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Compartmentalization of Phosphatidylinositol 4,5-Bisphosphate in Low-Density Membrane Domains in the Absence of Caveolin

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In cells that exhibit caveolae, the hormone-sensitive pool of PtdIns 4,5-P_2 is localized in a low density, caveolin-enriched membrane fraction (1). Neuro 2a cells do not express caveolin. Nonetheless, the PtdIns 4,5-P_2 in these cells is compartmentalized in a low density, detergent-insoluble domain that also contains other signaling-related molecules. Compartmentalization of PtdIns 4,5-P_2 was observed regardless of whether Triton X-100-containing or detergent-free methods were used to prepare the membranes. However, the partitioning of receptor tyrosine kinases and GPI-anchored proteins into the low density domains was dependent upon the method of membrane preparation. Treatment of Neuro 2a cells with cyclodextrin delocalized the PtdIns 4,5-P_2 and inhibited hormone-stimulated PtdIns turnover. These results suggest that compartmentalization of PtdIns 4,5-P_2 does not require caveolin but is necessary for the proper functioning of phosphoinositide-based signaling. © 1998 Academic Press

A variety of growth factors and hormones stimulate the hydrolysis of PIP_2 to produce the two intracellular second messengers, diacylglycerol and inositol trisphosphate. Diacylglycerol activates protein kinase C while inositol trisphosphate induces the release of calcium from intracellular stores. We have recently reported that PIP_2 is not randomly distributed in cells but instead is concentrated in a low density, Triton-resistant compartment that is also highly enriched in caveolin (1,2). Caveolin is a 21 kDa integral membrane protein that appears to be the main structural element of caveolae, small uncoated plasma membrane invaginations (3). Stimulation of cells with EGF or bradykinin led to the time-dependent loss of PtdIns 4,5-P_2 from the Triton-resistant fraction with no change in the level of PtdIns 4,5-P_2 in the detergent-soluble pool (1). These findings suggest that the detergent-resistant pool represents the primary source of PtdIns 4,5-P_2 hydrolyzed in response to hormones.

Cells that lack caveolin and do not exhibit non-clathrin-coated membrane invaginations nonetheless possess low density, Triton-resistant membrane domains (4,5). These non-invaginated, Triton-resistant domains have been referred to as DIGs (detergent-insoluble, glycosphingolipid-enriched domains) (6). If the localization of PtdIns 4,5-P_2 and signaling proteins to cholesterol/glycosphingolipid-enriched membrane domains is important for the regulation of PtdIns turnover, then cells that lack caveolae should still compartmentalize these molecules. In addition, disruption of these domains should lead to the inhibition of hormone-stimulated PI turnover. We have investigated this hypothesis by examining phosphoinositide-based signaling in Neuro 2a cells, a neuroblastoma cell line that does not express caveolin (5). Furthermore, we have directly compared the compartmentalization of polyphosphoinositides and other signaling molecules in low density membranes prepared using three different procedures, one involving extraction with Triton X-100 and two utilizing detergent-free protocols.

We report here that PtdIns 4,5-P_2 and other signaling proteins are localized to low density membrane domains in Neuro 2a cells that lack caveolae. However, the distribution of some signaling proteins is affected by the method used to isolate the low density domains indicating that not all preparations of caveolae/DIGs are biochemically equivalent. Disruption of the low density domains by treatment of cells with the cholesterol-binding drug, cyclodextrin, results in the delocalization of PtdIns 4,5-P_2 and a loss in the ability of hormones to stimulate PtdIns turnover. These results suggest that polyphosphoinositide compartmentalization

Abbreviations used are: DIGs, detergent-insoluble, glycosphingolipid enriched domains; GPI, glycosylphosphatidylinositol; lyso-PtdIns, lysophosphatidylinositol; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol phosphate; $PtdInsP_2$, phosphatidylinositol bisphosphate.

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does not require caveolin but is necessary for proper functioning of hormone-stimulated PI turnover.

MATERIALS AND METHODS

All anti-caveolin antibodies were from Transduction Laboratories. Anti-G_q and anti-Ret antibodies were from Santa Cruz. Anti-Grb2, anti-Shc, anti-MAP kinase and anti-PI 3-kinase antibodies were from Upstate Biotechnology, Inc. The anti-$G_{i/o}$ antibody was the generous gift of Dr. Maurine Linder (Washington Univ.) The anti-PrP antibody and the Neuro 2a cells transfected with chicken PrP were the kind gift of Dr. David Harris (Washington Univ.) *Myo*-[^{3}H]inositol and the Enhanced Chemiluminescence kit were from Amersham. All other chemicals were from Sigma.

