitioning between the plasma and ER membranes of DGK ϵ KO and WT MEF cells. The membranes of DGK ϵ KO and WT MEF cells were separated using an OptiPrep gradient. We used antibodies to the proteins GRP-94 and Na/K ATPase α as markers for the ER and the PM, respectively. The ER marker was found exclusively in the higher density fractions, but the PM has a broader distribution, with the major portion being in the lighter density fractions. The distribution of the PM was confirmed previously, using antibodies to caveolin-1, which showed a similar pattern to the distribution of Na/K ATPase α . The density of the bands was quantified by densitometry (Fig. 2). The standard curve, using different amounts of amino-terminal FLAG-BAP protein (Sigma), was plotted to show that loaded amounts of protein were in the linear range. The fractions containing the maximum amount of the marker proteins, were combined and used for mass spectrometry analysis. These were generally fractions 5–9 for the plasma membrane but varied by one or two fractions from one preparation to another and fractions 16–18 for the ER samples.

Phospholipid Composition of Plasma and ER Membranes of WT versus DGK ϵ KO Cells

Mass spectrometry analysis of plasma and ER membrane fractions of DGK ϵ KO and WT MEF cells showed a number of significant differences in PA and PI composition. Notably, the PM

of KO cells contains only 1/3 of the PI and PA, as does the ER (Fig. 3). Although the effect is modest, there is a close relationship between the level of PI enrichment in the PM versus ER to acyl chain length and fatty acid unsaturation in DGKe KO cells, but not in WT cells. In particular, the rank correlation (Spearman's rho) of the number of carbons to the PM/ER ratio for PI species in the DGKe KO case is -0.74 ($p < 0.01$), and is even more pronounced for the correlation with the number of double bonds (-0.88 , $p < 0.01$). These correlations are not significant for the WT cells.

When taken as a ratio of KO *versus* WT, several PA and PI species, such as 30:1 PA, 38:4 PA, 38:3 PA, 40:4 PA, and 36:4 PI, 38:6 PI, 38:3 PI, show about a two-fold decrease in the PM, whereas KO to WT ratios in the ER membrane show almost no significant changes (Fig. 4).

Comparison of PM versus ER membrane of MEF cells of molecular species of PA and PI

Although the levels of enrichment of PA and PI species in the PM versus ER in WT cells are similar across the acyl chain distribution (Fig. 3), the acyl chain profile for PI is very different from that for PA. The PI/PA ratio is >1 for 34:2, 36:1, 38:3 and 38:4, while for most of the other species it is <1 (Fig. 5). The overall PI/PA ratios in both the WT ER (1.79 ± 0.21 , mean \pm SEM) and PM (1.57 ± 0.12) are determined primarily by these major species of PI, which together account for more than half of the PI by mass in each fraction.

Also, it is of particular interest that several species are detected either in PI or in PA, but not in both (Table 1). With regard to PA, there are two species, 30:1 and 30:0 that make up 21% of the PA in the ER. These species are twice as abundant in the ER compared with the PM. With regard to the unique PI species, they are found equally in the ER and PM, like most other lipid species, but unlike the unique PAs.

Comparison of results to analysis using Relative Quantitation of molecular species of PA and PI versus Total Phospholipid

In order to safeguard against variable recovery rates across the subcellular fractions or genotypes, the analyses for Figure 3 and Figure 4 were repeated based on percent composition of the PA and PI molecular species, normalized by total phospholipid. These relative quantitation results are presented in Figures S1 and S2, and it is evident that no large differences exist between the respective analyses in Figure 3 and Figure 4, which use absolute quantitation.

Discussion

For most PA and PI species in WT cells, the ratio of PM/ER is close to one (Fig. 3), despite the fact that there is much more membrane in the ER than in the PM. Thus the concentration of PI and PA within the ER membrane must be less than in the PM. The equal amount of these lipids in the two compartments may be a consequence of the PI-cycle equalizing them. Although one would expect the rate of transfer from the more dilute PA and PI in the ER to be slower than the transfer to the ER from the higher concentration in the PM, this would be compensated by a larger amount of ER membrane, making the net flux of lipids in the two directions similar.

