Hormonal Regulation of Caveolae Internalization

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Abstract. Caveolae undergo a cyclic transition from a flat segment of membrane to a vesicle that then returns to the cell surface. Here we present evidence that this cycle depends on a population of protein kinase C-α (PKC-α) molecules that reside in the caveolae membrane where they phosphorylate a 90-kD protein. This cycle can be interrupted by treatment of the cells with phorbol-12,13-dibutyrate or agents that raise the concentration of diacylglycerol in the cell. Each of these conditions displaces PKC-α from caveolae, inhibits the phosphorylation of the 90-kD protein, and prevents internalization. Caveolae also contain a protein phosphatase that dephosphorylates the 90-kD once PKC-α is gone. A similar dissociation of PKC-α from caveolae and inhibition of invagination was observed when cells were treated with histamine. This effect was blocked by pyrilamine but not cimetidine, indicating the involvement of histamine H_1 receptors. These findings suggest that the caveolae internalization cycle is hormonally regulated.

THE caveola (47) is a membrane domain specialized for transcytosis (27) and endocytosis (3). The internalization process occurs in a cycle that begins with membrane invagination. The invaginated membrane then closes to form a compartment designated a plasmalemmal vesicle (27). Eventually the plasmalemmal vesicle returns to the cell surface to complete the cycle. The glycosyl phosphatidylinositol (GPI)[1]-anchored folate receptor has provided important clues to the dynamics of caveolae internalization (18–21). In MA104 cells, this receptor is internalized by caveolae and returns to the cell surface in ~1 h. Throughout this period, a constant number (50%) of receptors are maintained on the cell surface with an equal number remaining inside.

We have found the caveolae cycle to be influenced by three conditions. First, culturing cells in the absence of a source of cholesterol markedly reduces the number of caveolae and inhibits folate receptor internalization (6). Second, treatment of cells with phorbol esters prevents the invagination of caveolae without affecting the return of plasmalemmal vesicles to the cell surface (41). Third, elevation of cellular arachidonate levels with indomethacin blocks both plasmalemmal vesicle formation and the return of existing vesicles to the surface (40). These studies suggest that cholesterol is required for the structural integrity of caveolae, but that there is a set of resident caveolae molecules in control of the internalization cycle.

The internalization of the folate receptor by caveolae is an essential step in the potocytosis of 5-methyltetrahydrofolate (20, 31, 41). Recent biochemical and immunoelectron microscopic evidence now supports the view that caveolae also are important sites for compartmentalizing a variety of cell-signaling activities (1–3). Caveolae appear to be enriched in many different types of signaling molecules. These include: (*a*) GPI-anchored hormone receptors (44); (*b*) inositol 1,4,5-trisphosphate receptors (13); (*c*) protein kinase C (PKC) (24); (*d*) lipid second messenger molecules such as sphingomyelin (4) and gangliosides (28, 46); (*e*) G protein–coupled membrane receptors (8, 10, 30); (*f*) multiple heterotrimeric GTP-binding proteins (7, 24); (*g*) nonreceptor tyrosine kinases (24, 38); and (*h*) an ATP-dependent Ca^{2+} pump (12). None of these molecules is found exclusively in caveolae, which suggests that they have a special function in this location that cannot be performed elsewhere in the cell.

Signal transduction in caveolae could be regulated by controlling the internalization cycle (1). The recent finding that PMA inhibits invagination (41) while the phosphatase inhibitor okadaic acid prevents the return of internalized vesicles to the cell surface (29) has focused our attention on PKC as a possible regulator. We now present morphological and biochemical evidence that caveolae contain a resident population of PKC-α. PKC-α phosphorylates a 90-kD protein in caveolae. Phorbol-12,13-dibutyrate (PDBu) and AlF_4 prevent folate receptor internalization and at the same time stimulate the loss of PKC-α from caveolae. In the absence of the kinase the 90-kD protein becomes dephosphorylated. Finally, stimulation of histamine H_1 re-

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1. *Abbreviations used in this paper:* CHX, cycloheximide; DAG, diacylglycerol; GPI, glycosyl phosphatidylinositol; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; RACK, receptor for activated C kinase.

ceptors blocks internalization while causing the loss of PKC-α from caveolae.

Materials and Methods

Materials

Medium 199 with Earle's salts minus folic acid was prepared by standard methods (18). FCS was from Hazleton Research Products, Inc. (Lenexa, KS). Glutamine, trypsin-EDTA, and penicillin/streptomycin were from GIBCO-BRL (Gaithersburg, MD). The analytical silica gel TLC plates and the following solvents were from J. T. Baker, Inc. (Phillipsburg, NJ): heptane, petroleum ether, ethyl ether, acetic acid, and 2-propanol. Radiolabeled adenosine-5-triphosphate and orthophosphate were obtained from DuPont Co. (Wilmington, DE) with specific activities of ~6,000 and 9,000 Ci/mmol, respectively. The sulfuric-dichromate spray was from Supelco Inc. (Bellefonte, PA). Percoll was from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). [^{3}H]folic acid (sp act 27 Ci/mmol) was purchased from Moravek Biochemicals Inc. (City of Industry, CA). All PKC activators and inhibitors were purchased from Calbiochem-Novabiochem (San Diego, CA). Antibodies were obtained from the following sources: anti-caveolin IgG (mAb 2234) was a gift from Dr. John Glenney (Glentech, Inc., Lexington, KY); anti-clathrin IgG (mAb X-22) was a gift from Dr. Francis Brodsky (University of California, San Francisco); goat anti-mouse IgG conjugated to peroxidase was from Organon Teknika (West Chester, PA); anti-PKC-α IgG (mAb) and anti-receptor for activated C kinase (RACK)1 IgG (mAb) were from Transduction Laboratories (Lexington, KY); goat anti-mouse IgG (b2-6500) and gold-conjugated rabbit anti-goat IgG were from Sigma Chemical Co., St. Louis, MO. [^{3}H]acetate (4.13 Ci/mmol) was obtained from DuPont Co. Poly-L-lysine (P-1524) was from Sigma Chemical Co.

Methods

Cell Culture. The monkey kidney epithelial cell line, MA104, was grown as a monolayer in folate-free medium 199 supplemented with 5% (vol/vol) FCS and 100 U/ml penicillin/streptomycin. Cells for each experiment were set up according to a standard format. On day 0 cells were seeded into T-25 (1.5×10^5 cells) or T-75 (3×10^5 cells) culture flasks and cultured for 5 d without further feeding. For folate-binding studies, the medium was replaced with folate-free M199 containing 20 mM Hepes (pH 7.4) without serum and additions were made directly to the culture flasks.

