

Cholesterol Depletion Delocalizes Phosphatidylinositol Bisphosphate and Inhibits Hormone-stimulated Phosphatidylinositol Turnover*

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Caveolae and detergent-insoluble, glycosphingolipid-enriched domains (DIGs) are cholesterol-enriched membrane domains that have been implicated in signal transduction because a variety of signaling proteins as well as phosphatidylinositol bisphosphate (PtdInsP₂) are compartmentalized in these domains. We report here that depletion of cellular cholesterol leads to the inhibition of epidermal growth factor- and bradykinin-stimulated PtdIns turnover in A431 cells. This is associated with the loss of compartmentalization of epidermal growth factor receptors, G_q, and PtdInsP₂ in the low density membrane domains. Replacement of cellular cholesterol leads to the reorganization of signaling molecules in the low density domains and the reestablishment of hormone-stimulated PtdIns hydrolysis. Oxysterol derivatives show a variable ability to functionally replace the cholesterol in this system. These data are consistent with the hypothesis that localization of signaling proteins and lipids to cholesterol-enriched domains is required for the proper function of hormone-stimulated PtdIns turnover.

Caveolae are small, plasma membrane invaginations first identified by Palade and co-workers in the 1950s (1). Although caveolae are non-clathrin-coated invaginations, subsequent studies have shown that caveolae do possess a striated coat that appears to be comprised largely of caveolin (2). Caveolin is a 21-kDa integral membrane protein first described as a substrate for the tyrosine kinase, pp60^{src} (3, 4). It is a member of a family of three homologous proteins termed caveolin, caveolin-2, and caveolin-3 (5, 6).

Caveolae appear to represent specialized lipid domains that contain high levels of glycosphingolipids and cholesterol (7, 8). This unusual lipid composition renders caveolae and their constituent proteins resistant to solubilization with Triton X-100. Thus, caveolae can be isolated as a low density membrane fraction from cells lysed in the presence of Triton X-100 (7). Because proteins might be redistributed during extraction of membranes with Triton X-100, detergent-free procedures have also been developed to isolate caveolae (9, 10).

Cells that do not exhibit plasmalemmal caveolae nonetheless possess low density, Triton-resistant membrane domains (11) referred to as detergent-insoluble, glycosphingolipid-enriched

domains (DIGs)¹ (12) or lipid rafts (13). Cholesterol-enriched lipid rafts have been described in the Golgi and late endosomes suggesting that, among other things, these domains may participate in the apical sorting of proteins and glycolipids as well as in endocytic trafficking (13).

Substantial evidence also implicates caveolae/DIGs in signal transduction. Low density membrane fractions prepared in the presence or absence of Triton X-100 contain many proteins involved in signal transduction, including low molecular weight and heterotrimeric G proteins, *src* family kinases, EGF receptors, PDGF receptors, endothelin receptors, the phosphotyrosine phosphatase *syk*, Grb2, Shc, mitogen-activated protein kinase, protein kinase C, and the p85 subunit of PtdIns 3-kinase (9, 14–18). Treatment of fibroblasts with PDGF stimulates the tyrosine phosphorylation of proteins in the caveolin-enriched fraction but has little effect on the tyrosine phosphorylation of proteins in other membrane fractions (17). In addition, incubation of Rat 1 cells with EGF leads to the recruitment of Raf-1 to a caveolin-enriched fraction suggesting that the initial steps in the *ras* signaling pathway may originate in caveolae (18). Finally, caveolin has been shown to interact directly with G protein α subunits, *src* family kinases, EGF receptors, and protein kinase C, inhibiting their respective activities (19–21). Together these results have been used to support the hypothesis that caveolae/DIGs provide a site within the plasma membrane at which signaling molecules are concentrated and through which initial signaling events proceed.

Not only signaling proteins but also signaling lipids appear to be concentrated in low density membrane domains. Studies with Madin-Darby canine kidney, A431, and Neuro 2a cells indicate that as much as half of the cellular PtdIns 4,5-P₂ in cells is present in low density, detergent-insoluble domains that are enriched in caveolin (22–24).² Stimulation of A431 cells with either EGF or bradykinin led to the time-dependent loss of PtdIns 4,5-P₂ from the caveolin-enriched fraction with no change in the level of non-caveolar PtdIns 4,5-P₂, suggesting that these low density domains are the primary source of PtdIns 4,5-P₂ hydrolyzed in response to hormones (23).

If localization of PtdIns 4,5-P₂ and other signaling proteins in low density membrane domains is required for hormone-stimulated PtdIns turnover, then disruption of these domains should result in the ablation of this biological response. We report here that treatment of A431 cells with the cholesterol-binding agent methyl- β -cyclodextrin leads to depletion of

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¹ The abbreviations used are: DIGs, detergent-insoluble, glycosphingolipid-enriched domains; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; MES, 4-morpholineethanesulfonic acid; PDGF, platelet-derived growth factor; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol monophosphate; PtdInsP₂, phosphatidylinositol bisphosphate.

cellular cholesterol and results in the loss of compartmentalization of PtdIns 4,5-P₂, EGF receptors, and G_q. This delocalization of signaling components is associated with a loss of EGF- and bradykinin-stimulated PtdIns turnover. Replacement of cellular cholesterol leads to the reorganization of the low density domains and the reestablishment of hormone-stimulated PtdIns hydrolysis. These data implicate cholesterol in the process of hormone-stimulated PtdIns turnover and are consistent with the hypothesis that the localization of signaling proteins and lipids to cholesterol-enriched domains is required for the function of this signaling pathway.

