The polarity of epithelial cells derives from the selective distribution of their plasma-membrane proteins and lipids into distinct plasma-membrane domains (1, 2). Intracellular protein traffic from the endoplasmic reticulum (ER) to the cell surface through the Golgi apparatus is mediated by vesicles that transit between the organelles and different membrane domains. The newly synthesized membrane proteins may be directly addressed to their final destination from the ER (constitutive pathway) or stored in secretory vesicles until an extracellular stimulus induces their release to the membrane (regulated pathway) (1, 3). These pathways have crucial roles in the physiology of polarized cell function in the normal kidney and are perturbed in some pathological states. The asymmetry between basolateral and apical surfaces depends on mechanisms that control the movement of proteins to the correct target membrane in cells (1, 4). Despite intensive research, those processes remain poorly understood.

In addition to their classic roles in the production of second messengers in various aspects of signal transduction (5), lipids of intracellular compartments have been implicated in the regulation of membrane trafficking, vesicular fusion, and targeting (6). Phospholipases have also been implicated in this regulation. Phosphatidic acid, the main metabolite produced by hydrolysis of phospholipids by phospholipase D (PLD), has been proposed to have a direct action on target proteins involved in vesicle movement and to modify the lipid bilayer content and surface charge of membranes (7, 8). The group IV 85-kDa cytosolic phospholipase A2 (cPLA2) acts at the 50-2 position of phospholipids to generate free arachidonic acid (AA). AA and its metabolites are important lipid second messengers (5, 9, 10). cPLA2 is regulated by changes in intracellular free-calcium concentration within the physiological range. After release from phospholipids by PLA2, AA can be converted by cyclooxygenases, lipoxygenases, and cytochrome P-450 monooxygenase to prostaglandins, leukotrienes, and hydroxyeicosanoic acids, products that are involved in the regulation of a number of cellular processes (5, 11). Lysophospholipids generated in vesicle membranes by PLA2 may regulate the fusion of ER-transport vesicles with the Golgi apparatus (12). Furthermore, Tagaya et al. showed that PLA2 regulates intra-Golgi protein transport in vitro (13).

We have shown recently that aquaporin 2 (AQP2)/green fluorescent protein (GFP) chimeras are useful constructs for studying intracellular sorting signal transduction (5). Lipids of intracellular compartments have been implicated in the regulation of membrane trafficking, vesicular fusion, and targeting (6). Phospholipases have also been implicated in this regulation. Phosphatidic acid, the main metabolite produced by hydrolysis of phospholipids by phospholipase D (PLD), has been proposed to have a direct action on target proteins involved in vesicle movement and to modify the lipid bilayer content and surface charge of membranes (7, 8). The group IV 85-kDa cytosolic phospholipase A2 (cPLA2) acts at the 50-2 position of phospholipids to generate free arachidonic acid (AA). AA and its metabolites are important lipid second messengers (5, 9, 10). cPLA2 is regulated by changes in intracellular free-calcium concentration within the physiological range. After release from phospholipids by PLA2, AA can be converted by cyclooxygenases, lipoxygenases, and cytochrome P-450 monooxygenase to prostaglandins, leukotrienes, and hydroxyeicosanoic acids, products that are involved in the regulation of a number of cellular processes (5, 11). Lysophospholipids generated in vesicle membranes by PLA2 may regulate the fusion of ER-transport vesicles with the Golgi apparatus (12). Furthermore, Tagaya et al. showed that PLA2 regulates intra-Golgi protein transport in vitro (13).

We have shown recently that aquaporin 2 (AQP2)/green fluorescent protein (GFP) chimeras are useful constructs for studying intracellular sorting signal transduction (5). Lipids of intracellular compartments have been implicated in the regulation of membrane trafficking, vesicular fusion, and targeting (6). Phospholipases have also been implicated in this regulation. Phosphatidic acid, the main metabolite produced by hydrolysis of phospholipids by phospholipase D (PLD), has been proposed to have a direct action on target proteins involved in vesicle movement and to modify the lipid bilayer content and surface charge of membranes (7, 8). The group IV 85-kDa cytosolic phospholipase A2 (cPLA2) acts at the 50-2 position of phospholipids to generate free arachidonic acid (AA). AA and its metabolites are important lipid second messengers (5, 9, 10). cPLA2 is regulated by changes in intracellular free-calcium concentration within the physiological range. After release from phospholipids by PLA2, AA can be converted by cyclooxygenases, lipoxygenases, and cytochrome P-450 monooxygenase to prostaglandins, leukotrienes, and hydroxyeicosanoic acids, products that are involved in the regulation of a number of cellular processes (5, 11). Lysophospholipids generated in vesicle membranes by PLA2 may regulate the fusion of ER-transport vesicles with the Golgi apparatus (12). Furthermore, Tagaya et al. showed that PLA2 regulates intra-Golgi protein transport in vitro (13).
nals that direct this water-channel protein to specific cellular locations (14). When GFP is fused to the cytoplasmic amino-terminus of AQP2, GFP-AQP2(NT), the water channel enters a vasopressin-regulated (VP-regulated) pathway of plasma membrane insertion. In contrast, the COOH-terminal chimera, AQP2-GFP(CT), localizes constitutively on both apical and basolateral plasma membrane, independently of VP or forskolin stimulation, and traffics in a way that is indistinguishable from wild-type AQP1 (14).