Caveolae Isolation. The caveolae isolation procedure was a modification of the method developed by Sargiacomo et al. (37). Five T-75 flasks of confluent cells (a total 10 mg protein) were used for each sucrose gradient. Each flask was washed briefly with PBS at room temperature and the cells collected by scraping with 5 ml of ice-cold PBS. All subsequent steps were carried out at 4°C. The cells were pelleted in a table-top centrifuge for 5 min at 1,400 *g*. Cells were resuspended with a pipette tip in 1.0 ml lysis buffer (0.25 M sucrose, 5 mM Hepes, pH 7.4). The samples were then dounced 15 times in a 1-ml tight Dounce homogenizer. The suspension was transferred to a 1.5-ml tube and centrifuged at 1,000 *g* for 10 min. The supernatant fractions were removed and stored on ice. The pellets were suspended in 1.0 ml lysis buffer, dounced, and centrifuged as described above. The supernatant fractions were combined and layered on top of 23 ml of a 30% Percoll solution (in lysis buffer). The cell lysates were then centrifuged at 84,000 *g* for 30 min, in a Ti60 rotor (Ti60; Beckman Instruments, Inc., Fullerton, CA). The plasma membranes appeared as a visible band ~5.7 cm from the bottom of the Ti60 bottle. The liquid above the plasma membranes was removed and the membranes collected with a Pasteur pipette. The volume of the plasma membranes was adjusted to 2.0 ml with lysis buffer and 200 ml 10% Triton X-100 was added to each sample giving a final Triton X-100 concentration of 1% and protein concentration of 0.6 mg. The mixtures were gently inverted several times and incubated on ice for 30 min. Linear sucrose gradients (10–30%) were prepared in 10 mM Hepes (pH 7.4). The solubilized membranes (vol, 2 ml) were layered on top of the gradients (vol, 11 ml). The samples were then centrifuged at 143,000 *g* for 18–20 h. At the end of the centrifugation, the gradients were fractionated in 0.65-ml fractions and analyzed.

Membrane Cholesterol Measurement. TLC was carried out as previously described (43). Five confluent T-75 flasks of MA104 cells were used for each sample. 75 μCi of [^{3}H]acetate was added to the culture media on day 4 and cells were harvested on day 5. The cells were then washed extensively in PBS and placed in 3 ml of buffer (DMEM, 20 mM Hepes, pH 7.4). The caveolae were then isolated on sucrose gradients. Each gradient fraction was adjusted to 1 ml final vol with PBS and adjusted to 30% taurodeoxycholate. This sample was then mixed with 2 ml Dole reagent (78: 20:2; 2-propanol/heptane/water) plus 1 ml of heptane. The samples were vortexed and spun in a table-top centrifuge for 10 min (3,000 *g*). The heptane phase (upper) contained neutral lipids and was used for TLC. The aqueous phase (bottom) contained proteins and was used for immunoblots. The heptane phase was dried under N_2 and suspended in 50 μl of the solvent system (80:20:1; petroleum ether/ethyl ether/acetic acid). To determine the position of cholesterol after chromatography, 5 μg unlabeled cholesterol was dissolved in the same solvent system and run as a standard. 1 μg unlabeled cholesterol was also added to each fraction from the gradient. The lipids were spotted onto a silica gel G plate and developed in the solvent system. Lipids were visualized by charring with sulfuric acid–dichromate followed by heating at 180°C for 10 min. The appropriate spots were scraped and the amount of radioactivity was measured using liquid scintillation counting.

Electrophoresis and Immunoblots. Protein concentrations were determined by D.C. assay (Bio-Rad Laboratories, Hercules, CA). Proteins were concentrated by TCA precipitations and washed in acetone. Pellets were suspended in Laemmli sample buffer and heated at 95°C for 3 min before being loaded onto gels. Proteins were separated in a 12.5% SDS–polyacrylamide gel by the method of Laemmli (23). Proteins were then transferred to nylon membrane. The membrane was blocked in TBST (20 mM Tris, pH 7.6; 137 mM NaCl; 0.5% Tween-20) plus 5% dry milk for 1 h at room temperature. Primary antibodies were diluted in TBST + 1% dry milk and added to the incubation mixture for 1 h at room temperature. After the primary antibody incubation, the membrane was washed four times, 10 min each in TBST + 1% dry milk. The appropriate secondary antibody conjugated to HRP was diluted 1/30,000 and added to the incubation mixture for 1 h at room temperature. The membrane was then washed and processed to visualize reactive proteins by the enhanced chemiluminescence method.

Alkaline Phosphatase Enzymatic Assay. A piece of nylon membrane was soaked in methanol for 1 min and then washed twice in PBS. 10 μl of each fraction from the gradient was spotted on the membrane. The membrane was washed once in PBS and developed using an alkaline phosphatase substrate kit from Bio-Rad Laboratories. To 50 ml of alkaline phosphatase color development buffer we added 500 μl each of substrates A and B. After mixing, we incubated the membrane in this reagent for 5–30 min. The reaction was stopped by washing the membrane with distilled water.

Folate Receptor Internalization. The [^{3}H]folic acid–binding assay was used to measure internal and external folate receptors as previously described (21). MA104 cells were subjected to the indicated treatments and then incubated in the presence of 5 nM [^{3}H]folic acid (sp act 27 Ci/mmol) for 1 h at 37°C. External [^{3}H]folic acid corresponded to the amount released when cells were incubated on ice for 30 s in the presence of acid saline (0.15 M NaCl, adjusted to pH 3.0 with glacial acetic acid). Internal folate was the amount of [^{3}H]folic acid that remained associated with the acid-saline-treated cells. The latter was collected by adding 0.1 N NaOH to the flask to dissolve the cells. Radioactivity was measured by liquid scintillation counting using a liquid scintillation analyzer (Tri-carb 1900A; Packard Instruments Co., Inc., Downers Grove, IL). Nonspecific binding, which was measured by adding 100-fold excess unlabeled folic acid, was <5% of specific binding.

Phosphorylation Assay. For the in vivo phosphorylation assays, MA104 cells were grown under standard conditions. Five T-75 flasks were used for each sample. Each flask was washed three times with 10 ml of Krebs-Ringer bicarbonate, pH 7.4 (KRB). Cells were then incubated in 20 ml of KRB for 60 min, 37°C. The KRB was removed and 6 ml fresh KRB containing 1 mCi of [^{32}P]orthophosphate (sp act 9,000 Ci/mmol) was added to each flask and the cells were incubated for 60 min at 37°C. The cells were then subjected to the indicated treatments. At the end of the treatment, the flasks were washed three times with KRB and the plasma membrane caveolae isolated. The proteins were resolved by SDS-PAGE and the radiolabeled proteins visualized by autoradiography.

For the in vitro phosphorylation assays, caveolae were prepared by standard methods (fractions 5, 6, and 7) on sucrose gradients and the following components were added: 20 mM Hepes, pH 7.4; 5 mM $MgCl_2$; 1 mM $MnCl_2$; 10 mM unlabeled ATP; 10 μCi [γ-^{32}P]ATP (0.13 μM, sp act 6,000 Ci/mmol). The mixture was then incubated for 15 min at room temperature. The reaction was stopped by TCA precipitation. Radiolabeled proteins were detected by autoradiography after electrophoresis in 12.5% polyacrylamide gels.

Electron Microscopy. Immunogold localization of folate receptors was carried out as previously described (35). To localize caveolin, clathrin, and PKC-α, we used the whole mount, plasma membrane preparation of Sanan and Anderson (36). The grids with adherent membranes were incubated 30 min each with the indicated primary antibodies (mAb 2234 diluted 1/100; anti–PKC-α IgG diluted 1/100; mAb x-22 at 1 μg/ml), followed by 50 μg/ml of goat anti–mouse IgG, and finally a 1:30 dilution of gold-conjugated rabbit anti–goat IgG. All the antibodies were diluted in PBS containing 0.15% BSA. The grids were washed after each incubation three times for 30 min in this mixture. After a final wash, grids were fixed with 2.5% glutaraldehyde in PBS for 10 min followed by 1% osmium tetroxide in PBS for 10 min. The grids were stained sequentially for 10 min each with 1% tannic acid, 1% uranyl acetate. All samples were examined and photographed with an electron microscope (100 CX; JEOL U.S.A. Inc., Peabody, MA).