Our results also clearly show that DGKe is an important component of the PI-cycle since deletion of this enzyme decreases the amounts of both PI and PA in the PM to about 1/3 of that found in the ER of these cells (Fig. 3). Since PI is neither a substrate nor a product of the reaction catalyzed by DGKe, its concentration in a particular membrane could most likely change as a consequence of slowing the PI-cycle by deletion of DGKe, though we cannot rule out that the knockout might have more indirect impacts on the PA and PI distributions, as well. The direct effect of slowing the PI-cycle would be to specifically reduce the concentrations of

arachidonoyl-containing PA and PI. However, we observe that the reduction in relative concentrations of PA and PI in the ER vs. the PM extends essentially over all species (Fig. 3). This is most likely a result of the interconversion among species of PA and PI with different acyl chain compositions. This can occur by acyl chain remodeling through acylation/deacylation reactions. In addition, DGK ϵ can be bypassed in the PI-cycle through PLD-catalyzed formation of PA, including direct conversion of PI(4,5)P₂ to PA. Additionally, other isoforms of DGK, although they are not specific for 1-stearoyl-2-arachidonoyl glycerol (SAG), can still use it as a substrate to form 1-stearoyl-2-arachidonoyl-PA (SAPA) and the specificity of DGK ϵ for SAG is not absolute, but rather it is the preferred substrate. Hence the PI-cycle will not be completely isolated from other metabolic pathways. Nonetheless, these results show the importance of the association of DGK ϵ with the PI-cycle and this agrees with results reported earlier (11;12) and is consistent with the specificity of this enzyme for 1-stearoyl-2-arachidonoyl lipids (14). In particular, although differences in rates across PI species are relatively small (14), we find that there is a strong relationship (rank correlations with $p < 0.01$) between the level of PI enrichment in the PM versus the ER and acyl chain length and/or fatty acid unsaturation in DGK ϵ KO cells, but not in WT cells (Figure 3).

The PM and the ER have different roles in the PI-cycle. In the isolated wild-type PM, PI can be converted to PA, however there are no enzymes in the PM that can synthesize PI from precursors. In addition, in the ER but not in the PM, PA can be synthesized *de novo* from smaller precursors. However, in the DGK ϵ KO cells the formation of PA from arachidonoyl-rich DAG is reduced, hence there is little SAPA produced. PA can also be produced by phospholipase D, including a small amount of SAPA by PI(4,5)P₂-requiring phospholipase D isoenzymes. However, in the PM alone not all of the components are present to allow the functioning of a PI-cycle to regenerate the lipid intermediates of the cycle.

It is known that intermediates in biochemical cycles have the property of being catalysts. They get regenerated each time the cycle repeats. As a consequence of this cyclic nature the PI-cycle lipid intermediates become progressively enriched with 1-stearoyl-2-arachidonoyl acyl chains through multiple iterations of this cycle. The cycle also contributes to maintaining the steady state concentration of the intermediates of the cycle. When the cycle is damaged, as in this case of the KO cells, by eliminating DGK ϵ , these lipid intermediates get metabolized to other products. Furthermore, in the PM, several enzymes of the PI-cycle are activated by other lipids of the cycle. In particular, PIP-5-kinase, that produces PI(4,5)P₂, is activated by PA (20;21). Thus, in the absence of DGK ϵ , the functioning of the PI-cycle in the PM will also be slowed down by the lack of SAPA produced by DGK ϵ . Overall there will be a lowering of PI and PA levels in the PM, which we observed in DGK ϵ KO cells.

In the case of the ER membrane, the levels of most PI and PA species are slightly higher or remain the same in KO cells in comparison with WT cells. The level of PA in the ER can be maintained in part by an alternative pathway for the *de novo* synthesis of PA from glycerol-3-phosphate (22) (Fig. 1). Using acyl-CoAs, PA is first synthesized, and undergoes maturation in the remodeling pathway that includes acylation of lyso-PA (Lands' cycle) (23). This newly synthesized PA can then enter the PI-cycle in the ER through a CDP-dependent reaction catalyzed by CDP-diacylglycerol synthase. CDP-diacylglycerol synthase is not found in the PM nor can the PM synthesize PA from small molecule precursors. Hence PA and PI are more rapidly depleted in the PM in DGK ϵ KO cells. Within the ER, PI can be phosphorylated to PI(4)P by PI(4)K, to PI(3,4)P₂ or to PI(4,5)P₂ by PIP(5)K (24).