Another protein that is constitutively targeted to the cell membrane in polarized epithelial cells is Na+–K+–ATPase, whose correct targeting is critical to normal cell function. Dopamine, which increases cPLA2 activity, induces a decrease in Na+–K+–ATPase activity in proximal tubules with internalization of its α- and β-subunits into endosomes via a clathrin-dependent pathway (15).

The goals of this study were to evaluate the role of cPLA2 in membrane-protein trafficking in a polarized renal epithelial cell. In cells expressing cPLA2, cell volume is increased, and plasma membrane Na+–K+–ATPase α-subunit is markedly reduced. The amount of AQP2-GFP(CT) is decreased in the plasma membrane and increased in the rough ER. Golgi cisternae are markedly disrupted, and giantin and β-COP are dispersed from their normal, condensed Golgi localization. In contrast, there is normal VP-stimulated membrane localization of GFP-AQP2(NT) and normal constitutive membrane localization of a Cl−/HCO3− anion exchanger. Further, the COOH-terminal chimera, AQP2-GFP(CT), localizes on both apical and basolateral plasma membrane, independently of VP or forskolin stimulation, and traffics in a way that is indistinguishable from wild-type AQP1 (14).

Another protein that is constitutively targeted to the cell membrane in polarized epithelial cells is Na+–K+–ATPase, whose correct targeting is critical to normal cell function. Dopamine, which increases cPLA2 activity, induces a decrease in Na+–K+–ATPase activity in proximal tubules with internalization of its α- and β-subunits into endosomes via a clathrin-dependent pathway (15).

The goals of this study were to evaluate the role of cPLA2 in membrane-protein trafficking in a polarized renal epithelial cell. In cells expressing cPLA2, cell volume is increased, and plasma membrane Na+–K+–ATPase α-subunit is markedly reduced. The amount of AQP2-GFP(CT) is decreased in the plasma membrane and increased in the rough ER. Golgi cisternae are markedly disrupted, and giantin and β-COP are dispersed from their normal, condensed Golgi localization. In contrast, there is normal VP-stimulated membrane localization of GFP-AQP2(NT) and normal constitutive membrane localization of a Cl−/HCO3− anion exchanger. Furthermore, the distribution of tubulin and actin is not altered by cPLA2, indicating that the microtubule and actin cytoskeleton remain intact. These results suggest that cPLA2 plays an important role in the selective regulation of constitutive membrane-protein trafficking by its action on Golgi structure and function.

**Methods**

**Materials.** PMA, A23187, vasopressin, AA, indomethacin, SKF 525A, nordihydroguaiacetic acid (NDGA), 4-methylumbelliferone-α-d-glucoside, 4-methylumbelliferone-α-d-mannopyranoside, 4-methylumbelliferone-phosphate, and streptavidin-agarose were purchased from Sigma Biochemical Co. (St. Louis, Missouri, USA). The membrane-impermeant biotin analogue sulfo-NHS-biotin was purchased from Pierce Chemical Co. (Rockford, Illinois, USA). Secondary goat anti-rabbit or antimouse IgG Ab’s coupled to Cy3 were from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania, USA). Peroxidase-conjugated goat anti-rabbit or anti-mouse IgG and reagents for protein measurement based on Bradford’s assay were purchased from Bio-Rad Laboratories. Iodixanol solution for form-staining and assay of cPLA2 activity. AdcPLA2, the recombinant adenovirus, was then purified in high titer by density gradients, Optiprep, was obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Iodixanol solution for formation of density gradients, Optiprep, was obtained from Nycomed Pharma AS (Oslo, Norway).

**Cell culture.** Cells were maintained at 37°C in DMEM containing 1 g/l glucose and supplemented with 2 mM 1-glutamine and 10% FBS (LLC-PK1) or 10% horse serum (293 cells) and cultured in 95% air and 5% CO2. Cells were stably transfected with GFP/AQP2 chimeric constructs using the DOTAP transfection reagent (Boehringer Mannheim GmbH) according to the manufacturer’s recommendations. Transfected cells were selected and maintained with 1 mg/ml G418 (Geneticin; GIBCO BRL, Grand Island, New York, USA), and single clones were isolated using cloning rings. Cells were routinely confirmed to be mycoplasma negative.

**GFP-AQP2 and GFP-cPLA2 chimeric constructs.** The AQP2-GFP(CT), GFP-AQP2(NT), and GFP-cPLA2 fusion chimeras were prepared by ligation of the AQP2 cDNA into the EcoRI/BamHI sites of the pEGFP-N1 vector and the SalI/BamHI sites of the pEGFP-C1 vector (CLONTECH Laboratories Inc., Palo Alto, California, USA), as described previously (14).