Cell Culture

Neuro 2a cells were maintained in Minimal Essential Medium containing non-essential amino acids and 10% fetal calf serum.

Isolation of DIGs

All manipulations were carried out at 4° C.

Triton X-100 extraction procedure. One D150 plate of confluent cells was washed once in ice-cold phosphate-buffered saline and scraped into 1 ml of MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA) to which had been added 1% Triton X-100. The lysate was passed through a 23g needle 10 times and then mixed with an equal volume of 80% sucrose in MES-buffered saline. Six ml of 30% sucrose and 4 ml of 5% sucrose in MES-buffered saline were layered on top of the lysate-containing layer. Gradients were centrifuged for 3 hr at 175,000 × g and fractionated into 1.2 ml fractions. The small pellet was resuspended into 1.2 ml of MES-buffered saline.

Neutral pH detergent-free procedure. This is a modification of the procedure of Smart et al (7). One D150 plate of confluent cells was washed once in ice-cold phosphate-buffered saline and scraped into 1 ml buffer containing 25 mM Tris, pH 7.4, 250 mM sucrose, 1 mM EDTA. The cells were lysed by 10 passages through a 23 g needle followed by sonication 3 times for 15 seconds in a Branson 250 sonicator set at maximum power output for a microtip. The sonicate was mixed with an equal volume of 80% sucrose in MES-buffered saline. Gradients were prepared and processed as described above.

Alkaline pH detergent-free procedure. A procedure identical to that described for the detergent-free preparation of caveolae/DIGs at neutral pH was utilized except the cells were initially scraped into 1 ml 150 mM Na_2CO_3, pH 11, 1 mM EDTA. Lysis and sonication were carried out in this buffer. Gradient preparation and analysis were as described above. This represents a modified version of the preparation of Song et al (8).

Analysis of [^{3}H]Inositol-Labeled Phosphoinositides and Inositol Phosphates

Cells were labeled for 48 hr with 2 μCi/ml [^{3}H]*myo*-inositol in a 1:1 mix of Dulbecco's Modified Eagle's Medium and inositol-free RPMI containing 5% dialyzed fetal calf serum. Gradients were fractionated and phosphoinositides analyzed as described previously (2).

For analysis of inositol phosphate production, cells were plated in 6-well dishes and labeled with [^{3}H]*myo*-inositol as described above. Thirty minutes prior to use, 10 mM LiCl was added to each well. Cells were stimulated with 10 μM bradykinin for 10 min at 37° C. Assays were stopped by aspiration of the medium followed by the addition of 5% trichloroacetic acid. [^{3}H]Inositol phosphates were isolated on Dowex columns as described previously (9).

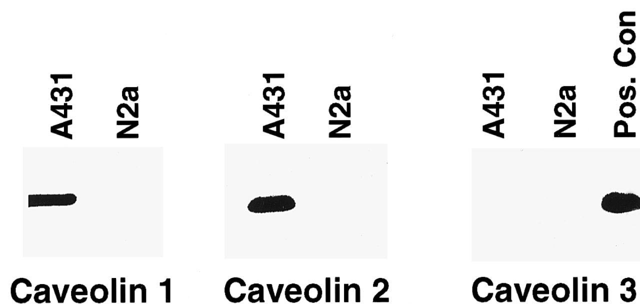

FIG. 1. Western blot analysis of cell lysates for caveolin. Neuro 2a cells (N2a) or A431 cells were lysed in RIPA buffer. Aliquots containing 100 μg protein were analyzed by SDS polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were subjected to Western blotting with caveolin 1, caveolin 2 or caveolin 3 antibodies. Pos. con. = positive control for caveolin-3.

RESULTS

Analysis of Neuro 2a Cells

Western blotting was used to analyze Neuro2a cells for the presence of caveolin 1, 2 and 3. A431 cells were used for comparison. As shown in Figure 1, Neuro 2a cells expressed neither caveolin 1 nor caveolin 2 though both of these isoforms were strongly expressed in A431 cells. Neither Neuro 2a cells nor A431 cells expressed the muscle specific form of caveolin, caveolin-3 (10). Thus, Neuro 2a cells fail to express any of the known forms of caveolin. Consistent with this observation, these cells have been shown to lack morphologically identifiable caveolae (11).