EXPERIMENTAL PROCEDURES

Materials—Anti-caveolin-1 and anti-caveolin-2 antibodies were from Transduction Laboratories. Anti-G_q antibody was from Santa Cruz. The polyclonal antibody to the EGF receptor (DB-1) was prepared as described previously (25). EGF was prepared according to the method of Savage and Cohen (26). *myo*-[³H]inositol and the Enhanced Chemiluminescence kit were from Amersham Pharmacia Biotech. Methyl- β -cyclodextrin was from Aldrich. All other chemicals were from Sigma.

Culture and Labeling of Cells—A431 cells were maintained in DMEM containing 7% newborn calf serum and 3% fetal calf serum. For labeling, subconfluent cultures were switched to inositol-free DMEM containing 5% newborn calf serum and 1 μ Ci/ml *myo*-[³H]inositol and grown for a further 48 h, at which point the cultures were confluent.

Isolation of Caveolin-enriched Membranes—One 150-mm plate of A431 cells was washed in phosphate-buffered saline and scraped into 1 ml of ice-cold 150 mM Na₂CO₃, pH 11, 2 mM EDTA. The cells were passed through a 23-gauge needle 10 times and subsequently sonicated 3 times for 15 s on ice using a Branson 250 sonicator set at maximum power output for a microtip. The lysate was mixed with an equal volume of 80% sucrose in MES-buffered saline (25 mM MES, pH 6.5, 150 mM NaCl, 2 mM EDTA) and placed in the bottom of a centrifuge tube. 6 ml of 35% sucrose in MES-buffered saline and 4 ml of 5% sucrose in MES-buffered saline were layered on top of the lysate. The gradient was centrifuged for 3 h at 175,000 $\times g$ and fractionated into 10 1.2-ml fractions. The small pellet was resuspended in 1.2 ml of MES-buffered saline.

Lipid Analyses—Aliquots (800 μ l) of each sucrose density gradient fraction were extracted in chloroform:methanol:HCl and inositol phospholipids analyzed as described previously (22). For analysis of cholesterol levels, lipids were extracted according to the method of Bligh-Dyer (27), and cholesterol was assayed using the Cholesterol CII assay kit (Wako).

PtdIns Turnover—Cells were plated in six-well dishes and labeled with *myo*-[³H]inositol as described above. Before the assay, cells were switched to inositol-free DMEM containing 1 mg/ml bovine serum albumin. Vehicle, EGF (50 nM), or bradykinin (10 μ M) was added to each well, and cells were incubated for 10 min at 37 °C in a CO₂ incubator. Assays were terminated by aspiration of the medium, washing once in cold phosphate-buffered saline, and addition of 1 ml of 5% trichloroacetic acid. Inositol phosphates were isolated on Dowex columns as described previously (28).

Western Blotting—Aliquots (100 μ l) of sucrose density gradient fractions were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically to nitrocellulose. Membranes were blocked using 10% powdered milk and then incubated for 2 h with primary antibody. Antibody detection was carried out using Enhanced Chemiluminescence according to the manufacturer's specifications.

Synthesis of Sterol-Methyl- β -Cyclodextrin Complexes—Sterol-methyl- β -cyclodextrin complexes were synthesized as described by Klein *et al.* (29). Briefly, 6 mg of sterol was dissolved in 80 μ l of isopropyl alcohol:CHCl₃ (2:1). Methyl- β -cyclodextrin (200 mg) was dissolved in 2.2 ml of water and heated to 80 °C with stirring in a water bath. The sterol was added in small aliquots and the solution stirred until clear. This yields a solution that contains 6.8 mM sterol. For use, complexes were diluted into inositol-free DMEM containing 1 mg/ml bovine serum albumin to a final concentration of 0.2 mM.

RESULTS

We have demonstrated previously that PtdInsP₂ is compartmentalized in caveolae/DIGs and that it is this compartmentalized PtdInsP₂ that is turned over in response to hormones (22, 23). Because caveolae/DIGs are membrane domains that are enriched in cholesterol, we wondered what effect chole-

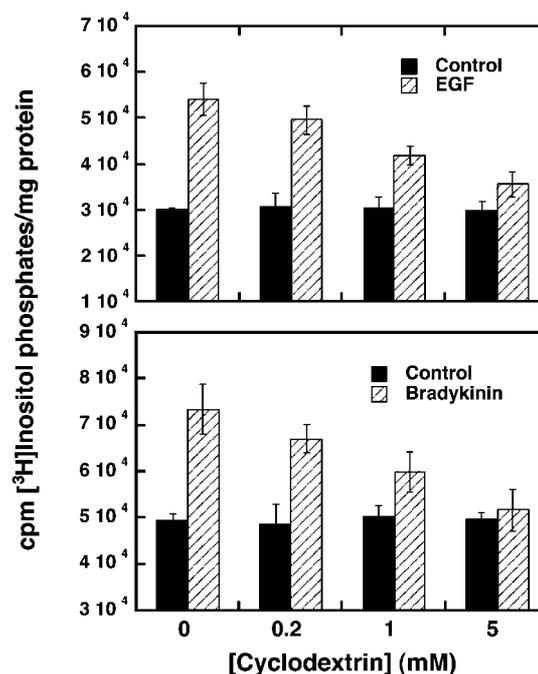


FIG. 1. Dose response to cyclodextrin for the inhibition of hormone-stimulated PtdIns turnover. A431 cells were labeled for 48 h with [³H]inositol and then switched into inositol-free DMEM containing the indicated concentration of methyl- β -cyclodextrin. Monolayers were incubated with cyclodextrin for 30 min at 37 °C. Subsequently, the cultures were treated with vehicle, 50 nM EGF (top panel) or 10 μ M bradykinin (bottom panel) for 10 min at 37 °C, and [³H]inositol phosphates were isolated as described under "Experimental Procedures." Data shown represent the mean \pm S.D. of triplicate determinations.

