Localization and Turnover of Phosphatidylinositol 4,5-Bisphosphate in Caveolin-enriched Membrane Domains*

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Linda J. Pike† and Laurieann Casey
From the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Caveolae are small, plasma membrane invaginations that have been implicated in cell signaling. In A431 cells, approximately half of the total cellular phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P$_2$) was found to be localized in low density, Triton-insoluble membrane domains enriched in caveolin. Treatment of cells with either epidermal growth factor or bradykinin for 5 min at 37°C resulted in approximately a 50% decrease in this caveolar PtdIns 4,5-P$_2$ with no change in the levels of plasma membrane PtdIns 4,5-P$_2$. These data suggest that the PtdIns 4,5-P$_2$ present in cells is largely compartmentalized and that the caveolar PtdIns 4,5-P$_2$ is subject to hydrolysis by hormone-stimulated phospholipase C. As growth factor receptors, seven transmembrane domain receptors, heterotrimeric G proteins, and the inositol trisphosphate receptor have all been shown to be enriched in caveolae, these findings suggest that both the generation and response to inositol trisphosphate is highly compartmentalized within the cell.

Caveolae are small, plasma membrane invaginations that are involved in apical protein sorting (1, 2) and the uptake of folates by potocytosis (3). In most cell types, they account for 1% or less of the total plasma membrane (4). Caveolin, a 21-kDa substrate for pp60$^{	ext{src}}$ (5), is localized almost exclusively to caveolae and probably represents a structural component of this plasma membrane domain (6–8). Caveolae are also enriched in glycosphingolipids and cholesterol, making these domains resistant to extraction in Triton X-100 (1). Caveolae are also enriched in glycosphingolipids and cholesterol, making these domains resistant to extraction in Triton X-100 (1).

Caveolae have been shown to contain a variety of molecules involved in cell signaling including low molecular weight and high molecular weight folates by potocytosis (3). In most cell types, they account for 1% or less of the total plasma membrane (4). Caveolin, a 21-kDa substrate for pp60$^{	ext{src}}$ (5), is localized almost exclusively to caveolae and probably represents a structural component of this plasma membrane domain (6–8). Caveolae are also enriched in glycosphingolipids and cholesterol, making these domains resistant to extraction in Triton X-100 (1).

Caveolae have been shown to contain a variety of molecules involved in cell signaling including low molecular weight and heterotrimeric G proteins (9, 10), Src family kinases (10), mitogen-activated protein kinase (10), the epidermal growth factor receptor (11, 12), and the platelet-derived growth factor receptor (13). These findings implicate caveolae in signal transduction and suggest that many of the molecular components for cell signaling are localized in this relatively small area of the plasma membrane.

We have recently shown that in Madin Darby canine kidney cells, a large proportion of the total cellular PtdIns 4,5-P$_2$ resides in detergent-insoluble lipid domains enriched in caveolin (14). Given this observation, the question arises as to whether the PtdIns 4,5-P$_2$ present in caveolae is subject to turnover in response to growth factors and hormones. We now report that, in A431 cells, at least 50% of the PtdIns 4,5-P$_2$ is present in caveolae, and this pool of phospholipid is reduced in cells treated with EGF or bradykinin. Polysphosphoinositides present in the plasma membrane fraction are not altered by hormone treatment. These findings suggest that PtdIns 4,5-P$_2$ is highly compartmentalized within cells and that caveolae are the primary site of agonist-stimulated PtdIns 4,5-P$_2$ turnover.

EXPERIMENTAL PROCEDURES

Materials—Anti-caveolin antibodies were from Transduction Laboratories (Lexington, KY). Anti-actin antibodies were from Chemicon (El Segundo, CA). The polyclonal anti-EGF receptor antibody, DB1, was described previously (15). myo$^3$HInositol and the Enhanced Chemiluminescence kit were from Amersham. EN$^3$HANCE was from DuPont NEN. EGF was prepared by the method of Savage and Cohen (16). All other chemicals were from Sigma.

Cell Culture—A431 cells were maintained in DMEM containing 7% newborn calf serum and 3% fetal calf serum.

