Chronic PKC-β2 activation in HT-29 Cl.19a colonocytes prevents cAMP-mediated ion secretion by inhibiting apical membrane CFTR targeting

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Broughman, James R., Limin Sun, Shahid Umar, Joseph H. Sellin, and Andrew P. Morris. Chronic PKC-β2 activation in HT-29 Cl.19a colonocytes prevents cAMP-mediated ion secretion by inhibiting apical membrane CFTR targeting. Am J Physiol Gastrointest Liver Physiol 291: G331–G344, 2006. First published March 30, 2006; doi:10.1152/ajpgi.00356.2005.—We investigated the effects of PKC-stimulated phorbol esters on subcellular CFTR expression and localization in polarized HT-29 Cl.19a monolayers. Modulation of PKC activity with the PKC-β-specific agonist 12-deoxyphorbol 13-phenylacetate 20-acetate (DOPPA) or nonspecific-selective PMA altered monolayer CFTR immunofluorescence. A decrease in the CFTR signal within the luminal cellular pole was noted with both phorbol esters. Volumetric analysis of the intracellular CFTR signal revealed that both compounds promoted CFTR accumulation into punctate vesicle-like structures found adjacent to the cellular tight junction (labeled with zona occludens (ZO)-1 antibody), extending basally (DOPPA) into the cell. Puncta were more frequent with DOPPA and larger in size with PMA. DOPPA also promoted ZO-1 accumulation at tricellular corners associated with enhanced CFTR puncta number. The observed loss of CFTR immunofluorescence signal induced by low-dose PMA was related to CFTR sequestration into fewer cytoplasmic puncta and correlated with larger increases in PKC substrate phosphorylation. Both phorbol esters downregulated steady-state cellular CFTR mRNA levels by 70%. However, the effects of DOPPA and PMA were largely independent of CFTR biosynthesis: expression levels were 80–85% of control, and the glycosylation status of immunoprecipitated protein remained largely unchanged. Thus changes in cellular CFTR localization correlated with our companion study showing that PMA-induced inhibition of transcellular cAMP-dependent short-circuit current (Isc-cAMP) was accompanied by cytoplasmic PKC-β2 accumulation and modest activation of PKC-β1 and PKC-ε. The inhibitory effect of DOPPA on Isc was related solely to increased cytoplasmic PKC-β2 levels. Thus PKC-β2 is hypothesized to participate in the regulation of CFTR apical plasma membrane targeting within the constitutive cellular biosynthetic pathway.

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medium was replaced with serum-free DMEM 2 h before experimental
treatment and then replaced with 1 ml of identical serum-free
medium containing PMA (0.01–1 μM) in DMSO, DOPPA (1–500
nM), or G69976 (1–5 μM) for 10 min to 48 h. To ensure that
significant cellular metabolism of DOPPA to the nonspecific activator
12-deoxyphorbol-13-phenylacetate (DOPP) did not occur (15), we
exchanged DOPPA incubation media every 4–6 h.

Northern blot analysis and RT-PCR. Poly(A)+ mRNA was isolated
from treated HT-29 Cl.19A cells using TRIZol reagent (GIBCO-BRL)
or the micro Fast Track kit (Invitrogen; San Diego, CA) according to
the manufacturer’s instructions. For Northern blot analysis, 2.5 μg
poly(A)+ mRNA was denatured and fractionated on a 1% agarose gel
containing formaldehyde. RNA was then transferred to GeneScreen
Plus nylon membranes (DuPont-New England Nuclear). The blot was
hybridized at 60°C in 10% dextran sulfate, 1 M NaCl, 1% SDS, and
100 μg/ml denatured salmon testes DNA with the use of a [α-32P]dCTP-labeled probe encompassing the regulatory domain of
CFTR (bases 1773–2654, 2 x 106 counts·min⁻¹·ml⁻¹) and subse-
quently with a probe against GAPDH (bases 163–608, 1 x 106
counts·min⁻¹·ml⁻¹). The latter was used to normalize mRNA levels.
The probe for CFTR detection was generated by a PCR of full-length
CFTR cDNA, and the GAPDH probe was generated by RT-PCR from
mouse colonic RNA (28). Both sequences were confirmed by oligo-
nucleotide sequencing before random primer labeling.

