

Protein Kinase C Activators Inhibit Receptor-Mediated Potocytosis by Preventing Internalization of Caveolae

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Abstract. Potocytosis is an endocytic pathway that utilizes glycosylphosphatidylinositol-anchored membrane proteins and caveolae to concentrate and internalize small molecules. We now report that activators of protein kinase C are potent inhibitors of potocytosis. Activators such as phorbol-12-myristate-13-acetate (PMA) inhibit the internalization of receptors for 5-methyltetrahydrofolate but allow the internal receptor pool to return to the cell surface. PMA does not affect

the clustering of the folate receptor but instead markedly reduces the number of caveolae. Exposure to PMA totally blocks the intracellular accumulation of 5-methyltetrahydrofolate without affecting receptor-independent uptake or the formation of polyglutamylated species of 5-methyltetrahydrofolate in the cytoplasm. These data suggest that PMA inhibits uptake by inactivating caveolae internalization.

CELLS concentrate and take up low molecular weight molecules by potocytosis (5, 6). The best studied example of this endocytic process is the receptor-mediated uptake of 5-methyltetrahydrofolate in MA104 cells. Morphological (28, 29) and biochemical (19–22) experiments have shown that this vitamin is delivered to the cell interior after binding to a glycosylphosphatidylinositol (GPI)-anchored membrane receptor that is concentrated in caveolae. The receptor-folate complex is then sequestered within closed caveolae. After the caveolae seal off from the extracellular space, the folate dissociates from the receptor and diffuses through a membrane carrier into the cytoplasm (21). The vitamin becomes polyglutamylated as soon as it reaches the cytoplasm, which prevents diffusion out of the cell. Finally, the closed caveolae open to expose the receptors for another round of folate uptake.

Evidence is beginning to accumulate that caveolae are not only a membrane vehicle for potocytosis; they also appear to play an important role in coordinating the receipt and the transmission of cellular messages. A number of molecules that have previously been implicated in several different cellular signaling cascades are being found associated with caveolae. These molecules include: an ATP-dependent Ca^{2+} pump (11); a form of the inositol 1,4,5-trisphosphate (IP_3)-sensitive, Ca^{2+} channel (12); members of the src kinase family of nonreceptor tyrosine kinases (32); heterotrimeric

GTP-binding proteins (32); 5'-nucleotidase, an enzyme involved in the production of the signaling molecule, adenosine (10); and inositolphosphoglycans (30). The space created each time a caveolae closes is a special environment where these molecules may generate or transmit messages.

There are other indications that caveolae are involved in cell signaling. A critical substrate for the transforming activity of pp60^{src} kinase is caveolin (13, 15), a major integral membrane protein of caveolae (14, 27). The function of caveolin is still not known. Most likely caveolae are functionally dependent on this protein so that phosphorylation interferes with some aspect of the internalization cycle. Therefore, there is a strong correlation between cell transformation, which involves the disruption of normal cell signaling pathways, and impaired caveolae function as a consequence of caveolin phosphorylation.

One way to further define the role of caveolae in potocytosis and cell signaling is to find reagents that inhibit caveolae function. Therefore, we used the folate internalization assay developed for MA104 cells (19) to identify reagents that prevent vitamin uptake. We found that activators of protein kinase C (PKC) are potent inhibitors of potocytosis because they prevent the internalization of caveolae. Caveolae were inactivated by phorbol-12-myristate-13-acetate (PMA) in several different cell types, which suggests that this membrane specialization is a general target for PKC.

Materials and Methods

Materials

Medium 199 with Earle's salts with and without folic acid was prepared in the laboratory by standard protocols. Glutamine, trypsin-EDTA, and peni-

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1. *Abbreviations used in this paper:* GPI, glycosylphosphatidylinositol; PKC, protein kinase C.

