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Phosphoinositides and Phosphoinositide-utilizing Enzymes in Detergent-insoluble Lipid Domains

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Recent evidence has implicated caveolae/DIGs in various aspects of signal transduction, a process in which polyphosphoinositides play a central role. We therefore undertook a study to determine the distribution of phosphoinositides and the enzymes that utilize them in these detergent-insoluble domains. We report here that the polyphosphoinositide phosphatase, but not several other phosphoinositide-utilizing enzymes, is highly enriched in a low density, Triton-insoluble membrane fraction that contains caveolin. This fraction is also enriched in polyphosphoinositides, containing approximately one-fifth of the total cellular phosphatidylinositol (4,5)P_2. Treatment of cells with the tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA), did not alter the distribution of polyphosphoinositides or the polyphosphoinositide phosphatase. However, PMA treatment did lead to a decrease in the mitogen-activated protein kinase and actin present in these domains. PMA also induced the recruitment of protein kinase Cα to the caveolae/DIGs fraction. These findings suggest that polyphosphoinositides, the polyphosphoinositide phosphatase and protein kinase C play an important role in the structure or function of detergent-insoluble membrane domains.

INTRODUCTION

Caveolae are smooth, nonclathrin-coated plasma membrane invaginations that have been implicated in a variety of cellular processes including apical protein sorting (Brown and Rose, 1992; Zurzolo *et al.*, 1994) and a form of nonclathrin-mediated endocytosis known as potocytosis (Anderson *et al.*, 1992). Caveolae are enriched in glycosphingolipids (Palade, 1953; Brown and Rose, 1992; Anderson, 1993) and as a result are resistant to solubilization with Triton X-100 (Yu *et al.*, 1973; Brown and Rose, 1992; Hanada *et al.*, 1995). Because caveolae retain their lipid components following extraction with Triton X-100, these low density protein/lipid complexes can be separated from the bulk of cellular proteins by sucrose density gradient centrifugation (Brown and Rose, 1992).

Caveolin is a 22-kDa, integral membrane protein that is found predominantly in caveolae (Glenney and Soppet, 1992; Rothberg *et al.*, 1992). Caveolin appears to be required for the formation of caveolae as expression of this protein in cells that lack caveolin leads to the formation of membrane invaginations that appear to be normal caveolae (Fra *et al.*, 1995). Lymphocytes and neuroblastoma cells have been shown to lack caveolin and caveolae but nonetheless contain detergent-insoluble glycosphingolipid-enriched membrane domains (Fra *et al.*, 1994; Gorodinsky and Harris, 1995), recently termed DIGs (Parton and Simons, 1995). Thus, there appear to be two types of Triton-resistant, low density lipid domains within cells. Recent evidence indicates that, at least in rat lung epithelial cells, caveolae and DIGs co-exist (Schnitzer *et al.*, 1995b).

Caveolae/DIGs contain a limited set of proteins. They appear to be enriched in GPI-anchored proteins such as the folate receptor, Thy-1, and decay-accelerating factor as well as caveolin (Anderson *et al.*, 1992; Anderson, 1993). Other proteins that have been localized to low density Triton-resistant domains include src family kinases (Lisanti *et al.*, 1994), protein kinase C (Lisanti *et al.*, 1994), mitogen-activated pro-

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tein (MAP)[1] kinase (Lisanti *et al.*, 1994), low molecular weight and heterotrimeric GTP-binding proteins (Chang *et al.*, 1994; Lisanti *et al.*, 1994), the inositol (1,4,5)P_3 [Ins(1,4,5)P_3] receptor (Fujimoto *et al.*, 1992), the epidermal growth factor receptor (Smart *et al.*, 1995b), gelsolin, and monomeric actin (Lisanti *et al.*, 1994). Many of these proteins participate in growth factor and hormone signaling, implicating caveolae/DIGs in signal transduction.

Within cells, signal transduction often involves membrane polyphosphoinositides. Phosphatidylinositol (4,5)P_2 [PtdIns(4,5)P_2] serves as a precursor for the generation of the two intracellular second messengers, Ins(1,4,5)P_3 and diacylglycerol. In addition, the polyphosphoinositides themselves act as regulatory molecules. PtdIns(4)P and PtdIns(4,5)P_2 bind to a variety of actin binding proteins, such as gelsolin and profilin, and induce their dissociation from actin (Lassing and Lindberg, 1985; Janmey *et al.*, 1987; Janmey and Stossel, 1987, 1989; Lind *et al.*, 1987). Polyphosphoinositides have also been shown to increase the activity of ARF-GAP and inhibit the activity of ras-GAP (Tsai *et al.*, 1988, 1990; Yu *et al.*, 1990; Randazzo and Kahn, 1994). PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are known to activate protein kinase Cζ (Nakanishi *et al.*, 1993). Polyphosphoinositides have also been implicated in protein trafficking, vacuole formation, and secretion (Bankaitis *et al.*, 1990; Herman and Emr, 1990; Salama *et al.*, 1990; Schu *et al.*, 1993; Hay *et al.*, 1995; Yamamoto *et al.*, 1995). Thus, these molecules play a role in the control of a large number of basic biological processes.

