Phosphatidylinositol 4-Phosphate Synthesis in Immunoisolated Caveolae-like Vesicles and Low Buoyant Density Non-caveolar Membranes*

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This study examined phosphatidylinositol 4-phosphate (PtdIns4P) synthesis in caveolae that have been suggested to be discrete signaling microdomains of the plasma membrane and are enriched in the marker protein caveolin.

Caveolin-rich light membranes (CLMs) were isolated from A431 cells by detergent-free, discontinuous density-gradient centrifugation method. The CLM fraction was separated from the bulk of the cellular protein and was greatly enriched in PtdIns, PtdIns4P, and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) and an adenosine-sensitive type II PtdIns 4-kinase activity. Preparation of CLMs by an OptiPrep-based cell fractionation procedure confirmed the co-localization of PtdIns 4-kinase and caveolin. Electron microscopy confirmed that an anti-caveolin antiserum immunopurified vesicles from CLMs that were within the size range described for caveolae in other systems. Co-immunoprecipitated PtdIns 4-kinase activity could utilize endogenous PtdIns, present within the caveolae-like vesicles, to produce PtdIns4P. The addition of recombinant phosphatidylinositol transfer protein increased PtdIns 4-kinase activity both in immunoisolated caveolae and CLMs. However, less than 1% of the total cellular PtdIns and PtdIns 4-kinase activity was present in caveolae-like vesicles, indicating that non-caveolar light membrane rafts are the main site for cellular PtdIns4P production.

A vast number of extracellular stimuli are transduced via plasma membrane-associated tyrosine kinase and G protein-coupled receptors to activate the phosphoinositidase C (PIC)1 signaling pathway. Activation of PIC leads to hydrolysis of PtdIns(4,5)P2 to generate inositol(1,4,5)P3 and diacylglycerol, which lead in turn to increases in intracellular Ca2+ and the activation of protein kinase C, respectively (reviewed in Refs. 1 and 2). In recent years other cellular roles for PtdIns(4,5)P2 have been identified. For example, PtdIns(4,5)P2 possesses biological activity in its own right in controlling the organization of the actin cytoskeleton (3–5) and binding a subset of pleckstrin homology domain-containing proteins (6). Furthermore PtdIns(4,5)P2 and its immediate metabolic precursor PtdIns4P are substrates for phosphorylation at the 3′ position by a family of enzymes known as the phosphoinositide 3-kinases (7). 3′-Phosphorylated polyphosphoinositides are thought to represent a novel class of signaling molecules that may regulate a range of intracellular target proteins, including, for example, the protein kinases Btk (8), PDK1 (9), and Akt (10). It is clear therefore, that levels of PtdIns4P and PtdIns(4,5)P2 may control multiple intracellular signal transduction cascades.

In A431 cells, occupation of epidermal growth factor (EGF) receptors leads to activation of phosphoinositide hydrolysis by PICγ (11) and stimulation of a type II PtdIns 4-kinase (12). In addition, stimulated EGF receptors co-purify (13) and co-immunoprecipitate (14) with PtdIns 4-kinase activity, and there is evidence to suggest that PtdIns 4-kinase (15) may bind to a basic juxtamembrane region of the EGF receptor. Work from this laboratory has also established that the phosphoinositide transfer protein (PITP) can be co-immunoprecipitated with both EGF receptors and PtdIns 4-kinase activity in EGF-treated A431 cells (14). This evidence, coupled with the observation that PITP is required to reconstitute PICg (16) and PICγ (17) signaling in cytosol-depleted cells, has led to the hypothesis that the receptor, phosphoinositide kinases, and PITP form a functional signaling complex in mitogen-activated cells (17).

Recent evidence has indicated that PtdIns4P, PtdIns(4,5)P2, and agonist-stimulated phosphoinositide hydrolysis may be highly compartmentalized in discrete Triton X-100-insoluble micro-domains of the plasma membrane that are enriched in caveolin, a recognized marker protein for caveolae (18). Caveolae, also known as plasmalemmal vesicles, are generally described as small flask shaped invaginations of the plasma membrane that are characterized enriched in the integral membrane protein caveolin (19). They also contain cholesterol, sphingolipids (20, 21), and possibly an array of proteins involved in signal transduction (21–27). However, in a previous study PtdIns 4-kinase activity was found not to be localized to caveolae (28) suggesting that PtdIns4P was made elsewhere within the cell and somehow sequestered by caveolae. In addition, recent studies have called in to question the equivalence of Triton-insoluble caveolae and caveolae. There is evidence to suggest that both receptors and G proteins may in fact localize to non-caveolar plasma membrane regions that do not contain caveolin but have a similar isopynic density to caveolae following equilibrium density gradient centrifugation (29, 30). These non-caveolar, low buoyant density membrane domains have...
been called detergent-insoluble, glycolipid-rich domains (DGAs) or membrane rafts, and it is possible that some of the functional properties previously assigned to caveolae may actually derive from molecules present in rafts as opposed to bona fide caveolae.