cillin/streptomycin were from GIBCO-BRL (Gaithersburg, MD). Fetal calf serum was from Hazleton Research Products, Inc. (Lenexa, KS). Antibodies were obtained from the following sources: anti-caveolin IgG (monoclonal No. 2234) was a gift from Dr. John Glenney (Glentech Inc., Lexington, KY); rat anti-Thy-1 IgG was prepared from the tissue culture supernatant of hybridoma cells 30-H12 (TIB-107; American Type Tissue Culture); rabbit anti-rat IgG was from Sigma (St. Louis, MO); goat anti-rabbit IgG conjugated to gold (10 nm) was from Biocell (Ted Pella, Redding, CA), mouse anti-dinitrophenol IgG was from Oxford Biomedical (Oxford, MI), goat anti-mouse IgG conjugated to gold (10 nm) was from Energy Beam Sciences (Agawam, MA). [^3H]folic acid (sp act 27 Ci/mmol) and 5-methyl[^3H]tetrahydrofolic acid (sp act 21 Ci/mmol) were purchased from Moravak Biochemicals (City of Industry, CA). All PKC activators and inhibitors were purchased from Calbiochem-Novabiochem (San Diego, CA). Probenecid was purchased from Sigma.

Cell Culture

MA104 cells, a monkey kidney epithelial cell line, were grown as a monolayer in folate-free medium 199 supplemented with 100 U/ml penicillin/streptomycin and 5% (vol/vol) fetal calf serum. NIH 3T3 cells, a mouse fibroblast cell line, were grown in Dulbecco's modified Eagle's medium supplemented with 100 U/ml penicillin/streptomycin and 5% (vol/vol) fetal calf serum (34). Cells for each experiment were set up according to a standard format. On day zero 2.5×10^5 cells were seeded into a T-25 culture flask and grown for 5 d. For binding and uptake studies, medium was replaced with M199 without folate, containing 20 mM Hepes (pH 7.4), and additions were made directly to the culture flask (22).

Folate Binding

The [^3H]folic acid-binding assay was carried out as previously described (19, 22). MA104 cells were grown for 5 d in low folate medium. At the beginning of the experiment, the cells were washed with PBS and then subjected to the indicated experimental treatment. Following the treatment, cells were chilled on ice for 20 min and washed twice with ice cold PBS. External-bound folate was the amount of [^3H]folic acid released when cells were incubated on ice for 30 s in the presence of 1.5 ml of acid saline (0.15 M NaCl, adjusted to pH 3.0 with glacial acetic acid). Internal-bound folate was the amount of [^3H]folic acid that remained associated with the acid/saline-treated cells. The latter was recovered by adding 0.1 N NaOH (15 min, room temperature) to the flask to dissolve the cells. Radioactivity was measured by liquid scintillation counting using a Tri-carb 1900A liquid scintillation analyzer (Packard Instruments Co., Downers Grove, IL). Nonspecific binding, which was measured by adding 100-fold excess unlabeled folic acid, was less than 5% of specific binding.

Immunoelectron Microscopy

Cells were processed for immunogold localization of either the folate receptor or Thy-1 as previously described (29, 34).

5-Methyltetrahydrofolate Acid Internalization

The 5-methyltetrahydrofolate internalization was measured as previously described (19, 22). Briefly, at the end of the indicated incubation time in the presence of 5-methyl[^3H]tetrahydrofolate, cells were washed with cold PBS (4×5 ml) before adding 1.5 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 0.02 mg/ml leupeptin, 0.02 mg/ml aprotinin, and 1 μM 5-methyltetrahydrofolic acid) to each flask. The flasks were placed at -80°C for 15 min and then immediately thawed on ice. The suspension was collected and the flask was washed with 1.0 ml of lysis buffer. The two were combined and centrifuged for 20 min at 100,000 g to separate the membrane (pellet) and cytoplasmic (supernatant) fractions. Radioactivity was measured by liquid scintillation counting. Nonspecific binding, which was measured by adding 100-fold excess unlabeled folic acid to dishes containing radiolabeled 5-methyltetrahydrofolate, was less than 5% of specific binding.