**Construction of a recombinant adenoviral vector carrying the cPLA2 cDNA.** The human cPLA2 cDNA was subcloned between the NotI and Xho1 sites of the bacterial plasmid vector pAdRSV4 to generate pAdRSV-cPLA2, which contains adenoviral sequences from 0–1.2 and 9.2–16.1 map units, the Rous sarcoma virus long-terminal repeat promoter, and the SV40 early-region polyadenylation signal. The position and the orientation of the cPLA2 cDNA were confirmed by sequencing. Using the calcium phosphate transfection technique, the plasmid vector containing cPLA2 was then cotransfected into 293 cells with pJM17, which encodes the adenoviral cDNA, as described previously (16). The homologous recombinants between the pAdRSV-cPLA2 and pJM17 contain the exogenous cDNA substituted for E1. An individual plaque was then purified and protein expression confirmed with immunoblotting and assay of cPLA2 activity. AdcPLA2, the recombinant adenovirus, was then prepared in high titer by propagation in 293 cells and purified by CsCl-gradient ultracentrifugation (17). The viral stock was determined to be 2.1 × 1012 particles/ml and 5 × 1010 plaque-forming units/cell (pfu/cell) with a plaque assay (17).

**Gene transfer into LLC-PK1 cells.** A recombinant adenovirus carrying the Escherichia coli LacZ gene encoding β-galactosidase (AdLacZ) (provided by David A. Dichek, Gladstone Institute for Cardiovascular Diseases, San Francisco, California, USA) was used as a control and to...
evaluate the ability of LLC-PK₁ cells to be infected by the adenoviral vector. This adenovirus is similar to AdcPLA₂. Cells were infected at different moi and those expressing β-galactosidase were detected 1, 2, 3, 5, 7, 15, and 21 days after infection using 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) substrate as described (18).

In other experiments, cells were transfected with pEGFP, pEGFP/cPLA₂, or pEGFP/cPLA₂(mut) constructs using the Superfect reagent (QIAGEN, Valencia, California, USA). The pEGFP/cPLA₂(mut) construct was generated by introducing the NH₂-terminal PstI/BamH1 fragment of human cPLA₂ (bp 22-375) into pEGFP-N1.

cPLA₂ activity. LLC-PK₁ cells were plated on 100-mm tissue-culture plates 2 days before infection with AdLacZ, AdcPLA₂, or vehicle. Two days after infection, the cells were washed, harvested, and lysed by sonication in 250 μl of a buffer containing 120 mM NaCl, 50 mM Tris, pH 9.0, and 1 mM EGTA. The lysate was centrifuged at 100,000 g for 1 hour at 4°C, and the supernatant was transferred to a new tube. The protein concentration of each sample was determined by Bradford assay. The cPLA₂ activity was assayed in duplicate at 37°C for 30 minutes in a 100-μl reaction volume that included 30 μg protein, 0.5 mM 1.3 phosphatidylcholine 1-stearoyl-2-[1-14C]arachidonyl, 2.5 mM CaCl₂, 0.2 mM EGTA, 50 mM NaCl, and 75 mM Tris, pH 9.0. The reaction was stopped by adding 800 μl Dole’s reagent (32% isopropyl alcohol, 67% n-heptane, 1% 1 H₂SO₄) and mixing using a Vortex-Genie (Fisher Scientific, Pittsburgh, Pennsylvania, USA). After centrifugation, 150 μl of the upper phase was transferred to a new tube containing 50 mg of silica gel and 800 μl of n-heptane. After vortexing and allowing the silica gel to settle, 800 μl of supernatant was counted for radioactivity in a liquid-scintillation counter.

AA release. Forty-eight hours after infection, subconfluent cells in 12-well plates were labeled for 18 hours with 0.30 μCi/ml [³H]AA in DMEM containing 0.1% FBS. The cells were then washed with DMEM containing 0.2% BSA and incubated with the same medium for 30 or 120 minutes or stimulated for 30 minutes with 200 nM PMA and 10 μM A23187. Medium was removed and radioactivity in 400 μl of supernatant was measured in a liquid-scintillation counter. The cells were solubilized with 1% Triton X-100, and the amount of [³H]AA released into the medium was expressed as a percentage of the total (cell-associated plus released).