Given this background, we sought to further investigate the compartmentation of cellular PtdIns4P synthesis by examining whether caveolae represent major sites for the generation of PtdIns4P. Indeed, despite its central role in the regulation of cellular polyphosphoinositide levels, and the observation that only a proportion of the total cellular PtdIns may be available to replenish PtdIns4P during PIC activation, little is known about the organization of PtdIns4P synthesis at the plasma membrane or how the reported cellular compartmentation of phosphoinositide turnover may relate to agonist- and PITP-dependent PtdIns4P production (16, 17, 31).

The current study utilizes subcellular fractionation to isolate caveolin-enriched light membranes (CLMs) from A431 cells. We demonstrate that PtdIns4P synthesis is indeed concentrated within CLMs and that following immunopurification only a small proportion of the CLM PtdIns 4-kinase activity is associated with caveolae. In addition, using both CLMs and purified caveolae, we show first that endogenous PtdIns in these membrane subdomains is a substrate for a co-localized PtdIns 4-kinase and second that this activity is enhanced by the addition of PITP.

EXPERIMENTAL PROCEDURES

Materials—[3H]Inositol with PT6–271 stabilizer (17.1 Ci/mmol), [γ-32P]ATP (4500–6000 Ci/mmol), and the ECL Western blotting detection system were purchased from Amersham Pharmacia Biotech. Monoclonal anti-caveolin IgG (mAb C060) and anti-caveolin polyclonal anti-serum were obtained from Transduction Laboratories. Cell culture reagents were from Life Technologies, Inc. Prestained molecular weight markers and protein assay reagents were purchased from Bio-Rad. Protease inhibitor mixture tablets (COMPLETE) were from Boehringer Mannheim. ENHANCE™ spray was purchased from DuPont. OsO4 and pelleted by centrifugation at 13,000 g for 30 min at 4 °C. Immune complexes were collected by magnetic separation and washed four times in 20 mM Tris-HCl, pH 7.4.

Analysis of [3H]inositol-labeled Phosphoinositides—A431 cells were cultured in inositol-free medium containing 1% fetal calf serum in the presence of 2 μCi/ml [3H]inositol for 48 h. Cell monolayers were then washed twice in ice-cold phosphate-buffered saline and the CLM fraction was isolated. All steps were carried out on ice. The carbonate buffer used in these experiments contained EGTA (10 mM) to inhibit phosphatase activity, and it was found that more than 90% of the [3H]inositol-labeled phosphoinositides in the cell lysate were recovered in the fractions following density gradient centrifugation. Samples from gradient fractions and immunoprecipitates on Dynabeads were extracted with chloroform/methanol:1 M HCl (60:36:4). Samples were vortexed and centrifuged for 10 s in a microcentrifuge at 10,000 rpm. Organic phases were collected and re-extracted twice with methanol:1 M HCl (1:1). Samples were vortexed and centrifuged as before and the organic phase from each tube collected. Lipids were resolved by TLC on Silica-60 plates (Merck) which had been pretreated with 1% potassium oxalate, 2 mm EDTA in 50% methanol and developed using an acid solvent system composed of 200 mM Tris-HCl, 40 mM MgCl2,1 mM EGTA, 0.6% Triton x-100, 200 μCi/ml [γ-32P]ATP. Samples were incubated at 37 °C for 30 min. Reactions were stopped and the samples extracted and separated by TLC as described for the analysis of [3H]inositol-labeled phosphoinositides. The amount of radioactivity associated with each spot was determined by counting scintillation fluid using the ECL system.

A similar protocol was employed for assaying endogenous PtdIns kinase activity, with the exception that the assay buffer did not include exogenous PtdIns or detergent.

Note that the density gradient buffers employed in this study were found not to interfere with PtdIns4P in gradient activity.