Results

Inhibition of Folic Acid Internalization

Folic acid is a high affinity ligand that remains bound to the folate receptor as it travels through the caveolae internaliza-

tion cycle (22). MA104 cells incubated in the presence of [^3H]folic acid at 37°C for several hours have 50% of the bound folate in an internal, acid-resistant compartment and 50% in an external, acid-releasable compartment. The internal- and external-bound folate exchange with each other once every hour. A reagent that inhibits the internalization of caveolae should increase the number of external receptors while, at the same time, decrease the internal receptor pool. The PKC activator PMA was found to have exactly this effect (Fig. 1). We incubated MA104 cells in the presence of various concentrations of PMA for 30 min at 37°C before adding [^3H]folic acid to the medium. After an additional 1 h at 37°C , we measured the internal- and external-bound [^3H]folic acid. The lowest concentration of PMA did not perturb the normal 1:1 ratio of internal to external receptors. As the concentration of the drug increased, however, there was a reciprocal decline in the internal receptor pool and increase in the external receptor pool. A concentration between 100 and 500 nM PMA was sufficient to cause a 90% shift of [^3H]folic acid from the internal to the external fraction. We choose to use 1 μM PMA for the remaining experiments because this was a concentration that insured complete inhibition without affecting cell viability (data not shown).

The shift of bound folate from the internal to the external fraction suggested that PMA did not prevent previously internalized receptors from returning to the cell surface. This was confirmed by analyzing the time course of the PMA effect. We incubated cells in the presence of 1 μM PMA for various times at 37°C , chilled them to 4°C , and then incubated them for an additional hour at 4°C after adding [^3H]folic acid to the dish. Fig. 2 shows the amount of receptor-bound ligand present in the internal and external compartments at each time point. As expected, [^3H]folic acid was not found in an internal compartment because 4°C prevents internalization (Fig. 2, *internal*). By contrast, between 0 and 30 min of exposure to PMA, there was a steady increase in the amount of [^3H]folic acid that bound to external receptors (Fig. 2, *external*). The amount of bound [^3H]folic acid plateaued after 30 min of incubation, indicating that virtually all of the receptors had come to the surface. Other experiments showed that PMA could be removed after as little as 5 min of incubation without affecting the rate at which internal receptors appeared at the cell surface.

We next wanted to see if the receptors that migrated to the

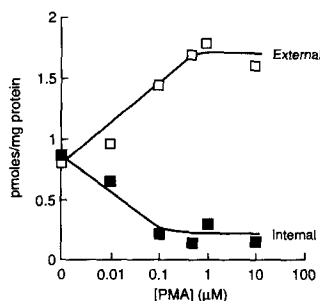


Figure 1. PMA inhibits the binding of [^3H]folic acid to internal receptors (\blacksquare) while at the same time increasing the binding to external receptors (\square). MA104 cells were incubated in the presence of the indicated amount of PMA dissolved in 0.02% ethanol for 30 min at 37°C before the addition of 5 nM [^3H]folic acid. Cells were further incubated

for 60 min at 37°C before the amount of radiolabel present on external and internal receptors was measured. Each point is the average of triplicate measurements.

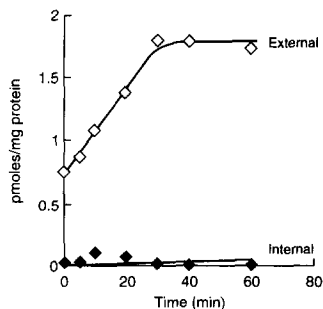


Figure 2. PMA allows internal receptors to come to the cell surface (\diamond). Cells were incubated in the presence of $1 \mu\text{M}$ PMA for the indicated time at 37°C and then chilled to 4°C for 20 min. Cells were then incubated for 60 min at 4°C in the presence of 5 nM [^3H]folic acid before assaying for the amount of label bound to internal (\blacklozenge) and external (\diamond) receptors. Each point is

the average of triplicate trials. Cells that were incubated for 60 min at 37°C in the absence of the drug bound 0.81 pmol/mg of protein to external receptors while cells incubated in the presence of $1 \mu\text{M}$ of the inactive isomer of PMA, 4α -phorbol-12,13-didecanoate, bound 0.77 pmol/mg of protein to external receptors.