Isolation of Caveolae—A431 cells were plated in 150-mm dishes, and, 24 h later, cultures were labeled with 50 μCi of myo$^3$HInositol in DMEM containing 10% dialyzed fetal calf serum. Cells were grown in labeling media for 48 h at which time the cultures were confluent. For hormone stimulation, EGF or bradykinin was added directly to the culture medium at final concentrations of 50 nM or 10 μM, respectively. Incubations were for 5 min at 37°C. Monolayers were washed once in ice-cold phosphate-buffered saline and scraped into 1 ml of lysis buffer containing 25 mM MES, pH 6.5, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 1 μg/ml leupeptin. Lysates were incubated for 10 min on ice with frequent agitation. One ml of lysate was mixed with 1 ml of 25 mM MES, pH 6.5, 150 mM NaCl, 1 mM EGTA containing 80% sucrose by passing the sample through a 22-gauge needle five times. Six ml of 25 mM MES, pH 6.5, 150 mM NaCl, 1 mM EGTA, 30% sucrose and, subsequently, 4 ml of 25 mM MES, pH 6.5, 150 mM NaCl, 1 mM EGTA, 5% sucrose were layered on top of the sample. The tubes were centrifuged for 3 h at 4°C in an SW41 rotor. Fractions of 1.2 ml were collected beginning from the top of the gradient. The pellet was resuspended in 1.2 ml of phosphate-buffered saline.

Analysis of $^3$HInositol-labeled Phosphoinositides and Inositol Phosphates—Aliquots (800 μl) of each fraction were removed, extracted with chloroform/methanol, and analyzed by thin layer chromatography on silica plates as described previously (14). Following autoradiography, bands corresponding to PtdIns, lypo-PtdIns, PtdInsP, and PtdIns 4,5-P$_2$ were identified by co-migration with standards, scraped from the plates, and counted for $^3$H.

For analysis of phosphoinositide turnover, A431 cells were plated in 6-well dishes and labeled for 48 h with myo$^3$HInositol as described above. Cultures were preincubated for 30 min at 37°C with 10 mM LiCl and subsequently incubated with vehicle, 50 mM EGF, or 10 μM bradykinin for the times indicated. $^3$HInositol phosphates were isolated on Dowex columns as described previously (17).

Detergent Extraction of Caveolae—A431 cells in D$_6$O plates were labeled for 2 days in myo$^3$HInositol to ~50% confluence. Cells were treated with 50 mM EGF or 10 μM bradykinin by direct addition of the hormone to the labeling medium. After the indicated period of time at 37°C, the plates were washed twice with ice-cold phosphate-buffered saline. Three ml of Triton extraction buffer (25 mM HEPES, pH 7.2, 250 mM NaCl, 2 mM MgCl$_2$, 2 mM MnCl$_2$, 1 mM CaCl$_2$, 1 mM EGTA) was added directly to the culture medium at final concentrations of 50 nM EGF or 10 μM bradykinin for the times indicated. 

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† To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 So. Euclid, Box 8231, St. Louis, MO 63110. Fax: 314-362-7183.
RESULTS

A431 cells were subjected to extraction in Triton X-100 followed by analysis of the lysates by sucrose density gradient centrifugation. Caveolae have been shown to be resistant to extraction in Triton X-100 and are separable from bulk cellular lipids and proteins by density gradient centrifugation. As shown in Fig. 1A, relatively little protein was found in the upper, low density fractions of the sucrose gradient. However, the vast majority of caveolin, the protein marker for caveolae, appeared in fraction 4 (Fig. 1B), a position corresponding to the interface between the 5% and 30% sucrose layers. A low and variable amount of caveolin was associated with the pellet fraction and is probably due to the association of caveolae with cytoskeletal elements or the presence of some unbroken cells. These findings identify fraction 4 as the fraction containing caveolae.

The bulk of the cellular protein was present in fractions 9, 10, and the pellet. Fractions 9 and 10 correspond to the position of the original lysate (mixed with sucrose) at the bottom of the gradient and would be expected to contain cytosolic proteins as well as any membrane proteins or lipids that were solubilized by treatment with Triton X-100. To substantiate this assignment, the gradient fractions were analyzed for the presence of actin, a cytosolic/cytoskeletal protein, and the EGF receptor, an integral membrane protein. Although some actin and some EGF receptor were found in the fraction enriched in caveolin, the bulk of both proteins appeared in fractions 9 and 10. These results confirm the conclusion that fractions 9 and 10 contain both solubile cytosolic proteins as well as detergent-solubilized integral membrane proteins.

Fig. 1. Distribution of phosphoinositides in A431 cells extracted with Triton X-100. A431 cells were labeled with myo-[3H]inositol, extracted with Triton X-100, and analyzed by sucrose density gradient centrifugation as described under “Experimental Procedures.” Aliquots of each fraction were subjected to protein analysis (A), Western blotting using anti-caveolin antibodies (B), anti-actin antibodies, or anti-EGF receptor antibodies (EGFR) (C), or thin layer chromatography to identify PtdIns (D), PtdInsP (E), and PtdInsP2 (F).