Immunoprecipitation of CFTR. Cells were lysed in 500 μl of
ice-cold buffer consisting of (in mM) 10 Na-HEPES, 150 NaCl, and
1 EDTA with 1% Nonidet P-40 (pH 7.0). The lysate was centrifuged
for 5 min at 4°C, and the supernatant was diluted with a 1:4 volume
of 250 mM Tris, 120 mM sodium deoxycholate, 750
mM NaCl, 5% Triton X-100, and 0.5% SDS (pH 7.5; 5 x radioim-
munoprecipitation (RIPA) buffer). Total protein was measured before
the addition of 5 × RIPA buffer by the bicinchoninic acid (BCA)
assay (Pierce Chemical). CFTR was immunoprecipitated with 1.5 μg
of monoclonal antibody directed against the regulatory domain of
CFTR (Genzyme; Cambridge, MA) and protein-G-Sepharose, accord-
ing to standard techniques. As the control, the CFTR antibody was
substituted with 1.5 μg of nonimmune mouse IgG (Pierce). Immuno-
precipitates were then washed, resuspended in 50 μl of 50 mM Tris
(pH 7.5), 10 mM MgCl2, and 0.01% BSA, and phosphorylated at
30°C by the addition of 10 μCi of [γ-32P]ATP (3,000 Ci/mmol, Du-
Pont-New England Nuclear) and 1 μl of 50 μg/ml PKA.

PKC activity assay. PKC activity was measured in cytosolic and
membrane fractions prepared using the GIBCO-BRL PKC assay
system. Subcellular fractions were prepared by homogenization of
HT-29 Cl.19A cells in 1.5 ml of ice-cold homogenization buffer [50
mM Tris·HCl (pH 7.5) containing 5 mM EDTA, 10 mM EGTA, 0.3%
mercaptoethanol, 10 mM benzamidine, 50 μg/ml PMSF, and 50
μg/ml leupeptin] using a Tekmar Tissuemizer (Tekmar; Cincinnati,
OH) rotating at 20,000 rpm for five 40-s periods. Cytosolic and
membrane fractions were separated by sedimentation for 30 min at
100,000 g. The pellet was then detergent extracted in Tris-buffered
saline (TBS) containing (in mM) 10 Tris·HCl, 140 NaCl, 25 KCl, 5
MgCl2, 2 EDTA, and 2 EGTA with 1% Triton X-100 (pH 7.5),
protease inhibitor cocktail, and the solubilized membrane fraction
collected by centrifugation. The protein content was determined using
the Bio-Rad DC protein assay kit. PKC activity was quantified by the
transfer of the terminal phosphate of [γ-32P]ATP (Amersham; Arling-
ton Heights, IL) to myelin basic protein synthetic peptide. PKC
specificity was determined using a PKC pseudosubstrate inhibitor
peptide (PKCᵣ₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋₋_-
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IgM antibody (Labvision Neomarkers; Union City, CA). Monolayers were then washed three times with PBS and coincubated overnight at 4°C with secondary Cy2-labeled goat anti-rabbit (Jackson Labs; West Grove, PA) and donkey anti-mouse Cy3-conjugated (Jackson Labs) antibodies. Before being mounted onto coverslips, monolayers were labeled with the dimeric cyanine nuclear acid stain TO-PRO-3 (Molecular Probes; Eugene, OR). After membranes had been washed and the autofluorescence quenched by treatment with 1 mg/ml NaBH₄ in PBS (Sigma), monolayers were mounted in Vectashield mounting medium (Alexis PLATFORM; San Diego, CA), and the overlying coverslip was sealed with clear nail polish. Samples were then imaged with a Zeiss LSM 510 META confocal microscope using a ×63 oil-immersion objective (Carl Zeiss Optical; Thornwood, NY) in multitrack scanning mode with excitation wavelengths set at 488 nm (Argon laser) and 543 and 633 nm (HeNe lasers). Emission wavelengths were 505–530, 560–615, and >650 nm for Cy2, Cy3, and TO-PRO-3 signal detection, respectively. Single optical slices were set to 0.8 μm, and Z-stack slices were set to 0.38 μM. Collected images were processed using a LSM Image three-dimensional bit plane and VisArt software (version 3.2, Carl Zeiss) and exported in a 16-bit TIFF RGB format.

Data analysis. All summary results are presented as arithmetic means ± SE. The differences between control and treatment data were analyzed using Student’s t-test (Excel, Microsoft; Redmond, WA). The probability of making a type I error < 0.05 was considered statistically significant.

RESULTS

PKC activation status during chronic phorbol ester exposure.

When PKC activity was measured in both cytoplasmic- and membrane-associated fractions by radiolabeled phosphate incorporation into a synthetic substrate peptide (see MATERIALS AND METHODS), 24-h DOPPA preincubation increased membrane-bound PKC activity by 2.2-fold (P < 0.001, n = 6) and cytosolic activity by 1.1-fold (Fig. 1A, bars i and ii, respectively). When the levels of each immunodetectable PKC isoform were determined from the same DOPPA-treated cellular extracts by Western blot analysis (Fig. 1B), we found that the levels of PKC-α, -γ, and -ε were unchanged relative to control (carrier-treated monolayers), whereas a modest decrease in PKC-β₁ (73% of control, P < 0.05) and a significant increase in total PKC-β₂ (122% of control, P < 0.01) levels were recorded. In our companion study (9a), we showed that DOPPA pretreatment of the same monolayers failed to induce PKC-α/β₁/γ or PKC-ε membrane translocation. DOPPA did, however, induce a significant decrease in the PKC-β₂ membrane translocation ratio by disproportionately increasing cytosolic PKC-β₂ levels while modestly increasing the size of the membrane-associated pool. Thus increased PKC catalytic activity in monolayers during DOPPA exposure (Fig. 1A) was caused by selective PKC-β₂ isoform activation. The accompanying increase in PKC-β₂ cytoplasmic translocation and chronic upregulation (Fig. 1B) further indicated that cytosolic PKC-β₂ protein in postactivation status was less susceptible to degradation than PKC-β₁ [whose cytosolic levels decreased rather than increased (9a)]. The PKC-β isoforms in active and inactive conformation therefore appeared to associate with different signaling partners (see DISCUSSION in Ref. 12).