surface in response to PMA (Fig. 2) were able to internalize [^3H]folic acid (Fig. 3). We incubated cells in the presence (\circ, \bullet) or absence (Δ, \blacktriangle) of $1 \mu\text{M}$ PMA for 30 min at 37°C and then chilled them to 4°C . We added [^3H]folic acid to each dish and incubated the cells for 60 min at 4°C . After the cells were washed, we warmed them to 37°C for the indicated time and assayed for external- and internal-bound [^3H]folic acid. Initially all of the bound folate in untreated cells (Δ, \blacktriangle) was on external receptors. With time at 37°C , there was a steady decline in the external [^3H]folic acid and a corresponding increase in the internal [^3H]folic acid. The internal to external ratio reached 1:1 at 60 min of incubation. The PMA-treated cells, by contrast, had nearly 40% more external-bound folate (\circ) than control cells, which indicates that internal receptors came to the surface during the incubation. These receptors did not internalize the vitamin since the bound [^3H]folic acid remained constant throughout the 60-min incubation period (Fig. 3, \circ).

PMA irreversibly activates PKC (18) and causes the down regulation of the enzyme within 24 h (35). This explains why MA104 cells exposed to PMA for just 5 min were unable to internalize [^3H]folic acid when assayed up to 6 h after the drug was removed (data not shown). We were concerned that the lack of reversibility might be due to a nonspecific toxic

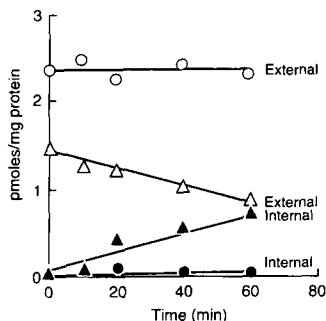


Figure 3. PMA prevents internalization of bound [^3H]folic acid. Cells were incubated in the presence, (\circ, \bullet) or absence (Δ, \blacktriangle) of $1 \mu\text{M}$ PMA for 30 min at 37°C and chilled to 4°C for 20 min. Cells were then incubated in the presence of 5 nM [^3H]folic acid for 60 min at 4°C , washed and then warmed to 37°C in the absence of [^3H]folic acid for the indicated time. The amount of radiolabel present on external (Δ, \circ) and internal receptors (\bullet, \blacktriangle) was measured as described.

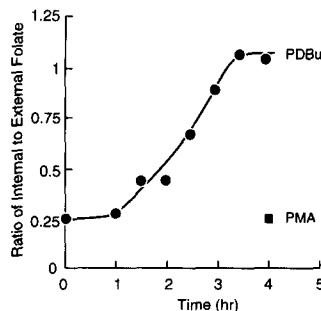


Figure 4. PDBu is a reversible inhibitor of folate internalization. Triplicate sets of MA104 cells were incubated in the presence of either $1 \mu\text{M}$ PDBu (\bullet) or $1 \mu\text{M}$ PMA (\blacksquare) at 37°C for 30 min. The cells were washed to remove the inhibitor and then incubated at 37°C in fresh media that contained 5 nM [^3H]folic acid for the indicated time. At the end of the incubation, the ratio of

internal- to external-bound [^3H]folic acid was measured as described. Untreated samples had a ratio of 1.02 and 1.04 at 1 and 4 h, respectively.

effect of the drug. For this reason, we tested the reversible PKC activator, phorbol-12, 13-dibutyrate (PDBu), to see if it inhibited [^3H]folic acid internalization. The design of the experiment was to incubate cells in the presence of PDBu for 30 min at 37°C and then wash the cells before adding fresh medium and incubating for various times (Fig. 4). At each time point, we measured the internal to external ratio of bound [^3H]folic acid after a 1-h exposure to the ligand at 37°C (Fig. 4, \bullet). PDBu was just as effective an inhibitor as PMA since both drugs caused a 75% reduction in the internal to external ratio (Fig. 4, compare \blacksquare at 4 h with \bullet on the ordinate). After a 1-h incubation in the absence of the drug, the ratio began to rise until at 3.5 h a normal ratio of 1:1 was observed. These results suggest that activation and deactivation of PKC can dramatically affect the caveolar internalization cycle.