sterol depletion would have on the integrity of these domains, their ability to compartmentalize PtdInsP₂, and the process of hormone-stimulated PtdIns turnover.

Methyl- β -cyclodextrin is a cholesterol-binding agent that removes cholesterol from intact cells (30, 31). A431 cells were treated with increasing concentrations of methyl- β -cyclodextrin for 30 min at 37 °C and then assayed for EGF- and bradykinin-stimulated PtdIns turnover. As shown in Fig. 1, cyclodextrin dose dependently inhibited inositol phosphate production stimulated by both hormones. The dose response to cyclodextrin for inhibition of PtdIns turnover was similar for both hormones, suggesting that the effect of cyclodextrin was not receptor-specific but rather more generally targeted.

The effect of methyl- β -cyclodextrin on hormone-stimulated PtdIns turnover was rapid and was associated with a decrease in total cellular cholesterol levels. The data in Fig. 2 demonstrate that both EGF- and bradykinin-stimulated PtdIns turnover were inhibited significantly within 10 min after the addition of cyclodextrin. Maximal inhibition was observed after 30–40 min of treatment with this agent. Over this same time period, cellular cholesterol levels declined almost linearly. Maximal inhibition of hormone-stimulated PtdIns turnover occurred when approximately half of the cellular cholesterol had been removed. However, 50% inhibition was observed when only 20% of the cellular cholesterol had been lost.

To determine the effect of methyl- β -cyclodextrin on the integrity of caveolae/DIGs, A431 cells were treated in the absence or presence of this reagent for 30 min. Low density membrane fractions were then isolated, and the distribution of several proteins enriched in caveolae/DIGs was assessed by Western blotting of the analytical sucrose density gradients (Fig. 3). Fraction 1 represents the top of the gradient, and fraction 10 is the bottom of the gradient. Fraction 4 contains the 5%/35% sucrose interface. In untreated cells, the majority of the caveo-

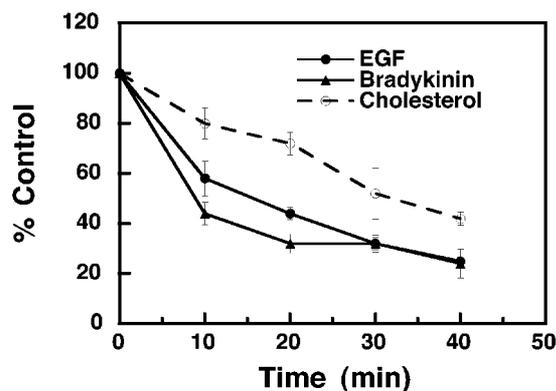


FIG. 2. Time course of cyclodextrin-induced cholesterol depletion and loss of hormone-stimulated PtdIns turnover. A431 cells were labeled for 48 h with [3 H]inositol and then switched into inositol-free DMEM containing 5 mM methyl- β -cyclodextrin. Cultures were incubated with cyclodextrin for the indicated length of time, stimulated with EGF or bradykinin, and inositol phosphates isolated as described under "Experimental Procedures." Hormone-stimulated inositol phosphate production was determined by subtracting the cpm of inositol phosphates produced in the absence of hormone from the cpm of inositol phosphates produced in the presence of hormone. EGF-stimulated inositol phosphate production in control cells was 31,765 cpm/mg of protein. Bradykinin-stimulated inositol phosphate production in control cells was 36,301 cpm/mg of protein. The results shown represent the mean \pm S.D. of triplicate determinations. For cholesterol determinations, A431 cells were treated for the indicated time with 5 mM cyclodextrin as described above. After washing the cultures with ice-cold phosphate-buffered saline, cellular lipids were extracted by the method of Bligh-Dyer, and cholesterol levels were determined as described under "Experimental Procedures." Results represent the mean \pm S.D. of triplicate determinations.

lin-1 was found in fraction 4, identifying this fraction as the caveolar fraction. After treatment with cyclodextrin, this tight compartmentalization was lost, and the bulk of the caveolin-1 was recovered in the high density region of the gradient, fractions 9, 10, and the pellet. Similarly, caveolin-2 was strongly localized to fraction 4 in control cells but was found almost exclusively in the high density fractions in cyclodextrin-treated cells. Treatment of A431 cells with cyclodextrin also led to the loss of compartmentalization of signaling-related proteins in the low density fractions. Both the EGF receptor and G_q were extensively localized to fraction 4 in untreated cells. However, after treatment with cyclodextrin both of these proteins were lost from the low density portion of the gradient and recovered instead in the high density region of the gradient.