When the substrate phosphorylating activity was estimated after a 24-h PMA preincubation, a 3.9-fold increase in membrane-bound PKC activity and a 2.2-fold increase in cytosolic activity were recorded (P < 0.001, n = 6; Fig. 2A). When cellular PKC isoform expression levels were quantitated by Western blot analysis, PMA did not significantly change PKC-α, -γ, and -ε levels (Fig. 2B), whereas total PKC-β₁ levels decreased significantly (50% of control, P < 0.01) and PKC-β₂ levels increased significantly (144% of control, P < 0.01). Total PKC activity and PKC-β isoform mass changes were therefore more pronounced but mirrored those recorded for DOPPA (Fig. 1). Similarly, when the membrane translocation status of individual isoforms was assayed (9a), PMA failed to significantly increase the membrane translocation status of either PKC-α and -γ, slightly increased PKC-β₁ and -ε membrane translocation, and modestly increased PKC-β₂ membrane association while strikingly promoting large changes in PKC-β₂ cytoplasmic partitioning. Changes in total extractable PKC activity promoted by PMA (Fig. 2A) were therefore also primarily attributed to increases in the activation status of the PKC-β₁ and -ε isoforms, with only PKC-β₂ exhibiting postactivation-dependent increases in total cell levels (Fig. 2B). After a 48-h preincubation with a high concentration (500 nM) PMA, substrate phosphorylation activity measured in both cytosolic and membrane fractions was reduced to 17% and 46%, respectively, of carrier-matched controls (n = 3, P < 0.001; Fig. 3A). When the total extractable PKC isoform amount under these conditions was measured by quantitative Western blot analysis (Fig. 3B), PKC-α, -β₂, and -γ levels were clearly significant (n = 3, P < 0.01).
remained similar to the control (102 ± 10%, 98 ± 9%, and 101 ± 6%, respectively). A 5-fold decrease in PKC-β1 (to 17 ± 5% of control) and a 2.4-fold decrease in PKC-ε levels (to 40 ± 9% of control) were measured. Under these conditions, the reduction in PKC phosphorylation activity did not correlate with the change in PKC-β2 but correlated with a chronic loss in the cellular PKC β1-ε isofrom mass.

Chronic monolayer exposure to low doses of both DOPPA and PMA reduced cellular CFTR mRNA levels but marginally affected cellular PKC α isoform protein expression. Shown in Fig. 4 are representative Northern and Western blots of CFTR mRNA and protein expression after 24 h in control and 50 nM DOPPA- and 50 nM PMA-treated HT-29 Cl.19A cells.

Cellular CFTR poly(A)+ mRNA abundance (n = 3) relative to the housekeeping gene GAPDH (n = 3) was observed to decrease after a phorbol ester preincubation (Fig. 4A). The intensity of the 6.5-kb CFTR band in DOPPA- and PMA-preincubated monolayers was 27% and 30% of that encountered in unstimulated conditions, respectively (n = 3, P < 0.001). In the phorbol ester-treated samples, GAPDH migrated with a slightly decreased apparent molecular mass. We attribute this change to reduced polyadenylation of the GAPDH transcript (20) after phorbol ester treatment. Densitometric line histograms for each lane line are shown (Fig. 4). Blots were captured with a 16-bit grayscale camera, and images were analyzed by Universal imaging Metamorph software (see MATERIALS AND METHODS, Densitometric analysis of PKC isofrom expression by Western blot analysis). Captured images were not saturated.

