Organized Endothelial Cell Surface Signal Transduction in Caveolae Distinct from Glycosylphosphatidylinositol-anchored Protein Microdomains*

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Regulated signal transduction in discrete microdomains of the cell surface is an attractive hypothesis for achieving spatial and temporal specificity in signaling. A procedure for purifying caveolae separately from other similarly buoyant microdomains including those rich in glycosylphosphatidylinositol-anchored proteins has been developed (Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995) Science 268, 1435–1439) and used here to show that caveolae contain many signaling molecules including select kinases (platelet-derived growth factor (PDGF) receptors, protein kinase C, phosphatidylinositol 3-kinase, and Src-like kinases), phospholipase C, sphingomyelin, and even phosphoinositides. More importantly, two different techniques reveal that caveolae function as signal transducing subcompartments of the plasma membrane. PDGF rapidly induces phosphorylation of endothelial cell plasmalemmal proteins residing in caveolae as detected by membrane subtraction and confocal immunofluorescence microscopy. This PDGF signaling cascade is halted when the caveolar compartment is disassembled by filipin. Finally, in vitro kinase assays show that caveolae contain most of the intrinsic tyrosine kinase activity of the plasma membrane. As signal transducing organelles, caveolae organize a distinct set of signaling molecules to permit direct regionalized signal transduction within their boundaries.

A fundamental question in signal transduction is how particular substrates are rapidly and specifically phosphorylated by select protein kinases. Because various signaling molecules can rather promiscuously activate a diverse group of effectors in different signaling pathways, a developing theme in signal transduction has been the importance of the intracellular localization of signaling molecules, especially protein kinases, through regulated anchoring to membranes (1). Restricted localization, for instance to the plasma membrane, of the kinases and substrates to their sites of action provides at least some of the specificity and efficiency in the effects mediated by each kinase. By extension, further compartmentalization of signaling mediators into specialized microdomains, for instance at the cell surface, may be required to direct a coordinated cascade that effectively creates a specific signal and effect on the cell. Here, we begin to investigate this hypothesis by examining cell surface signaling in endothelium and its caveolae.

Caveolae are specialized microdomains that appear as flask-shaped invaginations on the surface of many cells. Based primarily on morphological studies providing evidence for agonist-induced clustering in caveolae of G-protein coupled receptors (β-adrenergic and muscarinic acetylcholine receptors), Strosberg (2) hypothesized 6 years ago that caveolae may participate in cell surface signaling. Caveolae may contain the necessary molecular machinery for mediating compartmentalized signaling. They are reported to be resistant to Triton X-100 solubilization and can be found in Triton-insoluble membranes (TIM) (3–5). TIM isolated from MDCK cells contain large amounts of sphingomyelin, glycosphingolipids and cholesterol (6). TIM may also contain phosphoinositides, as reported by one group (7) but not another (6). Various TIM preparations (3, 4, 6, 8, 9) have been shown to contain molecules implicated in cell surface signaling including glycosylphosphatidylinositol (GPI)-anchored proteins, GTP-binding proteins, Src-like nonreceptor tyrosine kinases (NRTK), protein kinase C (PKC), and caveolin/VIP-21. Although Triton-insolubility of a membrane protein has been considered a standard hallmark of cytoskeletal association over 2 decades (10, 11), some investigators have chosen to consider TIM as equivalent to purified caveolae, leading to their proposal that caveolae function in cell surface signaling (4, 12, 13).

More recent work brings this presumed equivalence into serious doubt. Careful examination of the membrane topology and subcellular localization of caveolin/VIP-21, GPI-anchored proteins, and glycosphingolipids reveals that they are located both in the trans-Golgi network as well as in plasmalemmal microdomains (6, 14, 15), indicating that at least two different domains can contribute to the caveolin-containing TIM isolated from whole cells or tissues. Even when starting with highly purified plasma membranes, at least two TIM microdomains, namely caveolae and GPI-anchored protein microdomains, exist.
ist distinctly and have been purified separately (15). Caveolae not concentrated in GPI-anchored proteins have been purified both in the presence and absence of Triton X-100; they are highly enriched in all four caveolar markers previously identified by electron microscopy including caveolin, Ca$$^{2+}$$ ATPase, inositol triphosphate receptors, and the cholaer toxin-binding glycolipid GM$_1$ (15, 16). Colloidal gold localization by electron microscopy confirms that the TIM consist of many large vesicles (>200 nm) rich in GPI-anchored proteins along with smaller caveolar vesicles (<100 nm) lacking them (15). Moreover, caveolae need not be present for TIM isolation; TIM from cells without caveolae consist of many large vesicles (>200 nm) containing multiple GPI-anchored proteins and NRTK but not caveolin (17, 18). TIM isolated from cell membranes with or without caveolae have very similar buoyant densities (15, 18), thereby explaining the observed co-isolation of caveolae with GPI-anchored protein microdomains (15). Interestingly, lipid anchoring of the GPI-linked proteins and the dual acylated NRTK appear in both cases to be critical for their existence in TIM (19, 20). Finally, it has become apparent that antibodies previously used to immunolocalize GPI-anchored proteins can actually cross-link them and artifactualy induce sequestration into clusters associated with caveolae (21). Antibody sequestration of GPI-anchored proteins can induce cell surface signaling in cells without apparent caveolae leading to cell activation apparently via NRTK located in the same microdomains (22). Recently, another procedure for isolating caveolae has been developed using sonication in the absence of detergents to prepare vesicles that were presented as being quite similar to TIM and enriched not only in caveolin but also unfortunately GPI-anchored proteins (23). PDGF receptors and other signaling molecules are also found in these later vesicles (23, 24).