To determine the effect of cholesterol depletion on the compartmentalization of PtdIns P_2 , A431 cells were labeled with [3 H]inositol and subsequently treated with methyl- β -cyclodextrin for 30 min (Fig. 4). Treatment with cyclodextrin did not significantly alter the distribution of PtdIns or lyso-PtdIns in A431 cells (Fig. 4, A and B). However, this treatment was associated with the loss of PtdInsP and PtdIns P_2 from the low density, caveolin-enriched fraction of the sucrose gradients (Fig. 4, C and D). The PtdInsP and PtdIns P_2 lost from fraction 4 were recovered quantitatively in the high density fractions, indicating that there was not a general decrease of polyphosphoinositides but rather a specific relocalization of these lipids to other parts of the cell membrane. These data suggest that depletion of cellular cholesterol results in the loss of hormone-stimulated PtdIns turnover and the delocalization of caveolin, signaling proteins and PtdIns P_2 from the low density membrane fraction.

Cholesterol Reverses the Effects of Methyl- β -Cyclodextrin—Whereas methyl- β -cyclodextrin can remove cholesterol from cell membranes, soluble complexes of cholesterol and methyl- β -cyclodextrin can mediate the incorporation of cholesterol into

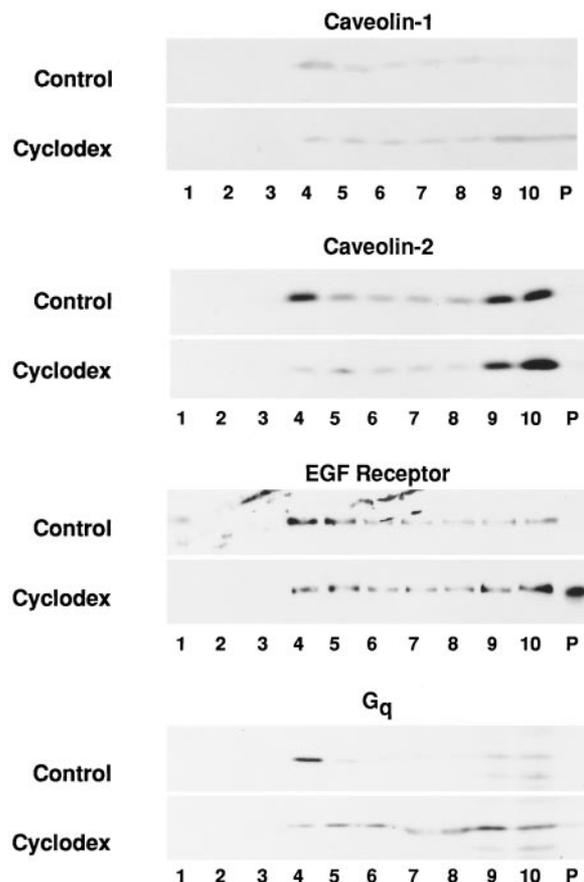


FIG. 3. Effect of cyclodextrin on the partitioning of proteins into the low density membrane fraction. A431 cells in 150-mm plates were treated in the absence or presence of 5 mM methyl- β -cyclodextrin for 30 min at 37 $^{\circ}$ C. Cells were lysed in 150 mM Na_2CO_3 , pH 11, sonicated, and subjected to sucrose density gradient centrifugation as described under "Experimental Procedures." Gradients were fractionated, and 100- μ l aliquots of each fraction were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting with the indicated antibody. *Fraction 1* represents the top of the gradient, and *P* indicates the small, resuspended pellet fraction.

membranes (29, 32–34). To determine whether the loss of hormone-stimulated PtdIns turnover and the loss of integrity of caveolin-enriched membrane domains were due to the depletion of cholesterol, A431 cells were first treated with cyclodextrin for 30 min at 37 $^{\circ}$ C. Subsequently, the cells were washed and incubated for increasing times with either 0.1% bovine serum albumin or cholesterol-methyl- β -cyclodextrin complexes in 0.1% bovine serum albumin and tested for bradykinin-stimulated PtdIns turnover. As shown in Fig. 5, treatment of cholesterol-depleted cells with cholesterol-methyl- β -cyclodextrin complexes rapidly reversed the cyclodextrin-induced loss of bradykinin-stimulated PtdIns turnover. Essentially complete recovery was observed within 20 min after the addition of cholesterol-methyl- β -cyclodextrin complexes. Some reversal occurred in the presence of bovine serum albumin alone, but it was slower and less extensive than that seen in the presence of the cholesterol-containing complexes. This may be due to redistribution of cholesterol from intracellular membranes to the plasma membrane. Treatment with the cholesterol-methyl- β -cyclodextrin complexes also reversed the effects of cyclodextrin treatment on EGF-stimulated PtdIns turnover (not shown). Measurement of the cholesterol levels of cells treated with cholesterol-methyl- β -cyclodextrin complexes confirmed that treatment with the complexes led to the repletion of cellular cholesterol, whereas incubation in bovine serum albumin-con-

FIG. 4. Effect of cyclodextrin on the partitioning of inositol phospholipids into the low density membrane fraction. A431 cells in 150-mm plates were labeled with [^3H]inositol for 48 h and then switched to inositol-free DMEM in the presence or absence of 5 mM methyl- β -cyclodextrin. Cultures were incubated for 30 min at 37 °C, and subsequently lysates were prepared and analyzed by sucrose density gradient centrifugation as described under "Experimental Procedures." Gradients were fractionated, and 800- μl aliquots of each fraction were extracted with chloroform:methanol:HCl to isolate inositol phospholipids. Phosphoinositides were separated by thin layer chromatography and visualized by autoradiography. Bands corresponding to PtdIns, lyso-PtdIns, PtdInsP, and PtdInsP₂ were identified by comigration with standards, scraped from the plates, and quantitated by scintillation counting.