We have recently purified a novel, membrane-bound polyphosphoinositide phosphatase that dephosphorylates several polyphosphoinositides including PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P_2. All substrates are converted to PtdIns, without the generation of a monophosphorylated intermediate (Hope and Pike, 1994). This unusual substrate specificity suggests that this enzyme may play a role in reversing or terminating the effects of polyphosphoinositides on the above physiological processes.

During the purification of this enzyme, we noted that the polyphosphoinositide phosphatase was unusually difficult to solubilize in Triton X-100. Because detergent resistance is a characteristic of proteins present in caveolae/DIGs and because polyphosphoinositides could play a role in the function of these domains, we undertook a study of polyphosphoinositides and polyphosphoinositide-utilizing enzymes in low density, Triton-insoluble membrane domains. We report here that the polyphosphoinositide phosphatase, but not several other inositol phospholipid-metabolizing enzymes, is highly enriched in a low density, Triton-insoluble membrane fraction. Furthermore, we find that this fraction is enriched in polyphosphoinositides, in particular PtdIns(4,5)P_2. Treatment of cells with the tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate (PMA) has been shown to flatten membrane caveolae and inhibit folate uptake (Smart *et al.*, 1994). We find that this treatment does not alter the distribution of polyphosphoinositides or the polyphosphoinositide phosphatase. However, PMA treatment does lead to a decrease in the MAP kinase and actin present in these domains and induces the recruitment of protein kinase Cα to these low density, Triton-insoluble domains. These findings suggest that polyphosphoinositides, the polyphosphoinositide phosphatase and protein kinase C may play an important role in the structure or function of detergent-insoluble membrane domains.

[1] Abbreviations used: Ins(1,4,5)P_3, inositol (1,4,5)P_3; MAP, mitogen-activated protein; MDCK, Madin-Darby canine kidney; PMA, phorbol 12-myristate 13 acetate; PtdIns(4,5)P_2, phosphatidylinositol (4,5)P_2.

MATERIALS AND METHODS

Materials

A polyclonal antibody to caveolin was obtained from Transduction Laboratories (Lexington, KY). Anti-actin monoclonal antibody was obtained from Chemicon (El Segundo, CA). The polyclonal antibody to MAP kinase and the monoclonal antibody to phosphatidylinositol 3-kinase were from UBI (Lake Placid, NY). The monoclonal antibody to protein kinase Cα was provided by Dr. David Silbert (Washington University, St. Louis, MO). [^{3}H]*myo*-inositol and the Enhanced Chemiluminescence kit were purchased from Amersham (Arlington Heights, IL). Silica gel thin layer 60A K6 chromatography plates were obtained from Whatman (Maidstone, UK). EN^{3}HANCE was purchased from DuPont-NEN (Boston, MA). All other reagents were purchased from Sigma.

Cell Lines and Culture Conditions

Madin-Darby canine kidney (MDCK) cells were kindly provided by Dr. Doug Lublin. The cells were maintained in DMEM containing 10% fetal calf serum. For experiments in which cells were treated with PMA, PMA was added to the media at a final concentration of 200 nM for the times indicated.

Isolation of DIGs

Confluent 150-mm dishes of MDCK were washed twice with ice-cold Hanks' buffered saline, then scraped into 1 ml of lysis buffer containing 25 mM MES, pH 6.5, 150 mM NaCl, 1% Triton X-100, 10 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 11 U/ml aprotinin. Lysates were incubated for 20 min on ice with frequent agitation of the tubes. The lysate was mixed with an equal volume 80% sucrose in MBS (25 mM MES, pH 6.5, 150 mM NaCl) and placed at the bottom of a centrifuge tube. Six milliliters of 30% sucrose in MBS were layered on top followed by 4 ml of 5% sucrose in MBS. The discontinuous gradients were then centrifuged for 3 h at 4°C at 175,000 × *g* in an SW41 rotor. Fractions of 1.2 ml were collected beginning at the top of the gradients. The insoluble pellet was resuspended in 600 μl of lysis buffer.

Immunoblotting

Aliquots of 100 μl of each of the fractions from the discontinuous sucrose gradients were analyzed by SDS-PAGE. Proteins were

transferred to nitrocellulose and the membrane was blocked with 10% powdered milk. Nitrocellulose membranes were incubated for 1 to 2 h with primary antibodies at appropriate dilutions, washed, and incubated with horseradish peroxidase-conjugated secondary antibody. Bands were visualized using enhanced chemiluminescence.

Polyphosphoinositide Phosphatase Assay

Polyphosphoinositide phosphatase activity was measured as the release of $^{32}PO_4^{3-}$ from [^{32}P]PtdIns(4)P as described previously (Hope and Pike, 1994). The reactions contained 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, and 2 μM [^{32}P]PtdIns(4)P (20,000–40,000 cpm/assay) plus enzyme in a final volume of 100 μl. The assays were started by the addition of the labeled lipid and the reaction mixtures were incubated for 30 min at 30°C. Reactions were stopped by the addition of 0.5 ml methanol:concentrated HCl (10:1). A 0.5-ml aliquot of water and a 1-ml aliquot of chloroform were added to each tube. The tubes were vortexed for 20 s to extract the lipids into the chloroform, and the phases were separated by centrifugation for 3 min at 1500 × *g*. A 0.5-ml aliquot of the aqueous phase was added to 5 ml of Econo-Safe scintillation fluid and counted for ^{32}P in an LKB 1217 Rackbeta liquid scintillation counter.