Electron Microscopy—Electron microscopic analysis of CLM fractions and intact immunoprecipitated caveolae was performed essentially as described by Stan et al. (30). CLMs were fixed in suspension with 1% OsO4 and pelleted by centrifugation at 13,000 × g for 30 min in a microcentrifuge. The pellet was then stained with 2% uranyl acetate. The caveolae immunolabeled on collagen Dynabeads were resuspended in 100 mM cacodylate buffer, pH 7.4, and fixed with 3% freshly prepared formaldehyde and 1.5% glutaraldehyde. The samples were treated with 1% OsO4, following by staining in 2% uranyl acetate prior to subsequent processing for electron microscopy using a Jeol 1010 electron microscope operating at 80 kV as described previously (34).

RESULTS

Isolation of CLM Domains—CLM domains were isolated according to the detergent-free method of Song et al. (32). This method was precleared with dynal 280 beads coated with sheep anti-caveolin IgG. The cleared sample was incubated with anti-caveolin polyclonal antiserum for 1 h at 4 °C, followed by the addition of Dynabeads for 30 min at 4 °C. Immune complexes were collected by magnetic separation and washed four times in 20 mM Tris-HCl, pH 7.4. 2

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method has been used previously to demonstrate the interaction of p21ras with caveolin (32) and to confirm the localization of the novel protein flotillin within caveolae (35). In addition, this method avoids the use of Triton X-100, which has been shown to extract acylated proteins (32, 36) and phospholipids (21) from CLM. Caveolin, a 21-kDa integral membrane marker for caveolae, was reproducibly enriched in fraction 5 following density gradient centrifugation of A431 cell homogenates solubilized in sodium carbonate buffer (Fig. 1A). The CLM fraction was visible as a light-scattering band at the interface of the 5 and 35% sucrose layers and contained within fraction 5. In common with the findings of other groups, this fraction was separated from the bulk of the cellular protein content, which remained in the 45% sucrose layer at the bottom of the ultracentrifuge tube. The CLM fraction was depleted in 5'-nucleotidase activity, a marker for the plasma membrane and NADPH-cytochrome c reductase activity, which is a marker for the endoplasmic reticulum (Fig. 1B). These data are consistent with previous reports demonstrating that CLMs prepared in this way contain a small fraction of the total cellular protein and are separate from other cellular membranes (32, 37).

Analysis of [3H]Phosphoinositide Distribution—The distribution of inositol phospholipids was assessed by density gradient centrifugation of an equilibrium [3H]inositol-labeled cell extract followed by TLC to identify the radiolabeled lipids in each gradient fraction. It was found that levels of [3H]PtdIns and [3H]PtdInsP were greatest in fractions 4–6, which corresponded to the CLM fraction (Fig. 2). This region of the gradient contained 68.3 ± 14.6% (n = 3) of the total radiolabeled PtdIns, 61.9 ± 14% (n = 3) of the PtdIns4P, and 61 ± 11.3% (n = 3) of the cellular PtdIns(4,5)P2 pool. These data are in concordance with recent studies (18, 28), demonstrating that PtdIns, PtdIns4P, and PtdIns(4,5)P2 are present in CLM fractions, but the proportion of each phosphoinositide within CLM is reproducibly greater using the detergent-free conditions employed in this study. Liu et al. (21) also found that Triton X-100 was able to reduce the amount of PtdIns in CLM purified using detergent-free buffers. These results imply that phosphoinositides are highly compartmentalized within CLM domains. Furthermore, these data indicate that a mechanism exists whereby polyphosphoinositides are (a) synthesized elsewhere in the cell and then transported to CLM domains or (b) the enzymes responsible for inositol phospholipid synthesis are concentrated within CLM domains. The latter possibility was investigated by examining the distribution of PtdIns 4-kinase activity in the density gradient.
PtdIns 4-Kinase Distribution—PtdIns 4-kinase activity was found to be highly concentrated within CLM-containing fractions (Fig. 3A). It was found that 60.1 ± 6.2% (n = 3) of the total PtdIns 4-kinase activity was present at the 5–35% sucrose interface. Note that these assays were carried out in the presence of a concentration of Triton X-100 that inhibits PtdIns 3-kinase activity.