The Neuro 2a cells used in these experiments had been transfected with the chicken prion protein (PrP), a GPI-linked protein. This protein has been shown to be localized to the detergent-insoluble domains present in these cells (5). To determine whether Neuro 2a cells compartmentalize their inositol phospholipids in a Triton-insoluble compartment as do caveolin-containing cells, Neuro 2a cells were labeled with [^{3}H]inositol and low density membrane fractions prepared by sucrose density gradient centrifugation of cells lysed in 1% Triton X-100. For comparison, membranes were also prepared from cells were lysed in the absence of detergent using an isotonic sucrose buffer at neutral pH. Gradients were fractionated and aliquots of each fraction were analyzed for phosphoinositide and protein content as well as for the presence of PrP (Figure 2). Fraction 1 represents the top of the gradient. P refers to the pelleted material at the bottom of the tube.

For both the detergent and the detergent-free preparations, the majority of the cellular protein was found in the 40% sucrose layer (fractions 9 and 10) at the bottom of the gradient (Figure 2E). Less than 1% of the total protein was in the low density fraction, fraction 4, that represents the 5%/30% sucrose interface. How-

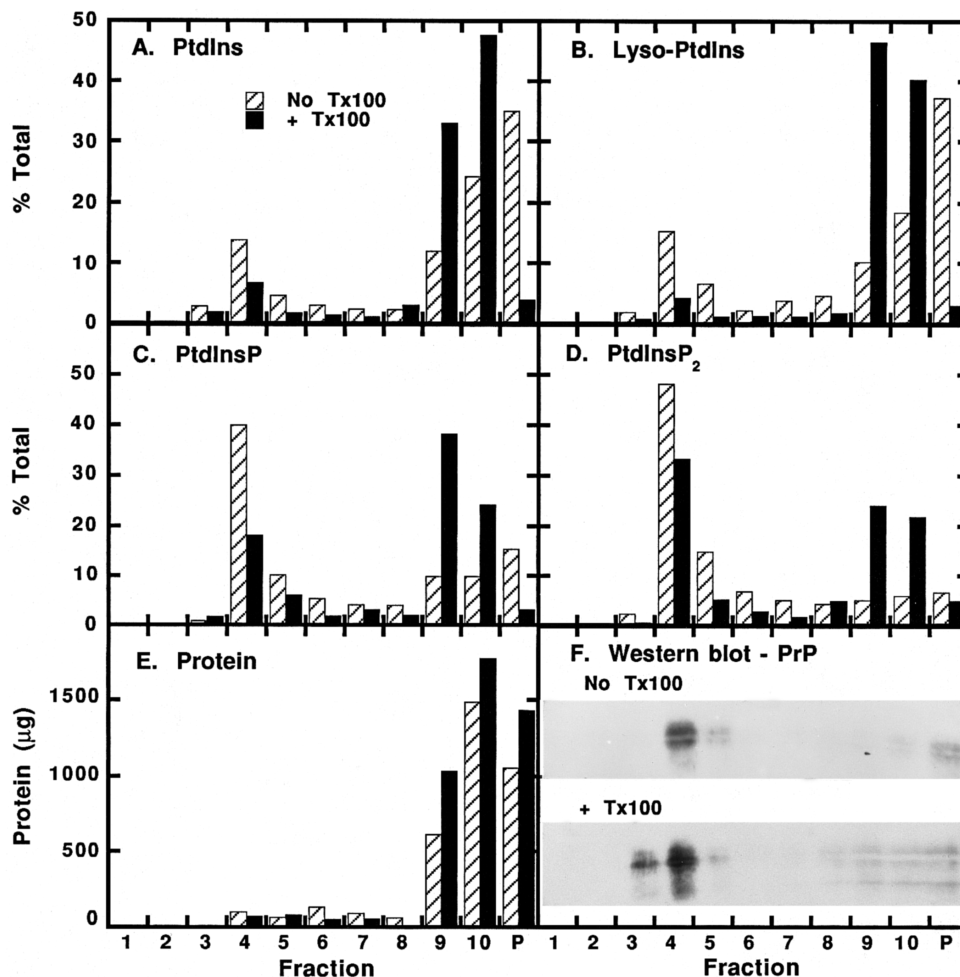

FIG. 2. Distribution of phosphoinositides in Neuro 2a cells. Neuro 2a cells were labeled with *myo*-[^{3}H]inositol, lysed in the absence (hatched bars) or presence (solid bars) of Triton X-100 at neutral pH and analyzed by sucrose density gradient centrifugation. Gradients were fractionated and aliquots of each fraction were analyzed for phosphoinositide content by thin layer chromatography (panels A through D), for protein content (panel E) and for the presence of PrP by Western blotting (panel F).

ever, the GPI-linked PrP was recovered almost exclusively in fraction 4 (Figure 2F).