With this current knowledge, it is quite unclear where various signaling mediators reside on the cell surface and what essential role, if any, caveolae really play in signaling. Do all lipid-anchored proteins such as the NRTK partition on the cell surface similarly to the GPI-anchored proteins? Or are NRTK unlike GPI-anchored proteins and reside concentrated in caveolae? What about lipid signaling molecules found enriched in the TIM? Do they reside in caveolae? And finally, even if many of these molecules reside in caveolae, can it be demonstrated more directly that distinct signal transduction really can occur in caveolae? Here, we will try to address these questions by utilizing both dual immunofluorescence confocal microscopy and a very selective procedure for purifying caveolae separately from GPI-anchored protein domains in order to define not only which, if any, signaling molecules truly reside in caveolae but also regionalized signal transduction possibly related to the presence of caveolae.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents and other supplies were obtained from the following sources: PDGF-BB (recombinant human PDGF) from R&D Systems (Minneapolis, MN); VEGF (human) from Pepro Tech Inc. (Rocky Hill, NJ); endothelin-1 from Sigma; protein A-Sepharose from Pharma- cia Biotech Inc.; [3H]choline chloride (80 Ci/mmol) from DuPont NEN; [3H]inositol (80 Ci/mmol), donkey anti-rabbit IgG conjugated with HRP, sheep anti-mouse IgG conjugated with HRP and the enhanced chemiluminescence (ECL) detection kit from Amersham; and the bicine-honin acid (BCA) protein assay kit from Pierce.

Monoclonal antibodies to the following antigens were purchased from the following vendors: caveolin (also polyclonal), PDGF β-receptor, phospho-tyrosine, PKCα, PKCβ, Fyn, Lck, and Lyn from Transduction Laboratories (Lexington, KY); Src (monoclonal) from Oncogene Science Inc. (Uniondale, NY); and PLCγ (monoclonal) from Upstate Biotechnology Inc. (Lake Placid, NY). Polyclonal antibody to 85-kDa subunit of PI 3-kinase was a kind gift from Dr. Lewis Cantley (Beth Israel Hospital, Boston, MA), polyclonal antibody to c-Yes was a kind gift from Dr. Marius Sudol (Rockefeller Institute, New York, NY). All other reagents and supplies were obtained as in our past work (16, 25, 26).

**Cell Culture**—Rat lung microvascular endothelial cells (RLMVEC) (a kind gift from Dr. Karen Guice, Duke University, Durham, NC) were grown as described originally (27). MDCK cells were grown in DMEM supplement with 10% fetal bovine serum as per ATCC instructions.

**Purification of Caveolae**—As described previously (15), caveolae were purified directly from lung microvascular endothelial cells isolated from rat lungs using an in situ silica-coating procedure. Briefly, the rat lung vasculature was perfused with a colloidal silica solution to coat selectively the luminal surface of the endothelium in situ and allow purification of the silica-coated endothelial cell plasma membrane (P) from the tissue homogenate (H) by centrifugation. P was rich in endothelial markers with little, if any, contamination from other tissue components. The numerous caveolae attached to these membranes were sheared off by homogenization in the presence or absence of 1% Triton X-100 at 4°C and then purified by sucrose gradient flotation. A membrane band (V) was easily detected at a density of 15–20% sucrose and contained a homogeneous population of caveolae amply enriched in caveolar markers with no detectable contamination from other sources (15, 16). The reisolated pellet of silica-coated membrane was devoid of caveolae (16) and was named P-V. In some experiments, P-V along with 0.5-ml fractions were collected from the top of sucrose gradient for lipid and protein analysis.

**Isolation of Low Density TIM**—The low buoyant density TIM were obtained from MDCK cells as described previously (6). Similarly, TIM were obtained from rat lungs by treating rat lung tissue with 1% Triton as described originally (5) and in our past work (26).

**Lipid Analysis**—Spingomyelin and phosphoinositides were measured as described previously (28, 29). Briefly, perfused rat lungs were incubated at 37°C for 3 h in Kreb’s solution containing 5% CO$_2$/95% O$_2$, and either [3H]choline chloride (10 μCi/ml) or [3H]inositol (15 μCi/ml). The lungs were processed for isolation of caveolae or TIM (see above). Fractions (0.5 ml) were collected from the sucrose gradients and 100 μl of each fraction was placed into 10 × 130-mm glass tubes for lipid extraction using chloroform/methanol/hydrochloric acid (1 N) (100:100:1). The lower organic phase was collected and dried under N$_2$. For the detection of spingomyelin, the lipid extract was subjected to alkaline hydrolysis (1 N methanolic KOH at 37°C for 1 h) to remove glycerophospholipids (29). Spingomyelin or phosphoinositides were then resolved by thin-layer chromatography using chloroform/methanol/acetic acid/water at the ratio of 50:30:8:4 or 55:43:3:4, respectively. The lipids were identified by iodine vapor staining and quantified by liquid scintillation counter at an efficiency of 48%. Phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate were identified by TLC initially assessing the lipid migration of Triton-labeled standards compared to the migration of the lipids detected in the sample.

**Protein Analysis**—Proteins of cells and selected tissue fractions were assessed and quantified by Western analysis as in our past work (15, 16, 30). Briefly, they were solubilized with cold solubilization buffer (SB) containing 0.17 M Tris-HCl (pH 6.8), 3% (w/v) SDS, 1.2% (v/v) β-mercaptoethanol, 2 μl urea, and 3 mM EDTA in double-distilled water. After incubation in boiling water for 4 min, they were separated by SDS-PAGE (5–15% gels) and electrotransferred to nitrocellulose filters for immunoblotting using enhanced chemiluminescence autoradiography followed by densitometric quantification using ImageQuant (Molecular Dynamics). The protein concentration of the samples was measured using the BCA method with bovine serum albumin as a standard.