To determine whether reductions in cellular mRNA content were reflected at the protein level under identical treatment conditions, CFTR was immunoprecipitated with the Genzyme anti-CFTR regulatory domain antibody and 32P labeled by PKA phosphorylation (see MATERIALS AND METHODS). A 24-h treatment with 100 nM PMA or DOPPA resulted in a modest but not significant reduction in labeled cellular PKC protein; levels fell to 80 ± 16% and 85 ± 18% of control values, respectively (Fig. 4B; n = 3, P > 0.01). The 32P-labeled immunoprecipitated product running at an apparent molecular mass of ~140 kDa (band A) and second broader immunoprecipitated phosphorylated band running at ~190 kDa (representing complex and mature post-Golgi-processed glycosylation patterns for CFTR, band C) exhibited a similar molecular mass to epithelial CFTR as detected by Western blot analysis with the TAM-18 antibody used previously in isolated colonic crypts (27). Densitometric analysis of the relative intensities for bands A and C within each lane [Fig. 4B, (–), (+d), and (+p) lanes] revealed no glycoprocessing differences. Thus, although low doses of both weak and strong phorbol esters shared the ability to partially downregulate anion channel message levels in confluent HT-29 Cl.19A monolayers, doses...
of either agonist at twice the concentration used to inhibit CFTR-mediated Cl\(^{-}\) section in our functional assays (9a) did not significantly affect cellular CFTR protein content. The effects of sustained PKC-\(\beta\) activation on apical plasma membrane \(I_{SC}\)-cAMP therefore must be occurring at another level. We therefore monitored the localization of CFTR in DOPPA- and PMA-treated monolayers.

Both DOPPA and PMA induce the loss of monolayer luminal pole CFTR IMF. Previously, we examined the cellular location of CFTR in HT-29 Cl.19A monolayers (22). Using the same immunostaining protocol and monoclonal antibody against CFTR, we quantified CFTR IMF within two-dimensional (XY) and three-dimensional (XYZ) cellular axial planes after 24 h of incubation with either carrier alone (PBS vehicle), vehicle plus 50 nM DOPPA, or vehicle plus 50 nM PMA (Fig. 5).

CFTR staining was first determined in nonphorbol ester-treated control monolayers in individual en face (XY-axes) planes throughout the cellular Z-axis at 1-\(\mu\)m intervals (Fig. 5A; see MATERIALS AND METHODS). The structures exhibiting the clearest labeling were the microvillus brush border and the subapical cytoplasm, which included a circumferential band of IMF within the region of the apical tight junction (Fig. 5A, images i and ii). A lower degree of staining was present in more basal subcellular regions and was absent from the nucleus (Fig. 5A, images iii and iv; \(n = 24\) monolayers). This localization of CFTR was consistent with its geographical role as an apical plasma membrane anion channel involved in apical membrane \(I_{SC}\)-cAMP generation. To quantify values from all areas within the 640 \((X) \times 480 \(Y\) pixel field of view, we collected individual XY-axes planes at 0.1-\(\mu\)m Z-axis intervals throughout the depth of the monolayer for the whole objective field (\( \approx 45 \mu\)m in \(X\) and \(Y\) planes; Fig. 5B; between 160 and 180 planes encompassing both basal and apical aspects were routinely sampled). To represent the three-dimensional data as a two-dimensional pictograph, we then rendered all 640 “\(X\)-axis” pixel values (\(X_{x-640}\) at every \(Y\)-axis position (\(Y_{y-480}\)) into single average values, \(P_{XY} = Y_{y}(X_{x-640}) \rightarrow Y_{x-640}(X_{y-480})\). These \(2X\) Y-axis slices were then stacked to form a \(P_{XYZ}\) matrix, where \(P_{XYZ} = (P_{XY} \times P_{Z})_{n} \rightarrow P_{XYZ} = P_{Zi} \rightarrow Z_{0}\) is the initial Z-axis (Fig. 6). Thus orthogonally constructed images were side-on views looking entirely through \( \approx 45 \mu\)m of the epithelial monolayer containing many cell bodies. For this reason, the resulting image, shown in Fig. 6A, appears more diffuse than would a single orthogonal plane image. However, because 640 pixel planes are averaged for each pixel displayed, this method is quantitatively more accurate for intensity measurement.

Starting points for the \(Z_{0}\)-axis were empirically judged by moving the focal plane in 0.1-\(\mu\)m steps away from the specimen-glass coverslip interface, which was visible as a change in background signal intensity at this boundary. However, when the 25-\(\mu\)m confocal slit width was used to collect sufficient light for immunodetection with minimal laser-induced photobleaching (see MATERIALS AND METHODS), planes close to this interface included an out-of-focus signal [light detection is a function of slit aperture, and wider apertures correspond to widening of the detection sensitivity but lowering of optical resolution (8)]. As a result, starting intensity along the glass-apposed \(P_{XYZ}\)-axis (Fig. 6, A, plane 0, and B, basolateral
membrane) was contaminated by internal reflection between the specimen and glass coverslip. This introduced variability between coverslips. As predicted, background intensity converged at high-

$P_{X/H2O}$

plane values above the luminal membrane of the epithelial cell monolayer (Fig. 6, A, plane 180, and B, apical membrane).