PI formed in the ER can be transferred to the PM by both vesicular transport or by specific lipid transporters. This process will also be slower in KO cells because of the lower level of PA in the PM of these cells. It has been shown that PA is required for vesicular trafficking, and that decreasing PA production results in reduced exocytosis (4). PA regulates fusion

through promotion of the negative membrane curvature (3). Therefore, in the PM of DGK ϵ KO cells, where the levels of PA are significantly reduced, the fusion process, where the vesicle membrane becomes contiguous with the PM, will be disrupted. Moreover, vesicular transport is also regulated by PI(4,5)P₂ (25). Thus, reduced levels of PI(4,5)P₂ and PA in the PM of KO cells would reduce the vesicle fusion with the PM, therefore impairing vesicular trafficking of PI from the ER, further reducing the levels of these phospholipids in the PM. This also can account for the slight accumulations of PI in the ER, which we observed in DGK ϵ KO cells. One interpretation is that the redistribution of PA and PI in the cell due to the knockout could largely be a result of the disruption of vesicular trafficking specifically, thereby altering the turnover of PI.

In further analysis, we also compared the distribution of different PI and PA species in DGK ϵ WT mouse embryonic fibroblasts. The data show that the acyl chain composition of the PI and PA is similar in both plasma and ER membranes of this cell line, and also about the same in the WT and KO cells. This suggests that DGK ϵ is not facilitating the transfer of specific species of PA between the PM and ER.

Furthermore, our results show that the acyl chain profile for PI is very different from that for PA. In both cellular fractions, virtually every species is either found with a PI/PA ratio significantly greater or less than 1. The PI/PA ratio is >1 for 34:2, 36:1, 38:3, and 38:4, while for most of the other species it is <1 (Fig. 5). These PI species, which together contain more than 50% of the PI mass in both fractions, have PI/PA ratios much higher than the other molecular species, and there is certainly no strict stoichiometry between PA and PI species across the acyl distribution. These data suggest that a narrow range of acyl chain lengths is enriched in PI relative to its precursor PA, and that PA is derived from other sources in addition to the action of DGK in the PI-cycle. Thus, the species of PA used for the synthesis of PI are either preferred substrates or modulators of the biosynthetic enzymes involved, or these lipids are physically segregated into specific membrane domains.

It is of particular interest that several species with particular acyl chains are detected either as PI or as PA, but not as both (Table 1). These lipids are examples of species of PA and PI that do not appear to participate in the PI-cycle since they do not have a corresponding partner, and thus, they should be somehow separated from lipids in the PI-cycle. With regard to PA, there are two species, 30:1 and 30:0 that constitute 21% of the PA in the ER. These species are twice as abundant in the ER compared with the PM. The 30:1 and 30:0 PA have a sum of 30 carbons in the acyl chains, which means that one acyl chain must be 14 or less. Only a minor fraction of acyl chains are this short, but these species are highly enriched in PA and in particular in the ER. We suggest that these short chain PA may concentrate on the outer monolayer of the ER. Since short acyl chains will facilitate positive curvature they would stabilize some of the folds in the ER. This would not be needed in the PM. These species have a decreased level in the KO cells, which may indicate a change in ER morphology in KO compared with WT cells, to a form that is less folded.

With regard to the unique PI species, they are equally distributed in the ER and PM, like most other species of PA and PI, but unlike the unique PA. Our data show that their levels do not differ in KO and WT cells. The results indicate that these lipids are not involved in the PI-cycle. In total, these unique PI species comprise about 30% of the total PI. They do not have a PA precursor for them to be synthesized from a CDP-dependent reaction catalyzed by CDP-diacylglycerol synthase, an essential step in the PI-cycle. Therefore, they could arise from an acyl chain exchange of one of the lipid intermediates of the PI turnover (through Lands' cycle), or by a PLD-catalyzed headgroup exchange from another lipid class. Thus, only a specific fraction of PI and PA participates in the PI-cycle and these pools are likely segregated from the other lipids with the same headgroup that are not intermediates in this cycle.