Results

In the current studies we modified the procedure of Sargiacomo et al. (37) to isolate caveolae from plasma membranes purified on Percoll gradients (15). Caveolae were isolated by separating the Triton X-100–insoluble and –soluble components of these membranes on a 10–30% sucrose gradient. Each fraction was assayed for the presence of three molecules that previously have been shown by morphological methods to be enriched in invaginated caveolae (Fig. 1): the 22-kD integral membrane protein, caveolin (34); the GPI-anchored membrane protein, alkaline phosphatase (17, 48); and cholesterol (39). Fractions 5, 6, and 7 contained all of the detectable caveolin (*Immunoblot*, Fig. 1) but only 1% of the total protein loaded on the gradient. These same fractions were enriched in alkaline phosphatase enzymatic activity (*A.P.*, Fig. 1). Soluble activity was also present in fractions 1, 2, and 3, which contained 95% of the protein. Fractions 5, 6, and 7 contained 6% of the total plasma membrane cholesterol (■, Fig. 1), which is an approximately sixfold enrichment relative to the plasma membrane.

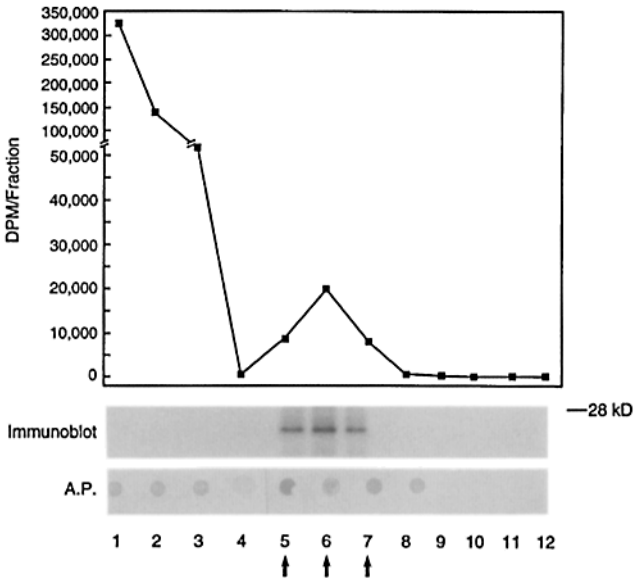

Figure 1. Characterization of Triton X-100–soluble and –insoluble plasma membrane. Plasma membranes were isolated from MA104 cells using a 30% Percoll gradient and then incubated with 1% Triton X-100 at 4°C. The sample was layered on the top of a 10–30% sucrose gradient (top is at the left) and soluble (fractions *1–3*) and insoluble material (fractions *5–7*) was separated by centrifugation. The gradient was fractionated and the concentration of cholesterol (■), caveolin (*Immunoblot*), and alkaline phosphatase (*A.P.*) was measured in each fraction as described. Typically the caveolae-rich fractions were 5, 6, and 7 (*arrows*). These fractions contained <1% of the total protein loaded on the gradient.

PKC-α Is in Caveolae

We used immunoblotting to determine if the caveolae fractions contained PKC-α. Equal amounts of protein (10 μg) from each fraction, plus an aliquot (10 μg) of the whole plasma membrane (lane *P*, Fig. 2) and the cytosol (lane *C*, Fig. 2), were separated by SDS-PAGE. Each sample was then immunoblotted either with anti-caveolin IgG (*anti-caveolin*, Fig. 2) or anti–PKC-α IgG (*anti-PKCα*, Fig. 2). The concentration of caveolin in the plasma membrane sample was not high enough to detect (lane *P*, Fig. 2) but it was easily visualized in the caveolae fractions 5, 6, and 7. The PKC-α concentration in whole plasma membranes was also too low to detect (*anti-PKCα*, lane *P*, Fig. 2). By contrast, there was a very strong signal in each caveolae fraction (*anti-PKCα*, lanes *5*, *6*, and *7*, Fig. 2). PKC-α was also detected in the sample of cytoplasm (lane *C*, Fig. 2), consistent with it being an abundant cytosolic protein (16). Therefore, the only place in the plasma membrane where we detected PKC-α was in the caveolae fractions.

To rule out the possibility that PKC-α was a contaminant of the caveolae fractions, we used immunogold to localize the kinase in isolated plasma membranes. The flattened morphology of normal human fibroblasts make them better suited for this analysis than the MA104 cell. Therefore, the upper membrane surface of these cells was attached to poly-L-lysine–coated electron microscope grids by the method of Sanan and Anderson (36) and processed to localize either caveolin (*a–d*, Fig. 3) or PKC-α (*b* and *c*, Fig. 3). Anti-caveolin IgG gold was exclusively associated with numerous dense regions of membrane that had the general morphology of invaginated caveolae (Fig. 3 *a*). Structures that labeled with anti–PKC-α IgG gold had exactly the same morphology (Fig. 3 *b*). Clathrin-coated pits did not label with either one of these antibodies (*arrows*, Fig. 3, *b* and *d*). Double labeling with a rabbit anti-caveolin IgG (5 nm gold) and a mouse anti-PKC IgG (15 nm gold) showed that the two antigens were both present in the same membrane structure (Fig. 3 *c*). Only the anti-caveolin IgG–gold (5 nm) labeling was seen when a non-

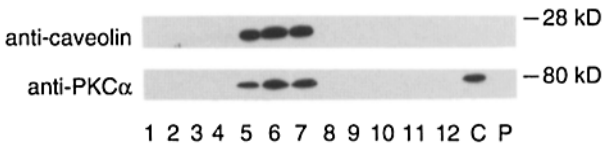

Figure 2. Localization of PKC-α in plasma membranes by immunoblotting. MA104 cell plasma membranes were fractionated as described in Fig. 1. Equal amounts of protein from each fraction (*1–12*) plus samples of cytoplasm (*C*) and plasma membrane (*P*) were separated by electrophoresis and immunoblotted with either anti-caveolin IgG (*anti-caveolin*) or anti–PKC-α IgG (*anti-PKC-α*).