Although fraction 4 contained very little protein, this fraction contained 40% to 50% of the total $PtdInsP_2$ recovered in the gradients from cells prepared using either the detergent or detergent-free method of preparation (Figure 2D). By contrast, only ~5% of the total PtdIns or lyso-PtdIns was recovered in fraction 4 in cells that had been lysed in the presence of Triton X-100. Approximately 15% of the PtdIns and lyso-PtdIns was recovered in the low density fraction isolated from cells using the detergent-free protocol (Figure 2A,B). For both preparations, the total recovery of PtdIns, lyso-PtdIns and $PtdInsP_2$ averaged about 90%. Strikingly, the recovery of PtdInsP was nearly 100% in gradients prepared using the detergent-free protocol but <20% in preparations using the Triton X-100 extraction procedure. This difference in recovery may be responsible for the observation that the low density fraction contained approximately 40% of the PtdInsP recovered from cells lysed in the absence of detergent but only 20% of the PtdInsP recovered from cells lysed in the presence of Triton X-100.

Western blot analyses of the sucrose gradient fractions demonstrated that, with one exception, the distribution of signaling proteins was essentially similar in the membranes prepared in the absence or presence of Triton X-100 (Figure 3). Heterotrimeric G proteins, $G_{i/o}$ and G_q, were extensively localized to the low density fraction containing the PrP. However, Grb2 and the PtdIns 3-kinase were largely excluded from this fraction in both membrane preparations. Low levels of Shc and MAP kinase were present in the low density domains, particularly in gradients derived from cells lysed in the absence of detergent. Only the distribution of the Ret tyrosine kinase, an enzyme that is homologous to the EGF receptor (12,13), was markedly different in the two preparations. In cells extracted with