**Protein Phosphorylation Assay in Cultured Cells**—RLMVEC were incubated overnight in DMEM containing 1% fetal calf serum before washing and treatment with DMEM containing PDGF (100 ng/ml), endothelin-1 (100 nM), VEGF (10 ng/ml), or insulin (100 nM). The stimulation was terminated by adding SB in preparation for SDS-PAGE and Western analysis to determine tyrosine phosphorylation of proteins using a monoclonal antibody specific for phosphotyrosines.

**Immunoprecipitation**—RLMVEC stimulated with PDGF (100 ng/ml, 10 min) were solubilized in lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 mM Na$_2$VO$_4$, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) on ice for 30 min. The cell lysate was collected and spun at 5,000 rpm for 10 min in a microcentrifuge to pellet cellular debris. The supernatant was subjected to immunoprecipitation using an antibody specific for phosphotyrosines, which was prebound to protein A-Sepharose beads. After extensive washing of the lysis buffer, the immunoprecipitated proteins were solubilized in SB for Western analysis. To verify the specificity of the antibody for phosphotyrosines, a parallel set of samples was incubated in lysis buffer in the presence of 40 μM phenylphosphate.

**Protein Phosphorylation Assay in Rat Lungs in Situ**—The pulmonary artery was perfused with: (i) Ringer’s solution (5 mM HEPES (pH 7.4),
 measure the area occupied by phosphotyrosine, caveolin, and those vesicles expressing both fluorescent probes.

RESULTS

Here, we attempted to examine the potential role of caveolae in compartmentalized signal transduction by utilizing two independent techniques: confocal immunofluorescence microscopy and plasmalemmal subfractionation analysis. A very selective subfractionation procedure was used for purifying caveolae from luminal plasma membranes of endothelium isolated directly from tissue. As shown in our past work (15, 16), this procedure, unlike various other methods (3–5, 9, 23), avoided: (i) contamination from intracellular compartments by starting with highly purified plasma membranes, (ii) potential artifacts induced by isolating and growing cells in culture, and (iii) contamination from other detergent-resistant microdomains including those rich in cytoskeletal or GPI-anchored proteins. It did not require the use of detergents. Briefly, the plasma membrane was coated with polycationic colloidal silica particles so that a stable silica-coated membrane pellicle of increased density was formed that allowed rapid effective isolation to high purity by sedimentation. Equally important, the silica particles uniformly coated the extracellular side of the plasma membrane opposite to the attached caveolae so that other noninvaginated microdomains including those rich in GPI-anchored proteins were firmly adherent, thus preventing their detachment during the shear-mediated stripping and isolation of the caveolae (15, 16). This technique was used in situ by intravascular perfusion to allow the purification directly from tissue of endothelial cell caveolae to homogeneity with significant enrichment in known caveolar markers (15, 16). It clearly separates caveolae from GPI-anchored protein domains. Here, we first established that these purified caveolae were indeed rich in various key signaling molecules including, surprisingly, certain lipid and lipid-anchored molecules previously not detected in the TIM because of their detergent solubility. Next, we show that a specific ligand can initiate signaling selectively in caveolae as detected by protein-tyrosine phosphorylation regionalized in caveolae and that this signal cascade is inhibited when caveolae are disrupted.

Lipid Analysis—We first examined the effects of Triton X-100 on two kinds of lipid signaling molecules, sphingomyelin (SM) and phosphoinositides, in cell membranes in general. Rat lungs were prelabeled with either [3H]choline or [3H]inositol before homogenizing the rat lung tissue. The homogenates were treated with Triton X-100 and subjected to sucrose density centrifugation to isolate a visible band of low density TIM floating at 15–20% sucrose (fractions 4–6). Lipid analysis revealed that TIM contained abundant [3H]sphingomyelin, but very little, if any, [3H]phosphatidylinositol (PI). As shown in Fig. 1A, approximately 50% of sphingomyelin in rat lung homogenate was found in TIM with 30% in Triton-soluble phase (T) (T1–T5) and 20% in pellet. When normalized by protein concentration (to allow a meaningful comparison of approximately equal amounts of membrane), SM was enriched 20-fold in TIM over starting homogenate (H) (Fig. 1B). By comparison, little PI was found in TIM (<5% of H). The major portion of PI was found in the Triton-soluble phase, which accounted for 71% of H. Most recent with our past results (15), immunoblotting showed that caveolin was enriched 3–5-fold in the TIM relative to H and it was distributed similarly to SM in the fractions collected from the sucrose gradients (data not shown). Our results were consistent with a previous report that SM, but not PI (<5%), was concentrated in the TIM isolated from cultured MDCK cells (6). When we used MDCK cells (see “Experimental Procedures”), we found about 60% of SM was recovered in TIM, with 20% each in the Triton-soluble phase and the pellet (data
For PI >95% was in the Triton-soluble phase. These results confirm that TIM are rich in SM but not PI. In contrast to these findings, a recent study (7) shows PI in TIM at levels varying from about 10 to 25%. This discrepancy may be explained by methodological differences such as the use of a 40 to 5% sucrose step-gradient in Ref. 7, rather than a 40 to 5% continuous gradient, which may improve separation.

To investigate whether SM is truly concentrated in caveolae, the lipid components were analyzed in highly purified caveolae. Rat lungs were radiolabeled as above with tritiated precursors and then processed for purification of the silica-coated luminal endothelial cell plasma membranes (P) and then the caveolae (V). As reported previously (15, 16, 30), Western analysis showed that the purified caveolae were rich in caveolin in V over P (data not shown). Very little caveolin (about 3% of P) remained in P-V (the repelled silica-coated plasma membranes after removing the caveolae). Lipid analysis showed that the major peak signal of [3H]SM radioactivity was recovered in the purified caveolae fractions. Fig. 1C shows the distribution of SM in each fraction of sucrose gradient. The amount of SM in fractions 4–5 (V) and P-V was approximately equal for each at 35–40% of P. Fig. 1D shows the signal for SM normalized to the protein content of the collected fractions. The enrichment of SM in caveolae (V) was 10-fold relative to P and 23-fold over P-V. In contrast to SM, PI was found mostly in the Triton-soluble phase (T1–T5), which accounted for 86% of P. Very little PI was detected in caveolae (approximately 2% of P). These results indicate that SM was concentrated in the caveolae of the plasma membrane.