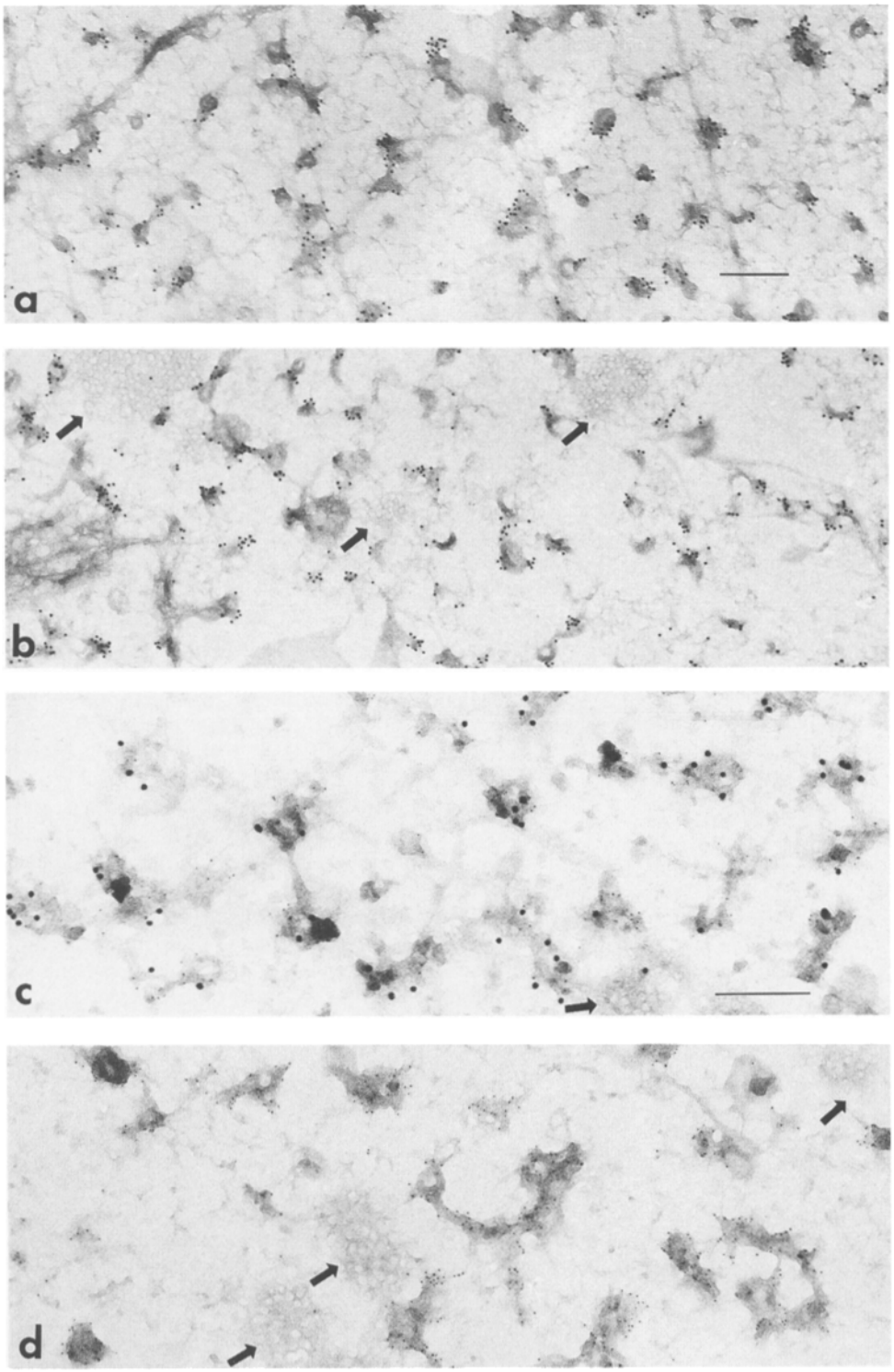

Figure 3. Localization of PKC-α in plasma membranes by immunogold. Whole plasma membranes from human fibroblasts were attached to Formvar-coated electron microscopy grids and immunogold stained using either (*a*) mAb anti-caveolin IgG (15 nm gold), (*b*) mAb anti–PKC-α IgG (15 nm gold), (*c*) rabbit anti-caveolin IgG (5 nm gold) plus mouse anti-PKC (15 nm gold), or (*d*) rabbit anti-caveolin IgG (5 nm gold) plus nonimmune mouse IgG (15 nm gold). Arrows indicate the location of clathrin-coated pits. *a* and *b* are the same magnification and *c* and *d* are the same magnification. Bars, 0.45 μm.

immune mouse IgG was substituted for the anti-PKC IgG (Fig. 3 *d*).

Inhibitors of Caveolae Internalization Displace PKC-α from Caveolae

Fig. 4 shows the effect that PDBu has on folate receptor internalization and on the presence of PKC-α in caveolae. Cells were incubated in the presence (*PDBu*, Fig. 4 *A*) or absence (*NT*, Fig. 4 *A*) of 1 μM PDBu for 1 h before measuring the internal (*R*) to external (*L*) folate receptor ratio. PDBu caused a 70% decline in this ratio by preventing receptor internalization. After we removed the drug and incubated the cells an additional 3.5 h (*wash-out*, Fig. 4 *A*), the receptor ratio returned to control values. Triton X-100–treated plasma membranes from a replicate set of cells were then fractionated on a sucrose gradient and each fraction was blotted with an anti–PKC-α IgG (Fig. 4 *B*).

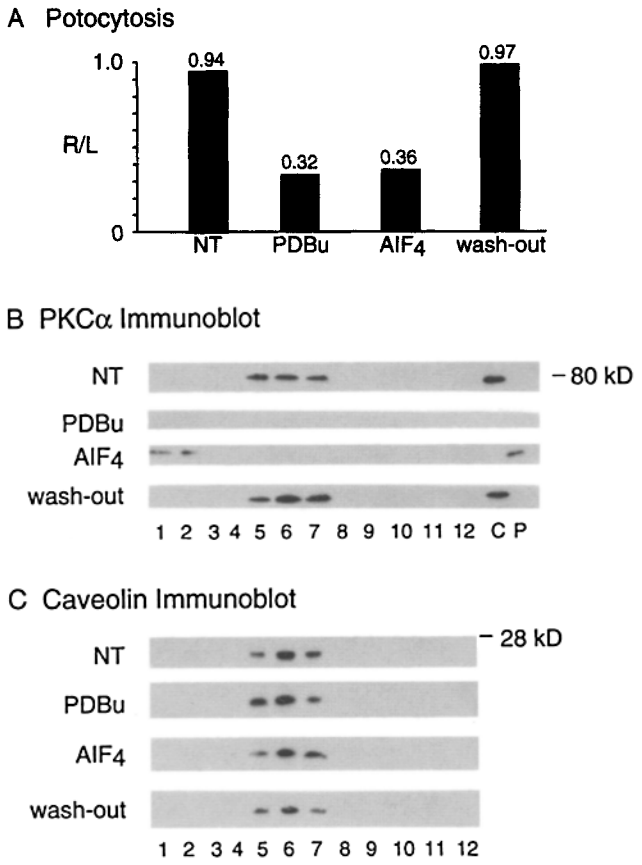

Figure 4. The effect of PDBu and AlF_4 on potocytosis (*A*) and the distribution of either PKC-α (*B*) or caveolin (*C*) in the plasma membrane. Either potocytosis was measured (*A*) or fractions of Triton X-100–treated plasma membranes from the sucrose gradients were immunoblotted (*B* and *C*) in cells incubated in the presence of media alone (*NT*); 1 μM PDBu (*PDBu*); 10 μM $AlCl_3$ plus 10 mM NaF (*AlF_4*); or PDBu followed by 3.5 h in the absence of the drug (*wash-out*). For the immunoblots, each lane contained an equal amount of protein (10 μg/ml). Each lane corresponds to the indicated fraction number and lanes *C* and *P* (*B*) are equal protein loads of cytoplasm and plasma membrane, respectively.

Nontreated cells had a prominent PKC-α band in the caveolae fractions (*NT*, *5*, *6*, and *7*, Fig. 4 *B*) and in the sample of cytosol (*C*, Fig. 4 *B*). PDBu-treated cells (*PDBu*, Fig. 4 *B*), by contrast, did not have detectable PKC-α in any of the fractions, indicating that it was no longer associated with the plasma membrane. The PKC-α returned to the caveolae fraction after the drug was removed (*wash-out*, Fig. 4 *B*). The distribution of caveolin in the sucrose gradient was not altered in PDBu-treated cells (Fig. 4 *C*).