Other Activators of Protein Kinase C

PMA binds to a conserved region of PKC that is normally used by diacylglycerol to regulate the enzyme (18). Homologous PMA-binding domains are found in several other proteins (1, 2, 26), which raises the possibility that PMA may sometimes affect cells by binding to molecules other than PKC. We tested several other activators of PKC and found that they all were equally effective at inhibiting [^3H]folic acid internalization. For example, $1 \mu\text{M}$ mezerein caused a 92% inhibition of internalization while $1 \mu\text{M}$ (-) indolactam V inhibited by 80%. The inactive stereoisomers of PMA (4α -phorbol-12,13-didecanoate) and (-) indolactam V ([+]-indolactam V) had no effect. We also found that after an overnight incubation in the presence of PMA, which stimulates the degradation of PKC (35), the inhibition was reduced by 80%. This was unchanged by the addition of fresh PMA. Finally, we found that the natural PKC activator, diacylglycerol, caused a 30% inhibition of internalization. The rapid hydrolysis and removal of diacylglycerol from the cytosol of intact cells is probably responsible for the lack of complete inhibition (18). In contrast to these results, the PKC inhibitors staurosporine, H-7, and calphostin C did not reverse the effects of PMA on folate uptake. Each inhibitor was used at a concentration substantially lower than that required for a complete inhibition of PKC to maintain cell viability (7, 33). Thus, although we can not entirely rule out the possibility that proteins other than PKC are the principle

