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Dominant-negative PKC-ε impairs apical actin remodeling in parallel with inhibition of carbachol-stimulated secretion in rabbit lacrimal acini


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Jerdeva, Galina V., Francie A. Yarber, Melvin D. Trousdale, Christopher J. Rhodes, Curtis T. Okamoto, Darlene A. Dartt, and Sarah F. Hamm-Alvarez. Dominant-negative PKC-ε impairs apical actin remodeling in parallel with inhibition of carbachol-stimulated secretion in rabbit lacrimal acini. Am J Physiol Cell Physiol 289: C1052–C1068, 2005. First published June 1, 2005; doi:10.1152/ajpcell.00546.2004.—We investigated the involvement of PKC-ε in apical actin remodeling in carbachol-stimulated exocytosis in reconstituted rabbit lacrimal acinar cells. Lacrimal acinar PKC-ε cosedimented with actin filaments in an actin filament binding assay. Stimulation of acini with carbachol (100 μM, 2–15 min) significantly increased PKC-ε recovery with actin filaments in two distinct biochemical assays, and confocal fluorescence microscopy showed a significant increase in PKC-ε association with apical actin in stimulated acini as evidenced by quantitative colocalization analysis. Overexpression of dominant-negative (DN) PKC-ε in lacrimal acini with replication-defective adenovirus (Ad) resulted in profound alterations in apical and basolateral actin filaments while significantly inhibiting carbachol-stimulated secretion of bulk protein and β-hexosaminidase. The chemical inhibitor GF-109203X (10 μM, 3 h), which inhibits PKC-α, -β, -δ, and -ε, also elicited more potent inhibition of carbachol-stimulated secretion relative to Gö-6976 (10 μM, 3 h), which inhibits only PKC-α and -β. Transduction of lacrimal acini with Ad encoding syncollin-green fluorescent protein (GFP) resulted in labeling of secretory vesicles that were discharged in response to carbachol stimulation, whereas cotransduction of acini with Ad-DN-PKC-ε significantly inhibited carbachol-stimulated release of syncollin-GFP. Carbachol also increased the recovery of secretory component in culture medium, whereas Ad-DN-PKC-ε transduction suppressed its carbachol-stimulated release. We propose that DN-PKC-ε alters lacrimal acinar apical actin remodeling, leading to inhibition of stimulated exocytosis and transcytosis.

lacrimal gland; acinar epithelial cell; exocytosis; polymeric immunoglobulin A receptor

PRODUCTION AND RELEASE of ocular fluid of appropriate composition is essential for maintenance of corneal health. The lacrimal acinar cells of the lacrimal gland are the major source of tear proteins released into ocular fluid. Most tear protein stores are released at the apical plasma membrane (APM) of lacrimal acini from mature secretory vesicles (SVs) that are coated with the small GTPase rab3D (27, 41). These SVs fuse with the APM rapidly on secretagogue stimulation (41). Additional tear proteins such as the extracellular domain of the polymeric immunoglobulin receptor (pIgR), either alone (secretory component, SC) or complexed to dimeric IgA (secretory IgA), can be released at the APM through the transcytotic pathway. The area under the APM in lacrimal acini is enriched in a dense network of actin filaments that appear to form a barrier preventing uncontrolled SV release in unstimulated acini. Several studies have shown that extensive remodeling of the underlying actin network accompanies stimulated secretion in acinar epithelial cells from pancreas (24, 38, 39), parotid gland (29), and lacrimal gland (Jerdeva GV and Hamm-Alvarez SF, unpublished observations). This remodeling includes increased actin filament turnover as well as transient formation of actin-coated invaginations thought to represent SV fusion intermediates. Stabilization of actin-coated structures is associated with inhibition of exocytosis (39). Little is known about the effectors that regulate actin filament remodeling in acinar exocytosis.

In this study, we have investigated the contribution of the novel PKC isoform PKC-ε to actin filament remodeling in apical exocytosis. Three subfamilies of PKC are distinguished based on their requirements for activation: 1) the Ca<sup>2+</sup>-dependent or conventional PKCs, 2) the Ca<sup>2+</sup>-independent or novel PKCs, and 3) the phorbol ester-insensitive or atypical PKCs (reviewed in Refs. 12 and 26). All PKCs consist of a single polypeptide chain containing regulatory NH2-terminal and catalytic COOH-terminal domains. Although conventional isoforms have domains for binding to diacylglycerol and Ca<sup>2+</sup+, novel PKC isoforms lack the Ca<sup>2+</sup>-binding domain, making them maximally responsive to diacylglycerol or phorbol esters. Uniquely, PKC-ε has an actin-binding site spanning amino acids 223–228 (Refs. 30, 31; reviewed in Ref. 2). Association of PKC-ε with actin filaments is triggered by binding of diacylglycerol or phorbol esters; conversely, association of PKC-ε with actin filaments maintains the kinase in the active state. It is thought that the phosphorylation of specific substrate proteins by PKC-ε is regulated by its targeting to actin filaments or its other partner, Golgi β'-coat protein (COP), which brings the activated kinase into close proximity to target molecules.

Secretagogue-mediated activation of diacylglycerol- and Ca<sup>2+</sup>-dependent pathways in the lacrimal gland and conse-
quent activation of Ca$^{2+}$- and phospholipid-dependent PKCs has long been associated with stimulation of exocytosis (11). At least five PKC isoforms are present in lacrimal gland acini: $\alpha$, $\epsilon$, $\delta$, $\mu$, and $\lambda$, each exhibiting unique locations and translocation patterns in response to activation (47). In rat lacrimal acini, the muscarinic agonist carbachol (CCH) evokes a secretory response by activation of PKC-$\alpha$ and PKC-$\epsilon$ (46). In contrast, the secretory response evoked by $\alpha_1$-adrenergic agonists such as phenylephrine (PE) in rat lacrimal acini occurs primarily through activation of PKC-$\epsilon$ (46). PKC-$\epsilon$ facilitates synaptic vesicle exocytosis (31) as well as prolactin secretion from pituitary cells (1). Given the important role for actin filament remodeling in lacrimal acinar exocytosis and the established link between PKC-$\epsilon$ and exocytosis in other systems, we hypothesized that PKC-$\epsilon$ participates in actin filament remodeling in lacrimal acinar exocytosis. Here we have used biochemical strategies, confocal fluorescence microscopy, and functional assays to demonstrate in lacrimal acini that 1) PKC-$\epsilon$ is an actin-binding protein; 2) PKC-$\epsilon$ association with actin is responsive to secretagogue stimulation; 3) introduction of dominant-negative (DN) PKC-$\epsilon$ with replication-defective adenovirus (Ad) elicits profound alterations in actin cytoskeleton including accumulation of actin-coated invaginations; and 4) expression of DN-PKC-$\epsilon$ significantly ($P < 0.05$) inhibits CCH-stimulated release of bulk protein, $\beta$-hexosaminidase, cotransduced syncollin-green fluorescent protein (GFP), and SC. We propose that, in stimulated acini, activated PKC-$\epsilon$ is targeted to apical actin, where it phosphorylates actin-associated proteins, leading to the changes in actin organization required for exocytosis. We further suggest that the impaired CCH-stimulated exocytosis at the APM of acini transduced with Ad-DN-PKC-$\epsilon$ is a consequence of the changes in apical actin remodeling exerted by DN-PKC-$\epsilon$.

MATERIALS AND METHODS

Reagents. CCH, PE, rhodamine-phalloidin, and goat anti-rabbit secondary antibody conjugated to FITC were obtained from Sigma (St. Louis, MO). GF-109203X and Go-6976 were obtained from EMD Biosciences (San Diego, CA). Sheep anti-rabbit antibody to SC was generated against purified SC from rabbit bile (Pel-Freez, Rogers, AR) by preparative gel electrophoresis and used to produce sheep antiserum with protein G-Sepharose (Amersham-Pharmacia). Mouse monoclonal antibody to actin was obtained from Roche Diagnostics (Indianapolis, IN). Goat anti-mouse, anti-rabbit, and donkey anti-sheep IRDye800-conjugated secondary antibodies were purchased from Rockland (Gilbertsville, PA). Cell culture reagents were from Life Technologies.