Although this technical limitation prohibited us from measuring quantitatively the area under the curve for each experimental condition, it was clear from $P_{X/Z}$ intensity matrixes (Fig. 6A) that control monolayers exhibited a node of IMF signal intensity within the cellular apical pole and that both 50 nM DOPPA and 50 nM PMA decreased the volume of CFTR within this region ($n = 4$ separate experimental procedures). The effects of DOPPA were subtle in that a higher accumulation of signal was detected in the cellular basolateral pole than within the cellular apical pole, an effect made more apparent...
when $P_{YXZ}$ intensity matrices were graphically plotted as mean $P_{XY}$ slice intensity (i.e., $\bar{x}(Y_{1-480}, X_{1-640})$ vs. $z$-axis graphs). The stronger phorbol ester PMA potentiated this effect but reduced IMF intensity throughout the monolayer volume. The reduction in the IMF signal detected when we used PMA-treated samples was unexpected, given that the level of protein remained relatively constant, as determined by CFTR immunoprecipitation studies (Fig. 4B).

Because quantitative intensity measurements using pixel averaging within monolayer planes includes areas where no CFTR fluorescence is found (both within and outside cells) as well as areas containing true CFTR intensity, alterations in the subcellular nature of CFTR protein fluorescence change the resulting averaged signal readout. For instance, CFTR existing in a diffuse cytoplasmic labeling pattern presents the ideal intensity measurement condition. However, when CFTR is volume constrained into structures where the intensity of CFTR fluorescence may be masked (such as when vesicles contain different amounts of protein), then averaged intensity measurements will not remain truly quantitative. Under these conditions, the extent of CFTR intracellular three-dimensional coverage or geography that is no longer intensity encoded but encoded by volume provides another measurement method.

Both DOPPA and PMA induce vesiculation of monolayer apical pole subcellular CFTR IMF into structures bordering the tight junction. To further investigate the changes in CFTR IMF intensity profiles recorded in fixed monolayers (Figs. 5 and 6), the murine monoclonal TAM-18 IgM anti-CFTR antibody used previously for intracellular CFTR fluorescence labeling was employed (27) together with a marker antibody for the cellular tight junction [ZO-1 (18)] and the nuclear stain (TOPRO-3). The choice of a different primary anti-CFTR antibody made multiple (triple) fluorescent staining in fixed monolayers possible (see MATERIALS AND METHODS). When triple-stained monolayers were visualized at high ($\times 63$) magnification on a Zeiss 510 META confocal microscope running in multiscan mode (see MATERIALS AND METHODS), the resulting Cy3-conjugated secondary anti-CFTR pattern exhibited clear diffuse apical localization within the top-most luminal (2 $\mu$m) aspect of the monolayer (Fig. 7Ai).

Deconvolved orthogonal YZ and XZ planes exhibited brush-border CFTR (red) labeling interspersed with a circumferential ZO-1 (green) staining pattern (Fig. 7). The stained caps of nuclei (blue) were found 4–6 $\mu$m below the majority of CFTR IMF (Fig. 7). When a scattergram [two-dimensional histograms mapping all the possible (red) CFTR/(green) ZO-1 pixel colocalization events] was constructed and color encoded by frequency of incidence (blue to red; Fig. 7Aii), no clear colocalization was found between CFTR and ZO-1 (complete XY colocalization produces a straight line through the origin). Thus CFTR staining was constrained above and by the ZO-1-labeled cellular tight junctions. When CFTR staining was visualized in monolayers treated for 24 h with 50 nM DOPPA, the CFTR signal within the monolayer was redistributed from the luminal (top 2 $\mu$m) aspect basally into the cell and exhibited areas of high intensity at intracellular structures closely apposed to, but not colocalized with, ZO-1-labeled tight junctions (Fig. 7Bi). Also seen were random blebs of ZO-1 staining within the apical cellular pole not associated with junctions (see also Fig. 10). When scattergrams were constructed, no clear colocalization between CFTR and ZO-1 IMF was found (Fig. 7Bii), although CFTR did appear to surround both tight junctions and the dispersed cytoplasmic blebs of ZO-1 (data not shown). In monolayers exposed to 50 nM PMA for 24 h, the punctate CFTR staining pattern appeared even more eroded and vesiculated. Apical pole CFTR IMF was close to background levels, and the cellular CFTR signal was concentrated into fewer areas (Fig. 7Ci). The scattergram of CFTR versus ZO-1 colocalization (Fig. 7Cii) continued to show no significant degree of colocalization, although, in this instance, there were more coincident red-green pixels. Thus the results confirmed the averaged intensity measurements made in Fig. 6 in that DOPPA and PMA induced subapical pole CFTR relocation into what appeared to be vesicle-like structures.

Further confirmation of the compartmentalized nature of the internal CFTR signal was sought by constructing bit plane shadow masks of the triple-labeled Z-axis stacks. This technique encodes (by $4 \times 4$ binning) each $2,048 \times 2,048$ bitmap into a $512 \times 512$ bitmap that is not voxel (three-dimensional pixel volume) rendered but retains the stack plane information. Because of the need for transparency in the top-most layers, the image is no longer rigidly intensity encoded. The shadow mask images shown in Fig. 8 are reconstructed from Fig. 7 with front plane (apical $\rightarrow$ basal) priority.