While screening agents that affect caveolae internalization, we found that AlF_4 was also a potent inhibitor of internalization. Fig. 4 (*AlF_4*, Fig. 4 *A*) shows that AlF_4, a general activator of heterotrimeric GTP-binding proteins (5), was as effective as PDBu at blocking folate receptor internalization. This treatment also stimulated the loss of PKC-α from the caveolae fractions (*AlF_4*, *5*, *6*, and *7*, Fig. 4 *C*). Unlike PDBu, however, there was an increase in the amount of PKC-α bound to the plasma membrane (compare P in control with P in AlF_4, Fig. 4 *B*). All of the additional PKC-α was in the soluble material at the top of the gradient (*AlF_4*, *1* and *2*, Fig. 4 *B*). The distribution of caveolin was not affected (compare NT with AlF_4, Fig. 4 *C*). Cholera toxin, which activates the $G\alpha_s$-coupled adenylate cyclase but not the $G\alpha_q$-coupled phospholipase Cβ, did not have any effect on caveolae function (data not shown). Therefore, two conditions that prevent caveolae internalization displace PKC-α from caveolae.

PKC-α Phosphorylates a 90-kD Substrate in Caveolae

We next looked for PKC-α substrates in caveolae. MA104 cells were incubated in the presence of [^{32}P]orthophosphate (1 mCi/ml) for 1 h before fractionating Triton X-100–treated plasma membranes on a sucrose gradient. Autoradiographic analysis of polyacrylamide gels from each fraction showed that the caveolae fractions contained a heavily phosphorylated, 90-kD protein (*NT*, Fig. 5 *A*; see Fig. 7 for an autoradiogram of the whole gel). We did not detect this protein in any other fraction. We could not detect this protein in caveolae fractions if the cells were treated with either PDBu (*PDBu*, Fig. 5 *A*) or AlF_4 (*AlF_4*, Fig. 5 *A*) before processing for ^{32}P incorporation. The subsequent removal of the PDBu from the media resulted in the return of the ^{32}P-labeled band to caveolae (*wash-out*, Fig. 5 *A*).

We prepared isolated caveolae to determine if they could support the phosphorylation of the 90-kD protein in vitro. Cells were incubated under various conditions before we separated Triton X-100–treated plasma membranes on a sucrose gradient (Fig. 5 *B*). We then incubated each fraction in the presence of 10 μCi/ml of [^{32}P]ATP for 15 min. In control cells, autoradiography detected the 90-kD phosphoprotein only in the caveolae fractions (*NT*, Fig. 5 *B*). This phosphoprotein was not seen in caveolae fractions prepared from cells that were exposed to either PDBu (*PDBu*, Fig. 5 *B*) or AlF_4 (*AlF_4*, Fig. 5 *B*). When we removed the PDBu from treated cells, the caveolae regained the ability to phosphorylate the 90-kD protein (*wash-out*, Fig. 5 *B*).

The isolated caveolae were used to verify that the 90-kD protein was a PKC-α substrate (Fig. 5 *C*). Caveolae fractions were prepared from control (*NT*, Fig. 5 *C*) and experimentally treated cells (*PDBu*, *AlF_4*, *wash-out*, Fig. 5 *C*). We then used [^{32}P]ATP to measure the phosphorylation of the 90-kD protein after adding either a PKC-α–specific inhibitor or purified PKC-α to the reaction mixture. Without any additions the 90-kD protein was phosphorylated (*Buffer*, Fig. 5 *C*) in caveolae from control and wash-out cells, but not from cells that had been treated with either PDBu or AlF_4. The addition of the PKC-specific inhibitor peptide (22) completely blocked phosphorylation in caveolae from control cells and wash-out cells (*Inhibitor Peptide*, Fig. 5 *C*). By contrast, the addition of purified PKC α (*PKCα*, Fig. 5 *C*) stimulated the phosphorylation of the 90-kD protein in the caveolae fractions from both PDBu- and AlF_4-treated cells. Thus, the 90-kD protein is a PKC-α substrate that is not displaced from caveolae when cells are incubated in the presence of either PDBu or AlF_4.

Phosphorylation of 90-kD Protein Is Linked to Caveolae Invagination

To link more firmly the activity of PKC-α to caveolae in-

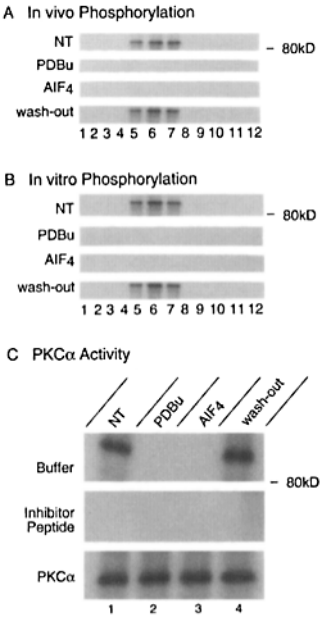

Figure 5. In vivo (*A*) and in vitro (*B* and *C*) phosphorylation of a caveolae substrate. (*A*) Cells were first incubated in the presence of orthophosphate ^{32}P for 1 h and then either not treated (*NT*) or incubated further in the presence of PDBu (*PDBu*), AlF_4 (*AlF_4*), or PDBu followed by 3.5 h in the absence of the drug (*wash-out*). Triton X-100–treated plasma membranes were fractionated on sucrose gradients, separated by electrophoresis, and exposed for autoradiography (2 h). (*B*) Cells were either not treated (*NT*) or incubated in the presence of PDBu (*PDBu*), AlF_4 (*AlF_4*), or PDBu followed by 3.5 h in the absence of the drug (*wash-out*). Membranes were fractionated and each fraction was incubated in the presence of 10 μCi of [^{32}P]ATP for 15 min at room temperature. Each sample was then separated by electrophoresis and analyzed by autoradiography. (*C*) Cells were incubated under the indicated conditions as described in *A* and *B*. The caveolae fractions from the sucrose gradient were collected and incubated in an in vitro phosphorylation assay buffer that contained no additions (*Buffer*), 10 μM PKC-α–specific inhibitor peptide (*Inhibitor Peptide*) or 0.25 U of rat brain PKC-α. Samples were separated by gel electrophoresis and analyzed by autoradiography.

vagination, we had to rule out the possibility that PDBu itself was an inhibitor. Therefore, we prepared PKC-α–deficient MA104 cells. Cells were first exposed to PDBu for 1 h to target the destruction of the kinase (Fig. 4 *B*). The PDBu was then removed and the cells were further incubated for 3.5 h in the presence of cycloheximide (CHX) to prevent the synthesis of PKC-α. PDBu-treated cells were unable to internalize [^{3}H]folic acid (compare NT with PDBu, Fig. 6 *A*) and neither PKC-α (compare NT with PDBu, Fig. 6 *B*) nor the 90-kD phosphoprotein (compare NT with PDBu, Fig. 6 *C*) were detectable in isolated caveolae. The caveolae from PKC-α–deficient cells also did not have any detectable PKC-α (*wash-out + CHX*, Fig. 6 *B*) or phosphoprotein (*wash-out + CHX*, Fig. 6 *C*). These cells were unable to internalize [^{3}H]folic acid (*wash-out + CHX*, Fig. 6 *A*), indicating that inhibition is not due to PDBu alone. CHX by itself had no effect on the association of PKC-α with caveolae or the ability of cells to internalize folate (*CHX*, Fig. 6, *A*, *B*, and *C*). Furthermore, cells washed free of PDBu but not exposed to CHX were normal (*wash-out*, Fig. 6, *A*, *B*, and *C*).