Isolation of lacrimal acini. Isolation of lacrimal acini from female New Zealand White rabbits (1.8–2.2 kg) obtained from Irish Farms (Norco, CA) was in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Protocols for isolation of rabbit lacrimal acini were approved by the University of Southern California Institutional Animal Care and Use Committee. Lacrimal acini were isolated as described previously (9, 12) and cultured for 2–3 days. Cells prepared in this way aggregate into acinus-like structures, and individual cells within these structures display distinct apical and basolateral domains and maintain a robust secretory response (9, 10, 41). CCH and PE were used at 100 $\mu$M.

Ad amplification and transduction. Replication-defective Ad constructs used in this study include Ad expressing DN-PKC-$\epsilon$ and GFP separately (Ad-DN-PKC-$\epsilon$), Ad encoding GFP alone (Ad-GFP), and Ad encoding a syncollin-GFP fusion protein (Ad-syncollin-GFP). QB1 cells were infected with Ad constructs and grown at 37°C and 5% CO$_2$ in DMEM (high glucose) containing 10% fetal bovine serum for 66 h until cells were completely detached from the flask surface. The virus was purified by cesium chloride ultracentrifugation as described previously (41). Viral titers were determined with the tissue culture infectious dose$_{50}$ assay on 293 cells. Transduction with Ad constructs involved exposure for 1–3 h on day 2 of culture at a multiplicity of infection (MOI) of 5, followed by washing well in Dulbecco’s PBS and incubation in fresh culture medium for 18–20 h at 37°C and 5% CO$_2$. Cells were analyzed on day 3 of culture. Transduction efficiency was maintained at ~80%, consistent with previous reports (41). For cotransduction studies, an MOI of 5 was used for each Ad construct. Dual transduction efficiency was difficult to quantify with flow cytometry because the constructs of interest both expressed GFP (Ad-DN-PKC-$\epsilon$ coexpressing GFP and Ad-syncollin-GFP). However, analysis by confocal fluorescence microscopy in fixed acini showed coexpression of cytosolic PKC-$\epsilon$ and large syncoll-containing vesicles in ~70% of lacrimal acini, consistent with efficiencies seen for cotransduction by other constructs in lacrimal acini.

Confocal fluorescence microscopy. For analysis of effector proteins and actin filaments in fixed cells, reconstituted rabbit lacrimal acini cultured on Matrigel-coated coverslips were fixed and processed as described previously (9, 10, 41). Acini were incubated with appropriate primary and fluorophore-conjugated secondary antibodies and rhodamine- or Alexa Fluor 647-phalloidin. Most confocal images were obtained with a Zeiss LSM 510 Meta NLO imaging system equipped with argon, red HeNe, and green HeNe lasers mounted on a vibration-free table and attached to an incubation chamber controlling temperature, humidity, and CO$_2$. The ability of this system to acquire fluorescence emission signals resolved within narrow ranges in multitrack mode, and the use of singly labeled control samples ensured the validity of colocalization studies. Panels were compiled in Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA).

For live cell imaging of acini expressing syncollin-GFP, rabbit lacrimal acini seeded on Matrigel-covered glass-bottomed round 35-mm dishes (MatTek, Ashland, MA) at a density of 4 $\times$ 10$^4$ cells/dish for 2 days were transduced with Ad-syncollin-GFP as described above and cultured in fresh medium for 18–24 h. On day 3, lacrimal acini were analyzed by time-lapse confocal fluorescence and differential interference contrast (DIC) microscopy with Zeiss Multiples Time Series V3.2 and Physiology V3.2 software modules. Live cell analyses were performed at 37°C. DIC images and GFP fluorescence were acquired simultaneously with the 488-nm line of the argon laser.

For analysis of transduction efficiency, rabbit lacrimal acini transduced with Ad-DN-PKC-$\epsilon$ coexpressing GFP as above were fixed in 4% paraformaldehyde for 15 min, followed by permeabilization with 0.5% Triton X-100. These acini were then incubated with appropriate primary and secondary antibodies to visualize PKC-$\epsilon$, as well as Alexa 647 phalloidin and DAPI to visualize actin filaments and nuclei, respectively. Most of the expressed GFP fluorescence was preserved with this fixation method. To evaluate transduction efficiency, 7–10
random fields per slide were chosen from each of 4 different preparations and all reconstituted acini in the field were subjected to quantitation of transduction efficiency. The cells in the acini expressing DN-PKC-ε were easily determined based on the increased intensity of PKC-ε labeling of apical and basolateral membranes in parallel with the extended process formation. The cells in the acini expressing GFP were also easily determined. The number of nuclei within the reconstituted acini was used for normalization to cell number. (The small numbers of single cells were not counted, because they did not represent reconstituted and functionally competent acini.)

Analysis of the extent of colocalization between apical actin and PKC-ε in confocal microscopy images was done with the Enhanced Colocalization tool available with the Zeiss LSM510 software. Fluorescence intensities shown in red (actin filaments labeled with rhodamine phalloidin) were recorded with the 543-nm laser at 100% transmission. Fluorescence intensities shown in green (PKC-ε labeled with appropriate primary and FITC-labeled secondary antibodies) were measured with the 488-nm laser at 2% transmission. Narrow band-pass filters were used to avoid bleaching through the channels, and the palette function of the software was used to eliminate intensity saturation. For measurement of colocalization, a region of interest was selected around each luminal area that extended beneath it by ~1 μm; the luminal area was defined by accumulation of actin filaments as previously described (9). The thresholding function was used to establish the background intensity. The colocalization coefficients obtained were calculated as follows, and they reflect the total number of colocalizing pixels within the region of interest for each marker divided by the total pixels in that channel:

\[
c_1 = \frac{\text{pixels}_{c1,\text{coloc}}}{\text{pixels}_{c1,\text{total}}} \quad c_2 = \frac{\text{pixels}_{c2,\text{coloc}}}{\text{pixels}_{c2,\text{total}}}
\]

**Biochemical analysis of PKC-ε-actin filament binding.** For actin filament binding assays, the nonmuscle actin-binding protein biochemical kit from Cytoskeleton (Denver, CO) was used. Cell lysates were prepared by lysing acinar cells (unstimulated or exposed to CCH for 2.5, or 15 min at 100 μM) in buffer A (5 mM Tris-HCl, pH 8.0, containing 0.2 mM CaCl₂ and protease inhibitor cocktail (12)) through a 21-gauge needle on ice and clarified by low-speed centrifugation as described above. The supernatant fraction was concentrated in Amicon 3000 filters before addition of polymerized actin and sedimentation according to the manufacturer’s protocol. PKC-ε and actin contents in supernatant and pellet fractions were measured by Western blotting.

Sequential detergent extraction was performed as described previously (41). The distribution of PKC-ε and actin across each of the pools was determined by SDS-PAGE and Western blotting. Western blots were processed with appropriate primary antibodies and either goat anti-mouse, anti-rabbit, or anti-sheep secondary antibodies conjugated to IRDye800. Blots were quantified with an Odyssey scanning infrared fluorescence imaging system (Li-Cor, Lincoln, NE). For display, fluorescent signals were converted digitally to black and white.