Phosphatidylinositol 4-Kinase Assay

PtdIns(4) kinase activity was measured by following the transfer of phosphate from [γ-^{32}P]ATP to PtdIns. The reactions contained 50 mM β-glycerol phosphate, pH 7.4, 0.2% Triton X-100, 10 mM $MgCl_2$, 100 μM PtdIns, 50 μM ATP plus sample to a final volume of 100 μl. Reactions were started by the addition of [γ-^{32}P]ATP and were incubated for 5 min at 30°C. Assays were stopped by the addition of 0.5 ml methanol:concentrated HCl (10:1) and were processed as described (Walker and Pike, 1987).

Diacylglycerol Kinase Assay

Diacylglycerol kinase activity was measured by assessing the formation of [^{32}P]phosphatidic acid from diacylglycerol and [γ-^{32}P]ATP as described (Grondin *et al.*, 1991).

Analysis of [^{3}H]Inositol-labeled Lipids

Log phase cells were shifted to labeling media containing DMEM: inositol-free RPMI (1:1) plus 5% dialyzed fetal calf serum and 1 μCi/ml [^{3}H]inositol. Cells were labeled for 48 h at 37°C to achieve steady state labeling before the preparation of lysates. After centrifugation to isolate the caveolae/DIGs as described above, 1-ml aliquots of each fraction were extracted by the addition of 1 ml of methanol:concentrated HCl (10:1) and 2 ml chloroform. The aqueous phases were removed by aspiration, and the chloroform phases were reextracted with 1 ml of methanol:1 N HCl (1:1). The aqueous phases were removed and the chloroform phases were evaporated to dryness using a Savant Speed Vac Concentrator. Samples were then analyzed by TLC as described previously (Pike and Eakes, 1987). The thin layer plates were sprayed with EN^{3}HANCE and exposed to x-ray film for 3 days. The bands corresponding to PtdIns, PtdInsP, and $PtdInsP_2$ were scraped and counted for ^{3}H.

RESULTS

Phosphoinositide-utilizing Enzymes in Caveolae/DIGs

To isolate caveolae/DIGs, MDCK cells were extracted in buffer containing 1% Triton X-100 and the lysate was subjected to sucrose density gradient centrifugation. The gradient was fractionated and the fractions analyzed for protein and various enzyme activities. The results are presented in Figure 1. Fractions 1 through 4 represent the 5% sucrose layer while fractions 9 and 10 contain the 40% sucrose layer that comprised the original cell lysate. The pellet that sedimented to the bottom of the gradient is indicated as P.

The bulk of the protein remained in the original 40% sucrose layer or was sedimented to the bottom of the tube as a pellet. However, a small fraction of the total protein moved up into the gradient and was present in the low density fractions (fraction 4). Western blot analysis demonstrated that caveolin was present in fraction 4, indicating that this fraction contains the

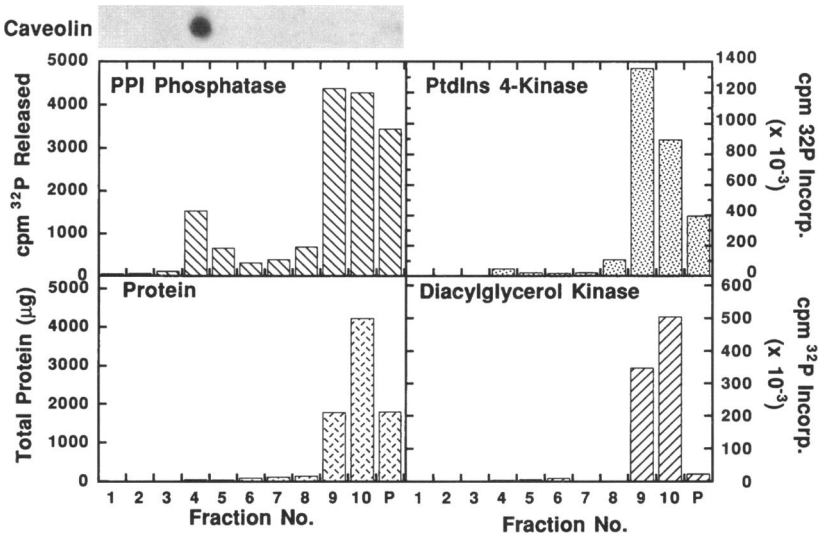

Figure 1. Distribution of polyphosphoinositide-utilizing enzymes in MDCK cells extracted with Triton X-100. MDCK cells were lysed in buffer containing 1% Triton X-100 and analyzed by sucrose density gradient centrifugation and described in MATERIALS AND METHODS. Gradients were fractionated and assayed for polyphosphoinositide phosphatase (PPI phosphatase) activity, phosphatidylinositol 4-kinase (PtdIns 4-kinase) activity, diacylglycerol kinase (DG kinase) activity, and protein. The data are presented as the total activity present in each fraction. Equal aliquots of each fraction were also analyzed by SDS-PAGE and Western blotting for the presence of caveolin.

low-density, Triton X-100–resistant lipid domains. Variable levels of caveolin were also recovered in the pellet, possibly as a result of incomplete cell lysis.