Immunofluorescence. Cells plated on glass coverslips were infected with either AdLacZ or AdcPLA₂ at a moi of 50 pfu /cell, or transfected with pEGF, pEGF/cPLA₂ or pEGF/cPLA₂(mut). In some experiments, after infection, cells were incubated with either 5 μM of A23187, 10 nM VP, 25 μM indomethacin, SKF 525A, ETYA, or 10 μM NDGA. After fixation for 20 minutes with 4% paraformaldehyde containing 5% sucrose in PBS, cells were permeabilized with 0.1% Triton X-100 for 5 minutes, followed by 10 minutes of blocking in 1% BSA. The inherent fluorescence of the GFP tag was used to detect chimeric proteins. The primary Ab’s used for double labeling were an anti-β-COP mAb (clone #M3A5; Sigma Biochemical Co.) to visualize Golgi apparatus–associated vesicles, anti-giantin to visualize Golgi cisternae, anti-tubulin to label microtubules, anti-AE1/2 to label the Cl–/HCO₃– anion exchanger, and anti-Na⁺/K⁺-ATPase. BODIPY (Molecular Probes, Eugene, Oregon, USA) 581/591 phalloidin was used to label F-actin. The β-COP and AE1/2 Abs were applied after treatment of cells with 2% SDS in PBS for 4 minutes to unmask antigens (19). All primary Ab’s were incubated for 1 hour at room temperature. Secondary Ab’s, either Cy3-conjugated goat anti-rabbit IgG or Cy3-conjugated donkey anti-mouse IgG (1:800), were incubated for 1 hour at room temperature. Rhodamine isothiocyanate-conjugated (RITC-conjugated) dextran (Mr 11,000; Sigma Biochemical Co.) was used as a fluid-phase marker of endocytosis. Coverslips were mounted on slides in Vectashield/Tris-Cl (pH 8.9) at a ratio of 1:1. Indirect immunofluorescence was visualized with a Nikon FXA photomicroscope (Nikon Inc., Melville, New York, USA) and a confocal laser-scanning microscope (MRC/600; BioRad Microscopy Division, Hemel Hempstead, United Kingdom). Generated computer images were analyzed using IP Lab Spectrum imaging software (version 3.1; Signal Analytics Corp., Fairfax, Virginia, USA).

Electron microscopy. Forty-eight hours after infection cells were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 1 hour at room temperature. After rinsing in sodium cacodylate, they were post-fixed in 1% OsO₄ in 0.1 M sodium cacodylate for 30 minutes and stained en bloc in 2% aqueous uranyl acetate for 30 minutes at room temperature. Cells were scraped, pelleted, and embedded in 2% agarose, which was minced into 2-mm³ blocks. Blocks were dehydrated in graded series of ethanol and propylene oxide and polymerized in Epon at 60°C for 24 hours. Thin sections were stained with uranyl acetate and propylene oxide and polymerized in Epon at 60°C for 24 hours. Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips CM 10 electron microscope (Philips Electronics Inc., Mahwah, New Jersey, USA) at 80 kV.

Subcellular fractionation of LLC-PK₁ cells. To localize AQP2-GFP(CT) and GFP-AQP2(NT) to plasma or intracellular membranes, total cellular membranes were separated according to the technique described by van’t Hof et al. (20). For each gradient, cells from three subconfluent 100-mm dishes were used. Two days after infection, cells were harvested in an isotonic buffer containing proteinase inhibitors and homogenized in a 1.5-ml Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation for 10 minutes at 500 g, and the supernatants were centrifuged for 60 minutes at 100,000 g at 4°C. The resulting pellet, containing total cellular membranes, was resuspended in 1 ml of the previous buffer and layered on top of continuous iodixanol density gradients (2.5–25% wt./vol. Optiprep in 0.25 M sucrose/TE). Gradients were centrifuged for 3 hours at 100,000 g and fractionated into 700-μl samples. For analysis of marker-enzyme activities, 50-μl samples were taken from each
fraction and mixed with 200 μl of enzyme assay buffer in 48-well plates. The α-glucosidase II activity (ER membranes) was assayed in 150 mM citrate/phosphate, pH 6.5, 1 mM 4-methylumbelliferone-α-D-glucoside, α-mannosidase II (Golgi membranes) in 150 mM phosphate buffer, pH 6.0, 1 mM 4-methylumbelliferone-α-D-mannopyranoside, and alkaline phosphatase activity (plasma membranes) was measured in 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl₂, and 1 mM 4-methylumbelliferone-phosphate. Reactions were allowed to proceed for 4 hours at 37°C and stopped by addition of 100 μl ice-cold 1 M glycine, pH 10, 1 M Na₂CO₃. Fluorescence was measured at λex 360 nm, λem 460 nm using a DNA fluorometer TKO 100 (Hoefer Scientific Instruments, San Francisco, California, USA).