It is known that phospholipids including PI can be extracted from cell membranes by Triton X-100 (10, 36). To avoid this detergent effect, we also purified caveolae in the absence of Triton X-100. Fig. 1E shows that caveolae purified without detergent were indeed rich in PI. The lipid signal in caveolae fraction (V) was enriched by 5–7-fold over both P and P-V. Phosphatidylinositol 4-phosphate and phosphatidylinositol
4,5-bisphosphate were also found enriched in purified caveolae (data not shown) along with caveolin as reported previously (15). By comparison, there was very little PI found in Triton-free 40% sucrose phase (V') (approximately 2% of P). As expected, the signal for SM was enriched in V', which was 11-fold more than P-V (Fig. 1E).

To verify the Triton X-100 solubility of the caveolar PI, the radiolabeled purified caveolae were treated with cold Triton X-100 for 10 min before sucrose gradient centrifugation. Fig. 1F shows the analysis of PI in each fraction of the gradient. Clearly, the Triton X-100 extracted PI with the majority being recovered in detergent-soluble phases (T1–T5), which accounted for 65% of total radioactivity. Hence, our lipid analysis recovered in detergent-soluble phases (T1–T5), which accounted for 65% of total radioactivity. Hence, our lipid analysis showed data for subfractionation performed in the presence of Triton X-100, while the right panel is in the absence of the detergent.

NRKT Reside Concentrated in Caveolae—Many past studies report NRKT are enriched in TIM in some cases with GPI-anchored proteins (5, 8, 19). This association for both types of key lipid signaling molecules, SM and PI, can both reside concentrated in caveolae. This shows that two types of key lipid signaling molecules, SM and PI, can both reside concentrated in caveolae. This shows that two types of key lipid signaling molecules, SM and PI, can both reside concentrated in caveolae. This shows that two types of key lipid signaling molecules, SM and PI, can both reside concentrated in caveolae.

TABLE I
Enrichment and total recovery of signaling molecules detected in endothelial cell caveolae

| Proteins | Enrichment (V/P-V) | % of total in P1 |
|----------|--------------------|------------------|
| Caveolin | 20                 | 60.8             |
| PDGF-R   | 5.7                | 46.6             |
| PI3-kinase| 7.22               | 51.0             |
| PLCγ     | 9.15               | 51.6             |
| PKCα     | 16.5               | NA               |
| PKCδ     | 19                 | 35.7             |
| Yes      | 9.5                | NA               |
| Fyn      | 5.5                | 36.5             |
| Lck      | 8.48               | 34.2             |
| Lyn      | 7.0                | 40.4             |
| Src      | 5.38               | 23.5             |

*NA, not available.

When caveolae were purified in the absence of Triton X-100, all of these NRKT were found in the detergent-free purified caveolae (V') at levels much greater than the plasma membrane stripped of the caveolae (P-V') (Fig. 2). Table I shows the relative enrichment and percent distribution of these proteins in the plasma membranes and its subcomponents. On the endothelial cell surface, much of Lck, Fyn, Lyn, and Yes resided concentrated in caveolae. Src also was detected concentrated in caveolae but to a lesser extent. Src was present significantly in other noncaveolar plasmalemmal domains remaining in P-V'. Immunofluorescence microscopy confirmed the presence of Yes, Lck, Lyn, and Src in endothelial cells and their caveolae by showing significant co-localization of these NRKT with caveolin on the surface of cultured lung endothelial cells (data not shown). Unfortunately, the antibody to Fyn did not detect its antigens in this assay. Thus, it appears that unlike GPI-anchored proteins, various NRKT reside on the endothelial cell surface concentrated in caveolae.

Other Signaling Molecules Found in Caveolae—We also have screened the endothelial caveolae purified from rat lung for the presence of other key signaling molecules. So far, we have found PDGF β-receptor, PI 3-kinase, PLCγ, and PKC α and β isoforms amply expressed on the silica-coated luminal endothelial cell plasma membranes and in their caveolae (Fig. 3A). Enzyme-linked immunosorbent assays were performed on the membrane pellet after removing the caveolae. As shown clearly in the examples given in Fig. 3B, the remainder of the signal was distributed over the other 20 fractions at much smaller levels ranging from 0 to nearly 4% of the signal.

Ligand-induced Signal Transduction Localized within Caveolae—Although we have shown that various key signaling molecules were found in caveolae, the necessary functional evidence for signal transduction in caveolae has been lacking. It is possible that caveolae might not transduce signals directly.
across the membrane into the cell and alternatively could function as some sort of storage or recovery site for signaling molecules. Therefore, in order to investigate whether signal transduction could occur specifically and directly in caveolae, we tested a panel of ligands (endothelin-1, PDGF, insulin, or VEGF) known to activate various signaling pathways in endothelium. These ligands bind to their respective receptors to transduce a signal into the cell through processes involving at least in part some of the molecules that we have found in caveolae; note we have shown previously that G proteins can be found but not enriched in the purified endothelial caveolae; Ref. 30). Using RLMVEC, we immunodetected tyrosine phosphorylation of proteins in response to these ligands with the phosphorylation pattern differing significantly among these ligands (Fig. 4). PDGF clearly provided the strongest signal. This finding was confirmed by immunofluorescence microscopy. Extensive tyrosine phosphorylation was visualized on the surface of the PDGF-stimulated cells but not the unstimulated cells (Fig. 5). With PDGF stimulation, abundant small but strong punctate labeling was easily observed at the cell surface and leading edge of the cell, which is consistent with phosphorylation events not randomly distributed over the cell surface but rather localized in discrete small patches that could be vesicular in nature. The signal detected for the cells treated with the other ligands was much less (consistent with our immunoblots), and this signal was not detected in control experiments performed without primary antibody (data not shown). From these experiments, it was quite clear that PDGF caused the more extensive rapid tyrosine phosphorylation of proteins in this system, and therefore we focused for the remainder of this study on PDGF.