The co-localization of caveolin and PtdIns 4-kinase activity in a light density fraction was confirmed using a non-carbonate, detergent-free method for preparing CLMs (23). Using this technique, caveolin (Fig. 3B) and PtdIns 4-kinase (Fig. 3C) had very similar distributions.

Characterization of PtdIns 4-Kinase Activity in the CLM Fraction—Adenosine sensitivity has been established as a criterion that can differentiate between type II and type III PtdIns 4-kinase activities (38). Type II PtdIns 4-kinase activity is characteristically sensitive to adenosine inhibition in the micromolar range, whereas type III PtdIns 4-kinase activity is relatively insensitive to adenosine inhibition. It was found that the PtdIns 4-kinase activity associated with the CLM fraction was inhibited by adenosine with a $K_i$ of 107 μM (n = 3) and a Hill slope of 1 (data not shown). This result suggested that the PtdIns 4-kinase activity under investigation was a type II isozyme. These data are in concordance with other studies that have shown that the membrane-associated PtdIns 4-kinase activity in A431 cells is predominately a type II activity (39). Addition of EGF (100 nM) for 2 min to the CLM fraction did not induce any significant increase in PtdIns4P synthesis. However, addition of recombinant PITPa (1 nM) resulted in a 40–60% increase in PtdIns 4-kinase activity in the CLM fraction (data not shown).

Immunopurification of Caveolae from the CLM Fraction—To immunopurify caveolae from the CLM fraction, we carried out an immunopurification procedure based on that described by Stan et al. (30), with the exception that our initial CLM preparation was produced using the sodium carbonate-based fractionation method. This method utilizes antibody-coated magnetic beads to isolate caveolae from CLM preparations. Under these conditions, caveolin was immunoprecipitated from CLMs (Fig. 4A), whereas no caveolin was immunoprecipitated by the magnetic Dynabeads alone, indicating the specificity of the immunoprecipitation reaction (Fig. 4A). Furthermore, immunoblotting with an anti-caveolin monoclonal antibody revealed that 70–100% of the caveolin was cleared from the CLM fraction using the polyclonal anti-caveolin antiserum. The antiserum used in these experiments recognized the intracellular amino terminus...
lipids were extracted from anti-caveolin and control immunoprecipitates. Neither \([3H]\)PtdIns4P nor \([3H]\)PtdIns(4,5)P2 were detected by TLC showing that PtdIns 4-kinase co-immunoprecipitates with caveolin but not with Dynabeads alone. No caveolin associates with the Dynabeads alone (anti-caveolin lanes). No caveolin was specifically co-immunoprecipitated with PtdIns4P by immunoblot showing that caveolin is immunoprecipitated from a CLM sample by an anti-caveolin antiserum (+ anti-caveolin lanes). No caveolin was found to co-immunoprecipitate \([3H]\)PtdIns4P but not with Dynabeads alone. C, assay of PtdIns4P synthesis against endogenous PtdIns present in the anti-caveolin co-immunoprecipitate. TLC showing that PtdIns 4-kinase co-immunoprecipitates with caveolin but not with Dynabeads alone.

Electron microscopy was employed to further characterize both the putative caveolae immunoisolated on Dynabeads and the CLM fraction. The CLM fraction was found to be composed of a heterogeneous mix of vesicles (Fig. 4A) composed of large size vesicles > 100 nm in diameter (22% of vesicles in CLM), interspersed with smaller electron lucent vesicles 50–100 nm of vesicles in diameter (33% of CLM vesicles) and a population of more electron-dense 50–100 nm vesicles (45% of CLM vesicles) wide. In contrast, the vesicle population isolated by immunoprecipitation with a caveolin antiserum on Dynabeads was much more homogeneous in size (Fig. 4B and C). The vesicles isolated by the anti-caveolin antiserum were electron lucent and 50–100 nm in diameter, which is similar to the size range of caveolae identified in situ (19, 40, 41). These results show that the vesicles isolated by an anti-caveolin antiserum resemble caveolae in size and buoyant density.

Caveolar PtdIns4P Synthesis—When caveolae were immunoprecipitated from \([3H]\)inositol-labeled CLMs, \([3H]\)PtdIns was found to co-immunoprecipitate (Fig. 5, A and B). The amount of \([3H]\)PtdIns that was specifically co-immunoprecipitated was not large enough to be reliably quantitated (<1% of the total cellular PtdIns), thus demonstrating that only a small proportion of the CLM-associated PtdIns was actually located in caveolae. Neither \([3H]\)PtdIns4P nor \([3H]\)PtdIns(4,5)P2 were detectable in the caveolin immunoprecipitate, indicating that the bulk of the cellular PtdIns4P and PtdIns(4,5)P2 are located in low buoyant density membranes that are distinct from caveolae.