To evaluate whether cPLA₂ localizes to the Golgi apparatus of native nontransfected LLC-PK₁ cells, the cells were grown to 80% confluence. Approximately 30 × 10⁶ cells were resuspended in 1 ml of homogenization buffer (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, with Complete cocktail of protease inhibitors) and homogenized by passing ten times through a ball-bearing homogenizer (EMBL Precision Engineering, Heidelberg, Germany). The postnuclear supernatant was generated by centrifugation at 1,000 g for 5 minutes and placed on a 5–20% linear Optiprep gradient formed in separation buffer (78 mM KCl, 4 mM MgCl₂, 8 mM CaCl₂, 10 mM EGTA, 50 mM HEPES/KOH, pH 7.0) using a gradient former (Model 385; Bio-Rad Laboratories). Gradients were spun at 100,000 g for 4 hours, washed twice with ice-cold PBS, and cell surface proteins were labeled by biotinylation, using the membrane-impermeant biotin analogue sulfo-NHS-biotin (0.5 mg/ml; Pierce Chemical Co.) in PBS-C/M (containing 0.1 mM CaCl₂ and 1 mM MgCl₂), for 30 minutes at 4°C. Cells were then washed four times with ice-cold PBS-C/M, harvested by scraping with a rubber policeman, pelleted by centrifugation, and stored at -80°C until analysis. Total (plasma membrane and intracellular) Na⁺-K⁺-ATPase and AE 1 protein was immunoprecipitated using monoclonal anti-Na⁺-K⁺-ATPase and AE 1 protein was immunoprecipitated using monoclonal anti-Golgi stacks and cytosol from kidney cortex was purified as described previously (21) and solubilized in SDS-PAGE sample buffer.

**Purification of Golgi stacks from kidney tissue.** Isolation of Golgi stacks and cytosol from kidney cortex was performed using a discontinuous sucrose gradient as described previously (22).

**Western blot analysis.** Cells and subcellular lysates were solubilized in buffer containing 20 mM HEPES, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM Na₃VO₃, 1% Triton X-100, 10% glycerol, and protease inhibitors. An aliquot of protein lysates was separated from nonbiotinylated (intracellular) Na⁺-K⁺-ATPase and AE 1 protein was precipitated with 4–20% SDS-PAGE and fluorography.

**Protein synthesis.** Protein synthesis analysis in LLC-PK₁ cells infected at a moi of 50 pfu/cell with AdLacZ and AdcPLA₂ or noninfected (control) was assessed by [³H]-leucine incorporation and expressed as cpm/10³ cells, as described previously (16). After stimulation, cell were pulse labeled with 1μCi/ml [³H]-leucine for 6 hours, washed twice with ice-cold PBS, and fixed on ice for 30 minutes in 10% trichloroacetic acid (TCA). The medium was then removed, the cells were washed twice with 5% TCA, and once with water. The radioactivity incorporated into the TCA-precipitated material was determined by liquid-scintillation counting after solubilization in 0.25 M NaOH.

**Statistical analysis.** Data are expressed as mean plus or minus SD. A Student’s t test was used to compare the means of normally distributed continuous variables. A value of P < 0.05 was chosen to determine statistical significance. Each experiment was performed independently at least three times.
Results

PLA₂ expression in cells infected with AdcPLA₂. There is a dose-dependent increase in cPLA₂ activity in AQP2-GFP(CT) LLC-PK₁ cells infected at a moi of 0 (uninfected controls), 10, 50, 100, 200, and 400 pfu/cell for 48 hours with AdcPLA₂. No increase is seen in uninfected cells or cells infected with AdLacZ. Normal MDCK and rat kidney mesangial cells have cPLA₂ activity within the range seen in LLC-PK₁ cells infected with AdcPLA₂ at a moi of 50 to 100 pfu/cell (Figure 1a). Because we wanted to establish conditions in which the expression of cPLA₂ did not exceed that normally measured in other renal epithelial and mesangial cells, we chose a concentration of 50 pfu/cell for our studies. PMA and A23187 treatment results in more AA release into the medium in AdcPLA₂-infected cells than in control cells or cells infected with AdLacZ (Table 1). In cells infected with AdcPLA₂, PLA₂ activity increases with time, with a peak of activity between 3 to 6 days after infection (Figure 1b). Immunoblot analysis confirmed that the progressive increase in cPLA₂ activity of cells exposed to increasing moi of AdcPLA₂ was a direct consequence of progressive increases in the expression of cPLA₂ (Figure 1c). When infected with 50 pfu/cell, the total amount of cPLA₂ protein was less in the infected LLC-PK₁ cells than it was in the MDCK or mesangial cells.

Greater than 90% of the cells infected with AdLacZ or AdcPLA₂ expressed either β-galactosidase or cPLA₂, respectively, as assessed by immunocytochemistry (data not shown). There was no measurable toxicity over 48 hours after infection as assessed by the absence of an increase of lactate dehydrogenase (LDH) release (data not shown).

AQP2-GFP(CT), but not GFP-AQP2(NT), trafficking is disrupted by cPLA₂. Constitutive and hormone-regulated pathways of protein trafficking were examined using GFP/AQP2 chimeras as model systems. The AQP2-GFP(CT) chimera has been shown previously to traffic constitutively to the membrane. Constitutive AQP2-GFP(CT)-membrane localization is normal in AdLacZ-infected cells (Figure 2, a and b). By contrast, in cells expressing cPLA₂, there is a considerable reduction of AQP2-GFP(CT) on the plasma membrane and an increase in perinuclear, intracellular staining both in the absence (Figure 2c), or presence, of VP (Figure 2d).