The gradient fractions were also assayed for enzymes involved in polyphosphoinositide metabolism. As shown in Figure 1, approximately 15% of the polyphosphoinositide phosphatase activity was recovered in the low-density, caveolin-enriched fractions while the remainder was present in the 40% sucrose layer and the pellet. As fractions 4 and 5 contained only 0.7% of the total cellular protein, this represents approximately a 20-fold enrichment of the polyphosphoinositide phosphatase in these fractions. By contrast, neither PtdIns 4-kinase nor diacylglycerol kinase activities were significantly enriched in the low density fractions. In addition, Western blotting failed to detect the p85 subunit of PtdIns 3-kinase, phospholipase Cγ or phospholipase Cβ in these fractions, although the proteins were present in the 40% sucrose layer (Figure 2).

Phosphoinositides in Caveolae/DIGs

The polyphosphoinositide phosphatase hydrolyzes the monoester phosphate bonds in PtdIns(3)P, PtdIns (4)P, and PtdIns(4,5)P_2 (Hope and Pike, 1994). To determine whether these substrates were present in the low density fractions, MDCK cells were labeled for 48 h with [^{3}H]inositol and lysates were fractionated by sucrose density gradient centrifugation as described above. Fractions were analyzed for polyphosphoinositide content as well as caveolin and general protein. The results are shown in Figure 3.

Fraction 4 contained high levels of caveolin, indicating that this fraction represents the caveolae/DIGs in this gradient. As before, the majority of the cellular protein was present in the 40% sucrose layer (fractions 9 and 10). Analysis of the inositol phospholipids demonstrated the presence of PtdIns, lyso-PtdIns, PtdIns(P), and PtdIns(4,5)P_2 in the low density fraction as well as the 40% sucrose fractions. The low density fraction contained a relatively high percentage of the total cellular phosphoinositides ranging from 8.5% for PtdIns(P) to 24% for PtdIns(4,5)P_2.

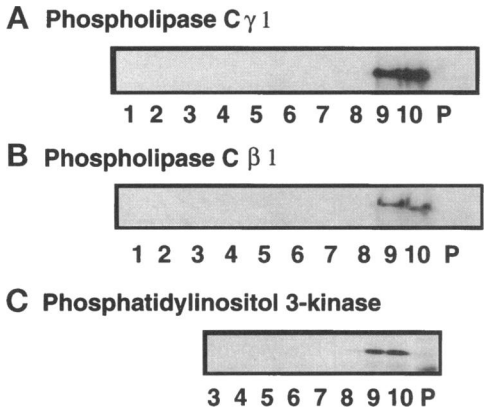

Figure 2. Distribution of phospholipase Cγ, phospholipase Cβ, and the p85 subunit of PtdIns 3-kinase in MDCK cells extracted with Triton X-100. MDCK cells were extracted with Triton X-100 and analyzed as described in MATERIALS AND METHODS. Equal aliquots of each fraction were subjected to SDS-PAGE and analyzed by Western blotting for phospholipase Cγ (A), phospholipase Cβ (B), and the p85 subunit of PtdIns 3-kinase (C).

Effect of Filipin on the Constituents of Caveolae/DIGs

To further examine the nature of the low density, detergent-resistant fraction, cells that had been labeled with [^{3}H]inositol were treated with or without 5 μg/ml filipin for 30 min before analysis by sucrose density gradient centrifugation. Filipin is known to bind cholesterol, a major lipid component of caveolae, and filipin treatment has been shown to impair caveolar function (Schnitzer, 1994). As shown in Figure 4, filipin treatment resulted in almost a complete loss of caveolin from the low density fraction. Likewise, approximately 60% of the polyphosphoinositide phosphatase activity was lost from this fraction. Interestingly, there was little loss of protein, PtdIns, or lyso-PtdIns from the low density fraction following treatment of cells with filipin. However, significantly less PtdInsP and PtdIns(4,5)P_2 were present in the caveolae/DIGs fraction from filipin-treated cells. All components lost from the low density fraction were recovered in the 40% sucrose and pellet fractions.

Effects of Phorbol Esters on Caveolae/DIGs

Previous studies have indicated that treatment of cells with the tumor-promoting phorbol ester PMA, leads to the flattening of caveolae and the inhibition of folate uptake mediated through caveolae (Smart *et al.*, 1994). To determine whether PMA affected the distribution of the polyphosphoinositide phosphatase and other proteins in caveolae/DIGs, MDCK cells were treated with 200 nM PMA for 30 min, extracted with buffer containing 1% Triton X-100, and analyzed by sucrose density gradient centrifugation. As shown in Figure 5, similar levels of caveolin were found in the low density fraction (fraction 4) of cells treated with and without PMA. Assay of the fractions for polyphosphoinositide phosphatase indicated that there was no change in total phosphatase activity following treatment with PMA and that the distribution of this enzyme was also unchanged.