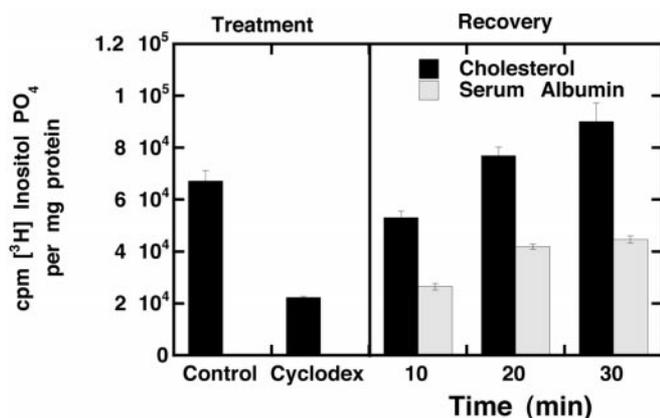
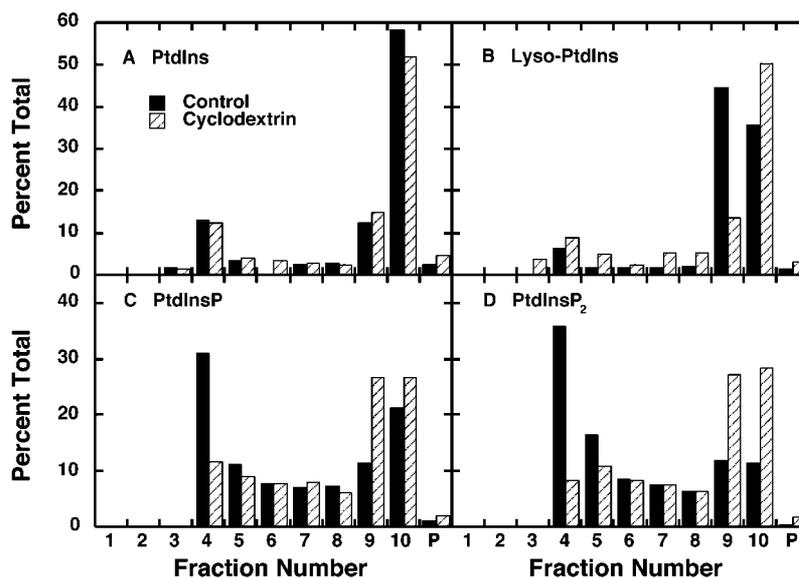


FIG. 5. Time course of the recovery of bradykinin-stimulated PtdIns turnover after incubation with cholesterol-methyl- β -cyclodextrin complexes. A431 cells were labeled for 48 h with [^3H]inositol and then switched to inositol-free DMEM in the absence or presence of 5 mM methyl- β -cyclodextrin. Cultures were incubated for 30 min at 37 °C. One control culture and one cyclodextrin-treated culture were stimulated immediately with or without 10 μM bradykinin, and inositol phosphates were isolated. The remaining cyclodextrin-treated cultures were switched to inositol-free DMEM containing 1 mg/ml bovine serum albumin in the absence or presence of 0.2 mM cholesterol in complex with methyl- β -cyclodextrin. Cultures were incubated with serum albumin or cholesterol for the indicated times at 37 °C, stimulated with or without bradykinin, and inositol phosphates isolated. Results represent the mean \pm S.D. of triplicate determinations.

taining medium did not lead to an increase in cholesterol levels (Table I).

Methyl- β -cyclodextrin complexes of several oxysterol analogs of cholesterol were also prepared to determine whether these cholesterol derivatives could reverse the effects of cyclodextrin on PtdIns turnover. As shown in Fig. 6, there was significant variation in the ability of the different analogs to restore hormone-stimulated PtdIns turnover. For both EGF- and bradykinin-stimulated inositol phosphate production, 25-hydroxycholesterol and cholestane-3 β ,5 α ,6 β -triol were unable to reverse the effects of cyclodextrin. However, other oxysterol derivatives were able to substitute for cholesterol. The 7-ketocholesterol derivative partially restored hormone-stimulated PtdIns turnover, while cholesterol 5 α ,6 α -epoxide was more effective than cholesterol in reversing the effects of cyclodextrin. Interestingly, while bradykinin-stimulated PtdIns turnover typically recovered to levels similar to or only slightly higher

TABLE I
Cholesterol levels in cells treated with cholesterol-methyl- β -cyclodextrin complexes

A431 cells were treated without (Control) or with 5 mM methyl- β -cyclodextrin (+Cyclodextrin) for 30 min at 37 °C. At the end of the incubation the medium was aspirated. Half of the plates were treated for the indicated times with DMEM containing 1 mg/ml bovine serum albumin, and half of the plates were treated with the same medium containing 0.2 mM cholesterol-methyl- β -cyclodextrin complexes. The medium was then removed and the monolayers washed with cold phosphate-buffered saline. Lipids were extracted and cholesterol levels determined as described under "Experimental Procedures." The values shown represent the mean \pm S.D. of triplicate determinations. * indicates that the value is significantly different at the $p < 0.05$ level from the untreated, control value.