DISCUSSION

Combining cell fractionation with immunoprecipitation revealed that PtdIns4P synthesis occurs in a CLM preparation that exhibits many of the characteristics that have previously been attributed to caveolae. In particular its low buoyant density, its enrichment in the caveolar marker protein caveolin, and the fact that it contains only a minute fraction of the total cellular protein all point toward the CLM fraction being analogous to purified caveolae. However, analysis of CLMs by electron microscopy revealed that this fraction is heterogeneous and is composed of a mixture of vesicles of different sizes. In particular the CLM preparation contained many vesicles with diameters ranging from 150 to 300 nm, which is greater than the expected 50–90 nm size of intact caveolae (19, 30, 40, 43). It is possible that these large clear vesicles may correspond to low density raft-type regions of the plasma membrane that pinch off during sonication to form vesicular structures. This contrasts with the preparation obtained by immunodepletion of caveolin-containing vesicles from the CLM. The vesicles that are specifically immunoprecipitated from the CLM fraction by an anti-caveolin antiserum are far more homogeneous in size, indicating that the antiserum selects for 50–100-nm diameter low density vesicles. Importantly, the vesicles that were isolated on magnetic beads by the caveolin antiserum are of a size range close to the observed dimensions of caveolae from studies with whole cells. We are, however, cautious in stating that we have succeeded in isolating intact, pure caveolae as we cannot rule out the possibility that small areas of the plasma mem-

![Figure 6: PITP stimulates endogenous PtdIns4P synthesis in immunoprecipitated caveolae. Anti-caveolin immunoprecipitates were prepared from A431 cells, and endogenous PtdIns 4-kinase activity was assayed under a variety of conditions. Phospholipids were then extracted and PtdIns4P detected by TLC. EGF (100 nm) stimulations were for 5 min. The concentration of PITP employed was (25 μg/ml).](http://www.jbc.org/content/171/11/19163/F6.large.jpg)
brane that are contiguous with caveolae may pinch off during the nonspecific process of sonication to form vesicles that contain both caveolae and non-caveolar membrane (for example see Fig. 4D). Notwithstanding such provisos, our characterization of the CLM and immunoesolated caveolae indicates that density gradient ultracentrifugation of sonicated membranes does not result in pure caveolae but in a heterogeneous mixture of light membrane vesicles. Conversely, it is clear that immunoprecipitation of caveolin-containing vesicles results in a much more homogeneous preparation that exhibits many of the characteristics expected of purified caveolae (19, 30, 40, 43). These results may have implications for other studies that have implied caveolar localization of proteins solely on the basis of their co-localization with caveolin in the CLM fraction.

Our main purpose in preparing caveolae and CLMs was to investigate the hypothesis that caveolae may be important domains for the compartmentation of polyphosphoinositide metabolism and PtdIns-dependent signaling pathways. We have demonstrated that a substantial proportion of the cellular PtdIns 4-kinase activity is localized to a non-caveolar membrane fraction that resembles, at least in buoyant density, light membrane rafts, which are likely to be analogous to DIGs (40, 44, 45). The identity of this low density receptor-rich compartment is not known, but it may be analogous to similar ill-defined plasma membrane domains distinct from caveolae that have been identified in lymphocytes (45), the epithelial Caco-2 cell line (46), neuroblastomas (47), and rat lung microvasculature (30). The low buoyant density of these membrane microdomains is thought to derive from their enrichment in cholesterol and glycosylphospholipids. There is some evidence to indicate that DIGs are contiguous with caveolae in the plasma membrane (40) and that in the presence of detergent DIGs and caveolae can fuse to form low density vesicles that render their separation purely on the basis of density gradient centrifugation difficult (40). The CLM fraction was found to contain many large vesicles, possibly derived from the plasma membrane, with diameters in the range of 100–350 nm, which were not immunoprecipitated with an anti-caveolin antiserum and may be derived from non-caveolar rafts.