An evenly diffuse apically oriented CFTR IMF signal was recorded in control monolayers treated with carrier alone (Fig. 8A, images i and ii). Shadowing effects revealed that CFTR was positioned uniformly above the plane of the tight junction, and, in some instances, structures resembling CFTR-labeled apical plasma membrane microvilli were visible. Apical priority shadow mask rendering of the 24-h DOPPA-pretreated monolayer demonstrated that less CFTR IMF was found in the top-most (apical) and that CFTR IMF accumulated within puncta that resembled cytoplasmic vesicles localized near the tight junctions (Fig. 8B, images i and ii). After 24 h of treatment with 50 nM PMA, CFTR was clearly found at or below the level of the ZO-1-labeled tight junction and was dispersed in less frequently occurring punctate vesicle-like structures. Comparative analysis of the overall size of the vesicular puncta under DOPPA and PMA conditions proved too difficult because of masking due to high levels of small (0.1–0.3 $\mu$m) apically oriented puncta in DOPPA-pretreated monolayers. However, when clear isolated vesicles below the ZO-1-labeled tight junction were measured, those found under PMA conditions were larger (>0.3 $\mu$m, data not tabulated).

When the same stacks were visualized with back plane shading (basal $\rightarrow$ apical priority), important geographical differences in CFTR location were observed after phorbol ester exposure (Fig. 9).

Shadowing of control Z-axis stacks (Fig. 9A, images i and ii) with back plane priority showed a CFTR signal below the planes of the nuclear (blue) and ZO-1 signal (green) (i.e., little CFTR IMF was detected in back planes), confirming the apical location of the CFTR signal. However, in DOPPA-treated monolayers, the back planes were now populated with red punctate structures. Rendering priority on CFTR now localized in more basal planes caused masking of the green circumferential ZO-1 label (Fig. 9B, images i and ii). Thus CFTR-labeled puncta were present within the central volume of the cell, oriented along basolateral membranes around and below the cellular tight junction. In PMA-treated monolayers viewed from the back plane (Fig. 9C, images i and ii), a
Fig. 7. Ai, Bi, and Ci: triple fluorescently labeled monolayer Z-axis stack and deconvolved YZ and XZ orthogonal planes [CFTR (red), zonula occludens (ZO)-1 (green), and TO-PRO-3 nuclear acid stain (blue)]. CFTR was detected with TAM-18 monoclonal antibody and Cy-3 secondary antibody. ZO-1 detected with Zymed polyclonal antibody and Cy2 secondary antibody. The far red fluorescence emission of TO-PRO-3 dye was pseudocolored blue (see materials and methods). Aii, Bii, and Cii: scattergrams of red/green (CFTR/ZO-1) intensity within the topmost Z-axis plane. A: 24-h pretreatment with carrier alone; B: 24-h pretreatment with 50 nM DOPPA; C: 24-h pretreatment with 50 nM PMA. Monolayers stained with nonspecific primary antibodies or with primary antibodies omitted failed to exhibit Cy2/Cy3 staining (data not shown). DOPPA and PMA reduced CFTR staining within the luminal (top 2 μm) aspect of the monolayer (n = 6, 30 × 0.35-μm Z-axis planes). Bars = 5 μm.
dispersed punctate vesicle-like staining pattern similar to that recorded in front plane prioritized stacks (see Fig. 8C, images i and ii) was observed. These findings, repeated in six different experiments, demonstrated that the apparent loss in signal-averaged luminal CFTR intensity after phorbol ester treatment (Fig. 6) was caused by a geographic shift of CFTR protein away from the apical cellular pole into vesicle-like structures found deeper within the cell and associated (DOPPA) with the cellular tight junction. Environmental differences in intracellular CFTR IMF detection may explain the differences between these higher resolution studies (Figs. 7–9) and the earlier intensity-averaged monolayers (Figs. 5 and 6).

Fig. 8. Stacks shown in Fig. 7 viewed “en face” as front plane prioritized (Ap → Bi) shadow projections (bit-plane mapped, 45° incidence light) at ×1 magnification in Ai, Bi, and Ci and ×2 magnification in Aii, Bii, and Cii [CFTR (red), ZO-1 (green), and TO-PRO-3 (blue)]. A: control monolayers treated for 24 h with carrier alone exhibited an evenly dense CFTR IMF signal at the Ap cell surface, bordered by ZO-1 staining. B: CFTR staining in monolayers treated for 24 h with 50 nM DOPPA exhibited a punctate staining pattern highest in areas adjacent to ZO-1-stained cellular borders. C: CFTR staining in monolayers pretreated for 24 h with 50 nM PMA exhibited a loss of the diffuse CFTR staining pattern and more eroded punctate staining adjacent to ZO-1-stained cellular borders. Underlying nuclear cap TO-PRO-3 fluorescence (blue) was visible in areas where CFTR IMF levels (and hence, protein accumulation) was low (30× 0.35-μm Z-axis planes). Bars = 5 μm.
The second important finding recorded from the triple-labeling studies related to changes in intracellular tight junction structure observed in DOPPA- and PMA-pretreated monolayers. Shown in Fig. 10 is a volumetric reconstruction of ZO-1-labeled tight junctions from the monolayers shown in Fig. 7.