Although PMA failed to alter the localization of caveolin and the polyphosphoinositide phosphatase, treatment of cells with this phorbol ester markedly affected the distribution of other proteins. As shown in Figure 6, MAP kinase and actin were both associated

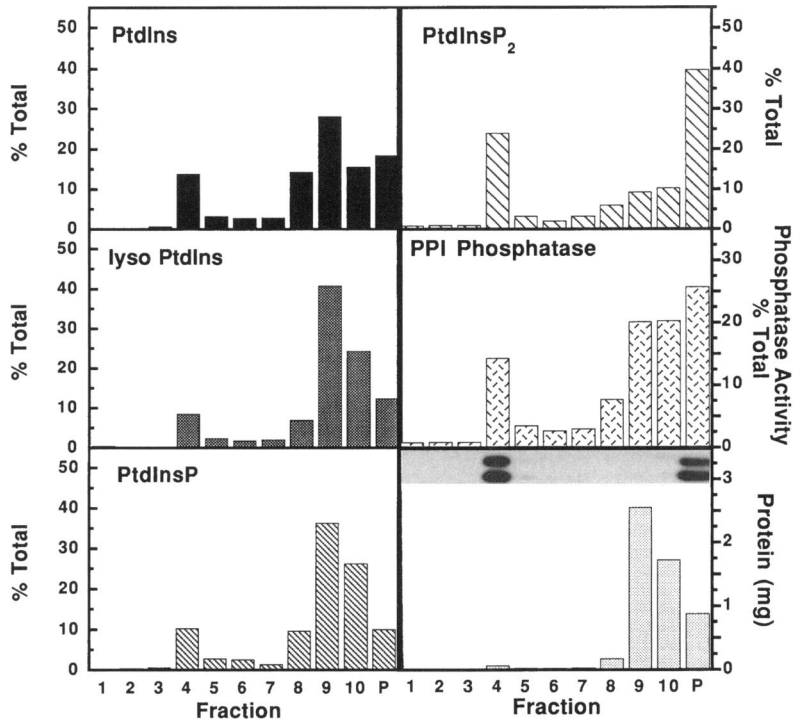

Figure 3. Distribution of polyphosphoinositides in MDCK cells extracted with Triton X-100. MDCK cells were labeled with 1 μCi/ml [^{3}H]inositol for 48 h, lysed in Triton X-100, and fractionated by sucrose density gradient centrifugation. The phospholipids in 90% of each fraction were extracted with $CHCl_3$/methanol and separated by TLC. Following autoradiography, the bands representing PtdIns, lyso-PtdIns, PtdInsP, and PtdIns (4,5)P_2 were scraped and counted. The data are presented as the percent of the specified phospholipid found in each fraction. The lower right panel indicates the total protein found in each fraction and the inset shows a Western blot analysis of the gradient fractions for caveolin.

with the low density fraction (fraction 4) in control cells. However, treatment with PMA led to the loss of these proteins from caveolae/DIGs. The presence of an additional, slower moving form of MAP kinase in the 40% sucrose fractions (fractions 9 and 10) indicates that the enzyme had been stimulated by the treatment with PMA. Remarkably, protein kinase Cα, which was only weakly detectable in caveolae/DIGs before treatment with PMA, was strongly recruited to this fraction after treatment. Analysis of the time course of recruitment indicated that protein kinase Cα levels increased within 15 min after treatment with PMA and remained elevated for 60 min (Figure 7). Densitometric analysis indicated a 3.3-, 3.1-, and 3.8-fold increase in protein kinase Cα levels in the low density fraction after 15, 30, and 60 min of treatment with PMA, respectively. Total cellular levels of protein kinase Cα remained relatively stable throughout the time course as judged by Western blot analysis of total cell lysates.

The effect of PMA treatment on the distribution of cellular inositol phospholipids was also examined. MDCK cells were labeled for 48 h with [^{3}H]inositol, treated with or without 200 nM PMA for 30 min, and the lysates were analyzed by sucrose density gradient centrifugation. The data in Figure 8 demonstrate that PMA did not significantly alter the distribution of phosphoinositides in the gradients. The low density fraction still contained a high percentage of all phosphoinositides and remained particularly enriched in PtdIns(4,5)P_2.

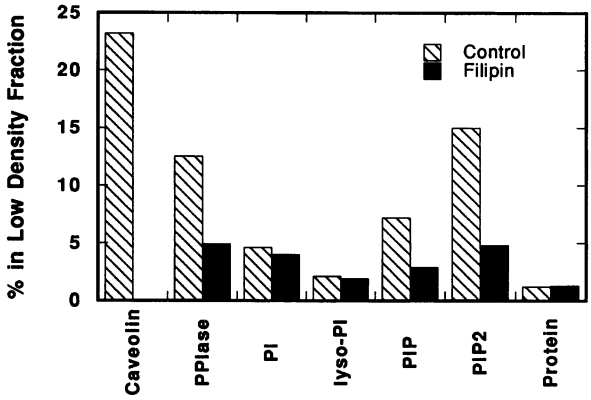

Figure 4. Effect of filipin on the distribution of proteins and lipids in the low density fraction. MDCK cells were labeled with 1 μCi/ml [^{3}H]inositol for 48 h. Cells were then transferred into inositol-free DMEM and incubated for 30 min at 37°C in the presence or absence of 5 μg/ml filipin. Cells were then washed in ice cold Hanks' buffered saline, lysed in Triton X-100-containing buffer, and subjected to sucrose density gradient centrifugation. Fractions were analyzed for caveolin content, polyphosphoinositide phosphatase activity, inositol phospholipid content, and protein as described in MATERIALS AND METHODS. Caveolin content was quantitated by densitometry. The results are presented as the amount of each constituent in the low density gradient fractions (fractions 4 and 5) as a percent of the total amount of that constituent recovered over the entire gradient.