We took advantage of the fact that PKC-α is rapidly degraded when cells are exposed to PDBu (data not shown) to determine if the 90-kD protein is dephosphorylated after PKC-α leaves caveolae. Cells were incubated in the presence of [^{32}P]orthophosphate to label the protein and then PDBu was added to the medium for various times (Fig. 7 *A*). Initially the 90-kD substrate was intensely labeled (time 0, Fig. 7 *A*). With time in the presence of PDBu, however, the intensity of the band declined. After 40 min the band was no longer present even though the [^{32}P]orthophosphate remained in the media throughout the incubation. The time sequence for the complete dephosphorylation of the 90-kD protein corresponded to the time required for internal folate receptors to recycle back to the cell surface during potocytosis (21).

We next used isolated caveolae to determine if they contain a resident phosphatase (Fig. 7 *B*). The caveolae were first incubated in the presence of [^{32}P]ATP to label the PKC-α substrate (see Fig. 5). We added excess unlabeled ATP to the reaction mixture to prevent further [^{32}P]ATP incorporation and incubated the sample for the indicated time (*Control*, Fig. 7 *B*). Initially the 90-kD protein was labeled (*0, control*, Fig. 7 *B*) but the intensity of the band declined rapidly after unlabeled ATP was added. The band was no longer evident after 9 min. The addition of 10 nM okadaic acid, a serine/threonine phosphatase inhibitor, to the reaction mixture inhibited the loss of phosphate from the protein (*Okadaic Acid*, Fig. 7 *B*). These data suggest that caveolae contain a protein phosphatase.

Histamine Regulates Caveolae Invagination

The differential response of caveolae to AlF_4 (an inhibitor) and cholera toxin (no effect) suggested that caveolae invagination might be regulated by a $G\alpha_q$-coupled hormone receptor. The histamine H_1 receptor belongs to this class of receptors. Therefore, we measured the effect of histamine concentration on folate receptor internalization

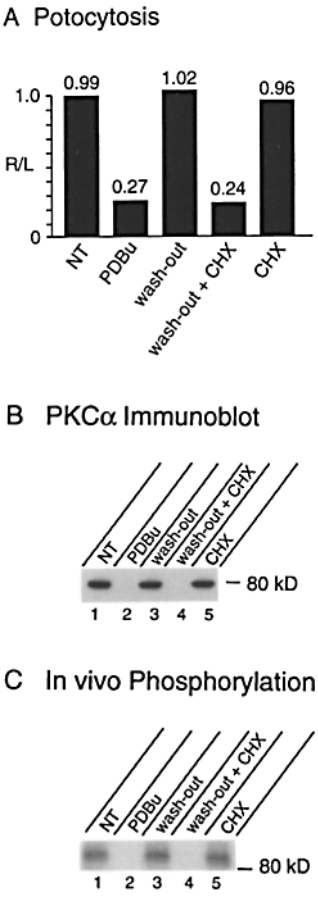

Figure 6. The effect of PKC-α depletion on potocytosis (*A*) and the presence of either PKC-α (*B*) or the 90-kD phosphoprotein (*C*) in caveolae. Either potocytosis was measured (*A*), caveolae fractions were immunoblotted with anti–PKC-α IgG (*B*), or fractions were analyzed for the presence of phosphoproteins (*C*). Cells were incubated in the presence of media alone (*NT*); 1 μM PDBu (*PDBu*); PDBu followed by 3.5 h in the absence of the drug (*wash-out*); PDBu followed by 3.5 h without PDBu in the presence of cycloheximide (*wash-out + CHX*); or 3.5 h in the presence of cycloheximide alone (*CHX*).

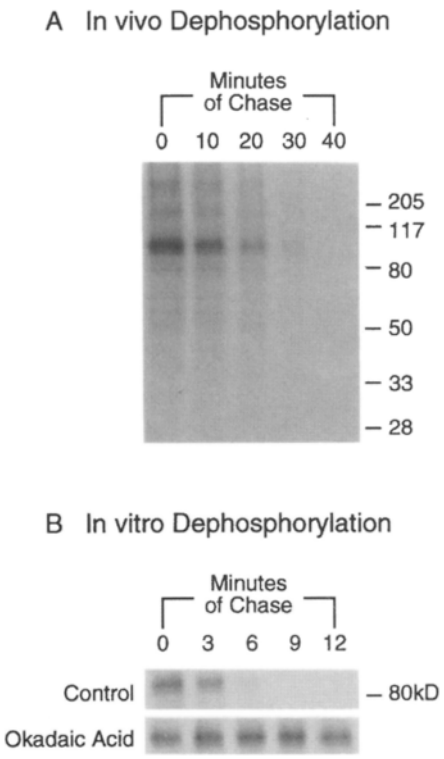

Figure 7. Dephosphorylation of the caveolae, PKC-α substrate in vivo (*A*) and in vitro (*B*). (*A*) Cells were first incubated in the presence of orthophosphate ^{32}P for 1 h to label phosphoproteins. PDBu (1 μM) was added to the dish and the cells were incubated for the indicated time. Caveolae were isolated, the proteins separated by gel electrophoresis, and analyzed by autoradiography. (*B*) Caveolae were isolated from MA104 cells and incubated in the in vitro phosphorylation mixture as described in Fig. 5. A 100-fold excess unlabeled ATP was added to the reaction mixture and each fraction was incubated for the indicated time in the presence (*Okadaic Acid*) or absence (*Control*) of 10 nM okadaic acid. The protein in each sample was separated by gel electrophoresis and analyzed by autoradiography.