High-precision volume rendering of control monolayers (Fig. 10A, images i and ii) demonstrated that the circumferential ZO-1-labeled tight junctions exhibited some degree of vertical-axis depth wandering (±0.5 μm) and appeared in most instances as a crinkled circumferential band. Under DOPPA conditions (Fig. 10B, images i and ii), the morphology of the ZO-1-labeled cellular tight junction was changed. The circumferential band appeared larger and more relaxed (reflecting a wider cellular neck region), smoother, and was more vertically
constrained (±0.2 μm vertical depth wandering). Also recorded were volume increases in ZO-1 staining at points where adjacent circumferential tight junctions from neighboring cells met (tricellular borders), suggesting that this relatively immobile cytoskeletal element was being redistributed intracellularly within the apical pole. In PMA-treated monolayers (Fig. 10C, images i and ii), the circumferential rings of the ZO-1 labeling were even larger but did not exhibit similar ZO-1 pooling at tricellular borders. Parallel electrical measurements of similarly treated monolayers mounted in Ussing chambers revealed little change in transcellular electrical resistance after DOPPA or PMA preincubation (9a). Thus the chronic inhibitory effects of both DOPPA and PMA on $I_{SC}$-cAMP (9a) appear related to structural changes in cellular architecture involving PKC-β2-dependent alterations in subcellular CFTR localization and the cellular tight junction.

Fig. 10. Ai, Bi, and Ci: high-resolution voxel-based image rendered from Z-axis stack data presented in Fig. 7 showing ZO-1-stained apically oriented circumferential and cellular tight junctions (green) together with nuclear caps (blue). For simplicity, the information encoded by CFTR (red channel) was omitted during rendering. Aii, Bii, and Cii: voxel rendering at × 2 magnification. The resulting images lack pixel intensity encoding but contain three-dimensional pixel (voxel) spatial information. A: 24-h exposure to carrier alone yielded monolayers with irregular ZO-1 lattices structurally defining apically oriented cellular tight junctions. B: 24-h exposure to 50 nM DOPPA resulted in circumferential rings that were larger and more regular with ZO-1 accumulation at tricellular borders. C: 24-h exposure to 50 nM PMA generated monolayers with even larger belts of ZO-1 staining but less ZO-1 accumulation at tricellular borders (30 × 0.35-μm Z-axis planes). Bars = 5 μm.
DISCUSSION

Significance to our recent in vivo findings. We (27, 28) have shown previously that PKC-β₁ activation in vivo leads to elevated colonocyte CFTR mRNA and protein levels. However, only modest increases in transepithelial cAMP-responsive anion secretion currently accompanied these changes. We thus hypothesized that this disparity was due to counteracting cellular homeostatic mechanisms. While a picture is emerging for such PKC-dependent effects operating at the level of the basolateral plasma membrane, the findings presented in our companion study (9a) identified an additional focus, incorporating new roles for three individual PKC isoforms (PKC-α, -β, and -ε) at the level of the apical plasma membrane. Using a unique experimental approach that allowed us to monitor the geography of cellular CFTR protein localization and CFTR protein/mRNA expression, we showed that chronic phorbol ester-induced activation and translocation of PKC-β₂ into the cellular cytoplasm, while unrelated to direct apical plasma membrane SC inhibition, posits a specific role for this isoform in CFTR targeting toward the apical membrane.

By measuring subcellular CFTR IMF in low-dose DOPPA- and PMA-preincubated monolayers, we found that both PKC agonists caused a specific redistribution of CFTR within the subcellular biosynthetic pathway, leading to a loss of CFTR from the cellular apical pole (Fig. 6) and an accumulation of the anion channel in vesicle-like structures within and below the plane of the circumferential tight junction (Figs. 7–9). Because the half-life of CFTR protein was not measured, these effects could have been due to either changes in subcellular CFTR turnover or movement. However, because neither agonist dramatically affected total cellular CFTR protein levels and failed to alter the ratio of high- to low-molecular-weight glycoprocessed forms of CFTR (indicating that the kinetics of immature endoplasmic reticulum/Golgi biosynthetic processing were not changing; Fig. 4), subcellular CFTR movement is predicted. We believe that chronic activation and the subsequent cytoplasmic partitioning of the PKC β₂-isofrom was causally associated with subcellular CFTR relocalization within the biosynthetic pathway because the only common correlates between both DOPPA and PMA treatment conditions were enhanced PKC substrate phosphorylation and cytoplasmic PKC-β₂ partitioning.