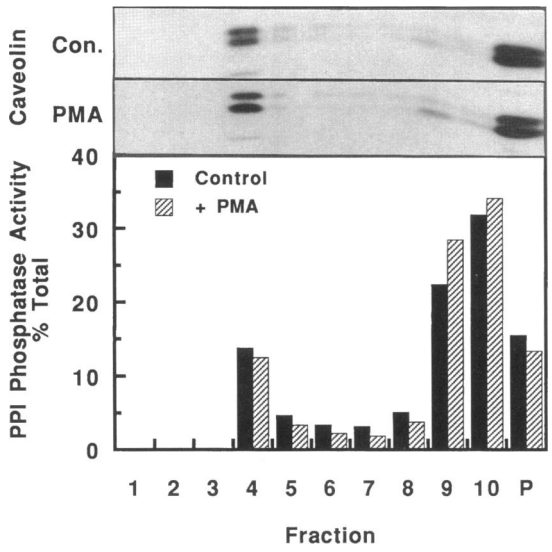

Figure 5. Effect of PMA on the distribution of caveolin and the polyphosphoinositide phosphatase in MDCK cells. MDCK cells were incubated in the absence (Control) or presence (PMA) of 200 nM PMA for 30 min at 37°C. Lysates were then prepared and fractionated by sucrose density gradient centrifugation. Equal aliquots of each fraction were then analyzed by Western blotting for caveolin (upper panels) or assayed for polyphosphoinositide phosphatase activity (lower panel). The data are presented as the percent of the total polyphosphoinositide phosphatase activity found in each fraction.

DISCUSSION

Recent evidence has implicated caveolae/DIGs in various aspects of signal transduction, a process in which polyphosphoinositides play a central role. We therefore undertook a study to determine the distribution of phosphoinositides and the enzymes that utilize them in these detergent-insoluble domains. Prior studies of the lipid content of Triton X100-insoluble vesicles using chemical methods of detection had indicated that these organelles contained very low levels of PtdIns but provided no information on the distribution of the biologically active polyphosphoinositides (Brown and Rose, 1992). Our results indicate that caveolae/DIGs contain not only PtdIns but also lyso-PtdIns, PtdInsP, and PtdInsP$_2$. A minimum of 5–10% of each of the phosphoinositides was found in the low density fraction. As caveolae have been estimated to account for 0.4–0.8% of the plasma membrane in MDCK cells (Sargiacomo *et al.*, 1993), this suggests that these low density domains are 10- to 20-fold enriched in phosphoinositides. On average, about one-fifth of the total cellular PtdInsP$_2$ was found in the caveolae/DIGs fraction. Thus, these detergent-insoluble domains appear to be glycosphingolipid islands that contain relatively high concentrations of inositol phospholipids, in particular PtdIns(4,5)P$_2$.

Caveolae are known to be involved in the apical sorting of proteins from the Golgi (Brown and Rose, 1992; Dupree *et al.*, 1993; Zurzolo *et al.*, 1994) and in the uptake of folates by potocytosis (Anderson *et al.*, 1992), two processes that involve membrane fusion. Consistent with the central role of membrane fusion in the function of caveolae, these lipid domains have been shown to contain proteins such as VAMP-2, SNAP, and annexins that are required to mediate vesicle formation, docking, and fusion (Schnitzer *et al.*, 1995a). Polyphosphoinositides have also been implicated in membrane trafficking and vacuole formation, as mutations in enzymes participating in PtdIns metabolism lead to defects in these processes (Herman and Emr, 1990; Cleves *et al.*, 1991; Schu *et al.*, 1993; Yamamoto *et al.*, 1995). The high concentration of polyphosphoinositides present in caveolae/DIGs suggests that these lipids may participate in the fusion and trafficking functions of caveolae.