**Secretion assays.** Lacrimal acini seeded in Matrigel-coated 24-well plates and either transduced with Ad-GFP or Ad-DN-PKC-ε or treated with GF-109203X and Go6976 for 3 h previously described in (41). The distribution of PKC-ε and actin across each of the pools was determined by SDS-PAGE and Western blotting. Western blots were processed with appropriate primary antibodies and either goat anti-mouse, anti-rabbit, or anti-sheep secondary antibodies conjugated to IRDye800. Blots were quantified with an Odyssey scanning infrared fluorescence imaging system (Li-Cor, Lincoln, NE). For display, fluorescent signals were converted digitally to black and white.

To further verify the apparent increased association of PKC-ε with actin filaments associated with CCH stimulation, we used an additional protocol based on isolation of subcellular protein pools with sequential detergent extraction to analyze PKC-ε partitioning among soluble (saponin solubile), membrane (Triton X-100 solubile), and cytoskeletal (SDS solubile) pools isolated from unstimulated and CCH-stimulated lacrimal acini. A representative blot of PKC-ε and actin contents of each pool from unstimulated and CCH-stimulated acini (100 μM, 15 min) is shown in Fig. 1D, and Fig. 1E shows summary data from several preparations. It should be noted that PKC-ε and actin in the cytoskeletal pool migrate more slowly, because of the presence of high amounts of detergent in this fraction necessary to solubilize filaments. In unstimulated acini, most PKC-ε (~60%) was recovered in the cytosolic pool, with only a trace amount in the membrane pool and the remainder (~30%) enriched in the cytoskeletal pool. In CCH-stimulated acini, a significant (P ≤ 0.05) shift in the partitioning of protein before normalization to control and comparison across treatments. Differences in experimental groups in all secretion assays were determined by a paired t-test with P ≤ 0.05.

**RESULTS**

Lacrimal acinar PKC-ε associates with actin filaments. To demonstrate that lacrimal acinar PKC-ε associates with actin, we performed several in vitro and intact cell assays. Actin binding proteins can be identified by their cosedimentation with polymerized nonmuscle actin. During establishment of the parameters of the actin sedimentation assays, control experiments revealed that added bovine serum albumin did not pellet with the polymerized actin but remained in the supernatant fraction, whereas added α-actinin, a known actin-binding protein, did partition with polymerized actin filaments in the pellet (Fig. 1A). A weaker protein signal showing the major band with a molecular mass of ~95 kDa that likely represented PKC-ε could also be detected by Coomassie blue staining of the gel in the lysate lane in the pellet fraction when nonmuscle actin was added to the reaction. As shown in Fig. 1B, analysis of the actin pellet by Western blotting revealed that PKC-ε was cosedimented in unstimulated or control (Con, +actin) acini, whereas equivalent sedimentation in the absence of exogenous polymerized actin (Con, −actin) did not result in sedimentation of PKC-ε. Analysis of PKC-ε in the supernatant remaining after sedimentation of actin also revealed depletion of the cytosolic pool (data not shown).

Because PKC-ε is activated by secretagogue exposure in lacrimal acini (46), including agents acting through M₃ muscarinic receptors, we investigated whether the interaction between PKC-ε and actin was increased in lysates from CCH-stimulated lacrimal acini. When actin filament binding assays were performed with lysates from acini exposed to CCH (100 μM) for 2.5, or 15 min, the amount of PKC-ε recovered with polymerized actin in the pellet was increased significantly at 2 and 15 min of CCH stimulation, as evidenced by an increase in the ratio of PKC-ε to actin signal on Western blots (Fig. 1, B and C). Although the increase in PKC-ε/actin signal was not statistically significant at 5 min of CCH stimulation, a trend toward an increase was evident at this time, consistent with the other time points chosen for analysis and with confocal fluorescence microscopy findings (see below).

To further verify the apparent increased association of PKC-ε with actin filaments associated with CCH stimulation, we used an additional protocol based on isolation of subcellular protein pools with sequential detergent extraction to analyze PKC-ε partitioning among soluble (saponin solubile), membrane (Triton X-100 solubile), and cytoskeletal (SDS solubile) pools isolated from unstimulated and CCH-stimulated lacrimal acini. A representative blot of PKC-ε and actin contents of each pool from unstimulated and CCH-stimulated acini (100 μM, 15 min) is shown in Fig. 1D, and Fig. 1E shows summary data from several preparations. It should be noted that PKC-ε and actin in the cytoskeletal pool migrate more slowly, because of the presence of high amounts of detergent in this fraction necessary to solubilize filaments. In unstimulated acini, most PKC-ε (~60%) was recovered in the cytosolic pool, with only a trace amount in the membrane pool and the remainder (~30%) enriched in the cytoskeletal pool. In CCH-stimulated acini, a significant (P ≤ 0.05) shift in the partitioning...
PKC-ε is an actin-binding protein in lacrimal acini. A: SDS-PAGE gel stained with Coomassie blue shows the supernatant (Sup; left) and pellet (right) fractions from actin-binding assays. Lysates from lacrimal acini (Lysate) were incubated without (−) or with (+) nonmuscle actin, and filaments were pelleted by centrifugation as described in MATERIALS AND METHODS. α-Actinin (an actin-binding protein) and BSA were used as positive and negative controls, respectively. α-Actinin was pelleted with actin filaments (arrow shows position in pellet fraction), whereas BSA was not (arrow shows position in Sup fraction). A weaker protein signal showing the major band with a molecular mass (MM) corresponding to PKC-ε of ~95 kDa could also be detected in the lysate lane in the pellet fraction when nonmuscle actin was added to the reaction (arrow labeled PKC-ε). B: Western blot (WB) analysis of the pellet fraction from a representative actin-binding assay when lysates (from equal amounts of cells) from acini stimulated without (Con) or with carbachol (CCH) for the indicated periods of time (2–15 min, 100 μM) were used for the actin binding assay, and pellet fractions were blotted for PKC-ε and actin as indicated. Duplicate samples were run in each assay as shown. C: summary of actin-binding experiments in B obtained from 3 independent preparations. *Significant at P < 0.05. D: lacrimal acini without or with CCH (100 μM, 15 min) were subjected to sequential detergent extraction to isolate soluble, membrane, and cytoskeletal pools as described in MATERIALS AND METHODS. Equal volumes of each of the fractions were resolved by SDS-PAGE, and the sample content of PKC-ε and actin was determined by Western blotting. Sap, saponin; Tx-100, Triton X-100. E: composite values reflecting PKC-ε enrichment within soluble, membrane, and cytoskeletal pools as described in C and expressed as % of total cellular PKC-ε. Stimulation did not affect the recovery of marker proteins in the 3 fractions (data not shown). Results are means ± SE from n = 3 experiments. *P < 0.05.
of PKC-ε was noted, with only ~30% recovered in the cytosolic fraction and ~55% detected in the cytoskeletal fraction containing most of the cellular actin. This finding, as well as the findings from actin sedimentation assays in Fig. 1, B and C, strongly suggested that PKC-ε translocation to actin filaments occurred in response to CCH stimulation. The α₁-adrenergic agonist PE showed only a modest trend toward an increase in PKC-ε association with actin in these assays (data not shown), consistent with previous findings that this agent is a weak secretagogue in rabbit lacrimal acini (32).