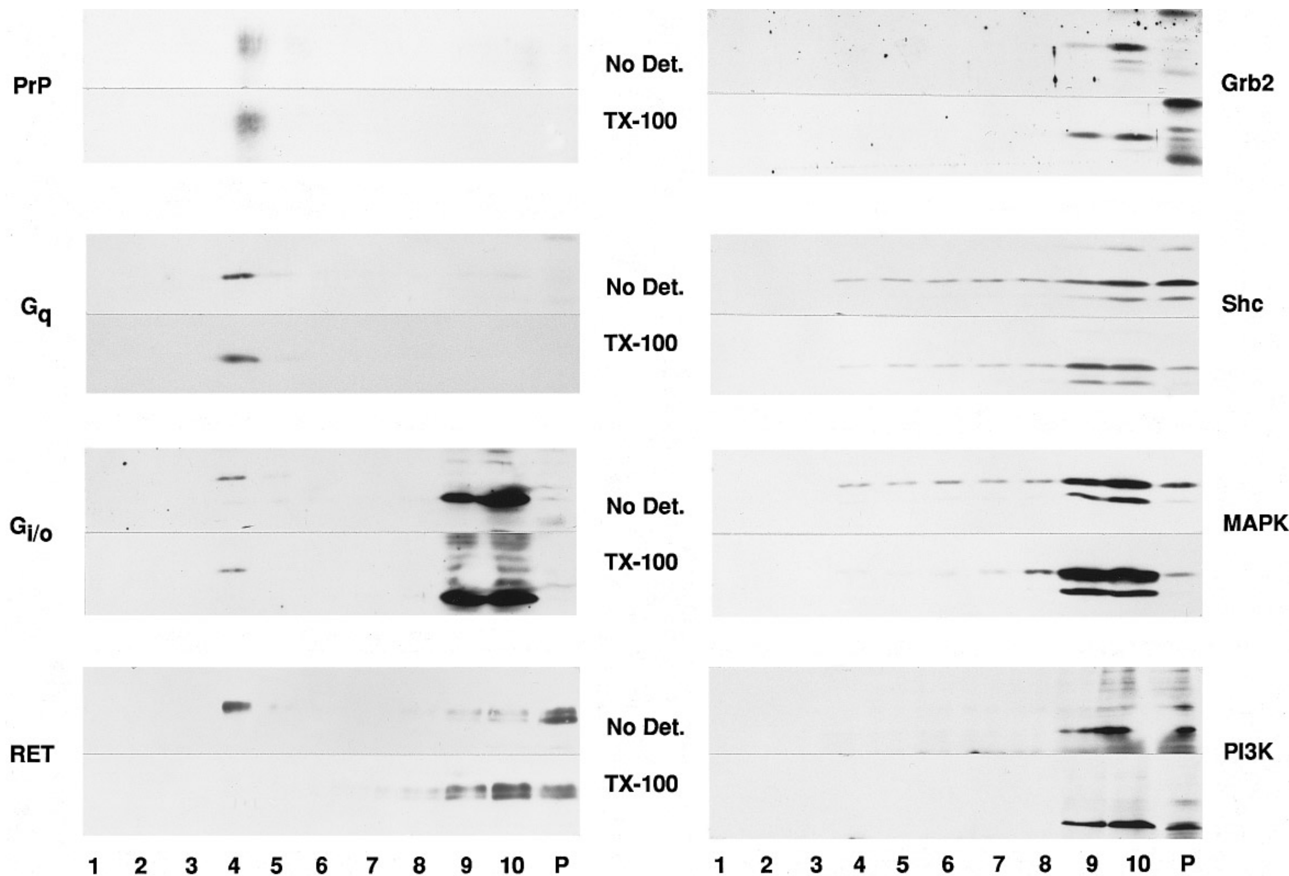

FIG. 3. Distribution of signaling-related proteins in sucrose density gradient fractions derived from Neuro 2a cells. Neuro 2a cells were lysed in the absence or presence of Triton X-100 at neutral pH and fractionated by sucrose density gradient centrifugation as described in Materials and Methods. Aliquots of each fraction were analyzed by SDS polyacrylamide gel electrophoresis and subjected to Western blotting using the indicated antibodies. Transferred proteins were detected using the Enhanced Chemiluminescence system. In each set, the upper panel shows the results obtained from cells that were lysed in the absence of Triton X-100 using an isotonic Tris buffer, pH 7.4. The lower panel of each set presents the results obtained from cells that were lysed in MES-buffered saline, pH 6.5 containing 1% Triton X-100.

Triton X-100, Ret was found almost exclusively in the high density fractions 9 and 10 that contain cytosolic proteins and solubilized membrane components. However, when cells were lysed in the absence of detergent, one-third to one-half of the Ret protein was found in the low density PrP-containing fraction.

In contrast to the concentration of GPI-linked proteins in low density domains noted above and by others (7), Song et al. (8) have reported that in cells lysed in the absence of detergent, GPI-linked proteins fractionated in the high density region of the sucrose gradient, well-separated from the caveolin marker. These workers lysed their cells in a sodium carbonate buffer, pH 11 whereas our experiments were carried out using cells lysed in a Tris buffer, pH 7.4. To determine whether the difference in the distribution of GPI-linked proteins was due to the different lysis conditions, Neuro 2a cells were labeled with [^{3}H]inositol and detergent-free membranes were prepared by lysing cells in a pH 11 sodium carbonate buffer. Density gradient fractions were analyzed for the presence of phosphoinositides, PrP, G_q and Ret (Figure 4).

Lysis of cells in pH 11, Na_2CO_3 buffer did not markedly alter the distribution of polyphosphoinositides. Approximately 40% of the $PtdInsP_2$ and 30% of the PtdInsP was recovered in the low density fraction whereas less than 10% of the PtdIns and lyso-PtdIns was found in this position. In addition, the partitioning of G_q and Ret into the low density fraction was similar to that seen when cells were lysed at neutral pH (compare with Figures 2 and 3). However, unlike the previous preparation, the GPI-linked PrP was found almost exclusively in the high density fractions containing the original lysate. Thus, lysis in the high pH Na_2CO_3 buffer led to the preferential loss of PrP from the low density fraction. This was not due to the loss of the GPI anchor following lysis of cells with pH 11 buffer. When membranes prepared from cells lysed with Na_2CO_3 buffer were extracted with Triton X-114 and subjected to temperature-induced phase separation,