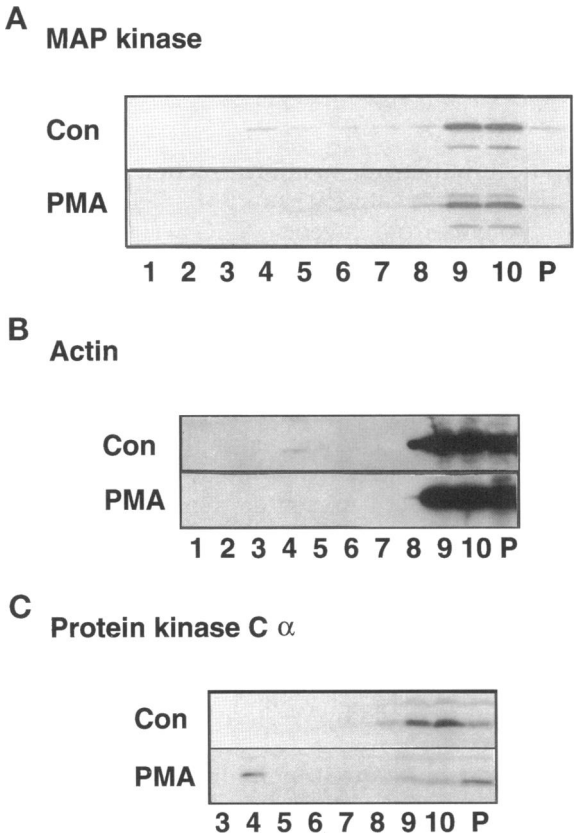

Figure 6. Effect of PMA on the localization of MAP kinase, actin, and protein kinase Cα in MDCK cells. MDCK cells were incubated in the absence (Con) or presence (PMA) of 200 nM PMA for 30 min at 37°C before analysis by sucrose density gradient centrifugation. Equal aliquots of each gradient fraction were subjected to SDS-PAGE followed by Western blotting for MAP kinase (A), actin (B), or protein kinase Cα (C).

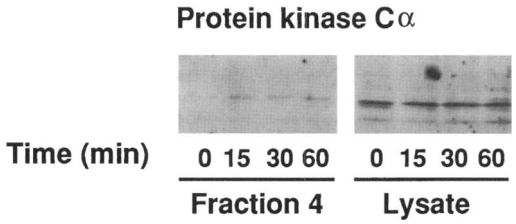

Figure 7. Time course of the PMA-dependent recruitment of PKCα to the low density fraction. MDCK cells were treated with 200 nM PMA at 37°C for the indicated times and then fractionated by sucrose density gradient centrifugation. Subsequently, 3 μg protein from fraction 4 of each gradient or 25 μg of protein from each cell lysate were subjected to SDS-PAGE followed by Western blotting for protein kinase Cα.

Although caveolae/DIGs contained high levels of polyphosphoinositides, they appear to have a relatively limited set of polyphosphoinositide-utilizing enzymes. Among the many enzymes examined, only the polyphosphoinositide phosphatase was found to be enriched in the low density fraction. We have previously shown that the polyphosphoinositide phosphatase is capable of converting PtdIns(3)P, PtdIns(4)P, and PtdIns(4,5)P_2 directly into PtdIns, a biologically inactive inositol phospholipid (Hope and Pike, 1994). Thus, this enzyme may act to terminate events stimulated by polyphosphoinositides. The presence of the polyphosphoinositide phosphatase within caveolae/DIGs is consistent with a role for this enzyme in regulating polyphosphoinositide levels in this domain and could provide a target for the control of phosphoinositide-mediated vesicle fusion.

The observation that treatment of cells with the cholesterol-binding agent filipin resulted in the loss of caveolin, polyphosphoinositide phosphatase, PtdInsP, and PtdIns(4,5)P_2, suggests that the presence of these components in the caveolae/DIGs fraction is dependent upon the availability of uncomplexed cholesterol. This implies that these compounds are constituents of caveolae, which require cholesterol for their integrity. The fact that there was residual phosphatase and polyphosphoinositides in the low density fraction after filipin treatment may be a reflection of the presence of two different types of low density membrane domains in this fraction. Alternatively, it could be an indication that some of the components of caveolae/DIGs do not require cholesterol for their maintenance in a glycosphingolipid-enriched domain.

Consistent with the findings of Lisanti *et al.* (1994), we found no evidence for the presence of phospholipase Cγ in caveolae/DIGs from MDCK cells. We also found no phospholipase Cβ in these domains. Thus, despite high concentrations of their phosphoinositide substrates, little phospholipase C activity appears to be associated with caveolae/DIGs under resting conditions. Despite the fact that the p85 subunit of PtdIns 3-kinase was reported to be present in mouse lung caveolae (Lisanti *et al.*, 1994), we were unable to detect this protein in our caveolae/DIGs fraction. This may be due to differences in cell type or could reflect the fact that p85 is only weakly associated with caveolae/DIGs and may be removed by Triton extraction.