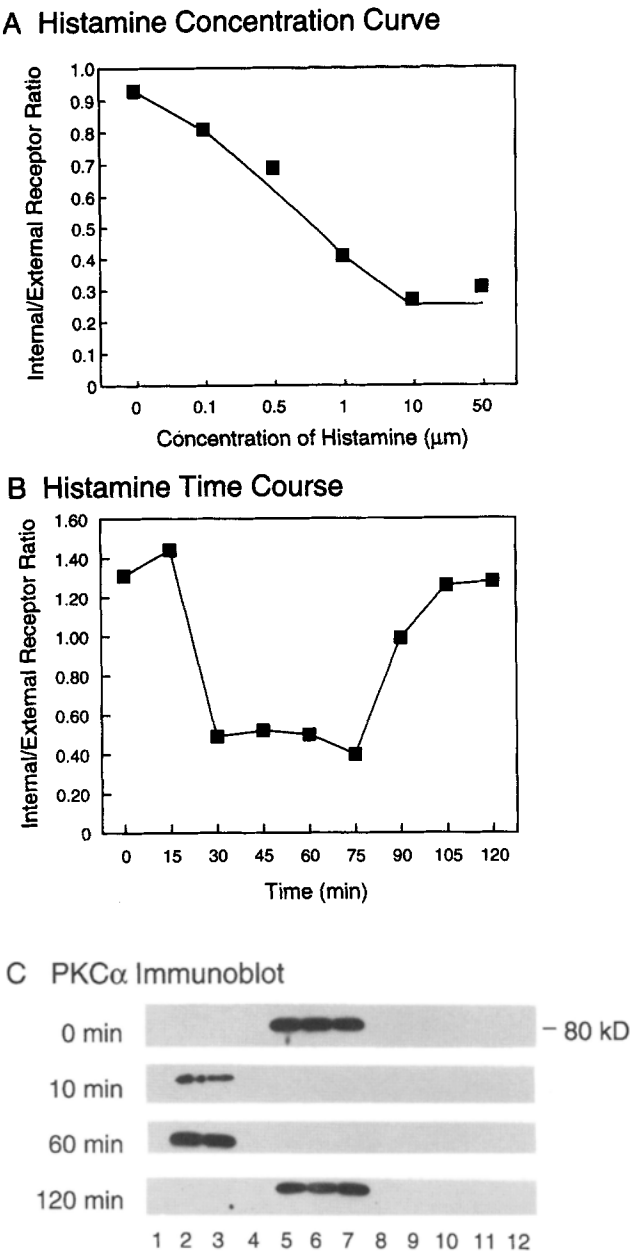

Figure 8. The effects of histamine on potocytosis (*A* and *B*) and the distribution of PKC-α in the plasma membrane (*C*). (*A*) MA104 cells were incubated in the presence of the indicated concentration of histamine for 1 h and the internal to external folate receptor ratio was measured as described. (*B*) Cells were incubated in the presence of 10 μM histamine for the indicated time and the folate receptor ratio was measured. (*C*) Cells were incubated in 10 μM histamine for the indicated time. Plasma membranes were fractionated on sucrose gradients and equal amounts of protein from each fraction (*1–12*) were separated by gel electrophoresis. Each fraction was then immunoblotted with anti–PKC-α IgG.

(Fig. 8). We observed a dose-dependent decline in receptor internalization (Fig. 8 *A*) without any change in the total number of receptors. Maximum inhibition occurred at 10 μM histamine (~70%). This concentration was as effective as either PDBu or AlF_4 (see Fig. 4 *A*). Incubation for 30–45 min in the presence of histamine was required to reach maximum inhibition (Fig. 8 *B*). After 90 min of incubation, the inhibition of receptor internalization began to decline. The receptor internalization returned to normal after 105 min. The loss of inhibition was apparently due to histamine receptor desensitization (45). The histamine H_1 receptor antagonist pyrilamine prevented the inhibitory effects of histamine (Table I) while the H_2-specific blocker cimetidine had no effect. For some reason pyrilamine also caused an increase in the total amount of bound [^{3}H]folic acid (Table I). These results suggest that histamine H_1 receptors can regulate caveolae internalization.

Fig. 8 *C* shows that like PDBu and AlF_4, histamine also caused a loss of PKC-α from caveolae. Initially all of the detectable plasma membrane PKC-α was in the caveolae fractions (*0 min*, Fig. 8 *C*). 10 min after 10 μM histamine was added to cells PKC-α appeared in the Triton X-100–soluble membrane fractions at the top of the gradient. At the same time, the kinase disappeared from the caveolae fractions (*10 min*, Fig. 8 *C*). More PKC-α was detected in the Triton X-100–soluble fractions after a 1-h incubation (*60 min*, Fig. 8 *C*), indicating that additional cytoplasmic PKC-α was recruited to the membrane. The PKC-α returned to the caveolae fractions after 2 h of incubation

Table I. The Effects of Histamine Receptor Antagonists on the Ability of Histamine to Regulate Folate Receptor Sequestration

Treatment	Internal	External	Internal/ external
	(pmol/mg protein)	*(pmol/mg protein)*	
None	1.04	1.08	0.96
Histamine	0.59	1.40	0.42
Histamine + pyrilamine	1.45	1.48	0.98
Histamine + cimetidine	0.61	1.43	0.43

MA104 cells were incubated in the presence of 10 μM histamine in the absence or presence (+) of either 1 μM pyrilamine or 1 μM cimetidine for 1 h and then the internal to external folate receptor ratio was measured as described. Neither pyrilamine nor cimetidine alone had any effect on the the internal to external folate receptor ratio. All values are the average of triplicate measurements.

(*120 min*, Fig. 8 *C*). The distribution of caveolin was not affected by the hormone (data not shown).

Histamine also reduced the number of invaginated caveolae on the surface of MA104 cells (Fig. 9). Cells were subjected to the indicated treatments and then processed for immunogold localization of the folate receptor. Control cells had normal numbers of caveolae (*arrowheads*, Fig. 9 *A* and Table II) and clusters of receptor-specific gold (*arrows*, Fig. 9 *A* and Table II). Cells exposed to histamine for 1 h, by contrast, had few caveolae but the same number of receptor clusters (*arrows*, Fig. 9 *B* and Table II). Normal numbers of invaginated caveolae were present after 2 h of incubation (*arrowheads*, Fig. 9 *C* and Table II). Histamine, therefore, appears to inhibit caveolae invagination by removing PKC-α from caveolae membrane.

Discussion

Three independent lines of evidence support the view that PKC-α is an integral component of the molecular mechanism responsible for caveolae invagination. First, both biochemical and morphological methods show PKC-α to be constitutively present in plasma membrane caveolae. Second, caveolae contain a PKC-α substrate that appears to be phosphorylated and dephosphorylated during the caveolae internalization cycle. Third, three different experimental treatments that cause the loss of PKC-α from caveolae inhibit internalization. We have detected PKC-α in caveolae isolated from normal human fibroblasts, MA104 cells, rat 1 cells, and NIH 3T3 cells (data not shown). In addition, we have found that PMA prevents caveolae invagination in MA104 cells, normal human fibroblasts, and NIH 3T3 cells (41 and data not shown).

PKC-α and the protein phosphatase may be the key regulatory molecules that control the caveolae internalization cycle. Initial invagination may require the phosphorylation of the 90-kD protein by PKC-α. Although we do not know the identity of this substrate, it may be a molecule involved in changing the shape of the plasma membrane. Once the plasmalemmal vesicle has formed, the PKC-α kinase activity declines. The 90-kD phosphoprotein is then dephosphorylated by an okadaic acid–sensitive, resident protein phosphatase. Since okadaic acid appears to block vesicle recycling after caveolae internalize cholera toxin (29), the dephosphorylation of the 90-kD protein may be required for the return of vesicles to the cell surface.

The enzymatic activities of the phosphatase and the kinase appear to operate at opposite ends of the caveolae cycle. Therefore, they are likely targets for regulatory hormones. Histamine appears to be one such hormone. The H_1 receptor is the only member of the seven transmembrane, histamine receptor family that uses $G\alpha_q$ to activate phospholipase C-β (9, 45). Histamine might be acting through H_1 receptors that are in caveolae since $G\alpha_q$ appears to be enriched in this membrane domain (37). The hormone clearly stimulated the recruitment of PKC-α to the plasma membrane (Fig. 8 *C*), which is the expected consequence of an increase in cellular diacylglycerol (DAG) (16). Paradoxically the increase in DAG also stimulated the loss of PKC-α from caveolae. Exactly how DAG can have these opposing effects on the membrane localization of PKC-α is not known.