The acyl chain composition is very similar for PI or PA in the ER vs. PM and for WT vs. KO. However, the acyl chain profile for PI is very different from that for PA. Our findings also reveal that DGK ϵ plays an important role in inositol lipid turnover and regulates the lipid composition of the PM in mouse embryonic fibroblasts. The PI-cycle is selective for lipids with specific acyl chains in both the PM and ER.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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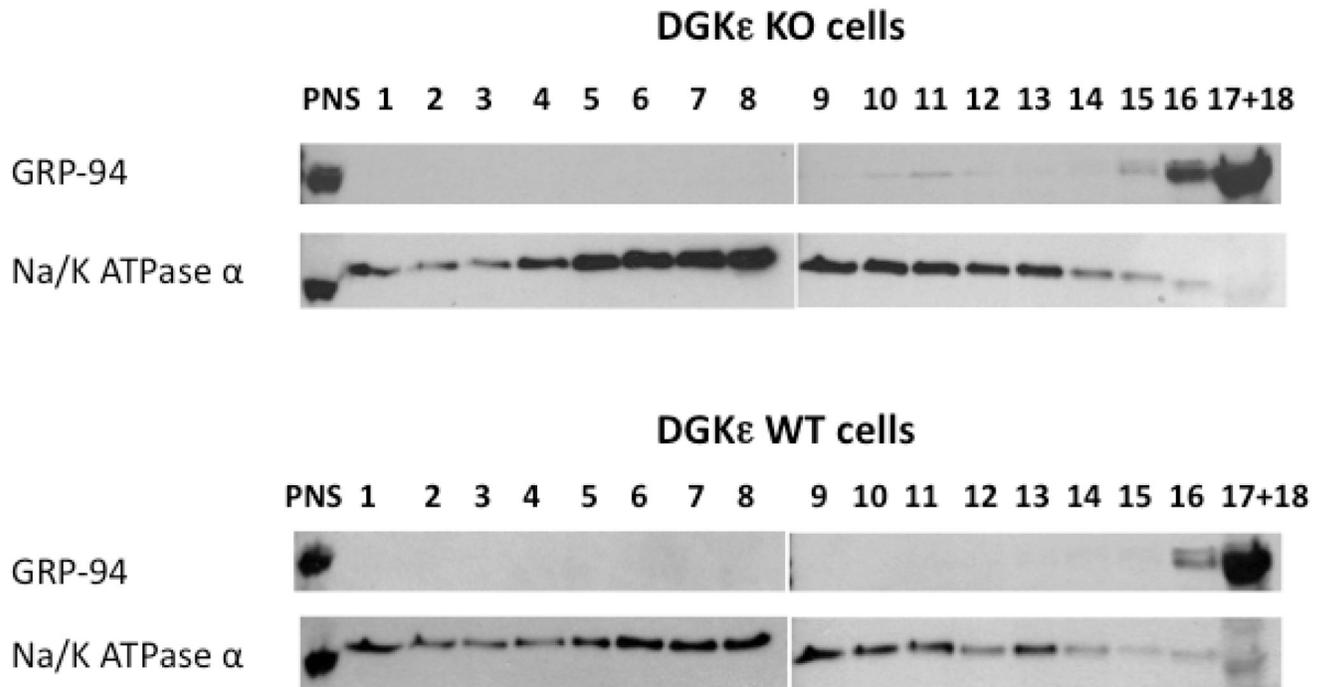


Fig. 2. Isolation of PM and ER membrane fractions by iodixanol gradient centrifugation. Fractionation was performed using a 3–25% OptiPrep gradient, and fractions were analyzed by immunoblotting with antibodies against GRP-94 (ER marker) and Na/K ATPase α (PM marker). PNS – postnuclear supernatant.