Before trying to localize the cell surface signaling, we first further characterized the PDGF-induced protein phosphorylation. Fig. 6 shows that the PDGF-induced tyrosine phosphorylation was time- and concentration-dependent. It increased rather linearly to reach an apparent maximum at about 10–15 min and then decreased afterward (Fig. 6, A and B). PDGF-induced protein phosphorylation was easily detectable at 10 ng/ml, while a PDGF concentration of 100 ng/ml seemed to give a maximal response (Fig. 6, C and D). Treatment of RLMVEC with PDGF phosphorylated several proteins with apparent molecular masses of 180–200, 120, 85, 60, and 40 kDa (Fig. 6, A and F). Immunoprecipitation with antibody to phosphotyrosine followed by immunoblotting revealed that PDGF β-receptor and PI 3-kinase (85-kDa subunit) were among the phosphorylated proteins (data not shown). Pretreatment of these cells with a specific tyrosine kinase inhibitor, genistein, or less-specific inhibitor, staurosporine, substantially inhibited PDGF-induced protein-tyrosine phosphorylation, suggesting that protein phosphorylation observed resulted from a tyrosine kinase activity induced by PDGF (Fig. 6E). Preincubation of the cells with staurosporine alone also reduced tyrosine phosphorylation signal, suggesting a basal tyrosine kinase activity in these cells.

Next, we utilized confocal immunofluorescence microscopy to ascertain whether PDGF caused signal transduction specifically within caveolae located on the surface of cultured endothelial cells. Fig. 7 shows that double immunostaining for phosphotyrosines and caveolin in the PDGF-stimulated RLMVEC revealed extensive co-localization at the cell surface. Tyrosine-phosphorylated proteins were detected in cell surface structures that also labeled with caveolin. Image analysis, in which two-dimensional pixel intensity histograms from both the phosphotyrosine and caveolin images were compared, allowed quantification of overlapping signals (see “Experimental Procedures”). Pixels with signal from phosphorytosine or caveolin were identified using high stringency criteria and converted into a binary section to produce area measurements of the regions occupied by phosphotyrosine, caveolin, or both signals. Fig. 7 shows a representative image. By quantifying the cell surface signal in 0.5-μm optical sections of 20 different cells in this detailed manner, we found an average of 82.4 ± 13.3% (with a median of 90) of the phosphotyrosine signal to be co-localized in caveolin-containing caveolae at the cell surface. Interestingly, only 59.8 ± 26.0% of the caveolae detected at the plasma membrane with the caveolin antibodies exhibited detectable tyrosine phosphorylation. This latter finding suggested the presence of significant caveolar subpopulations: one...
induced to signal and the other not induced by PDGF. It was interesting to note that, unlike the first colocalization category, a rather large variation was observed in the latter, which ranged from a low of 10% to high of 90% overlap of the caveolin-containing structures also exhibiting phosphotyrosine signal. Thus, the caveolar subpopulations probably are distributed in patches rather than uniformly over the cell surface. At least in cultured cells, regions with a high density of caveolae tend to be interspersed with long stretches of plasmalemma having few caveolae. These findings indicate that PDGF-induced signaling as detected by protein-tyrosine phosphorylation can indeed occur directly in caveolae.

Isolating and growing cells in culture might induce extensive phenotypic drift, easily detected at the morphological level, for instance, in endothelial cells by a significant reduction in the surface density of caveolae. Therefore, it was imperative that our in vitro findings be tested on endothelium in tissue in vivo. We examined possible signaling in endothelium under more native conditions by Western blotting using the phosphotyrosine-specific antibody to assess the tyrosine phosphorylation of endothelial plasmalemmal and caveolar proteins in response to PDGF administered in situ. We perfused rat lungs with and without PDGF and then performed our usual subfractionation procedure using the silica-coating method. Fig. 8A shows that under base-line conditions, little tyrosine phosphorylation was detected in the whole lung homogenates (H) or its subfractions (P, T, or P-V) with the possible exception of two proteins of about 50 and 52 kDa detected most clearly in the purified caveolae (consistent with their apparent significant enrichment in caveolae). Perfusion of PDGF dramatically increased the phosphorylation detected in all of the fractions. PDGF induced the tyrosine phosphorylation of multiple proteins easily detected as seven bands by Western analysis of P. There was a considerable increase in the detected signal in P over H as would be expected for a cell surface-mediated event. All seven phosphorylated plasma membrane proteins were found primarily in the purified caveolae (V), with little remaining behind in the plasma membrane stripped of caveolae (P-V). Very little, if any, signal was detected in either T or P-V. In addition to the seven bands, several phosphorylated proteins were detected in V but not in P, which is consistent with a significant overall enrichment of the proteins phosphorylated by PDGF. Finally, we were able to immunoprecipitate the phosphotyrosine-containing vesicles from V and find abundant caveolin in them, thereby providing further assurance that the caveolae did indeed contain the proteins phosphorylated by PDGF (Fig. 8B). It would appear that the signaling as detected by the PDGF-induced phosphorylation occurred selectively and directly in the caveolae. These observations were quite consistent with the confocal immunofluorescence image analysis. Both techniques demonstrated that PDGF initiated signaling events transduced specifically in caveolae.