The cellular localization of PKC-ε in unstimulated and CCH-stimulated acini was investigated in parallel. Figure 2 shows results from a representative preparation with actin filament labeling in red and PKC-ε in green. Cytoskeletal organization in reconstituted lacrimal acini has been characterized previously (9, 10). Briefly, the apical or luminal regions in lacrimal acini can be distinguished by the more intense actin filament labeling detected in roughly circular regions (marked by asterisks in Fig. 2A) associated with actin filament enrichment beneath the APM and within microvilli. As shown in the images in Fig. 2A, apical actin filaments in unstimulated acini appear continuous, without the appearance of multiple invaginations. Fainter actin filament labeling can also be detected beneath basolateral membranes. In unstimulated acini, some PKC-ε was colocalized with apical actin and also in the cytosol in punctate structures. CCH stimulation (100 μM, 5 min) appeared to increase the colocalization of PKC-ε with apical actin at the lumen and also within transient actin-coated structures, seen as apparent invaginations of the apical actin array in the luminal region. The intensity profile in Fig. 2B depicts the fluorescence in each channel across the region marked in each image by the line in Fig. 2A, confirming, by the increase in coincident peaks of fluorescence pixel intensity, the increased colocalization. Colocalization of these two markers beneath the lumen was detected as early as 2 min of CCH stimulation and was sustained to 15 min (data not shown). To quantify the colocalization of apical PKC-ε and actin across multiple samples, we measured the extent of colocalization of luminal PKC-ε pixels with actin pixels as a percentage of total PKC-ε pixels and the colocalization of luminal actin pixels with PKC-ε pixels as a percentage of total actin pixels (Fig. 2C). These measurements were conducted in resting (control) acini as well as in acini exposed to CCH (100 μM) for 5 min, within the time interval associated with microscopic and biochemical colocalization and well within the initial secretory burst from 0 to 10 min (9). Although some colocalization was seen in resting acini, CCH caused a significant increase in colocalization of each marker with the other. PE treatment caused a modest increase in colocalization of PKC-ε with apical actin (data not shown). Biochemical and confocal fluorescence microscopy data in Figs. 1 and 2 collectively suggested a strong interaction between lacrimal acinar PKC-ε and actin filaments that was increased in acini exposed to CCH.

**Introduction of DN-PKC-ε alters acinar actin organization.**

DN mutations in PKC isoforms utilize a point mutation in the ATP binding domain that renders the enzyme inactive (35). Such a mutation in PKC-ε has been generated (K437R) and inserted into an Ad expression vector (17). We transduced lacrimal acini with Ad-DN-PKC-ε coexpressing GFP and examined the efficiency of transduction. Although expression of GFP was clearly high, as evidenced by fluorescence microscopy (data not shown), GFP is the second gene inserted into the Ad expression vector and would not be expected to show as much expression as DN-PKC-ε, the first gene expressed by the Ad expression vector. We therefore conducted additional evaluations of transduction efficiency based on confocal fluorescence microscopy. Using acini fixed and processed to label PKC-ε, actin filaments, nuclei, and GFP as shown in Fig. 3A, we quantified DN-PKC-ε and GFP overexpression in multiple fields chosen randomly from each of several preparations. All reconstituted acini in the field were subjected to quantitation of transduction efficiency. The number of nuclei within the reconstituted acini was used for normalization. The percentage of cells within the reconstituted acini overexpressing DN-PKC-ε was 85 ± 2%, and the percentage expressing GFP was 62 ± 2% (n = 4). Therefore, in excess of 80% of reconstituted acini were transduced with Ad-DN-PKC-ε at a MOI of 5. Analysis of PKC-ε content in lysates of acini transduced at different MOIs revealed considerable overexpression of the DN-PKC-ε at a MOI of 5, with little additional expression elicited above that dose (Fig. 3B). Subsequent experiments used a MOI of 5 for transduction with Ad-DN-PKC-ε or Ad-GFP (as a control). The increased PKC-ε immunofluorescence detected in transduced acini was extensively colocalized with apical and basolateral actin filaments, whereas organization of actin at these domains was markedly different (Fig. 3, C and D). At the APM, actin accumulation was evident in acini overexpressing DN-PKC-ε, and lumina had a compressed appearance. Additionally, actin-coated invaginations were abundant (Fig. 3D, arrowheads) and were similar to the actin-coated structures detected transiently in CCH-stimulated acini (Fig. 2A). However, the actin coats in acini expressing DN-PKC-ε were always detected. Transduced acinar cells also exhibited increased attachment and spreading at the basolateral domains, resulting in an extended or elongated appearance (Fig. 3D, arrows) relative to their normally globular shape (Fig. 2). In some cases, small processes could be detected extending from the basolateral surface. Transduction with Ad-GFP alone elicited no change in acinar cytoskeleton (data not shown), as previously published (41).

Control experiments revealed that overexpression of DN-PKC-ε did not alter the normal recruitment of the conventional PKC isoform PKC-α to basolateral membranes in response to CCH stimulation for 15 min (data not shown), as previously reported (47). Moreover, the normal binding of the actin-binding protein ezrin to apical actin filaments in acini was not disrupted by overexpression of DN-PKC-ε (data not shown). These control studies suggested that the overall cellular signaling pathways involving diacylglycerol were not disrupted by DN-PKC-ε overexpression and, furthermore, that the apical actin filaments were not decorated with DN-PKC-ε to such an extent that other actin-binding proteins were unable to bind.

To better visualize the morphological changes associated with overexpression of DN-PKC-ε in the lacrimal acini, we performed three-dimensional (3D) reconstruction of serial sections acquired in lacrimal acini. Figure 4 shows the 3D reconstructed shapes of nontransduced acini and acini overexpressing DN-PKC-ε (Ad-DN-PKC-ε) rotated at different angles for better visualization of the features induced by DN-PKC-ε [movie 1 (control) and movie 2 (Ad-DN-PKC-ε); supplemental data for this article may be found at http://ajpcell.physiology.org].
The straight arrows in Fig. 4 indicate elongated neuritelike projections extending from the basolateral surface that are commonly detected in acini transduced with DN-PKC-ε but not in nontransduced acini. Although clearly revealing the more convoluted lumina associated with accumulation of actin-coated invaginations in acini transduced with DN-PKC-ε, these and additional projections did not reveal systematic evidence for alterations in the...
apparent accessibility of the lumina to the culture medium associated with DN-PKC-ε overexpression.

The effects of DN-PKC-ε overexpression on basal and stimulated secretion of bulk protein and the secretory product β-hexosaminidase are shown in Fig. 5A. Many lumina in reconstituted acini are open to the culture medium, so that secretion can be measured by collecting culture medium, measuring the marker of interest, and normalizing the signal to pellet protein as previously described (9, 10, 41). CCH is a robust secretogogue in reconstituted rabbit lacrimal acini, eliciting ~3.5-fold increase in both bulk protein and β-hexosaminidase release. GFP overexpression minimally affected this pattern of release. In contrast, DN-PKC-ε caused a significant ($P \leq 0.05$) increase in basal protein and β-hexosaminidase release that was significant relative to Ad-GFP-treated acini, with a concomitant significant decrease in both total and stimulated components of the release. We tried to evaluate the effects of DN-PKC-ε on secretion evoked by PE in parallel. Consistent with previous results (32), PE only weakly increased secretory functions, promoting only a 0.5 × increase in bulk protein release and minimally affecting β-hexosaminidase release. A trend toward increased basal release relative to Ad-GFP-transduced acini and a complete inhibition of PE-stimulated release were noted in these assays, although the magnitude of the effect made it difficult to distinguish actual changes from the normal variation inherent in the assay.