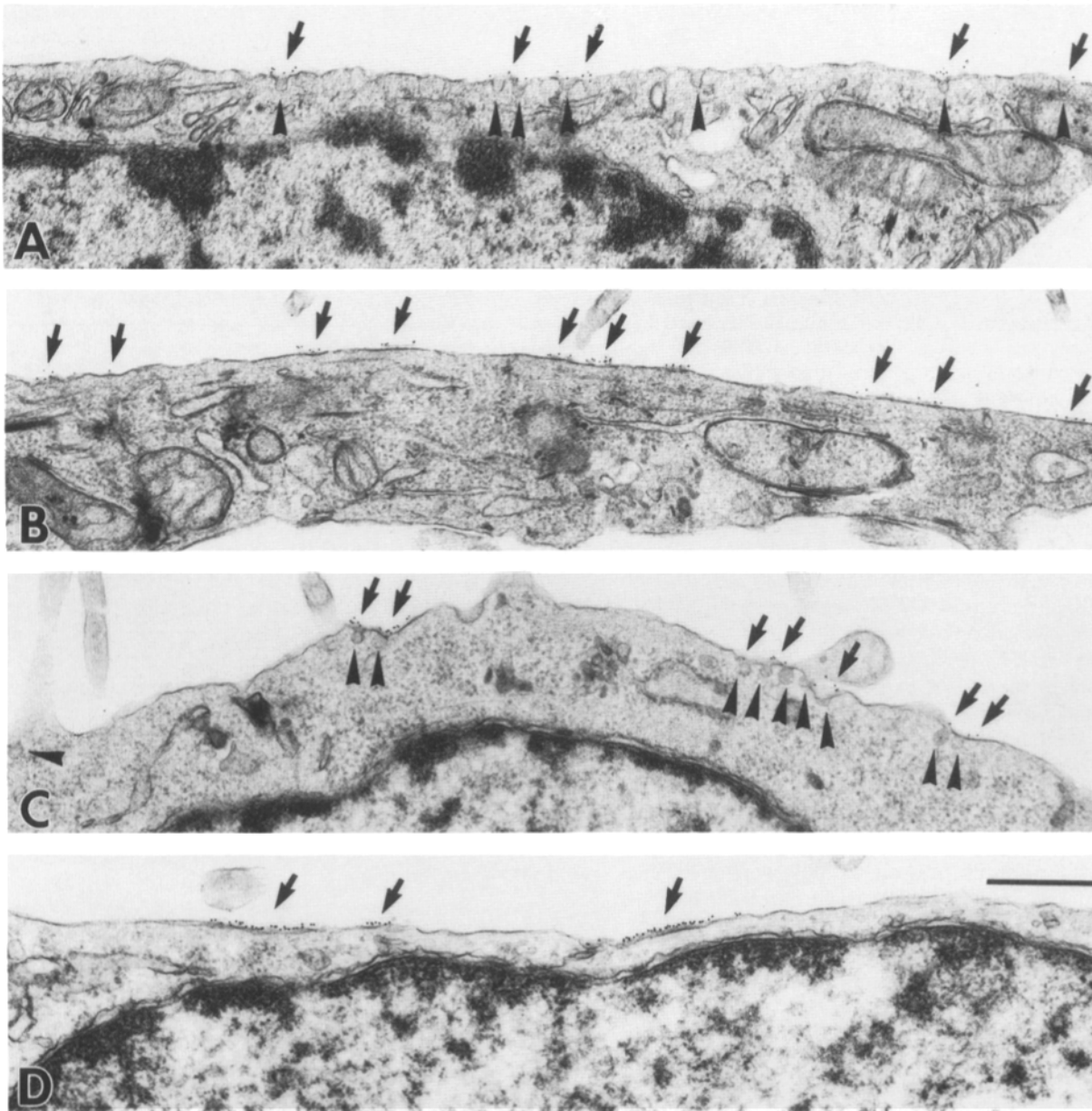


Figure 5. PMA inhibits the internalization of caveolae in MA104 cells (*A* and *B*) and NIH 3T3 cells (*C* and *D*). Both sets of cells were incubated in the presence (*B* and *D*) or absence (*A* and *C*) of $1 \mu\text{M}$ PMA for 30 min at 37°C . The cells were then chilled to 4°C and incubated in the presence of either $100 \mu\text{g/ml}$ of rabbit anti-folate receptor IgG (*A* and *B*) or a 1:1 dilution of hybridoma culture media from cells producing anti-Thy-1 IgG (*C* and *D*) for 1 h at 4°C . The cells were then processed for indirect immunogold localization of bound IgG. Black arrows mark the clusters of gold while the arrow heads indicate caveolae. Bar, $0.5 \mu\text{m}$.

effector of inhibition, the majority of the data supports a role for the kinase.

PMA Inhibits the Invagination of Caveolae

Previously, we showed that cholesterol-lowering drugs inhibit folate internalization by reducing both the density of clustered folate receptors and the number of caveolae on the cell surface (9, 28). To determine how PMA was affecting the plasma membrane, we incubated cells in the presence of $1 \mu\text{M}$ PMA at 37°C and then chilled the cells to 4°C before

using immunogold to localize folate receptors by electron microscopy (Fig. 5). Untreated MA104 cells (Fig. 5 *A*) had numerous clusters of anti-folate receptor IgG-gold and often they were associated with caveolae (Fig. 5 *A*, compare *arrows* with *arrow heads*). By contrast, PMA-treated cells had normal appearing gold clusters, but there was nearly a complete absence of caveolae (Fig. 5 *B*, compare *arrows* with *arrow heads*). Quantification of gold clusters and caveolae in the two sets of cells (Table I) showed that while PMA did not affect folate receptor clustering, there was a 10-fold reduction in the number of visible caveolae. The number of caveolae in cells that were incubated in the presence of 4α -

Table 1. Quantitative Analysis of the Effects of PMA on the Distribution of Anti-folate Receptor IgG-gold and on the Number of Invaginated Caveolae

	Number of clusters	Number of caveolae	Number of labeled caveolae	Gold/caveolae
Control	214	174	108	3.81
PMA	193	18	8	4.13

Cells were prepared and processed for localization of anti-folate receptor IgG. Random pictures of each sample were taken at a constant magnification of 10,800 \times and quantification was carried out directly on the negative. The linear distance of membrane evaluated in 40 cells was 385 μ m for control and 380 μ m for PMA-treated cells.

phorbol-12,13-didecanoate (PDD) were the same as control cells (data not shown).

These results suggested that PMA was preventing the uptake of [3 H]folic acid in MA104 cells by inhibiting the invagination of caveolae. We carried out exactly the same experiments on NIH 3T3 cells using anti-Thy-1 IgG-gold as a marker for GPI-anchored membrane proteins (Fig. 5, C and D). Thy-1 was clustered on the surface of both untreated (Fig. 5 C, arrows) and PMA-treated cells (Fig. 5 D, arrows). However, there was a nearly complete absence of caveolae in the treated cells (compare arrow heads in Fig. 5, C and D). Caveolae are organized into patches on the surface of 3T3 cells (Fig. 5 C) and as a consequence, PMA treatment caused the individual clusters of anti-Thy-1 gold to merge into giant clusters of gold (Fig. 5 D, arrows). We also examined normal human fibroblasts by both thin section and rapid-freeze, deep-etch electron microscopy. PMA appeared to cause the caveolae to flatten without disrupting the characteristic spiral coat (data not shown). This is in agreement with the finding that PMA did not alter the surface distribution of caveolin (data not shown).