**Purified Caveolae Rich in Tyrosine Kinase Activity**—To test further caveolar function in signaling and examined whether caveolae have endogenous kinase activity when separated from other plasma domains, the silica-coated rat lung endothelial plasma membranes were subfractionated and the basal tyrosine kinase activity of the important fractions was examined in vitro. Consistent with the observations described above, there was very little base-line tyrosine phosphorylation detected in the fractions in the absence of ATP (Fig. 9). By comparison, tyrosine phosphorylation was significantly increased in P in the presence of ATP, suggesting endothelial plasma membrane possesses intrinsic tyrosine kinase activity. Notably, most of the protein-tyrosine phosphorylation occurred in the caveolae, which was detected as a 30-fold enrichment of overall phosphorylation in V relative to P or P-V. Little to no tyrosine phosphorylation was detected in T. Several phosphorylated proteins with molecular masses of 180, 140, 36–40, and 18–20 kDa, which were not readily detected in P and P-V, were found in caveolae. Hence, these results indicate that tyrosine kinase(s) and their substrates are both quite concentrated in caveolae. In contrast to the PDGF-induced tyrosine phosphorylation, the intrinsic basal kinase(s) in this assay recognized a different subset of proteins (compare Figs. 8A and 9). The proteins phosphorylated with PDGF stimulation had molecular masses of 180–200, 120, 85, and 48–50 kDa, whereas proteins with molecular masses of 85–116, 48–50, 36–40, and 18–23 kDa were the preferred substrates for the intrinsic kinase(s). More specifically, caveolin was one of the major proteins being phosphorylated in caveolae by this in vitro kinase assay, whereas very little phosphorylation of caveolin was detected by in situ stimulation with PDGF.
Disassembly of Caveolae Prevents Propagation of PDGF-induced Signaling Cascade—If caveolae are cell surface microdomains concentrating protein kinases and their substrates and if this compartmentalization is important in signaling, then the loss of the caveolar subcomponent of the plasma membrane may prevent efficient access of kinases to their substrates and thereby abrogate or even interrupt the signaling cascade of the activated PDGF receptor. We have shown previously that filipin, which is a polyene antibiotic that binds and removes cholesterol from membrane, reversibly causes disassembly of caveolae resulting in dispersion of caveolar proteins over the endothelial cell surface (25). Using filipin concentrations causing more than a 75% decrease in the endothelial cell surface density of caveolae (25), we treated RLMVEC first with filipin before stimulation with PDGF. The effects of filipin first were examined by immunofluorescence microscopy. Filipin caused a striking change in the immunofluorescence staining pattern for protein-tyrosine phosphorylation induced by PDGF. The effects of filipin first were examined by immunofluorescence microscopy. Filipin caused a striking change in the immunofluorescence staining pattern for protein-tyrosine phosphorylation induced by PDGF. The starting cell lysate (lanes 1 and 2) and immunoprecipitated proteins (lanes 3–6) were subjected to SDS-PAGE and Western analysis with phosphotyrosine antibody. Arrows indicate proteins being phosphorylated by PDGF (see "Results").

In agreement with these findings, we also found by immunofluorescence that PDGF receptors clustered to give a punctate staining pattern on the cell surface, which was dispersed to a diffuse pattern with filipin treatment of the cells. Unfortunately, this antibody, like most antibodies to growth factors, was not monospecific. It recognized by Western analysis not only the 180-kDa PDGF receptor band but also other protein bands. Thus, definitive interpretation of the immunofluorescence microscopy results was not possible (data not shown).

Detection of tyrosine phosphorylation by Western analysis showed more directly that filipin substantially reduced the PDGF-stimulated tyrosine phosphorylation of proteins (Fig. 10, A and B). Little phosphorylation was detected with filipin pretreatment. Only the PDGF receptor appeared to be phosphorylated, which was also much less than the control. Both 2 and 5 ng/ml filipin were effective with the latter being a bit more inhibitory. When we used vanadate to stimulate tyrosine phosphorylation as a control, filipin did not reduce the phosphorylation (Fig. 10B). In fact, surprisingly there was a moderate increase, which might be caused by the redistribution of kinases, phosphatases, and their substrates. Although there is no indication that filipin is a general tyrosine kinase inhibitor, we still tested it along with staurosporine in our in vitro kinase assay using V. Tyrosine phosphorylation of proteins was not diminished by filipin but was almost eliminated by staurosporine (Fig. 10C). The disruption by filipin of the caveolae as an
organized subcompartment of the plasmalemma appeared to prevent the PDGF-induced signal from efficiently and rapidly propagating past the autophosphorylation of its receptor, to downstream targets necessary for the robust protein-tyrosine phosphorylation normally seen in the endothelial cells. Without proper subcompartmentalization, the usual PDGF-signaling cascade was interrupted.

**DISCUSSION**

**TIM, Caveolae, and Lipid-anchored Proteins**—Here, we focus on one specific type of TIM, namely the caveolae, which we have separated distinctly from a number of other possible Triton-insoluble microdomains including, but probably not limited to, those on the cell surface rich in GPI-anchored proteins or cytoskeletal elements along with those inside the cell such as the trans-Golgi exocytic vesicles rich in glycolipids, caveolin, and GPI-anchored proteins (15). Not surprisingly, these other noncaveolar domains can be found in cells apparently with few or no caveolae using the standard TIM isolation procedure (6, 17, 18). The caveolae purification procedure used here is the only one to date that inherently prevents co-isolation of other noncaveolar domains that are physically very similar to caveolae in their detergent resistance and buoyant densities (15). Thus, these two physical criteria when used for isolating caveolae (3, 4, 39) cannot inherently separate such similar domains. Another procedure for isolating caveolae has been proposed very recently, with similar results to the TIM, because again unfortunately only two nondiscriminating criteria are used for separation: buoyant density, coupled in this case with membrane disruption by sonication rather than detergent treatment (23).