Condition	Cholesterol $\mu\text{g}/\text{well}$
Control	11.1 \pm 0.5
+Cyclodextrin	7.5 \pm 0.3*
+Bovine serum albumin	
10 min	7.4 \pm 0.1*
20 min	6.7 \pm 0.3*
30 min	7.0 \pm 0.4*
+Cholesterol complexes	
10 min	9.4 \pm 0.3*
20 min	9.8 \pm 1.0
30 min	10.3 \pm 0.6

than control values, EGF-stimulated PtdIns turnover was consistently higher in sterol-replenished cells than in control cells. Thus, although the order of potency of the different oxysterols for restoring PtdIns turnover was similar for both hormones, the effect of sterol replacement was quantitatively different for the two hormones.

Treatment of A431 cells with cholesterol-methyl- β -cyclodextrin complexes also reversed the cyclodextrin-induced loss of integrity of the caveolin-enriched membrane fraction. The data in Fig. 7 demonstrate that although caveolin-1 was lost from the low density fraction after a 30-min treatment with cyclodextrin, addition of cholesterol-methyl- β -cyclodextrin complexes led to the recovery of the bulk of the caveolin in the low density fraction of the gradient. Similarly, EGF receptors and G_q were lost from the low density domains upon treatment with cyclodextrin but were found to relocate to the caveolin-containing fraction after incubation with cholesterol-methyl- β -cyclodextrin complexes.

The cyclodextrin-induced PtdInsP₂ delocalization was also reversed after treatment with cholesterol-methyl- β -cyclodex-

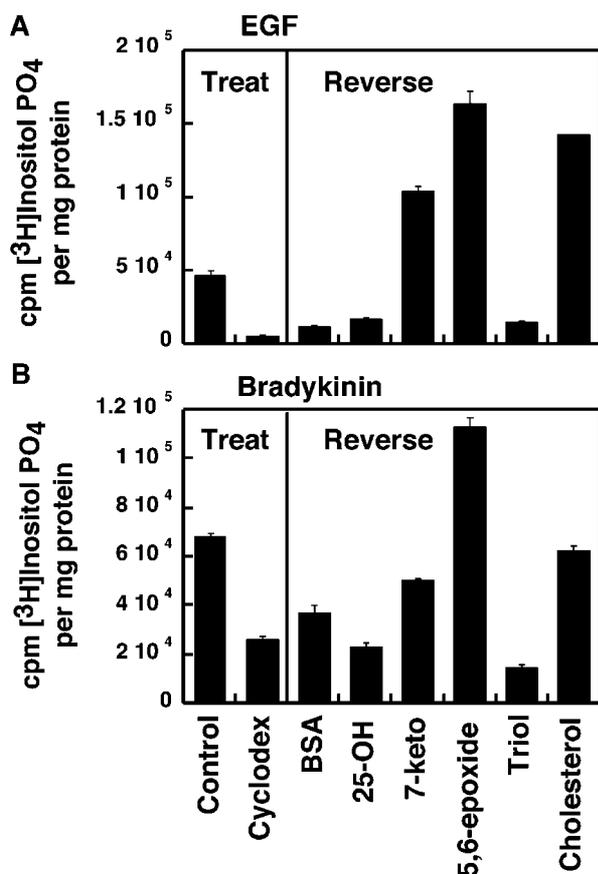


FIG. 6. Reversal of cyclodextrin-induced inhibition of PtdIns turnover by oxysterols. A431 cells were labeled for 48 h with [³H]inositol and then switched to inositol-free DMEM in the absence or presence of 5 mM methyl- β -cyclodextrin. Cultures were incubated for 30 min at 37 °C. One control culture and one cyclodextrin-treated culture were stimulated immediately with vehicle, 50 nM EGF (*top panel*) or 10 μ M bradykinin (*bottom panel*) and inositol phosphates isolated. The remaining cyclodextrin-treated cultures were switched to inositol-free DMEM containing 1 mg/ml bovine serum albumin in the absence or presence of a 0.2 mM concentration of the indicated oxysterols in complex with methyl- β -cyclodextrin. Cultures were incubated for 15 min at 37 °C, stimulated with or without bradykinin, and inositol phosphates isolated. Results represent the mean \pm S.D. of triplicate determinations.

trin complexes (Fig. 8). Approximately two thirds of the PtdInsP₂ originally present in the low density domain was lost after treatment of cells with cyclodextrin. However, after removal of the cyclodextrin and incubation with cholesterol-methyl- β -cyclodextrin complexes, PtdInsP₂ was re-compartmentalized to the low density domains, and the level of this lipid in the caveolin-enriched fraction was similar to that seen in untreated cells. Neither cyclodextrin treatment nor treatment with cholesterol-methyl- β -cyclodextrin complexes significantly affected the fraction of PtdIns that was recovered in the low density domains (not shown).

DISCUSSION

The data presented here demonstrate that hormone-stimulated PtdIns turnover requires appropriate levels of cholesterol in cells. Removal of as little as 20% of the cellular cholesterol led to an ~50% loss in EGF- or bradykinin-stimulated PtdIns turnover. Additional extraction of cholesterol from the cells resulted in a further loss of hormone-stimulated inositol phosphate production. These data suggest that this biological response is dependent on the presence of cholesterol in cell membranes.