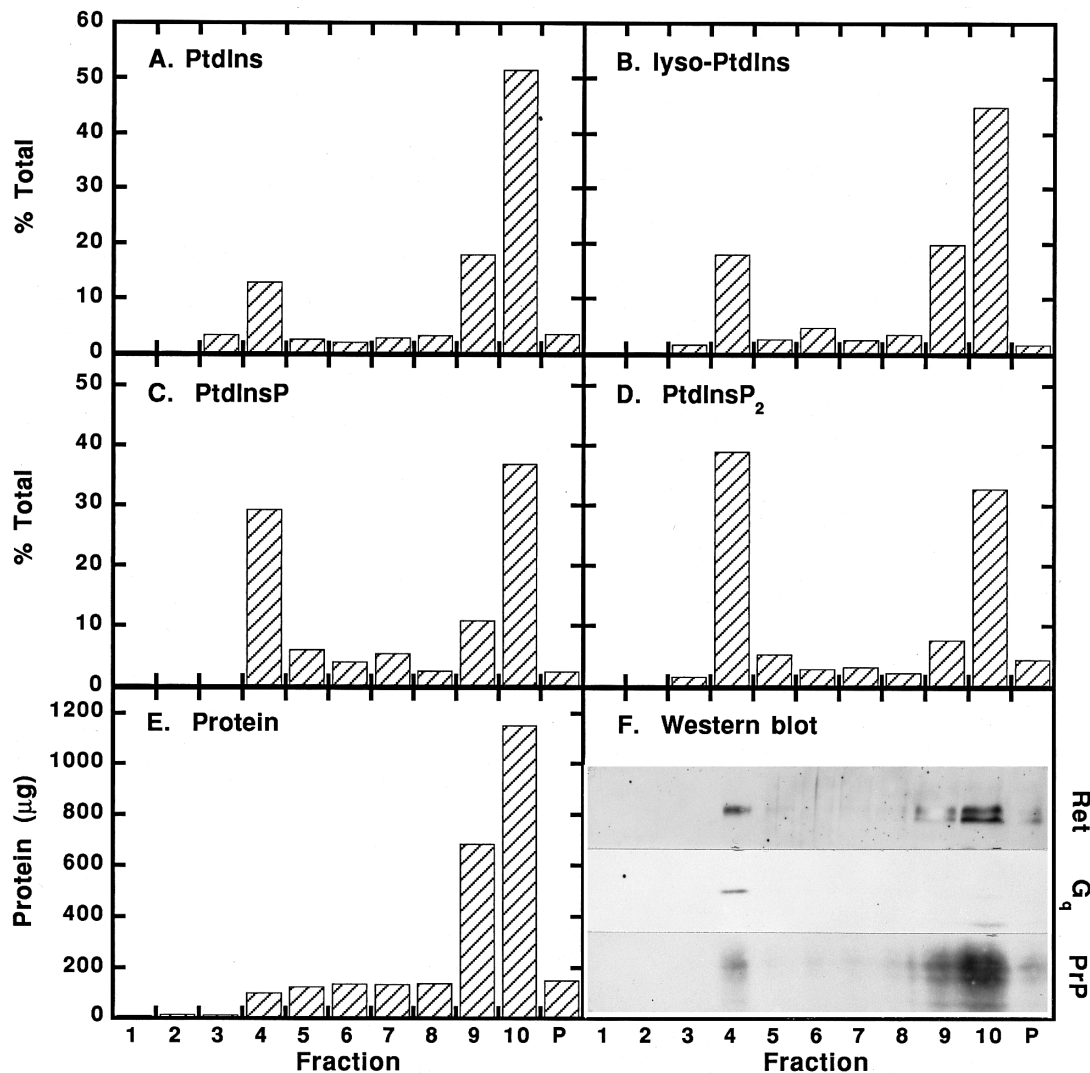

FIG. 4. Distribution of phosphoinositides and signaling proteins in Neuro 2a cells lysed using an alkaline buffer. Neuro 2a cells were labeled with *myo*-[^{3}H]inositol, lysed in 150 mM Na_2CO_3, pH 11 and analyzed by sucrose density gradient centrifugation. Aliquots of each fraction were analyzed for phosphoinositide content (panels A through D), protein content (panel E) and the presence of Ret, G_q and PrP (panel F).

the PrP partitioned into the detergent phase (not shown). As removal of the GPI anchor from GPI-linked proteins results in their partitioning into the aqueous phase, these data suggest that extraction of cells with Na_2CO_3 buffer does not result in the hydrolysis of its GPI anchor (14).

Effects of Cyclodextrin on PtdIns Turnover

If localization of signaling proteins and $PtdInsP_2$ to low density, detergent-resistant domains is important for the proper functioning of PtdIns turnover, then disruption of these domains should impair the ability of hormones to regulate inositol phosphate production. The cholesterol binding drug, cyclodextrin, has been shown to disrupt the structure of caveolae and induce the flattening of these invaginated domains.[1] Therefore, Neuro 2a cells were treated with or without 5 mM cyclodextrin for 30 min at 37°C and low density membrane fractions were prepared. Figure 5 shows the distribution of $PtdInsP_2$ and G_q in control and cyclodextrin-treated Neuro 2a cells.