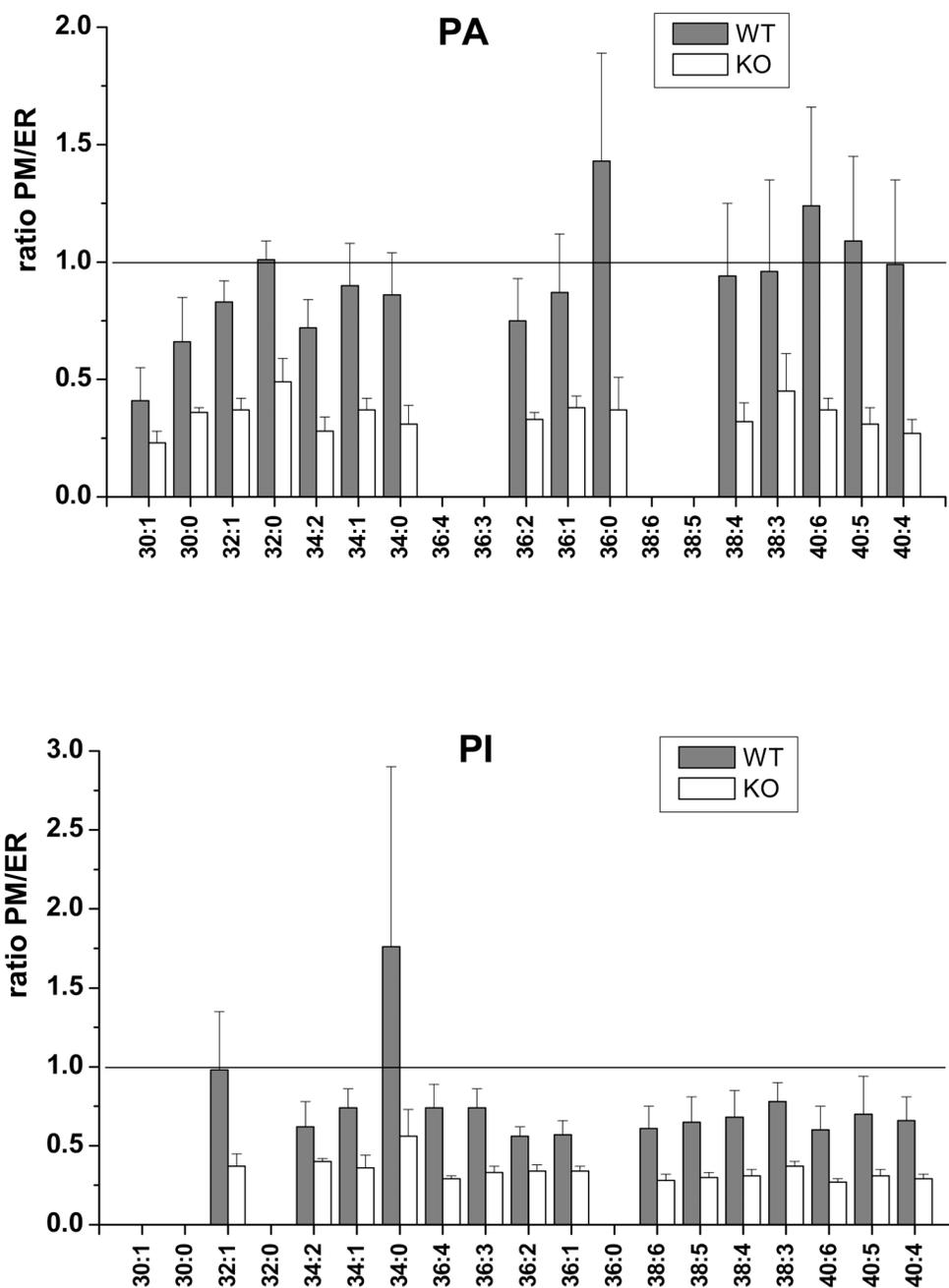


Fig. 3. Comparison of ratios of PA or PI in the PM to ER for DGK ϵ KO and WT cells. Results are presented as a mean of PM/ER ratio \pm SEM. In the KO case, all PM/ER ratios shown are significantly less than one with $p < 0.05$ except for PA 32:0 ($p=0.06$), PA 36:0 ($p=0.08$), PA 38:3 ($p=0.08$), and PI 34:0 ($p=0.19$). The only PM/ER ratios in the WT case which are significantly less than one are PI 36:1 ($p=0.01$) and PI 36:2 ($p=0.03$).