GFP-AQP2(NT) localization in intracellular vesicles is unchanged under unstimulated conditions in cells infected with AdcPLA₂ (Figure 3a) (14). A shift from intracellular vesicles to the basolateral membrane is observed in these cPLA₂-expressing cells upon stimulation with 10 nM VP for 30 minutes (Figure 3b) in a pattern identical to that seen in noninfected cells (14).
The loss of AQP2-GFP(CT) basolateral-membrane staining. Bar, 5 μm.

PLA2, cells were fixed and examined for GFP expression using indirect immunofluorescence. In control cells (a) and cells expressing β-galactosidase (b), AQP2-GFP(CT) is localized on the basolateral and apical membrane. By contrast, in cells expressing cPLA2 in the absence (c) or presence (d) of VP (10 nM, 30 minutes), there is a complete loss of AQP2-GFP(CT) basolateral-membrane staining. Bar, 5μm.

Immunoblot analysis of membrane fractions. To determine the intracellular localization of AQP2-GFP(CT) and GFP-AQP2(NT) in cells expressing LacZ or cPLA2, total cellular membranes were separated by ultracentrifugation on a continuous iodixanol density gradient according to the technique described by van’t Hof et al. (20). Fractions were analyzed for enzyme activities identifying plasma membrane, Golgi apparatus, and ER by the markers alkaline phosphatase, α-mannosidase, and α-glucosidase II, respectively. An equal sample of each fraction was separated by SDS-PAGE and blotted with a monoclonal anti-GFP Ab. In cells expressing either LacZ or cPLA2, GFP-AQP2(NT) was localized principally within the intracellular fractions as expected, although some plasma-membrane localization was apparent (Figure 4a). In contrast, AQP2-GFP(CT) was expressed mainly in the plasma-membrane fractions in cells expressing LacZ, whereas in cells expressing cPLA2, AQP2-GFP(CT) was shifted markedly to the ER fractions (Figure 4a). Marker-enzyme activities of each of the fractions are presented in Figure 4b.

Effect of cPLA2 on membrane localization of Na+-K+-ATPase and AE 1. To test whether cPLA2 had an effect on the trafficking of other constitutive membrane proteins, LLC-PK1 cells infected with either AdLacZ or AdcPLA2 were stained with Abs against the α-subunit of Na+-K+-ATPase or AE1/2. In cells expressing cPLA2, the normal Na+-K+-ATPase basolateral membrane localization (Figure 5a) was almost completely abolished, and it was difficult to tell where the protein was relocated (Figure 5b). However, AE1/2 basolateral localization was unaffected (Figure 5, c and d).

To confirm that the effect of cPLA2 on trafficking of the Na+-K+-ATPase and the anion exchanger was not related to a difference in the stability of the proteins at the plasma membrane, cells expressing either LacZ or cPLA2 were labeled with [35S]-methionine. Two days after infection with either AdLacZ or AdcPLA2, specific activities of postnuclear supernatants of LLC-PK1 cells (Figure 6a), SDS-PAGE profiles of the metabolically labeled proteins (Figure 6b), and total amount of cellular Na+-K+-ATPase (Figure 6c) are not affected by expression of cPLA2. Expression of cPLA2 results in a marked inhibition of trafficking of Na+-K+-ATPase to the plasma membrane of LLC-PK1 cells (Figure 6d). The trafficking of AE1 protein to the plasma membrane is not affected in cPLA2-expressing cells.

Figure 3
Normal vesicular trafficking of GFP-AQP2(NT) in cPLA2-expressing LLC-PK1 cells. Two days after infection with AdcPLA2, cells were fixed and examined for GFP expression by immunofluorescence. Under nonstimulated conditions (a), GFP-AQP2(NT) was localized to intracellular vesicles. After stimulation with 10 nM VP for 30 minutes (b), staining shifted to the plasma membrane.
cycloheximide treatment (25 μg/ml) for 1–3 hours had no detectable effect on the distribution of GFP-AQP2(NT) and AQP2-GFP(CT) (data not shown).

**Cell morphology and Golgi apparatus organization.** The actin cytoskeleton and microtubule network appeared unaffected by cPLA2, as determined by anti-tubulin and phalloidin labeling (data not shown). To examine the structure of the Golgi apparatus, anti-β-COP Ab was used to label coat proteins on transporting vesicles within the cis–medial Golgi apparatus, and anti-giantin Ab was used to label the Golgi cisternae. In LacZ control cells, β-COP and giantin are present in condensed, perinuclear patterns, as expected (Figure 7, a and c, respectively, for β-COP and giantin). In contrast, in cells expressing cPLA2, β-COP and giantin localization is dramatically dispersed in a manner that suggests disruption of the Golgi organization (Figure 7, b and d).