The cholesterol dependence of hormone-stimulated PtdIns

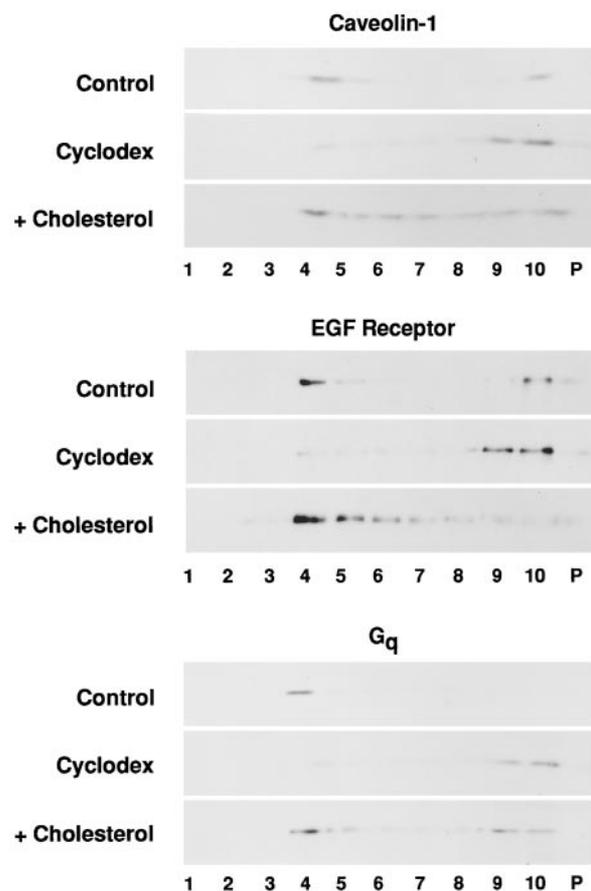


FIG. 7. Cholesterol-methyl- β -cyclodextrin complexes reverse the effect of cyclodextrin on the partitioning of proteins into low density domains. A431 cells in 150-mm dishes were treated in the absence (*Control*) or presence (*Cyclodextrin* and *+Cholesterol*) of 5 mM methyl- β -cyclodextrin for 30 min at 37 °C. Subsequently, the medium was removed and replaced with inositol-free DMEM containing 1 mg/ml bovine serum albumin in the absence (*Control* and *Cyclodextrin*) or presence (*+Cholesterol*) of 0.2 mM cholesterol in complex with methyl- β -cyclodextrin. After 30 min at 37 °C, cultures were washed with ice-cold PBS, lysed, and analyzed by sucrose density gradient centrifugation as described under "Experimental Procedures." Equal aliquots of each fraction were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting for caveolin, EGF receptor, or G_q.

hydrolysis may be caused by the localization of this signaling pathway to cholesterol-enriched membrane domains. We have shown previously that the hormone-sensitive pool of PtdInsP₂ is localized in a caveolin-enriched membrane fraction that also contains EGF receptors and G_q (23).² These domains typically contain high levels of cholesterol and glycosphingolipids (7). We show here that the inhibition of PtdIns turnover that results from depletion of cell cholesterol is associated with a decrease in the compartmentalization of PtdInsP₂ as well as caveolin, EGF receptors, and G_q. Treatment with cyclodextrin resulted in the loss of all of these components from the low density fraction and their recovery in the high density portion of the sucrose gradients. These data suggest that the removal of cholesterol disrupts the integrity of the low density domains. This results in the delocalization of these molecules and the inhibition of hormone-stimulated PtdIns turnover. Bradykinin- and EGF-stimulated PtdIns turnover was inhibited over the same dose range of cyclodextrin and showed a similar time course of inhibition at a fixed concentration of cyclodextrin. While these data suggest that cyclodextrin treatment affects a component that is common to both pathways, it is not clear from these data whether the decrease in hormone-stimulated PtdIns turnover is due to the loss of compartmentalization of