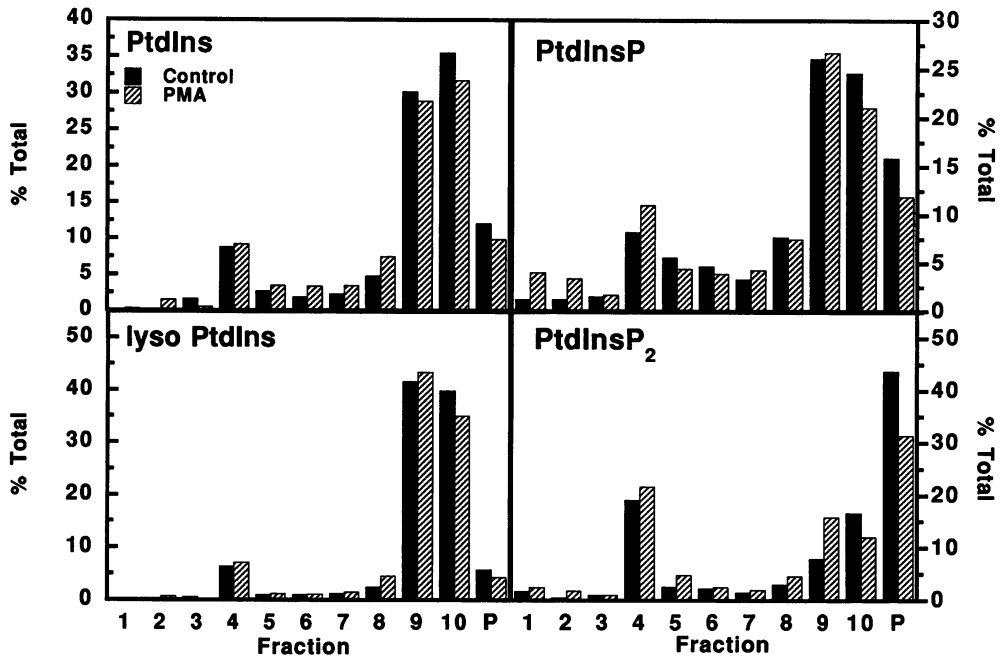

Figure 8. Effect of PMA on the distribution of polyphosphoinositides in MDCK cells. MDCK cells labeled with [^{3}H]inositol for 48 h were incubated with 200 nM PMA for 30 min at 37°C. Following analysis by sucrose density gradient centrifugation, the phospholipids in 90% of each fraction were extracted with chloroform/methanol and separated by TLC. The data are presented as the percent of the specified phospholipid found in each fraction.

PMA has been shown previously to inhibit folate uptake by inhibiting the internalization of caveolae (Smart *et al.*, 1994). Although the caveolae were no longer invaginated in PMA-treated cells, the folate receptor remained clustered, suggesting that the glycosphingolipid-enriched domains had remained intact following this treatment. We found that the distribution of caveolin, apparently a structural protein of caveolae, was unchanged in cells incubated with phorbol ester, a result also obtained by Smart *et al.* (1995a). Similarly, the polyphosphoinositide phosphatase was retained in the low density fraction following treatment with PMA, suggesting that it is also an intrinsic protein component of caveolae/DIGs. Polyphosphoinositide levels in caveolae/DIGs were also unaffected by PMA treatment, again implying that these lipids are an integral structural component of caveolae/DIGs that are maintained even when other aspects of caveolar structure are disrupted.

Although both caveolin and the polyphosphoinositide phosphatase were retained in caveolae/DIGs from PMA-treated cells, several other proteins were lost from these domains, specifically MAP kinase and actin. Actin has previously been reported to be strongly associated with caveolae (Chang *et al.*, 1994) and has been implicated in internalization of these invaginations (Parton *et al.*, 1994). In our experiments, PMA treatment induced rounding of the MDCK cells and thus clearly affected the entire cellular cytoskeleton. The loss of actin from caveolae may reflect this generalized effect of PMA on the cytoskeleton and is almost certainly related to the ability of PMA and cytochalasin to inhibit caveolar internalization (Parton *et al.*, 1994; Smart *et al.*, 1994). It is possible that in the absence of an organized cytoskeletal network, the detergent extraction of loosely bound proteins, such as MAP kinase, from the caveolae/DIGs is facilitated. A more interesting possibility is that activation of MAP kinase by PMA induces its release from this compartment.

Surprisingly, we found that PMA treatment induced the recruitment of protein kinase Cα to caveolae/DIGs. This observation is in disagreement with the recent report that treatment of cells with phorbol dibutyrate leads to a loss of protein kinase Cα from caveolae (Smart *et al.*, 1995a). This discrepancy is most likely due to differences in the reagents and protocols used in the two studies. In our experiments, cells were treated with 200 nM PMA whereas in the experiments of Smart *et al.* (1995a), cells were treated with 1 μM phorbol dibutyrate. Under our conditions, we saw a limited decrease in total cellular protein kinase Cα levels. By contrast, Smart *et al.* reported that protein kinase Cα was absent not only from the caveolae but also from the cytoplasm of the phorbol dibutyrate-treated cells (Smart *et al.*, 1995a). Thus, the enzyme had been degraded and removed from the cell by their treatment. Apparently, our treatment with a lower dose of PMA limited the loss of cellular protein kinase Cα, enabling us to observe recruitment of the enzyme to caveolae/DIGs.

The data reported here support the hypothesis that polyphosphoinositides play an important role in the structure or function of caveolae/DIGs. Whether the inositol phospholipids undergo turnover as a part of signal transduction or participate in the vesicular trafficking remains to be determined. The enrichment of the polyphosphoinositide phosphatase, but not other phosphoinositide-utilizing enzymes, in the low density fraction implicates this protein in caveolar function and suggests that remodeling rather than turnover may be the predominant fate of polyphosphoinositides in caveolae. Since polyphosphoinositides are known to be important regulators of membrane fusion and vesicle trafficking, dephosphorylation of these lipids may be important for the control of this caveolar function.

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