PMA Inhibits Receptor-mediated Potocytosis

The function of the folate receptor is to concentrate 5-methyltetrahydrofolate and deliver it to cells by potocytosis (6). All of the biochemical and morphological evidence indicates that this process takes place in caveolae. The ability of PMA to inactivate caveolae offered the first opportunity to determine directly if this organelle is involved in potocytosis. We depleted MA104 cells of endogenous folate and incubated

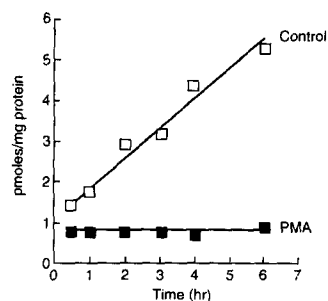


Figure 6. PMA prevented the delivery of 5-methyl[3 H]tetrahydrofolic acid to the cytoplasm. Triplicate sets of dishes were incubated either in the presence (■) or absence (□) of 1 μ M PMA for 30 min at 37°C. 5-methyl[3 H]tetrahydrofolic acid (10 nM) was added directly to each dish and the incubation was continued for the indicated times at 37°C.

At the end of the incubation, the cells were chilled on ice for 20 min and the amount of 5-methyl[3 H]tetrahydrofolic acid in the cytoplasm was determined by standard methods (22).

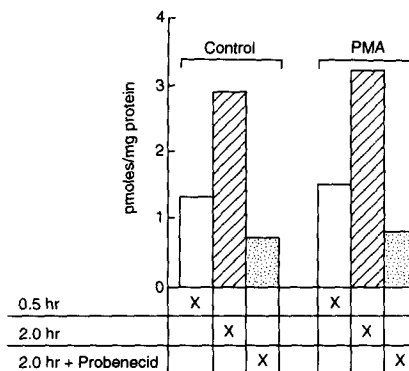


Figure 7. PMA does not inhibit receptor-independent uptake of 5-methyl[3 H]tetrahydrofolic acid. Triplicate sets of dishes received 1 μ M 5-methyl[3 H]tetrahydrofolic acid and were incubated for the indicated times in the presence or absence of 10 mM probenecid. The amount of 5-methyl[3 H]tetrahydrofolic acid in the cytoplasm was determined by standard methods.

them in the presence (Fig. 6, ■) or absence (Fig. 6, □) of 1 μ M PMA for 30 min before adding 10 nM 5-methyl[3 H]tetrahydrofolate to the dish and incubating for the indicated time. At each time point, the total cellular folate was measured. Control cells accumulated 5-methyl[3 H]tetrahydrofolate at a normal rate of \sim 1 pmol/mg of protein/h (21). PMA-treated cells, by contrast, did not accumulate any 5-methyl[3 H]tetrahydrofolate during the incubation period. We noticed that there was a constant amount of 5-methyl[3 H]tetrahydrofolate associated with PMA-treated cells (Fig. 6, PMA). Chromatographic analysis of the radioactivity from these cells showed that all of the label was folylmonoglutamate (data not shown), which indicates that it never reached the cytoplasm of the cell where polyglutamylation takes place (21). Virtually all of the label taken up by control cells, on the other hand, was folylpentaglutamate.