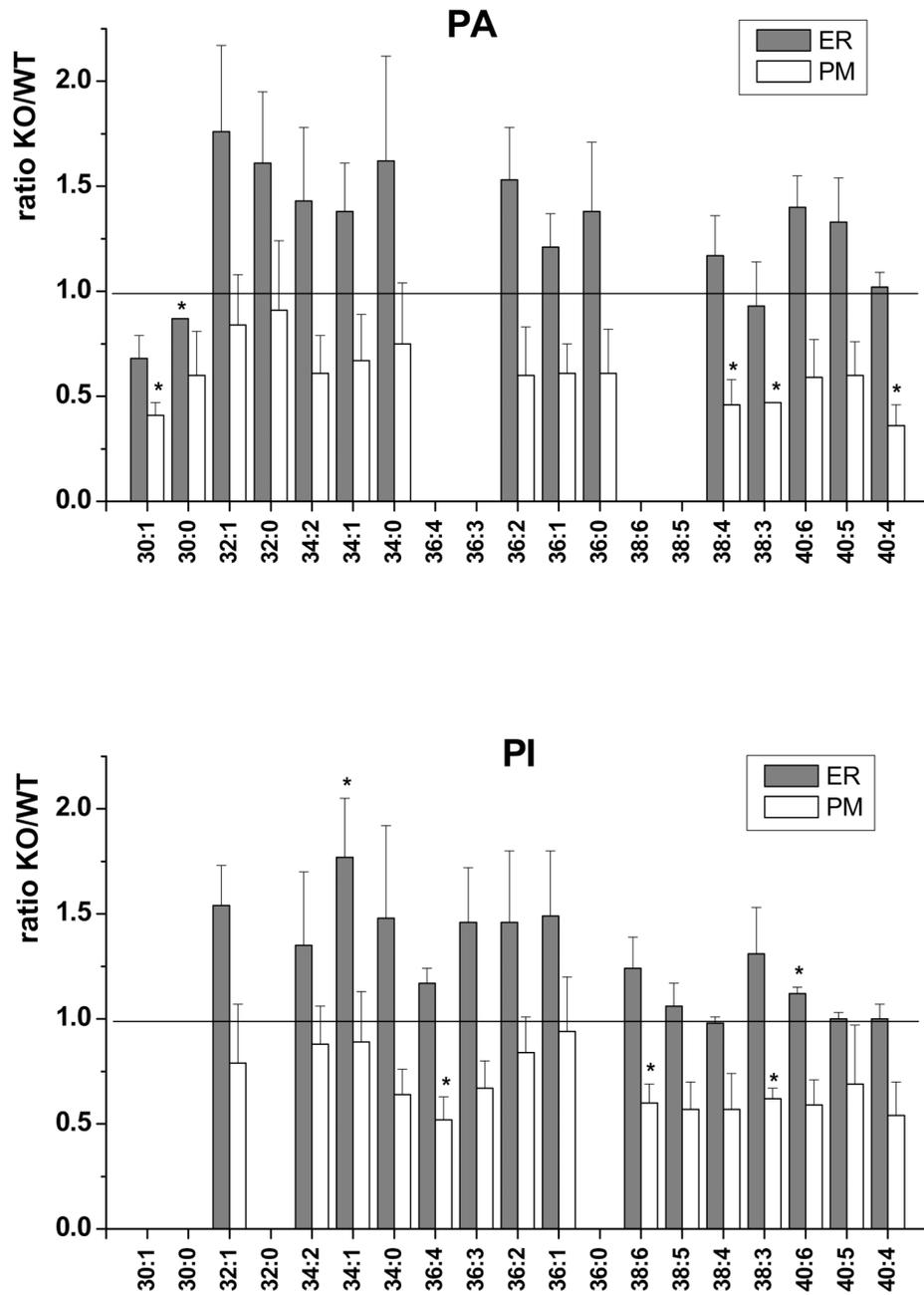


Fig. 4. Comparison of ratios of PA and PI in DGK ϵ KO to WT cells in plasma and ER membranes. Results are presented as a mean of KO/WT ratio \pm SEM. Values statistically differed with $p < 0.05$ are labelled with asterisks.