0.5% Triton X-100 was added to each plate which was then incubated for 10 min on ice with gentle rocking. At the end of the extraction, the Triton-soluble supernatant was collected and 3 ml of stop solution (methanol:concentrated HCl, 10:1) were added to the sample. The plates containing the Triton-insoluble material were washed twice in phosphate-buffered saline, and the residual cell material was scraped into 1 ml of stop solution. Lipids were extracted and analyzed as outlined above.

Immunoblotting and Protein Analysis—Aliquots of 50 μl of each fraction were separated on either a 5% SDS-polyacrylamide gel or a 15% SDS-polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose. Western blotting for caveolin, actin, and EGF receptors was carried out as by Hope and Pike (14). Protein was determined by the precipitation Lowry method (18).

In cells, the effects of EGF are mediated by the EGF receptor, a member of the tyrosine kinase family of growth factor receptors. Bradykinin stimulates biological responses via a seven transmembrane domain, G protein-coupled receptor. Treatment of A431 cells with either EGF or bradykinin resulted in a time-dependent increase in the production of inositol phosphates (Fig. 2). EGF stimulated a 2-fold increase and bradykinin a 3-fold increase in inositol phosphate production. These results indicate that, in A431 cells, PtdIns turnover can be stimulated either through a receptor tyrosine kinase or a G protein-coupled receptor.

To determine the subcellular location of the PtdInsP2 that is hydrolyzed in response to hormones, A431 cells were labeled with [3H]inositol for 48 h and subsequently treated with vehicle, EGF, or bradykinin for 5 min at 37 °C. The cells were then solubilized in Triton X-100 and subjected to sucrose density gradient centrifugation. Fig. 3A and B, shows the distribution of PtdIns and PtdInsP2 in these gradients. The results are presented as the total counts of each inositol phospholipid recovered in the fractions. C and D compare the levels of PtdIns...
and PtdInsP₂ present in fraction 4 (the caveolar fraction), fractions 9 + 10 (the cytosolic/solubilized plasma membrane fraction), or the pellet (cytoskeletal fraction) after normalization to the protein present in the control sample from each fraction.

Neither EGF nor bradykinin induced a significant change in the level of PtdIns or lyso-PtdIns in any of the subcellular fractions. By contrast, both hormones promoted a substantial decrease in the PtdInsP₂ present in the caveolar fraction. Surprisingly, neither hormone appeared to induce a loss of PtdInsP₂ from the solubilized plasma membrane fraction (fractions 9 + 10). In four separate experiments, EGF induced an average decrease of 47% in PtdInsP₂ in fraction 4 with a range of 30% to 60% (p < 0.05, paired t test). In the same experiments, bradykinin also stimulated an average decrease of 47% with a range of 40% to 50% (p < 0.01, paired t test). The decline in PtdInsP₂ levels seen in the presence of agonist cannot be attributed to decreased recovery of this lipid as analyses indicated 80%, 92%, and 100% recovery of PtdInsP₂ in the gradients derived from control, EGF-, and bradykinin-treated cells, respectively. In addition, treatment with hormones did not alter the distribution of protein, caveolin, actin, or EGF receptor in the gradients (not shown).

Because the use of sucrose gradients to separate caveolar lipids does not permit the simultaneous analysis of large numbers of samples, an alternate method of sample preparation was devised to permit further characterization of the effect of hormones on phosphoinositide levels in the caveolin-enriched fraction. This involved incubation of cell monolayers with Triton X-100-containing buffers on ice for 10 min followed by the recovery and analysis of lipids in the Triton-soluble supernatant and the Triton-insoluble pellet. Total PtdInsP₂ levels were calculated as the sum of the soluble and insoluble PtdInsP₂. The data shown represent the mean ± S.D. of triplicate determinations.

As shown in Fig. 4A, extraction of unstimulated cell mono-
layers with Triton-containing buffer resulted in the release of approximately 50% of the total PtdInsP\(_2\) into the Triton-soluble supernatant with the retention of ~50% of this lipid in the Triton-insoluble pellet. This compares favorably with the observed distribution of PtdInsP\(_2\) between Triton-soluble and Triton-insoluble fractions isolated by sucrose gradients. Under these same conditions, only 3% of the PtdIns and 6% of the lyso-PtdIns were retained in the pellet. Approximately 20% of the PtdInsP remained in the Triton-insoluble pellet. The inset in panel A shows a Western blot for caveolin in total cell lysate as well as the Triton-soluble and Triton-insoluble fractions. The data demonstrate that essentially all of the caveolin was retained in the Triton-insoluble pellet. Thus, the distribution of caveolin and phosphoinositides in the Triton-insoluble fraction mirrors the distribution of these components in the low density, Triton-resistant fraction from the sucrose density gradients.