After transfection with a construct encoding a GFP-cPLA2 fusion protein, there is marked disruption of the Golgi apparatus of cells that express the protein, whereas a mutant of cPLA2, which is catalytically inactive, has no effect on β-COP distribution (Figure 8).

Using electron microscopy, the morphology of the Golgi apparatus was analyzed in LacZ- and cPLA2-expressing cells. The Golgi stacks were well-formed cisternae in the β-galactosidase–expressing cells (Figure 9a, arrows), whereas they were scattered and vesiculated in cells expressing cPLA2 (Figure 9b, arrows). Nuclear membranes and ER are intact.

cPLA2 is localized to Golgi apparatus of kidney cortex and noninfected LLC-PK1 cells. Given the dramatic effects of cPLA2 on Golgi structure, it is of importance to evaluate whether cPLA2 is associated with the Golgi apparatus in normal renal epithelium. cPLA2 colocalizes with post-nuclear fractions that stain positively with anti–GP-58, a marker of the Golgi complex (Figure 10, a and b) in LLC-PK1 cells. CA-2 was used as cytosolic marker and Rab-11 as recycling endosome marker. In addition,
cPLA₂ is associated with Golgi complex isolated from the rat kidney cortex, which consists primarily of proximal epithelial cells (data not shown).

Cell volume and endocytosis in cells expressing cPLA₂. The size of the cells increased after cPLA₂ expression. To quantitate this effect, 50 β-galactosidase-expressing cells and 50 cPLA₂-expressing cells were measured. By determining the two-dimensional size of cells expressing cPLA₂, the estimated volume was eightfold larger than that of cells infected with AdLacZ. Rhodamine conjugated to the cell-impermeant agent dextran was used to evaluate endocytosis. Normal endocytosis is found in cells expressing cPLA₂ (Figure 11). The glycoprotein characteristics of the apical surface of β-galactosidase- and cPLA₂-expressing cells are similar as evaluated by staining with wheat germ agglutinin conjugated to rhodamine (data not shown).

Discussion
The polarized renal epithelial cell is an excellent model for studying differential targeting of proteins to cell membranes. Normal cell-membrane trafficking is necessary for the maintenance of transepithelial transport and other cellular functions such as volume regulation. Abnormal cell trafficking, including abnormal localization of the Na⁺-K⁺-ATPase, is characteristic of several renal diseases, including polycystic kidney disease (25) and pathophysiological states, such as ischemia/reperfusion injury (26).

Replication-deficient recombinant adenoviral vectors facilitate the titrated expression of a transgene in a high percentage of cells (> 90% in these studies) so that “physiological” levels of a protein are produced (27, 28). Using stable transfectants of GFP-AQP2 fusion proteins (14), we have found that cPLA₂ alters constitutive [AQP2-GFP(CT)] but not VP-regulated [GFP-AQP2(NT)] intracellular trafficking. To confirm that this effect was also relevant to endogenous proteins, it was demonstrated that basolateral membrane localization of Na⁺-K⁺-ATPase was almost completely abolished, whereas localization of the Cl⁻/HCO₃⁻ exchanger (AE1/2) was unaffected. [³⁵S]-methionine labeling experiments confirm that trafficking of the α-subunit of Na⁺-K⁺-ATPase to the cell membrane is altered, whereas AE1 trafficking is intact, confirming that there is a level of specificity to the effects of cPLA₂ on constitutive trafficking pathways. The absence of an effect of cPLA₂ on VP-induced membrane expression of GFP-AQP2(NT) may be explained by the localization of this protein in preformed post-Golgi vesicles that traffic to the membrane upon addition to VP. This process is unaffected by cPLA₂ activity.

While the redistribution of basolateral Na⁺-K⁺-ATPase in the proximal tubule with ischemia has been attributed to disruption of epithelial-cell cytoskeletal structural organization (29, 30), our data suggest that cPLA₂-induced changes in intracellular vesicle-trafficking processes may also account for changes in distribution of Na⁺-K⁺-ATPase.
The cPLA2 activity is significantly increased in rat proximal tubule-enriched kidney homogenates after ischemia and reperfusion (31). The decrease in plasma membrane Na+-K+-ATPase cannot be due to proteolysis, since equivalent amounts of the protein are found in control and cPLA2–expressing cells. The α-subunit is localized to intracellular membranes in cPLA2–expressing cells (data not shown). The decrease in Na+-K+-ATPase activity likely accounts for the increase in cell volume measured in the absence of morphological changes in actin cytoskeleton and microtubule network (32).

The cPLA2–induced changes in trafficking are possibly due to a direct effect of the enzyme on lipid composition of the Golgi membrane or may be an effect of AA itself. The asymmetry of the lipid bilayer, with choline-containing phospholipids located preferentially in the external leaflet and the aminophospholipids in the cytoplasmic leaflet, is necessary to maintain the fundamental properties of the different intracellular compartments within the cell (33). Hydrolysis of membrane phospholipid into lysophospholipid and free fatty acid by cPLA2 may modify the composition of the two leaflets of the membrane.