Once 5-methyltetrahydrofolate dissociates from the folate receptor within closed caveolae, it moves into the cell through an anion carrier in the membrane (21). Micromolar concentrations of the vitamin easily enter cells through this carrier without the need of a folate receptor (21). We wanted to rule out the possibility that PMA was preventing uptake by blocking this carrier. Therefore, we incubated treated and untreated cells in the presence of 1 μ M 5-methyl[3 H]tetrahydrofolate for 0.5 and 2.0 h (Fig. 7) and then measured the amount of cell-associated radioactivity. Fig. 7 shows that PMA had no effect on receptor-independent uptake of 5-methyl[3 H]tetrahydrofolate. Probenecid, a general inhibitor of anion carriers, blocked uptake in both sets of cells (Fig. 7). Finally, we determined by liquid chromatography that the cytoplasmic 5-methyl[3 H]tetrahydrofolate was polyglutamylated regardless of whether or not the cells were treated with PMA (data not shown).

Discussion

Potocytosis is a special name that was selected to distinguish the uptake of small molecules and ions from the uptake of macromolecules by receptor-mediated endocytosis (16), because entrance into the cell appeared to depend on caveolae

rather than clathrin-coated pits (3). The discovery of a class of potocytosis inhibitors that work by preventing the internalization of caveolae reaffirms the uniqueness of this pathway.

Clathrin-coated pits, lamellipodia, and caveolae are three regions of the plasma membrane that seem to be specialized for endocytosis (3). The only known way to inhibit endocytosis by coated pits and lamellipodia is to physically disrupt the structural molecules that control invagination and budding (3, 17, 25, 31). Endocytosis by caveolae seems to be different. We previously showed that depletion of intracellular cholesterol in MA104 cells selectively reduces the number of caveolae (28) and causes a marked inhibition of potocytosis (9). In addition, treatment of purified plasma membranes with cholesterol-binding drugs such as filipin causes invaginated caveolae to become flat (27). Therefore, potocytosis appears to be controlled by the lipid phase of the caveolar membrane.

The effects of PMA and other PKC activators help to further define how caveolae work. PMA inhibits folate receptor internalization almost instantaneously, but allows the closed caveolae to open at a normal rate (Fig. 2). Electron microscopic observations (Fig. 5, B and D) show that the drug prevents the invagination of the caveolar membrane. The curvature of coated pits is not affected (data not shown), which is consistent with previous studies (7, 24, 33) showing that receptor-mediated endocytosis is not inhibited by PMA. PMA may have its effect by directly or indirectly controlling the invagination process. The profound dependence of caveolae architecture on cholesterol suggests that a candidate target for PMA is an enzyme involved in membrane lipid metabolism. PMA is known to affect both the biosynthesis and the hydrolysis of phospholipids (23).

There are several reasons to suspect that PMA has its effects on caveolae by activating PKC: inhibition was rapid and occurred at nanomolar concentrations of the drug; caveolae were no longer inactivated after overnight incubation in the presence of PMA; PKC activators that are structurally unrelated to PMA were equally effective; inactive isomers of these PKC activators had no effect on folate uptake. The PKC family of isoenzymes are generally recognized to be kinases that control different signal-transducing pathways (18). Normally these kinases are activated by extracellular signaling ligands such as hormones, growth factors, and cytokines (18). We now must consider the possibility that these ligands sometimes exert their effects on cells by controlling caveolae internalization (4). For example, methacholine-induced Ca^{2+} oscillations in mouse lacrimal acinar cells appear to be caused by periodic fluctuations in PKC activity (8). Caveolae contain both an IP_3 -regulated Ca^{2+} channel and a Ca^{2+} ATPase. If sinusoidal changes in Ca^{2+} influx and efflux depend on the cyclic opening and closing of caveolae, then activated PKC would inhibit Ca^{2+} oscillations. Many of the early effects that PMA has on cells may ultimately be traced to the inhibition of potocytosis.

PMA, as well as other activators of PKC, is a new tool for studying the potocytosis of small molecules and ions. These reagents should be useful for identifying other ligands that are internalized by this route. In addition, they should help us determine the molecular basis of caveolar internalization because the controlling molecule(s) may be substrates for PKC.

We would like to thank William Donzell for helping with the rapid-freeze, deep-etch electron microscopy, Angela Smith for doing the HPLC analysis of 5-methyl[3H]tetrahydrofolate, and Kathy Estes for assistance with the internalization assays.

This work was supported by grants from the National Institutes of Health, HL-20948 and GM-43169, and the Perot Foundation.

Received for publication 18 October 1993 and in revised form 8 November 1993.

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