To confirm that this inhibition of CCH-stimulated protein and β-hexosaminidase secretion was caused specifically by the inhibition of cellular PKC-ε activity caused by introduction of the DN variant, we also utilized chemical inhibitors of PKC isoforms in these assays. The PKC inhibitor G6-6976 specifically inhibits only the Ca$^{2+}$-dependent PKC isoforms, α and β, without exerting any effects on PKC-δ, -ε, or -ζ isoforms (21). In contrast, the PKC inhibitor GF-109203X inhibits PKC-α, -β, -δ, and -ε isoforms with equimolar potency (21). As shown in Fig. 5B, pretreatment of lacrimal acini with G6-6976 elicited a small but significant increase in basal release of protein and β-hexosaminidase but caused no suppression of total release. CCH-stimulated release was significantly reduced. In contrast, pretreatment of lacrimal acini with GF-109203X elicited no effects on basal release of protein or β-hexosaminidase but promoted a significant inhibition of total and CCH-stimulated release. The CCH-stimulated decrease in protein release promoted by GF-109203X was also significantly reduced relative to the decrease promoted by G6-6976. Relative to the spectrum of PKC isoforms expressed in lacrimal acini (α, ε, δ, μ, and η/λ), GF-109203X should elicit additional inhibition of PKC-δ and PKC-ε, relative to G6-6976 at these doses, suggesting that the impaired secretion was due to effects on one or both of these kinases. Because comparable effects on CCH-stimulated protein and β-hexosaminidase secretion were noted in acini transduced with Ad-DN-PKC-ε, it was reasonable to conclude

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Fig. 3. High-efficiency transduction of lacrimal acini with a construct of replication-deficient adenovirus (Ad) expressing dominant-negative (DN) PKC-ε (Ad-DN-PKC-ε) results in colocalization of overexpressed DN-PKC-ε with actin filaments. A: PKC-ε (red), actin filaments (pink), nuclei (blue), and green fluorescent protein (GFP; green) in acini transduced with Ad-DN-PKC-ε coexpressing GFP at a multiplicity of infection (MOI) of 5 and fixed and processed for quantitation of transduction efficiency as described in MATERIALS AND METHODS. B: Western blot showing the expression of PKC-ε in lysates of lacrimal acini transduced with Ad-DN-PKC-ε at the indicated MOI. Equal amounts of protein were loaded in each lane. C: confocal fluorescence micrographs of lacrimal acini transduced with Ad-DN-PKC-ε and fixed and processed as described in MATERIALS AND METHODS to label actin filaments (red) and PKC-ε (green). Note that cytosolic GFP fluorescence coexpressed by the Ad-DN-PKC-ε construct is destroyed during fixation. D: confocal fluorescence micrographs showing actin filament organization in lacrimal acini transduced with Ad-DN-PKC-ε. Arrowheads in C and D indicate accumulation of actin-coated structures at the apical plasma membrane (APM); arrows indicate basolateral actin filaments associated with areas of cell spreading and process formation; asterisks indicate luminal regions; Scale bars = 5 μm in all panels.
that PKC-ε was at least a major target associated with the inhibition of secretion by GF-109203X.

Investigation of actin filament organization in lacrimal acini exposed to these broad-spectrum inhibitors revealed that pretreatment of lacrimal acini with GF-109203X also caused changes similar to those elicited by DN-PKC-ε transduction, including accumulation and bundling of apical actin filaments and elongation of cellular actin-enriched processes from the basolateral membrane (Fig. 5C). In contrast, actin filament organization in acini pretreated with Gö-6976 showed some evidence for increased apical actin, but not to the same extent as that caused by GF-109203X or DN-PKC-ε; no significant changes in basolateral actin or process formation were noted (Fig. 5C).

These results suggested that the normal pathways of CCH-stimulated secretion were inhibited by introduction of DN-PKC-ε. However, because bulk protein is a relatively nonspecific marker of secretion, it was difficult to discern whether the inhibition was exclusively due to alterations in apical targeting and release or whether it was complicated by changes in the basolateral release profile. Although β-hexosaminidase is a secretory protein, it is also present in basolateral membrane compartments that may, under some conditions, contribute to basolateral exocytosis. We therefore investigated whether DN-PKC-ε could modulate the CCH-stimulated release of two apically targeted proteins, syncollin-GFP and SC, into culture medium. PE treatment was not further pursued because of its limited efficacy as a secretagogue.

*CCH-stimulated release of syncollin-GFP is inhibited by DN-PKC-ε.* Studies with a syncollin-GFP fusion protein have shown labeling of large protein-enriched SVs in diverse systems including zymogen granules in pancreatic acini (15), large dense core vesicles in AT-20 cells (15), insulin granules in pancreatic β-cells (20), but not synapse-like or smaller SVs. These findings suggested syncollin-GFP as an excellent candidate for labeling of protein-enriched mature lacrimal acinar SVs. Figure 6A shows a 3D reconstructed image of the sub-apical region and interior cytoplasm from a live reconstituted acinus transduced with syncollin-GFP [see movie 3 (syncollin-GFP)]. A number of large spherical vesicles delineated by syncollin-GFP fluorescence were detected throughout the acini, with a particular enrichment beneath the APM (asterisk, Fig. 6A). Some of these vesicles appeared to have “tails” due to saltatory movement during image acquisition. The diameter of the vesicles ranged from 0.2 to 1 μm, with most vesicles (60%) sized from 300 to 500 nm in diameter. The 1-μm vesicles, representing ~30% of the total, were more apically localized. This fluorescence pattern suggested specific labeling of a subpopulation of mature SVs, consistent with previous work (15, 20).

Figure 6B shows selected images from live rabbit lacrimal acini transduced with Ad-syncollin-GFP and acquired by time-lapse microscopy over a 20-min interval within a single focal plane. As in the 3D reconstruction, syncollin-GFP was detected in large apparent vesicles throughout the acini that were particularly abundant (arrowheads, Fig. 6B) beneath the luminal regions (asterisk, Fig. 6B) detected in DIC micrographs acquired in parallel. Fainter, more diffuse syncollin-GFP labeling was detected above the basolateral membrane adjacent to the nucleus (arrow, Fig. 6B), likely reflecting enrichment in biosynthetic membrane compartments located in these regions. On
DOMINANT-NEGATIVE PKC-ε INHIBITS APICAL EXOCYTOSIS

A

CCH

Protein Release (A.U.)

bas tot stim bas tot stim bas tot stim bas tot stim
CON Ad-GFP Ad-DN-PKCε

β-hexosaminidase Activity (A.U.)

bas tot stim bas tot stim bas tot stim bas tot stim
CON Ad-GFP Ad-DN-PKCε

PE

Protein Release (A.U.)

bas tot stim bas tot stim bas tot stim bas tot stim
CON Ad-GFP Ad-DN-PKCε

β-hexosaminidase Activity (A.U.)

bas tot stim bas tot stim bas tot stim bas tot stim
CON Ad-GFP Ad-DN-PKCε

B

CCH

Protein Release (A.U.)

bas tot stim bas tot stim bas tot stim bas tot stim
DMSO Gö 5 μM Gö 10 μM GF 10 μM

β-hexosaminidase Activity (A.U.)

bas tot stim bas tot stim bas tot stim bas tot stim
DMSO Gö 5 μM Gö 10 μM GF 10 μM

C

Actin

CON Gö 10 μM GF 10 μM
CCH stimulation, a depletion of the subapical stores of syncollin-GFP was detected over time (arrowheads, Fig. 6B). The graph in Fig. 6C plots the fluorescence intensity of the entire region at 0 s and at 1,189 s. The subapical syncollin-GFP localized adjacent to the lumina in the untreated acinus was clearly the most intense (red labeling, Fig. 6C). At 1,189 s of CCH stimulation, this intensity was clearly diminished.

We were unable to analyze lacrimal acini dually transduced with Ad-synccollin-GFP and Ad-DN-PKC-ε (which coexpresses GFP) by live cell confocal microscopy because syncollin-GFP-enriched vesicles could not be resolved above the strong cytosolic GFP fluorescence. Syncollin-GFP can be visualized in fixed, transduced acini under fixation conditions associated with quenching of GFP and with appropriate primary and fluorescently labeled secondary antibodies in parallel with other markers. Figure 7A shows syncollin-GFP (green) detected by immunofluorescence in lacrimal acini co-transduced with Ad-synccollin-GFP and Ad-DN-PKC-ε (red) relative to Ad-synccollin-GFP alone. These images show that Ad-DN-PKC-ε-overexpressing acini (detected easily by the intense PKC-ε immunofluorescence associated with apical and basolateral actin) do not affect the abundance of syncollin-GFP-enriched vesicles or their apical enrichment in the same acinus. Interestingly, some colocalization of syncollin-GFP was detected with the actin-coated structures induced by overexpression of DN-PKC-ε (arrows and green and purple labels, Fig. 7A), consistent with our proposal that these actin-enriched structures were SV fusion intermediates.