**Figure 8**
Catalytically deficient mutant of cPLA2 has no effect on β-COP localization. Kidney LLC-PK1 epithelial cells were transfected with pEGFP only (a), pEGFP/cPLA2 (wt) (b), or with pEGFP/cPLA2 (mut), encoding a fusion protein of GFP with the calcium lipid-binding region of cPLA2 (c) and stained with Ab’s against β-COP. Left column represents GFP-expressing, GFP-cPLA2 (wt)–expressing, and GFP-cPLA2 (mut)–expressing cells, middle column shows endogenous β-COP distribution, and right column shows the merging of the two images in each row. Asterisks indicate transfected cells. Arrowheads indicate Golgi complex of nontransfected cells, and arrows indicate the Golgi structure in transfected cells in the same cell culture. Bar, 5 μm. wt, wild-type.

**Figure 9**
Electron microscopy examination of cellular morphology AQP2-GFP(CT) LLC-PK1 cells. (a) The Golgi complex (arrow) appears normal in cells infected with AdLacZ. (b) In contrast, the Golgi complex in cPLA2-expressing cells was markedly disrupted and vesiculated. ER and nuclear membranes appear normal. Bar, 1 μm.
phospholipid bilayer. In addition, the modulation of the lipid content induced by cPLA2 could alter the substrate of phospholipase D, thus affecting phosphatidic acid production locally, with secondary effects on trafficking due to the change in this important lipid (33, 34).

The complex organization and dynamic nature of the Golgi subcompartments make it very difficult to study their lipid composition (35). Phospholipase D is present in Golgi membranes (36), is activated by ADP-ribosylation factor (37), and is responsible for catalyzing the transphosphatidylation reaction of phosphatidic acid and diacylglycerol to form bisphosphatidic acid (38). The removal of an acyl chain from bisphosphatidic acid leads to the production of semilysobisphosphatidic acid, which has been localized to the most dynamic pleomorphic regions of the Golgi complex, the transvesicular networks on both sides of the Golgi stacks (35). Thus, cPLA2 activity might alter the lipid balance of the Golgi apparatus in such a way that stack formation or steady-state integrity is not favored. Since inhibitors of cyclooxygenases, lipoxygenases, and P-450 monooxygenase, or incubation of the cells with AA for 2–4 days, did not modify or mimic the effects observed, it is unlikely that an AA product, or AA itself, is responsible for the effects observed.

Membrane-associated or secreted proteins contain structural information that is recognized by the intracellular sorting machinery (39). Usually, this information is conveyed by short hydrophobic amino acid sequences in the cytoplasmic tail or phosphorylation of sugar residues or specific patterns of glycosylation, mainly in the rough ER and the Golgi apparatus (40). It is possible that cPLA2 could act on enzymes involved in posttranslational glycosylation within the Golgi apparatus. In addition, by modifying Golgi lipid composition, cPLA2 could modify the conformational environment of Golgi membranes, which may alter critical membrane-fission events (41) or composition of lipid rafts (42).

The cPLA2-induced modification of the Golgi apparatus is similar to the Golgi complex fragmentation that occurs in mitotic cells (43). Since cPLA2 is constitutively phosphorylated in mitotic cells (44), and cPLA2 activity increases in proliferating cells (data not shown), it is possible that cPLA2 is responsible, at least in part, for the Golgi complex fragmentation observed in mitosis.

As reported by de Figueiredo et al., the ability of brefeldin A to stimulate Golgi and trans-Golgi network tubulation was inhibited by a number of antagonists of PLA2 (45). However, because some of the antagonists used in this work are not specific and could have exerted their effects through changes in phosphatidic acid metabolism and on more than one form of PLA2 (46), it is not possible to attribute the changes observed to a particular enzymatic activity.

In conclusion, our studies demonstrate the potential importance of intracellular group IV cPLA2 activity for
Golgi apparatus structure and function. Our results also demonstrate the selectivity of these effects of cPLA2 for the constitutive pathway of membrane-protein trafficking and for a subset of polypeptides. These effects of cPLA2 are likely mediated by changes in Golgi membrane lipid composition and may also explain changes observed in the Golgi structure during the cell cycle.

Acknowledgments

This work was supported by grants from the NIH: DK-39773, DK-38452, and NS-10828 (to J.V. Bonventre); HL-50361 and HL-57623 (to R.J. Hajar); HL-54202, HL-59521, and AI-40970 (to A. Rosenzweig); and DK-38452 (to D. Brown). G. Choukroun was supported, in part, by a Lavoisier grant of the French Government and by the International Study and Research grant of the French Lilly Institute. C. Gustafson was supported by an award (to D. Brown). G. Choukroun was supported, in part, by grant no. 59521, and AI-40970 (to A. Rosenzweig); and DK-38452 (to D. Brown).}