Treatment of Neuro 2a cells with cyclodextrin led to approximately a 50% decrease in the fraction of PtdIns 4,5-P_2 recovered in the low density fraction of the sucrose gradient. Similarly, a portion of the G_q was lost from the low density fraction and recovered in the high density portion of the gradient. By contrast, there was

[1] Chung, K.-N., Roth, R., Morisaki, J. H. and Heuser, J., submitted for publication.

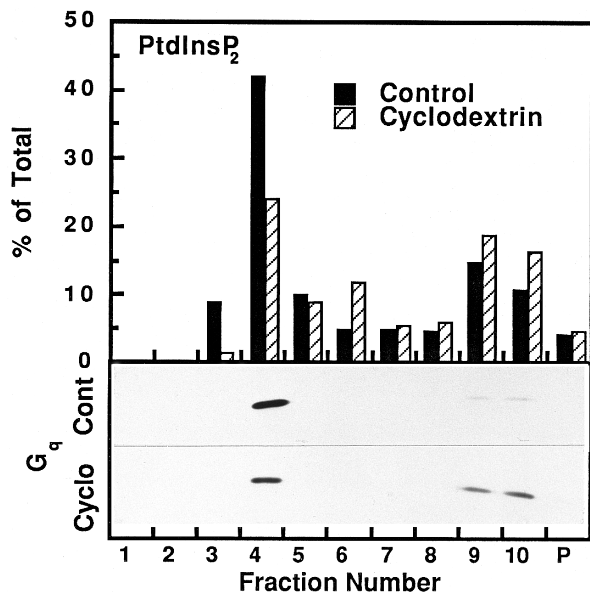

FIG. 5. Effect of cyclodextrin on PtdIns 4,5-P_2 and G_q distribution in Neuro 2a cells. Cells were labeled with [^{3}H]*myo*-inositol for 48 hr. Just prior to use, cultures were incubated in the absence or presence of 5 mM cyclodextrin for 30 min at 37°C. Cells were lysed in Na_2CO_3 buffer, pH 11, and lysates were subjected to sucrose density gradient centrifugation as described in Materials and Methods. Aliquots of each fraction were analyzed for [^{3}H]phosphoinositides using thin layer chromatography and for G_q by Western blotting.

little change in the distribution of PtdIns in the treated cells (not shown). As shown in Figure 6, the cyclodextrin-induced disruption of the low density domains was associated with a significant reduction in the ability of bradykinin to stimulate PtdIns turnover.

DISCUSSION

We have previously shown that in A431 cells a large portion of $PtdInsP_2$ is compartmentalized in a low density domain that is also enriched in caveolin (1). In this manuscript, we demonstrate that significant amounts of $PtdInsP_2$ are also localized to a low density fraction in Neuro 2a cells, a line that does not express caveolin and that lacks caveolae. We have documented localization of $PtdInsP_2$ in other cell lines including MDCK cells (2), NIH 3T3 cells, bEND cells, FRT cells and KB cells,[2] the last two of which do not express caveolin. Together with the results presented here, these data indicate that the compartmentalization of $PtdInsP_2$ is a general feature of most cells and does not require the presence of caveolin.

Biochemical characterization of DIGs prepared from Neuro 2a cells using detergent and detergent-free procedures indicates that G_q and $PtdInsP_2$ consistently localize to the low density membrane fraction regardless of the method used to prepare these domains. The finding that $PtdInsP_2$ is concentrated in low density domains isolated using either detergent or detergent-free procedures suggests that this localization is not the result of the method used for membrane isolation but instead reflects the distribution of this lipid *in vivo.* In contrast to $PtdInsP_2$, the association of several proteins with the low density domains was dependent on the method by which the domains were prepared. For example, the Ret receptor tyrosine kinase was present in the low density fraction in membranes prepared in the absence of detergent but was largely excluded from this fraction when the preparation involved the use of Triton X-100. Similarly, the GPI-linked PrP was present in the low density fraction in membranes prepared using neutral pH buffers but was shifted to the higher density fractions when a Na_2CO_3 buffer at pH 11 was used for cell lysis. This variability could reflect differences in the mechanisms through which these proteins associate with DIGs but clearly indicates that the protein content of DIGs can be markedly affected by the method used to isolate these membrane domains.