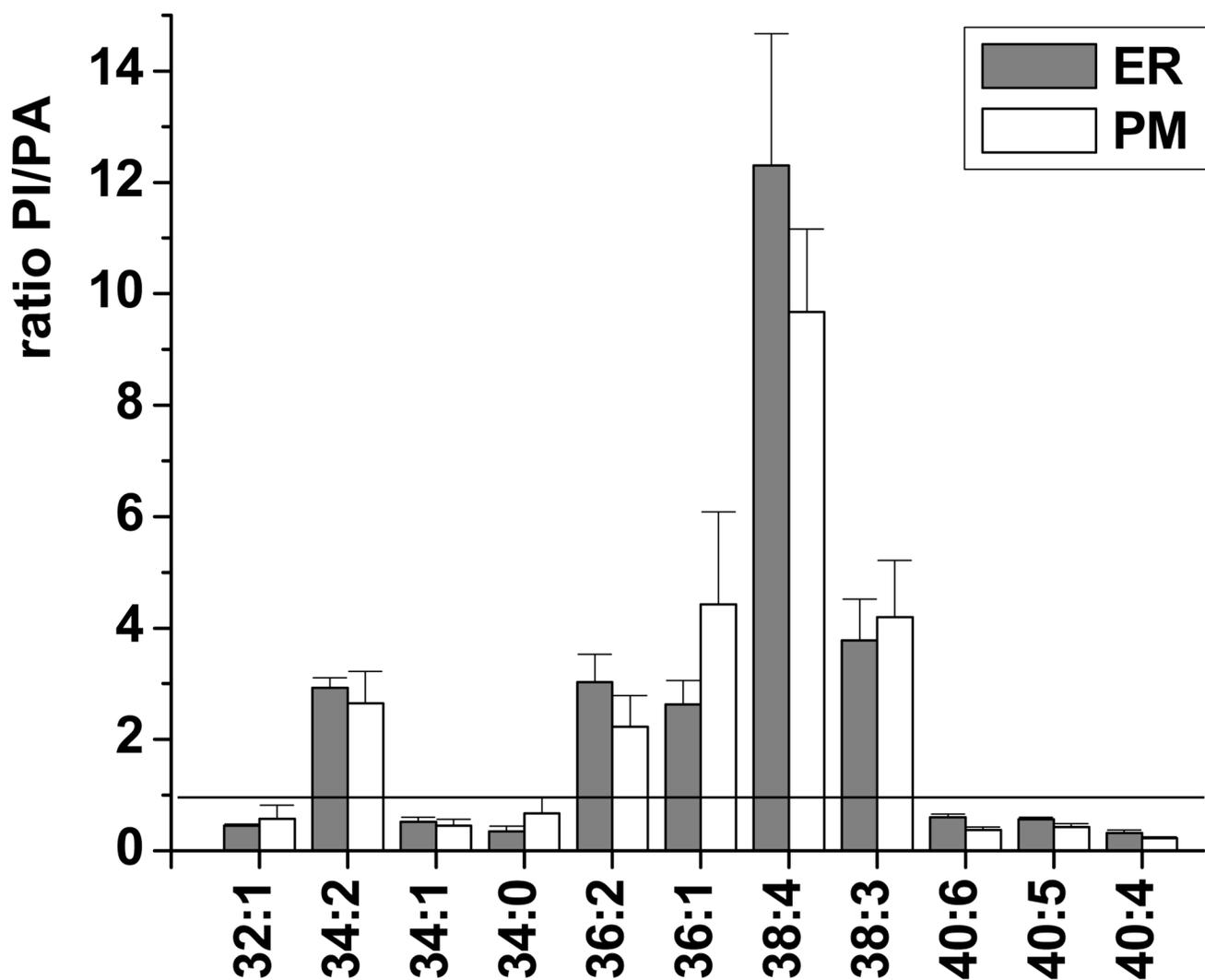


Fig. 5. Ratio of PI to PA in the plasma and ER membranes of DGK ϵ WT cells. Results are presented as a mean of PI/PA ratio \pm SEM. All ratios are statistically different from 1.0 with $p < 0.05$ except for 32:1 and 34:0 in the PM.