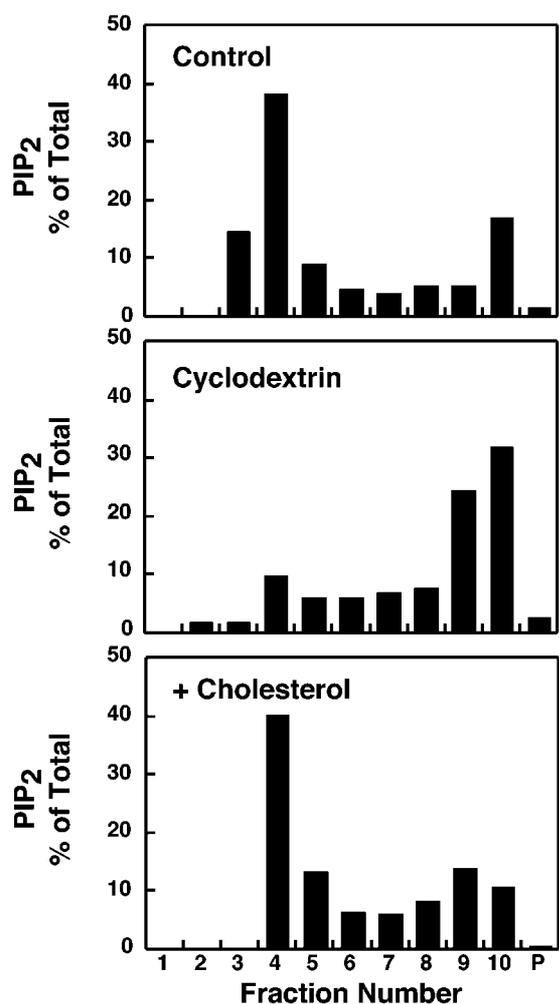


FIG. 8. Cholesterol-methyl- β -cyclodextrin complexes reverse the effect of cyclodextrin on the partitioning of PtdInsP₂ into low density domains. A431 cells in 150-mm dishes were labeled for 48 h with [³H]inositol. The labeling medium was removed and replaced with inositol-free DMEM in the absence (*Control*) or presence (*Cyclodextrin* and *+Cholesterol*) of 5 mM cyclodextrin. Cultures were incubated for 30 min at 37 °C. Subsequently, the medium was removed and replaced with inositol-free DMEM containing 1 mg/ml bovine serum albumin in the absence (*Control* and *Cyclodextrin*) or presence (*+Cholesterol*) of 0.2 mM cholesterol in complex with methyl- β -cyclodextrin. After 30 min at 37 °C, cultures were washed with ice-cold PBS, lysed, and analyzed by sucrose density gradient centrifugation. Inositol phospholipids were extracted from each fraction, analyzed, and quantitated as described under "Experimental Procedures."

PtdInsP₂, the delocalization of EGF receptors and G_q, or a combination of these events.

The association between domain integrity and hormone-stimulated PtdIns turnover is strengthened further by the results of the experiments in which cholesterol was replaced in cyclodextrin-treated cells. Replenishing cellular cholesterol resulted in the re-formation of the caveolin-enriched membrane domains and the relocalization of PtdInsP₂, EGF receptors, and G_q to the low density membrane fraction. Hormone-stimulated PtdIns turnover was restored concomitant with the re-formation of the low density domains. These data are consistent with the conclusion that the localization of PtdInsP₂ and signaling proteins to cholesterol-enriched domains is required for hormone-stimulated PtdIns turnover. Disruption of the domains results in loss of hormonal responsiveness, while restoration of the domains is associated with the reinstatement of hormone effects. Because the effects of cyclodextrin can be reversed by the replacement of cholesterol, these results also suggest that

the effect of cyclodextrin is due to its capacity to remove cholesterol from cells rather than by some other activity of this compound.

Cholesterol replacement in cyclodextrin-treated cells consistently resulted in a marked increase in the ability of EGF to stimulate PtdIns hydrolysis compared with control, untreated cells. Although bradykinin-stimulated PtdIns turnover was also increased after cholesterol replacement, the enhancement was significantly less than that seen for EGF-induced PtdIns turnover. The generally greater ability of cholesterol and oxysterols to restore EGF-stimulated compared with bradykinin-stimulated PtdIns turnover suggests that sterols may have effects on the EGF pathway other than simple restoration of low density domain integrity. Cholesterol has been shown to modulate binding to the cholecystokinin and oxytocin receptors by both changes in membrane fluidity and specific molecular interactions (34). Thus, the observed effects of sterols on hormone-stimulated PtdIns turnover may be due not only by their capacity to re-form caveolae/DIGs but also to their ability to induce changes in membrane fluidity or interact directly with receptors or other signaling components.

The observation that some but not all oxysterols are capable of reversing the effects of cyclodextrin is consistent with this possibility. If the hydrophobic/hydrophilic characteristics of the oxysterols were the only consideration in their ability to restore PtdIns turnover, then many of the compounds tested should have exhibited similar restorative properties. The vastly different capacities of the 7-keto, 5,6-epoxide, and 3,5,6-triol derivatives of cholesterol to reverse the effect of cyclodextrin on PtdIns turnover suggest that these compounds may regulate PtdIns hydrolysis by a mechanism other than simple structural replacement of cholesterol in cholesterol-enriched domains.

Previous workers have reported inhibitory effects of cholesterol-reducing agents on signaling pathways. Liu *et al.* (35) reported that filipin reduced the PDGF-stimulated tyrosine phosphorylation of proteins. Similarly, McGuire *et al.* (36) showed that lovastatin inhibited the ability of PDGF to stimulate PtdIns 3-kinase activity and the association of p85 with the PDGF receptor. Cyclodextrin treatment was shown to reduce the affinity of oxytocin for its receptor, and this was associated with a decrease in the ability of this hormone to stimulate inositol phosphate production (29, 34). These findings suggest that many aspects of hormone signaling are dependent on cholesterol and may be mediated through low density, cholesterol-enriched domains.

In summary, the data reported here demonstrate that hormone-stimulated PtdIns turnover is dependent upon the presence of sufficient cholesterol in the cell membrane. The decrease in cellular cholesterol is associated with the loss of compartmentalization of PtdInsP₂, G_q, and the EGF receptor in low density, caveolin-enriched membrane fraction. Replacement of cholesterol leads to the re-formation of these domains, the relocalization of these signaling components, and the reestablishment of hormone-stimulated PtdIns turnover. These findings suggest that hormone-stimulated PtdIns turnover requires the integrity of cholesterol-enriched domains and imply that localization of both protein and lipid components to these low density domains is necessary for the proper function of this signaling system.

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