It is the actual coating of the plasma membrane with the silica particles that provides two additional separation criteria to the purification procedures: (i) it allows isolation to high purity of the pertinent starting material, namely the plasma membranes, thereby avoiding contamination from other sources; and (ii) it stably attaches to the plasmalemma proper, thereby preventing excision of the flat noninvaginated domains, which can be detergent-resistant, are rich in GPI-anchored proteins or cytoskeletal elements, and are not by definition the invaginated caveolar microdomains (15–18).

A potential disadvantage of the silica-coating method for purifying caveolae includes the possibility that the mobile GPI-anchored proteins may move from the caveolae and bind to the silica particles. In a recent paper (40), we have shown that GPI-anchored proteins are not present in caveolae that bud from plasma membranes and instead remained behind with the plasmalemma proper. Interestingly, the budding of caveolin-rich vesicles lacking GPI-anchored proteins has been detected using not only the purified silica-coated plasma membranes but also plasma membranes not coated with silica and isolated on a Percoll gradient. Thus, it is not the silica-coating that specifically contributes to the lack of GPI-anchored proteins observed in our highly purified caveolae. Furthermore, immuno-electron microscopy shows that GPI-anchored proteins are not actually in the caveolae isolated using the Triton...
methodology (no silica present) but rather are located in the larger vesicles contaminating this preparation (15).

The silica-coating technique is quite effective in purifying caveolae in the absence of detergents (15, 16). Detergent-free purification can be quite important in light of the increasing number of caveolar molecules that, at least in part, can be extracted from the caveolae by Triton X-100 and therefore may in some cases be poorly detected or even absent in isolated TIM. If so, then at least some of the molecules solubilized into the detergent phase may be originally from the caveolae. Caveolin, which appears to polymerize around the bulb of the caveolae (41, 42), is quite resistant to detergent extraction at low temperatures, but that does not mean that all caveolar molecules need be the same, especially lipids and lipid-anchored proteins. We have found in this study that this is indeed true. Although sphingomyelin is quite resistant to Triton extraction and enriched in both TIM and purified caveolae, we have found that phosphoinositides, which reside concentrated in caveolae, are easily extracted from plasma membranes and the purified caveolae. To a lesser extent, the same is true for the Src-like kinases, which exhibit a wide range of sensitivity to Triton solubilization and yet they can be found in caveolae to varying degrees. On the cell surface, Yes, Lck, and, to a lesser extent, Fyn and Lyn seem to reside mostly in caveolae. Src is also found in caveolae but in addition appears to reside elsewhere in a detergent-resistant noncaveolar microdomain of the plasma membrane.

Some of the distributional diversity of the Src-like kinases in the plasma membrane may relate to differences in their lipid anchoring, especially dual acylation and direct interactions with other more firmly entrenched detergent-resistant caveolar proteins. It has been reported that G proteins, which also are dual acylated and present not only in TIM (3, 4) but also in purified caveolae (30), can interact with caveolin itself (43). Interestingly, G proteins can exist in TIM regardless of whether they are isolated from cells with or without caveolae (3, 4, 44). They can be immunoprecipitated with GPI-anchored proteins and most probably can also reside in the small GPI-anchored protein microdomains (44). These findings are consistent with the concept that the binding to caveolin and the local lipid milieu causing detergent resistance contribute independently to the preferential partitioning of these molecules into these distinct microdomains. Could it be that caveolin or even other detergent-resistant resident proteins of caveolae provide the anchoring necessary to keep some, but not other, lipid-anchored proteins selectively in caveolae over other microdomains when present? It will be interesting to determine why nature has created this distinction. Recently, eNOS, another dually acy-
Signaling in Caveolae

This section discusses the role of caveolae in signal transduction and the localization of signaling molecules within them. Caveolae are small invaginations of the plasma membrane that are rich in cholesterol and sphingomyelin. They function as signal transduction compartments and are involved in the regulation of cell growth and proliferation.

Caveolae are enriched in sphingomyelin, which activates a ceramide-activated protein kinase (31). This activates a ceramide-activated protein kinase (31). Our finding of enrichment of sphingomyelin in purified caveolae demonstrates the topology of this lipid signal molecule in plasma membranes, although its function there remains unclear. Recent studies using normal human fibroblasts show that interleukin-1β stimulates ceramide and diacylglycerol production primarily in sphingomyelin-rich TIM (47).

Phosphoinositides are essential signaling molecules mediating several receptor transduction pathways, including G-protein coupled receptor and tyrosine kinase receptor pathways (48). Ligand-activated receptors stimulate PLC to hydrolyze phosphatidylinositol 4,5-bisphosphate to generate two second messengers: diacylglycerol, which activates PKC, and inositol 1,4,5-trisphosphate (IP₃), which binds to an IP₃ receptor to mobilize stored Ca²⁺ and promote an influx of external Ca²⁺ (48, 49). Interestingly, caveolae purified from endothelium possess the IP₃-sensitive channel and Ca²⁺ ATPase (16), suggesting that caveolae play an important role in the regulation of intracellular calcium levels. Consistent with a role for caveolae in signaling, caveolae also are quite enriched in other signaling molecules found in the plasma membranes including PDGF β-receptor, PKC, PI 3-kinase, and PLCγ. Caveolae appear to be rich in the lipid precursors and proteins necessary for regionalized signal transduction for several different signaling pathways.

Caveolae as Signaling Subcompartments of the Plasma Membrane—The concept of signaling compartments within the cell has evolved into a current theme in signal transduction and is well supported by selective subcellular distribution of many signaling molecules (1). The observed promiscuity of kinases, especially in reconstituted assays, suggests that the intramolecular mechanisms for specificity are not absolute and that another level of organization may be required for more specific directed vential signaling. Compartmentalization creates appropriate access to the necessary substrates for initiation of the pertinent regionalized cascade reaction. Intracellular localization creating the suitable molecular proximity may be required to achieve fully the specificity necessary for balancing downstream pathway diversity with selectivity in effects and responses. Elements of various signaling pathways including G-proteins and many protein kinases and phosphatases have been shown to be compartmentalized intracellularly, for instance at the nucleus, plasma membrane, or Golgi (1, 50, 51).