Because we were unable to use live cell confocal microscopy to investigate the effect of DN-PKC-ε on exocytosis of syncollin-GFP in real time, we used Western blotting to measure syncollin-GFP release into culture medium under different experimental conditions. As shown in Fig. 7B, CCH stimulation caused a threefold increase in the recovery of syncollin-GFP in the culture medium of Ad-synccollin-GFP-transduced acini, which paralleled the loss of subapical fluorescence seen in live acini exposed to CCH (Fig. 6). Cotransduction of acini with Ad-DN-PKC-ε but not Ad-GFP significantly inhibited CCH-stimulated release of syncollin-GFP into culture medium (Fig. 7, B and C). Cotransduction of acini with Ad-DN-PKC-ε and Ad-synccollin-GFP moderately reduced syncollin-GFP expression relative to cotransduction with GFP and synccollin-GFP (data not shown), an effect that was likely due to nonspecific changes associated with overexpression of three exogenous proteins in cotransduced acini (GFP and PKC-ε by Ad-DN-PKC-ε and synccollin-GFP by Ad-synccollin-GFP). The percentage of total cellular syncollin-GFP released in response to CCH under each experimental condition represented ∼20–30% of total cellular syncollin-GFP, suggesting that sufficient cellular stores were available for exocytosis under all conditions (data not shown). Abundant syncollin-GFP immunofluorescence could also be detected in acini cotransduced with Ad-DN-PKC-ε (Fig. 7A). Finally, acini dually transduced with Ad-synccollin-GFP and Ad-DN-PKC-ε or Ad-GFP exhibited the same general profiles of β-hexosaminidase release reported for acini transduced with Ad-DN-PKC-ε or Ad-GFP alone in Fig. 5 (Table 1). These findings indicated that the overall acinar secretory pathway was intact in acini expressing syncollin-GFP and that DN-PKC-ε had the same magnitude of inhibitory effect on β-hexosaminidase release, regardless of syncollin-GFP coexpression. We also determined that GF-109203X but not Gö-6976 was able to exert the same significant inhibition of CCH-stimulated syncollin-GFP release, additional evidence that this change was reliant on the reduction in PKC-ε activity (Table 2). From these data, we concluded that DN-PKC-ε significantly and specifically inhibited apical exocytosis of syncollin-GFP in lacrimal acini.

Fig. 5. PKC-ε inhibition impairs secretagogue-stimulated release of protein and β-hexosaminidase in lacrimal acini. A: lacrimal acini grown on Matrigel-coated dishes were transduced with Ad-DN-PKC-ε or Ad-GFP on day 2 of culture as described in MATERIALS AND METHODS and analyzed on day 3 for secretion. Bulk protein secretion and β-hexosaminidase activity released into culture medium from transduced acini exposed without or with CCH (100 μM, 30 min) or phenylephrine (PE; 100 μM, 30 min) is shown. B: lacrimal acini grown on Matrigel-coated dishes were treated with Gö-6976 (Gö; 5 or 10 μM) or GF-109203X (GF; 10 μM) for 3 h before stimulation with CCH (100 μM, 30 min). Bulk protein and β-hexosaminidase activity released into culture medium is shown. A and B: open bars, basal (unstimulated) release; gray bars, total release (basal + stimulated); filled bars, stimulated component (total − basal). Values were normalized to cell protein before comparison across samples. Values are means ± SE expressed in arbitrary units (AU); n = 7 separate preparations for CCH stimulation and 4 separate preparations for PE stimulation in acini transduced with Ad-DN-PKC-ε or Ad-GFP; n = 5 separate preparations for acini treated with Gö or GF. *Significant decrease at P < 0.05 from paired control; †significant increase at P = 0.05 from paired control; ‡significant decrease at P ≤ 0.05 from Gö-treated, CCH-stimulated acini. C: confocal fluorescence micrographs of lacrimal acini without (control) or with treatment with Gö (10 μM, 3 h) or GF (10 μM, 3 h) fixed and processed for labeling of actin filaments with rhodamine phalloidin as described in MATERIALS AND METHODS. Scale bar = 5 μm.
with Ad-DN-PKC-ε revealed abundant pIgR immunoreactivity detected at and beneath the apical actin array of both unstimulated and CCH-stimulated acini, similar to that seen in the CCH-stimulated acini in Fig. 8 and consistent with accumulation of pIgR at the APM or subapical stores (data not shown). Finally, we determined that GF-109203X but not Gö-6976 was able to significantly reduce CCH-stimulated SC release, additional evidence that this change was reliant on changes in PKC-ε activity (Table 3).

DISCUSSION

Inspired by the fact that PKC-ε is the only PKC with an actin-binding site (2), and by observations that it is activated both by muscarinic and α₁-adrenergic receptor agonists in lacrimal gland (46), we investigated whether PKC-ε participates in regulation of apical actin and/or apical exocytosis. We found that the association of PKC-ε with apical actin filaments and actin-coated structures was increased after CCH stimulation in lacrimal acini. To probe its functional role in exocytosis, we needed to selectively inhibit its activity. We chose a DN strategy for inhibition of PKC-ε activity in acini, using replication-defective Ad to introduce the DN-PKC-ε. Lacrimal acini were readily transduced at high efficiency with Ad constructs, enabling us to express the DN-PKC-ε in essentially all cells and then to identify individual transduced cells by immunofluorescence for examination of other features. Overexpression of DN-PKC-ε resulted in profound changes in apical and basolateral actin filament organization in parallel with inhibition of the CCH-stimulated secretion of protein and β-hexosaminidase. Independent verification of the ability of PKC-ε inhibition to affect CCH-stimulated secretion and actin filament organization was obtained by our findings comparing the effects of the chemical PKC inhibitors Gö-6976 and GF-109203X in acini. Although CCH-stimulated protein secretion was significantly reduced by both of these inhibitors, the decrease promoted by GF-109203X was significantly reduced relative to that elicited by Gö-6976. These studies showed an inhibitory effect of GF-109203X but not Gö-6976 on CCH-stimulated β-hexosaminidase secretion in parallel with changes in apical and basolateral actin filament organization elicited by GF-109203X. The additional effects of GF-109203X are attributable to the ability of this inhibitor to inhibit PKC-ε and -δ in addition to PKC-α and -β, which are also inhibited by Gö-6976. The greater inhibitory effect of GF-109203X on CCH-stimulated protein and β-hexosaminidase secretion relative to DN-PKC-ε suggests the additional involvement of PKC-δ in facilitating an aspect of exocytosis.

After confirmation that the effects on actin filament organization and impaired exocytosis could be elicited through inhibition of PKC-ε either by introduction of DN-PKC-ε or by treatment with GF-109203X, we continued to explore the cellular effects of DN-PKC-ε overexpression. Acini transduced with DN-PKC-ε exhibited inhibition of the CCH-stimulated release of two markers of apical secretion newly established in this study, synccollin-GFP and SC. These effects could likewise be mimicked by GF-109203X but not Gö-6976. We propose a direct relationship between the alterations in apical actin filaments and the inhibition of exocytotic and transcytotic traffic.

Early work on PKC-ε implicated its increased expression in the transition to a transformed cell phenotype. The oncogenic activity of PKC-ε has been reported in several fibroblast and colonic as well as prostate epithelial cell lines (2, 23, 28, 42). Epidermis-specific transgenic overexpression of wild-type PKC-ε caused development of metastatic carcinomas in mice (16). Convergence of PKC-ε signaling events on the actin cytoskeleton is likely to explain many of the oncogenic effects reported for this kinase.