Significant proportions of the cellular PtdIns 4-kinase activity, PtdIns, PtdIns(4,5)P_2, and PtdIns(4,5)P_2 were present in the CLM fraction, but only a small fraction of the total cellular pool of PtdIns 4-kinase activity and its cognate substrate, PtdIns, were present in immunoesolated caveolae-like structures. Hence, the current study provides evidence that the localization of PtdInsP in CLM fractions results from co-localization of the enzyme responsible for its synthesis. These findings are significant, because they may indicate that receptor-activated phosphoinositide-hydrolysis previously reported in CLM (18) and phosphoinositide resupply may be coordinated within a discrete compartment of the plasma membrane. Furthermore, given the numerous reports of transmembrane receptors being present within caveolin-rich DIG preparations (21–23), the results presented here substantiate the idea that such receptors may be able to access discrete pools of phospholipids and that only a proportion of the total cellular phosphoinositide pool is immediately accessible to receptors (48). Such structural compartmentation could facilitate more efficient signaling than if the molecules were randomly distributed throughout the plasma membrane.

Studies from this group and others have led to the suggestion that PITP may be important for the continual supply of PtdIns to PtdIns 4-kinase (17, 49). A role for PITP in phosphoinositide metabolism is likely on the grounds that the endoplasmic reticulum is believed to be the primary site of PtdIns formation within the cell, necessitating that PtdIns be transported to the plasma membrane and at some stage undergo phosphorylation to form PtdInsP and PtdIns(4,5)P_2. It has also been shown that in permeabilized cell preparations depleted of PITP, there is a requirement for PITP to be added back to maintain agonist-stimulated PtdIns(4,5)P_2 hydrolysis (16, 50). In concordance with these ideas we found that the CLM-associated PtdIns 4-kinase activity could be enhanced by the addition of exogenous PITP. It is therefore tempting to speculate that CLMs may represent a site for PITP-dependent supply of PtdIns to the plasma membrane-associated PtdIns4P synthesis machinery.

Whereas the CLM fraction is shown to contain impure caveolae, the immunoprecipitated vesicles that we isolate from CLMs fulfill certain criteria, which indicate that they are derived from plasma membrane caveolae. In particular, the immunoesolated vesicles are of low density, of a size analogous to caveolae in intact cells (40, 41, 51, 52), and can be purified using an anti-caveolin antiserum. Significantly, specifically co-immunoprecipitated PtdIns 4-kinase activity could phosphorylate endogenous PtdIns present within the anti-caveolin immunocomplex. Furthermore, this activity was stimulated by physiologically relevant concentrations of PITP, thereby indicating that PITP can supply PtdIns directly to caveolae. These observations indicate that PtdIns and PtdIns 4-kinase activity are tightly associated with caveolae in A431 cells.

An important consideration is the observation that PtdIns4P generation in our caveolae preparation accounts only for a small proportion of the total cellular PtdIns4P synthesis. These results suggest that the agonist-sensitive pool of PtdIns resides elsewhere in the cell, most likely in the low density membranes that we found to contain the bulk of the cellular PtdIns, PtdIns4P, PtdIns(4,5)P_2, and EGF receptors. Previous studies have demonstrated that stimulation of PIC-linked receptors results in substantial turnover of polyphosphoinositides (11, 16, 49), therefore it is unlikely that the level of PtdIns4P resynthesis in caveola-like domains would contribute significantly to such an effect. These observations argue against a major role in signal transduction for the pool of PtdIns and PtdIns 4-kinase activity associated with the caveolar fraction.

Caveolar PtdIns4P production may be required for the internalization (52–54) and exocytosis (55) roles described for caveolin-rich vesicles derived from the plasma membrane and the trans-Golgi network, respectively. In line with this view, there are precedents for a functional relationship between polyphosphoinositide synthesis and vesicular trafficking (reviewed in Ref. 56). In particular, there is evidence that PITP-dependent polyphosphoinositide production is an essential element in secretory vesicle function in neuroendocrine and myeloid cells (57–59).

In conclusion, our data indicate that caution needs to be exercised in implying caveolar compartmentation of signaling molecules solely on the basis of co-localization in caveolin-rich membranes of low buoyant density. Indeed, we demonstrate that the bulk of the cellular PtdIns pool and PtdIns4P synthesizing machinery co-fractionates with low density membranes that can be distinguished from caveolae on the basis of immunoprecipitation. Further work will be aimed at characterizing the low density membrane rafts and to analyze their contribution to the organization of PIC-dependent signaling pathways.

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