In this study, we have extended this concept of signaling compartments by further dissecting the plasmalemmal compartment into subcompartments. Using both new subfractionation techniques and confocal immunofluorescence image analysis, we provide compelling direct evidence for signal transduction at the plasma membrane subcompartmentalized in caveolae. As discussed above, many key signaling molecules found at the cell surface actually appear to reside clustered in caveolae. Many other laboratories have also shown signaling molecules in various membrane subfractions such as TIM (3, 4, 6, 8, 9). Although these findings support the hypothesis that caveolae function as specific microdomains on cell surface in compartmentalized signal transduction, they do not directly demonstrate functionality. To test this hypothesis more directly, we choose to examine ligand-induced protein phosphorylation in endothelium found both in tissue in situ and grown in culture. PDGF induces protein-tyrosine phosphorylation on the luminal endothelial cell surface rather selectively, if not exclusively, in the purified caveolae. The PDGF receptor located in caveolae is quickly autophosphorylated by its ligand. The proteins, which are phosphorylated rapidly in response to PDGF, are also found in the caveolae. Many known targets of the PDGF receptor tyrosine kinase including PI 3-kinase, PLCγ, Src, Yes, and Fyn (37) are found in the purified caveolae. Our PDGF-related findings agree substantially with a very recent study (24) that showed PDGF receptors and associated signaling molecules in a caveolar preparation that, although isolated without detergent, was characterized by the investigators to be similar to TIM including, unfortunately, its enrichment in GPI-linked proteins (23). Thus, it appears that the PDGF signaling cascade does indeed reside in the caveolae and is not being detected in our preparations because of the presence of what may be associated but clearly distinct GPI-anchored proteins domains (15). Moreover, our immuno-affinity isolations provide direct evidence that the tyrosine-phosphorylated proteins are present in the same vesicles containing caveolin (Fig. 8B).

The localization of these signaling molecules within a small invaginated microdomain is likely to provide the proximity necessary for rapid, efficient, and specific propagation of the transduced kinase activity to immediate nearby substrates that appropriately promote downstream signaling events. Consistent with this concept, PDGF signaling was halted at the level of autophosphorylation of its receptor and was not capable of propagating the signal efficiently to the usual downstream targets when the caveolae with their organized subset of plasmalemmal signaling molecules were disrupted. Filipin treatment of the cells reversibly causes a very significant loss of caveolae at the endothelial cell surface and results in the dispersion of proteins normally found in caveolae (25). Without the properly organized caveolar subcompartment, the PDGF signaling cascade was interrupted. Finally, we have found that caveolae have both significant intrinsic tyrosine kinase activity and multiple different kinase substrates. Our in vitro kinase assay using purified plasma membranes and caveolae reveals that caveolae have intrinsic kinase activity so that other plasmalemmal components are not required for tyrosine phosphorylation. In fact, most of the intrinsic kinase activity of the plasmalemmal compartment is derived from the caveolar subcompartment. The proteins phosphorylated in vitro are different from those induced by PDGF, indicating that PDGF initiates a specific cascade by only phosphorylating a subset of potential substrates in caveolae. Thus, the evidence to date supports the existence of caveolae as organized subcompartments of the plasmalemma functioning in signal transduction.

Cells seeming to lack caveolin expression and apparent caveolae on their cell surface, such as lymphocytes, appear fully functional in responding to growth factors and in all aspects of cell growth and signaling. Interestingly, these cells have microdomains resistant to Triton X-100 solubilization.
that can be isolated similarly to caveolae as low density membranes in a sucrose gradient. These TIMs are rich in Src-like kinases and GPI-anchored proteins, which when cross-linked with antibodies cluster on the cell surface and induce signaling events (17, 18, 22). Thus, not surprisingly, it appears that caveolae are not the only type of plasmalemmal subcompartment organized to transduce specific signaling cascades. More work will be needed to provide the distinctions necessary to resolve why two distinct microdomains have evolved with what may be similar functions.

Relationship of Signaling to Caveolar Function in Transport—It is thought that caveolae may also function in transport via fluid-phase and receptor-mediated endocytosis and transcytosis (for review, see Ref. 52). The debate in this area has centered on whether caveolae are dynamic vesicular carriers capable of budding, docking, and fusion like other transport vesicles or rather permanent static structures of the plasma membrane. It could be argued that the latter is more apt to be the case at least for caveolae that function in signal transduction; however, transport of the caveolar signaling subcompartment into the cell may provide an important regulatory function that eliminates, augments, or otherwise changes signaling, possibly by targeting specific intracellular compartments. Recently, our laboratory has shown that caveolae have the molecular machinery necessary for transport via vesicular budding, docking, and fusion (26, 30). More importantly, they can bud directly from the plasma membrane via a fission process requiring GTP hydrolysis (40).

If signaling caveolae can be internalized, then caveolae may function not only in compartmentalized signal transduction but also in a broader sense as complete signaling processing centers. Maybe specific signaling events are required to initiate caveolae budding and transport. It will be important in the future to assess the possible interrelationship between signaling and transport by determining whether, and if so how, caveolae integrate signal transduction with their ability to function as dynamic carrier organelles. As signaling processing centers, caveolae organize signaling molecules to provide a topographic compartment at the cell surface that may determine substrate specificity and ultimately define the signaling pathway, message and even cellular response. In turn, caveolae may regulate cell surface signaling by escorting the signaling molecules into the cell to sustain, consume or otherwise change its effect.

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