Caveolae could contain a membrane receptor for PKC-α. A high-affinity membrane-binding site has been identified that binds DAG/Ca^{2+}-activated PKC (RACKs) (26, 33). We immunoblotted fractions from the sucrose gradient with an anti-RACK IgG and observed a strong band of the correct molecular weight in the caveolae fraction (data not shown). The protein was also abundant in the soluble membrane fractions, suggesting that it is in noncaveolae membrane as well. If RACK is the molecule that targets PKC-α to caveolae, then somehow it recognizes endogenous PKC-α. Recently we found that isolated caveolae contain diacylglycerol (25), which could serve to activate a portion of the cytoplasmic PKC-α.

We have proposed that an important function of caveolae is to compartmentalize signal transduction at the cell surface (1). We think that this is an important mechanism for integrating many different sources of spatial and temporal information as the cell responds to an environmental stimulus. An important source of cellular information are the hormone receptors that reside in caveolae. These include receptors for insulin (14), EGF (42), cholecystokinin (32), endothelin (8), and epinephrine (10, 30). Sometimes these receptors are also found at other locations in the same cell. For example, cholecystokinin stimulates the internalization of ~60% of its receptors by coated pits and ~20% by caveolae (32). The signaling activities of these two populations of receptors may be quite different. Furthermore, those signals originating in caveolae have the additional feature that they can be modulated by changing the sequestration state of the receptor–ligand complex. Hormonal inactivation of the protein phosphatase in caveolae, for example, would trap the complex in plasmalemmal vesicles thus potentially preserving the activity of the receptor for extended periods. In some cases, the hormone could act globally on all caveolae in the cell. Other times a neighboring cell might locally release a hormone and only affect a subpopulation of caveolae nearby. Each situation would have a different consequence for cell behavior.

The two markers that are most often used to define caveolae are the characteristic invaginated morphology (47) and the presence of caveolin (34). Recently we showed that in human fibroblasts caveolin is not a permanent structural molecule of caveolae because it can move to the Golgi apparatus without a loss of invaginated caveolae

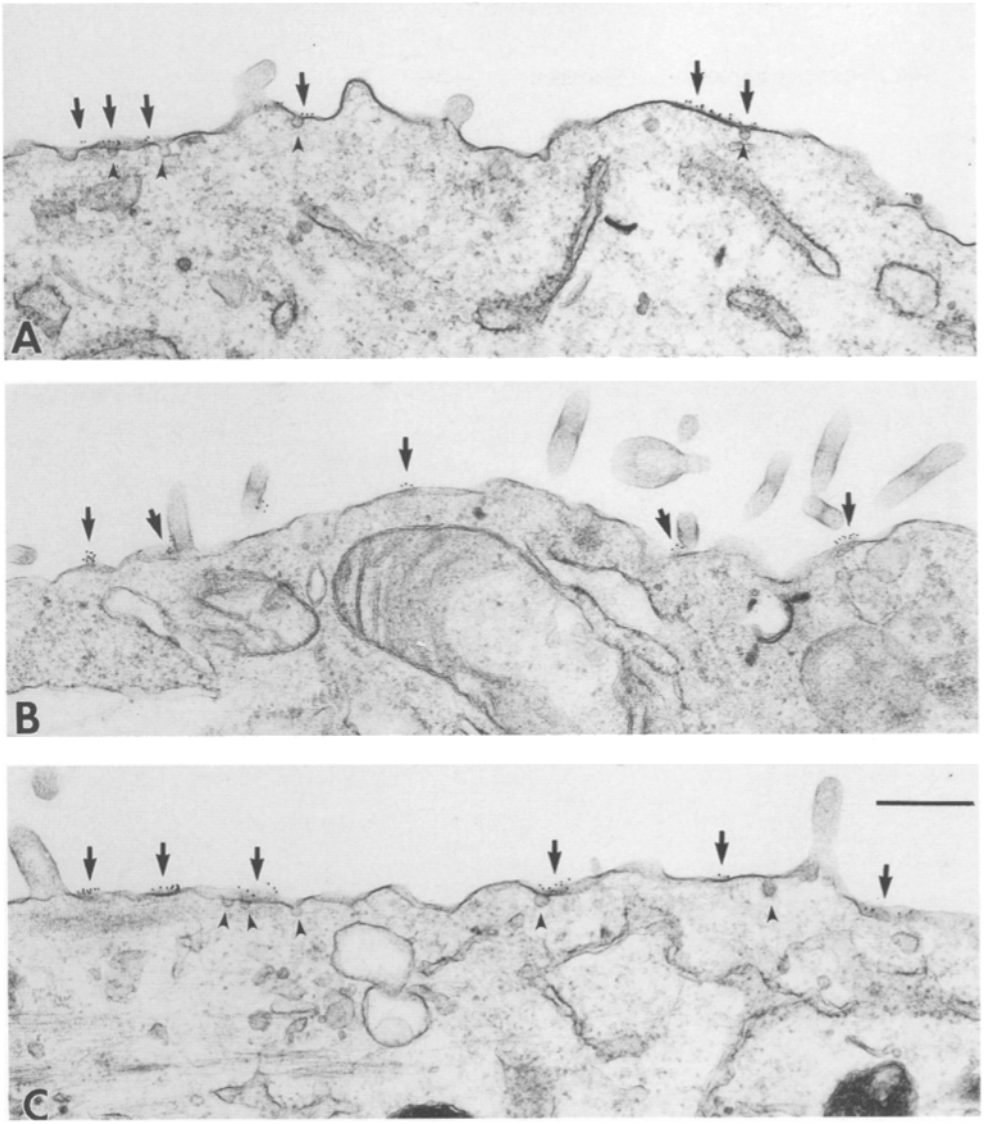

Figure 9. The effects of histamine on the distribution of folate receptors and caveolae in MA104 cells. Cells were either not treated (*A*) or incubated for 1 h (*B*) and 2 h (*C*) in the presence of histamine. The cells were immunolabeled with anti-folate receptor IgG and prepared for electron microscopy. Arrows indicate the position of folate receptor–specific gold clusters on the surface and the arrowheads mark the caveolae. Bar, 0.5 μm.

(43). Rapid-freeze, deep-etch images of the plasma membrane show a characteristic coat structure associated with both invaginated and uninvaginated membrane (34), yet normally all coats with this morphology appear to contain caveolin. This suggests that the flask-shaped morphology is also not an invariant feature of caveolae. Now we have found that the internalization cycle is regulated. This raises the possibility that in many cells caveolae membrane is present but not visible in thin section electron microscopic images because it is not engaged in an internalization cycle. This would explain, for example, why in lymphocytes (11) very few invaginated caveolae can be detected, yet membrane with similar biochemical properties to caveolae can easily be isolated. This suggests that caveolae are a complex membrane domain, defined by a unique lipid and protein composition, with many tissue specific functions. These functions may or may not make use of the internalization cycle.

Table II. The Effects of Histamine in the Number of Invaginated Caveolae and the Density of Anti-folate Receptor IgG

Treatment	No. of clusters	No. of caveolae	No. of labeled caveolae	Gold/labeled caveolae
None	176	131	92	4.27
Histamine 1 h	170	11	9	3.91
Histamine 2 h	173	92	62	4.11

MA104 cells were incubated in the presence of 10 μM histamine for the indicated time. Cells were then processed to immunogold label the folate receptor. Quantitative data was gathered from photographs taken at random of each sample (48).

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