If compartmentalization of PtdIns 4,5-P_2 in a low density fraction is important for PtdIns turnover, then all cells that signal via PtdIns turnover should exhibit a similar localization of $PtdInsP_2$, regardless of the presence of caveolin and caveolae. The observation that polyphosphoinositides are localized to a low density, detergent-insoluble domain in Neuro 2a cells that do not express caveolin and lack caveolae supports this hypothesis. Also consistent with this hypothesis are the results of the experiments examining signaling in cells treated with the cholesterol-binding drug, cyclo-

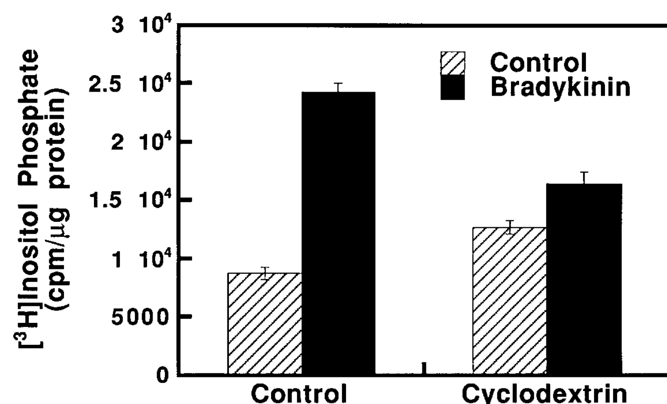

FIG. 6. Effect of cyclodextrin on hormone-stimulated PtdIns turnover. Neuro 2a cells grown in 35 mm dishes were labeled with *myo*-[^{3}H]inositol for 48 h. The cultures were treated for 30 min at 37°C in the absence or presence of 5 mM cyclodextrin. Cells were then stimulated for 10 min with vehicle or 10 μM bradykinin. Monolayers were processed and inositol phosphates isolated as described in Materials and Methods. Results represent the mean ± S.D. of sextuplicate determinations.

[2] Liu and Pike, unpublished observations.

dextrin. Cyclodextrin treatment led to the delocalization of PtdIns 4,5-P_2 and the depletion of G_q from the low density fraction of the cells. Whether this is due to a general decrease in the size of such domains or to a disruption of their overall structure is not clear. However, loss of the localization of PtdIns 4,5-P_2 and signaling proteins was associated with an inhibition of the ability of bradykinin to induce PtdIns turnover. These findings imply that localization of signaling molecules in cholesterol-rich domains is required to achieve proper function of the PI turnover pathway.

In summary, our data demonstrate that polyphosphoinositides are compartmentalized in low density domains in cells that lack caveolae as well as cells that contain caveolae. A variety of other signaling molecules including heterotrimeric G proteins and receptor tyrosine kinases also localize to low density domains regardless of the presence of caveolin. Thus, caveolin does not appear to be required for the localization of either lipids or proteins to these cholesterol/glycosphingolipid-enriched domains. However, the compartmentalization of signaling proteins and lipids appears to be required for appropriate regulation of PtdIns turnover as disruption of DIGs with cyclodextrin leads to a loss in the ability of hormones to promote PtdIns turnover.

REFERENCES

1. Pike, L. J., and Casey, L. (1996) *J. Biol. Chem.* **271,** 26453–26456.
2. Hope, H. R., and Pike, L. J. (1996) *Mol. Biol. Cell* **7,** 843–851.
3. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 8655–8659.
4. Fra, A. M., Williamson, E., Simons, K., and Parton, R. G. (1994) *J. Biol. Chem.* **269,** 30745–30748.
5. Gorodinsky, A., and Harris, D. A. (1995) *J. Cell Biol.* **129,** 619–627.
6. Parton, R. G., and Simons, K. (1995) *Science* **269,** 1398–1399.
7. Smart, E. J., Ying, Y.-S., Mineo, C., and Anderson, R. G. W. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 10104–10108.
8. Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 9690–9697.
9. Downes, C. P., and Michell, R. H. (1981) *Biochem. J.* **198,** 133–140.
10. Tang, Z., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) *J. Biol. Chem.* **21,** 2255–2261.
11. Shyng, S.-L., Huber, M. T., and Harris, D. A. (1993) *J. Cell Biol.* **125,** 1239–1250.
12. Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H., and Hiai, H. (1988) *Oncogene* **3,** 571–578.
13. Takahashi, M., Buma, Y., and Hiai, H. (1989) *Oncogene* **4,** 805–806.
14. Hooper, N. M., and Turner, A. J. (1988) *Biochem. J.* **250,** 865–869.