More recent studies on the cellular roles of PKC-ε have focused on its role in actin filament remodeling associated with a variety of intracellular events. PKC-ε was first identified in association with actin as an effector of exocytosis in hippocampal neurons (31). It has subsequently been shown to also have a role in actin filament remodeling in endocytosis in epithelial cells (36). Cell spreading is dependent on actin cytoskeleton reorganization, and integrins play an important role in linking the extracellular matrix with the cytoskeleton and signaling machinery of the cell. PKC-ε is known to influence cell adhesion and motility through its interactions with β₁-integrin through receptor for activated C kinase 1 (RACK1) and F-actin (2, 6, 37). Coexpression of PKC-ε can restore cell spreading inhibited by tac-β₁, a DN inhibitor of integrin function in normal human fibroblasts and Chinese hamster ovary cells; an intact kinase domain of PKC-ε was required for this process (5). Finally, PKC-ε was recently suggested to be a signaling mediator in the Toll-like signaling receptor pathway and to mediate macrophage and dendritic cell activation in response to lipopolysaccharide (3).

Our studies add to the body of literature implicating PKC-ε in actin filament remodeling in membrane trafficking, specifically exocytosis. In our working model, release of second messengers triggered by CCH stimulation normally results in PKC-ε activation followed by its translocation to apical actin filaments. The activated PKC-ε acts by phosphorylating key targets associated with actin filaments that result in the remodeling of apical actin in ways that facilitate SV exocytosis, including the transient formation of actin-coated structures. We (Jerdeva GV and Hamm-Alvarez SF, unpublished observations) and others (24, 29, 38, 39) have shown that actin filament remodeling is an integral part of apical exocytosis in acinar epithelial cells. Furthermore, stabilization of actin-coated structures, thought to represent fusion intermediates, was correlated with inhibition of acinar exocytosis (Ref. 39; Jerdeva GV and Hamm-Alvarez SF, unpublished observations).

In acini transduced with Ad-DN-PKC-ε, overexpressed PKC-ε was detected with actin filaments of altered organization even in unstimulated acini. The changes caused by DN-PKC-ε included accumulation of actin at the APM in the underlying filament network as well as accumulation of actin-coated structures representing prospective fusion intermediates. We hypothesize that the inhibitory effects of DN-PKC-ε on apical exocytosis were caused by stabilization of apical actin, either in the underlying actin filament network or in actin-coated structures, either of which could impair SV exocytosis. These effects are likely due to the absence of catalytic activity in DN-PKC-ε, which, when recruited to apical actin, is unable to appropriately phosphorylate actin-associated proteins that are normally required for its remodeling. The inhibitory effect on DN-PKC-ε on SC exocytosis suggested a comparable role for actin filament remodeling in the terminal events associated with its release (through transcytotic or exocytotic path-
Fig. 6. CCH stimulation of lacrimal acini transduced with Ad-syncollin-GFP depletes subapical syncollin-GFP fluorescence. Lacrimal acini grown on Matrigel-covered glass-bottomed round 35-mm dishes were transduced with Ad-syncollin-GFP on day 2 of culture as described in MATERIALS AND METHODS and imaged on day 3 of culture. A: 3D reconstruction at high magnification of the interior regions of a reconstituted acinus formed by 3 lacrimal acinar cells organized around a central lumen (*), each expressing syncollin-GFP. The reconstruction was obtained by compression of x-y images acquired at z-intervals of 0.5 μm. Dashed line, boundary of uppermost cell relative to the other 2, deduced by comparison to differential interference contrast (DIC) image. Scale bar, 1 μm. B: live acini were imaged in the presence of CCH (100 μM) at the indicated times by time-lapse confocal fluorescence microscopy. Arrowheads indicate regions of major loss of syncollin-GFP intensity surrounding luminal regions (*). Arrows indicate traces of basolateral syncollin-GFP. No major changes in syncollin-GFP intensity were observed when acini were imaged without CCH. Scale bar = 5 μm. C: 2.5D graphic reconstruction of the overall intensity profile of the imaged areas presented in A at 0 and 1,189 s of stimulation with CCH, illustrating individual intensities per pixel with the rainbow scale. The resolution is ~10 pixels/μm.
ways), a hypothesis supported by the colocalization of pIgR and PKC-ε with actin-coated structures in CCH-stimulated acini. Our studies implicating PKC-ε as a major effector of apical actin remodeling in exocytosis in lacrimal acini are consistent with findings from other systems. Prekeris et al. (31) found that PKC-ε was necessary for exocytosis of synaptic vesicles in hippocampal neurons. DN-PKC-ε also significantly reduced glucose-stimulated exocytosis of insulin in insulin-secreting INS-1E cells (22). The latter study also demonstrated glucose-induced association of endogenous PKC-ε with insulin granules, confirming a specific translocation to vesicles involved with exocytosis. Although its function in exocytosis was not specifically investigated, PKC-ε was also detected at the APM of pancreatic acini (4).

Fig. 7. DN-PKC-ε inhibits CCH-stimulated release of syncollin-GFP into culture medium in cotransduced lacrimal acini. A: high-magnification view of the APM region of acini cotransduced with Ad-syncollin-GFP and Ad-DN-PKC-ε or Ad-syncollin-GFP alone. Transduced acini were fixed and labeled as described in MATERIALS AND METHODS to detect syncollin (green), PKC-ε (red), and actin filaments (purple). Overlay shows all 3 fluorescence labels as well as the paired DIC image. Fixation quenches the intrinsic GFP fluorescence present in cytosol and on syncollin in these transduced acini. Arrow, colocalization of syncollin-GFP with an actin-coated imagination; *, luminal region. Scale bar = 5 μm. B: Western blots showing syncollin-GFP release into culture medium in the absence (−) and presence (+) of 100 μM CCH for 30 min in lacrimal acini transduced with Ad-syncollin-GFP without or with Ad-GFP or Ad-DN-PKC-ε. Syncollin release was detected with an anti-syncollin antibody combined with an appropriate IRDye800-conjugated secondary antibody. C: syncollin-GFP release under each experimental condition was quantified as shown in A, normalized to cell protein in the pellet, and compared across treatments. Open bars, basal (Con) release; gray bars, CCH-stimulated release. Values are means ± SE; n = 3 separate preparations. *Significant at P ≤ 0.05 from samples cotransduced with Ad-GFP.
Our previous investigations on the mechanisms of exocytosis in lacrimal acini have been hampered by our inability to track specific secretory products released exclusively at the APM. Syncollin-GFP, previously established as a marker of large dense core vesicles and zymogen granules in related systems (15, 20), was enriched in large, 1-μm diameter, spherical structures beneath the APM, consistent with its incorporation into lacrimal acinar mature SVs. Additional syncollin-GFP was also present in smaller vesicles ranging from 300 to 500 nm in diameter; these vesicles were detected in the cytoplasm as well as beneath the APM. This pattern suggested specific labeling of a subpopulation of mature 1-μm SVs that might be generated from the smaller, more abundant 300- to 500-nm SVs representing ~60% of the syncollin-GFP population. The fluorescence associated with these subapical vesicles was substantially diminished after CCH stimulation in parallel with the increased recovery of syncollin-GFP in the culture medium, confirming that the syncollin-GFP-enriched vesicles were in fact SVs. The inhibition of syncollin-GFP release in acini cotransduced with Ad-DN-PKC-ε but not Ad-GFP enabled us to conclude that apical exocytosis, in particular, was affected.

Little is known about the cellular mechanisms underlying the release of SC and secretory IgA at the APM of lacrimal acinar cells, although common wisdom suggests that some stores are transported via the transcytotic pathway as in other epithelial cells. One previous study investigated the release of SC from cultured lacrimal acini over 4–7 days, demonstrating that CCH modestly but significantly inhibited its release (18). The inhibitory effect of CCH in this previous study might have been due to the long incubation time, which allowed for the depletion of SC stores. In contrast, our shorter incubation time, performed in the presence of CCH, showed a more pronounced effect on SC release, consistent with the results obtained with acini transfected with Ad-DN-PKC-ε.