Using this procedure, the time course of EGF- and bradykinin-stimulated PtdInsP\(_2\) turnover was examined. The data in Fig. 4B demonstrate that both EGF and bradykinin stimulated a time-dependent loss of PtdInsP\(_2\) from the Triton-insoluble pellet. The level of PtdInsP\(_2\) in the Triton-soluble supernatant (total minus Triton-insoluble) remained essentially constant throughout the time course, indicating that the loss of PtdInsP\(_2\) was principally from the Triton-insoluble compartment.

**DISCUSSION**

The results presented in this report reveal two important features of the biology of cellular PtdInsP\(_2\). First, PtdInsP\(_2\) appears to be largely compartmentalized within A431 cells. At least half of this phosphoinositide is contained within a low density, detergent-resistant, caveolin-enriched fraction that almost certainly corresponds to caveolae. Less than 20% of the PtdInsP\(_2\) fractionated with plasma membrane components. The phosphoinositides are not uniformly concentrated in caveolae as these domains contain only about 10% of the cellular PtdIns and lyso-PtdIns. Nonetheless, the fact that, in most cells, caveolae represent less than 1% of the plasma membrane, indicates that even PtdIns and lyso-PtdIns are highly enriched in these domains. In addition, the higher abundance of PtdIns in cells as compared to PtdInsP\(_2\) means that in absolute terms there is more PtdIns than PtdInsP\(_2\) in caveolae. Our results suggest that these domains contain an order of magnitude more PtdIns than PtdInsP\(_2\). Caveolin has been shown to cycle between the Golgi, the plasma membrane, and the endoplasmic reticulum (19). Since the endoplasmic reticulum is the site of PtdIns synthesis, the high levels of PtdIns in caveolae may be derived from this source. As such, caveolae may play a role in the transport of PtdIns from its site of synthesis in the ER to the plasma membrane where it is presumably phosphorylated and used for signaling.

The second important observation is that the PtdInsP\(_2\) localized to the caveolae appears to turn over in response to EGF and bradykinin. No consistent decrease in PtdInsP\(_2\) levels was observed in any other gradient fraction suggesting that caveolae are the primary site of PtdInsP\(_2\) hydrolysis. The time course of PIP\(_2\) turnover in the caveolar fraction was consistent with the rate of hormone-stimulated inositol phosphate production observed in these studies as well as in previous work (20–22). Similar to other reports (23), the recovery of cellular PIP\(_2\) levels was relatively slow due to the persistent effects of EGF and bradykinin on turnover. The observation that both EGF and bradykinin stimulated PtdInsP\(_2\) turnover in caveolae indicates that pathways involving the activation of phospholipase C\(\gamma\) via tyrosine kinases and the activation of phospholipase C\(\beta\) by G protein-coupled receptors converge in caveolae and lead to the turnover of lipids in this compartment.

The finding that EGF and bradykinin-stimulate PtdInsP\(_2\) turnover in caveolae is consistent with reports that G protein-coupled receptors (24), heterotrimeric G proteins (4, 9, 10, 12), and the EGF receptor (12) are localized in caveolae. In our studies, EGF receptors were definitely present in the caveolar fraction; however, they represented only a small proportion of the total cellular EGF receptors. This may be due to the fact that A431 cells possess an extraordinarily high number of EGF receptors (1–3 × 10\(^5\)/cell) and their number may exceed the capacity of the caveolae. However, we cannot rule out the possibility that the detergent extraction method for isolating caveolae used in our studies and the detergent-free procedure used by Mineo et al. (12) lead to the different recoveries of EGF receptors in this fraction.

Previous experiments in a variety of cell types have indicated the presence of metabolically distinct pools of inositol phospholipids. In many cases, the pools have been identified as being hormone-responsive or hormone-unresponsive (reviewed in Ref. 25). The findings reported herein provide a framework for understanding this body of work. The data suggest that metabolic compartmentalization of PtdInsP\(_2\) may result from the sequestration of a portion of this lipid in caveolae which is then subject to hormone-stimulated hydrolysis. The remaining PtdInsP\(_2\) present within the plasma membrane and perhaps other cellular membranes, would appear to comprise the hormone-unresponsive pool of this polyphosphoinositide.

Our findings document the physical compartmentalization and turnover of PtdInsP\(_2\) in caveolae. The observation that the inositol trisphosphate receptor is also concentrated in this domain (26) indicates that both the production of and response to InsP\(_3\) is highly compartmentalized within cells. This suggests that the localization of signaling is an important aspect of intracellular communication.

**REFERENCES**