### Table 1. Effects of Ad-syncollin-GFP on lacrimal acinar secretion of β-hexosaminidase

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Experimental Treatment</th>
<th>β-Hexosaminidase Release, % of Nontransduced Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Ad</td>
<td>Basal</td>
<td>100 ± 8</td>
</tr>
<tr>
<td></td>
<td>Total (in presence of CCH)</td>
<td>174 ± 34</td>
</tr>
<tr>
<td></td>
<td>Stimulated (difference)</td>
<td>74 ± 34</td>
</tr>
<tr>
<td>Ad-syncollin-GFP + Ad-GFP</td>
<td>Basal</td>
<td>97 ± 2</td>
</tr>
<tr>
<td></td>
<td>Total (in presence of CCH)</td>
<td>159 ± 29</td>
</tr>
<tr>
<td></td>
<td>Stimulated (difference)</td>
<td>62 ± 27 (84% of value elicited in nontransduced)</td>
</tr>
<tr>
<td>Ad-syncollin-GFP + Ad-DN-PKC-ε</td>
<td>Basal</td>
<td>106 ± 6</td>
</tr>
<tr>
<td></td>
<td>Total (in presence of CCH)</td>
<td>149 ± 25</td>
</tr>
<tr>
<td></td>
<td>Stimulated (difference)</td>
<td>43 ± 21 (58% of value elicited in nontransduced)</td>
</tr>
</tbody>
</table>

Results are means ± SE from n = 3 separate preparations and errors. Ad, adenovirus; GFP, green fluorescent protein; DN, dominant negative. Carbachol (CCH) stimulation was for 30 min at 100 μM.

### Table 2. Effects of Go−6976 and GF-109203X on lacrimal acinar secretion of syncollin-GFP

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Resting, % of Control</th>
<th>CCH Stimulated, % of Resting Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±49</td>
<td>183±26</td>
</tr>
<tr>
<td>DMSO</td>
<td>100±8</td>
<td>176±4</td>
</tr>
<tr>
<td>Go−6976</td>
<td>111±22</td>
<td>156±15</td>
</tr>
<tr>
<td>GF-109203X</td>
<td>73±8</td>
<td>109±19*</td>
</tr>
</tbody>
</table>

Results are means ± SE from n = 3 separate preparations. CCH stimulation was for 30 min at 100 μM. *Significant reduction from DMSO/CCH at P ≤ 0.05.
DN-PKC-ε was markedly decreased relative to its content in untreated acini (data not shown), suggestive of increased sedimentation with actin filaments and also consistent with the existence of a pool of DN-PKC-ε that was still recruited to actin by CCH stimulation. Acini may maintain a certain percentage of total PKC-ε in association with the actin cytoskeleton even in the resting state to maintain unique structures or morphology. The substitution of DN-PKC-ε for wild-type PKC-ε in transduced acini may underlie some of the remarkable changes in apical actin morphology seen even in the resting state. However, it is clear that the amount of DN-PKC-ε associated with actin cytoskeleton in resting transduced acini is far greater than the amount of endogenous PKC-ε associated with actin cytoskeleton in resting acini.

PKC-ε was not detected with basolateral actin in resting or CCH-stimulated nontransduced acini. However, DN-PKC-ε was extensively colocalized with basolateral actin in parallel with a profound change in the organization of basolateral actin filaments into neuritelike extensions. As discussed above, PKC-ε overexpression is well established as a contributor to cell transformation and cell metastasis (2), processes that involve extensive actin filament remodeling, branching, and extension. However, recent work on the role of PKC-ε in neurite outgrowth has reported that overexpression of PKC-ε and overexpression of the regulatory domain of PKC-ε (containing the actin-binding but not catalytic domains) exerted the same effects on outgrowth (43, 44). It is possible that association of the regulatory domain present in the DN-PKC-ε with the acinar actin cytoskeleton may be able to modulate actin filament reorganization in the absence of catalytic activity, influencing both basolateral and apical actin filament dynamics.

Although a complete consideration of the mechanisms underlying the increased association of DN-PKC-ε with basolateral actin is beyond the scope of this study, it should be noted that such changes could influence apical exocytosis. Interactions between lacrimal acini and extracellular matrix can regulate exocytosis, whereas integrins, in concert with actin cytoskeleton, are thought to modulate some of these interactions (7). Previous work has established that β1-integrin interacts with PKC-ε and actin filaments through its receptor RACK1 and that these interactions regulate cell migration and adhesion (2, 6, 8, 37). Recent work has also suggested that the regulatory (actin binding) domain of PKC-ε can inactivate RhoA and that this mechanism is involved in the switch from an adherent (stress fiber enriched) to a motile (neuritic process) state in neuronal cells (19). RhoA was recently implicated as a positive effector in the formation of actin-coated fusion intermediates in pancreatic acini (25).

Table 3. Effects of Gö-6976 and GF-109203X on lacrimal acinar secretion of SC

<table>
<thead>
<tr>
<th>Cell Treatment</th>
<th>Resting, % of Control</th>
<th>CCH Stimulated, % of Resting Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±57</td>
<td>223±26</td>
</tr>
<tr>
<td>DMSO</td>
<td>109±14</td>
<td>217±32</td>
</tr>
<tr>
<td>Gö-6976</td>
<td>113±27</td>
<td>257±43</td>
</tr>
<tr>
<td>GF-109203X</td>
<td>63±29</td>
<td>120±19*</td>
</tr>
</tbody>
</table>

Results are means ± SE from n = 3 separate preparations. SC, secretory component. CCH stimulation was for 30 min at 100 μM. *Significant reduction from DMSO/CCH at P ≤ 0.05.
Interestingly, DN-PKC-ε overexpression also elevated basal release of β-hexosaminidase. We have observed (41) a similar effect by overexpression of dynamin, an effector of the cytoplasmic dynein complex, in lacrimal acini with an Ad-expression system. Dynamin overexpression inhibits cytoplasmic dynein activity that is essential for apical exocytosis, but it also elevates basal release of β-hexosaminidase. This suggests that β-hexosaminidase already present in basolateral membrane compartments (33) may be released at an increased rate when the secretory pathway is inhibited, even under resting conditions. The altered actin filament organization noted at basolateral membranes may contribute to this phenomenon. This scenario is consistent with the observation that basal release of synccollin-GFP, which is exclusively present in secretory vesicles, was not influenced by Ad-DN-PKC-ε. This latter alternative is also consistent with previous observations that the endosomal and lysosomal compartments in acini are highly enriched in β-hexosaminidase. It is interesting that the inhibition of PKC-ε (and δ) associated with GF-109203X shown in Fig. 5B does not cause increased basal release of β-hexosaminidase in parallel with the decreased CCH-stimulated release. This phenomenon of altered basolateral release of β-hexosaminidase therefore seems to occur primarily in acini transduced with Ad vectors expressing proteins that have an impact on the secretory pathway, suggesting that this phenomenon may be triggered by alterations in the secretory pathway as well as cellular stress elicited by protein overexpression driven by the Ad vectors.

We have established that PKC-ε is an actin-binding protein recruited transiently to apical actin filaments and actin-coated structures, possibly representing fusion intermediates, in CCH-stimulated lacrimal acini. We have further established that its inhibition, through overexpression of DN-PKC-ε and by use of chemical inhibitors, stabilized actin-coated structures in parallel with inhibition of stimulated exocytosis of a variety of secretory products at the APM. Future work will focus on the identification of actin-associated proteins that are likely to facilitate apical actin remodeling and that serve as targets of PKC-ε.

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