The tight junction protein ZO-1 has been proposed to be part of a cytoplasmic junctional “waystation” or nexus for membrane trafficking to both apical and basolateral membranes. ZO-1 is known to bind a large variety of cytoskeletal elements (31) and acts as a scaffolding protein for signaling molecules. Coimmunoprecipitation studies have shown that ZO-1 associates with Go-subunits of heterotrimeric G proteins (19), which are known regulators of targeting within the constitutive biosynthetic pathway (22). It was therefore interesting to note that low (50 nM) concentrations of both DOPPA and PMA affected the shape and appearance of ZO-1-labeled circumferentially oriented apical tight junctions without affecting monolayer electric resistance. High-dose PMA is well characterized as an agent that inhibits cAMP-regulated Cl⁻ secretion as well chronically affecting colonocyte tight junctional integrity, leading to a gradual reduction in transepithelial resistance through PKC-α activation (26) and the multilayering of cells (13). A more recent study (30) has demonstrated that PKC agonists such as bryostatin-1, a nonphorbol ester activator of conventional and novel PKC isoforms, can increase transepithelial resistance in the colonocyte T84 cell line by specifically activating PKC-ε, a phenomenon associated with the translocation of ZO-1 and claudin-1 from cytoplasmic to membrane pools. The fact that we did not observe transepithelial resistance changes (9a) reinforces the argument that DOPPA-dependent ZO-1 pooling at tricellular corners, associated with the appearance of CFTR in intracellular vesicles below the apical membrane, supports a structural role for the PKC β₂-isofrom in the regulation of CFTR trafficking within a constitutive apical membrane targeting pathway. Figure 11 summarizes our working model for long-term PKC-β₂-dependent inhibition of apical membrane-generated IₑC-cAMP common to both DOPPA and PMA monolayer exposure.

Evidence supporting a role for PKC-β₂ as a modulator of constitutive CFTR movement within the trans-Golgi network/apical targeting pathway. Although PKC-β₁ and -β₂ are 96% identical [differing in only a small stretch of amino acids within the most terminal region of the catalytic domain (5)], they exhibit homolog-specific subcellular localization. Whereas PKC-β₁ partitions predominantly between the membrane and cytosol, the amino acid differences in the PKC-β₂ COOH-terminal region confer on this molecule the ability to bind to cytoskeletal actin with much greater avidity than PKC-β₁. A number of groups have investigated the signaling aspects of cytoskeletal PKC-β₂ association, and a picture is emerging supporting a role for this isoform in vesicular trafficking to and from the trans-Golgi network (TGN) and at other locales within epithelial cells. A role for PKC in constitutive vesicle release from TGN stacks has been identified in cell-free in vitro systems (10, 29). Furthermore, in

Fig. 11. Working model of DOPPA- and PMA-induced CFTR redistribution in HT-29 Cl.19A monolayers. Top: control. The diffuse luminal CFTR staining pattern (red) in untreated monolayers localizes predominantly above the plane of the cellular tight junction (green ovals). Left: DOPPA. After DOPPA treatment, CFTR redistributes into vesicular structures apposed to and found both above and below the plane of the cellular tight junction. Right: PMA. The less specific but more potent phorbol ester induced a more reduced CFTR staining pattern with less spatial distribution within the cell. CFTR was often juxtaposed with both ZO-1 staining at the cellular tight junctions and within cytoplasmic ZO-1-labeled vesicles (Figs. 7–9). The observed coalescence of CFTR into vesicles after PKC-β activation accounts for the apparent differences in mean overall CFTR IMF intensity X-axis summed YZ planes (Fig. 5) and protein expression levels assayed by ³²P radiolabeling (Fig. 4).
polarized MDCK epithelial cells, nonspecific PKC β-isof orm depletion (11) inhibits the appearance of glycoprotein markers at the apical plasma membrane. These inhibitory effects are functionally similar to those recorded for brefeldin A, a fungal antibiotic that prevents anterograde vesicle movement within the constitutive biosynthetic pathway by inhibiting guanine nucleotide exchange factor modulation of ADP-ribosylation factor-1-dependent coat protein binding (6). We (21) have previously shown that a 24-h brefeldin A preincubation of HT-29 Cl.19A monolayers downregulates apical membrane-generated I_sc-cAMP by preventing CFTR-containing vesicle movement between the TGN and apical plasma membrane. A separate group (2) has reported a temperature sensitivity to this phenomena corresponding to a post-TGN trafficking block, but they, unlike us, only recorded brefeldin A effects on PMA-stimulated I_sc. The differences may relate to basal levels of PKC activity between each laboratories’ cell lines. More recently, post-TGN apical glycoprotein glucose transporter GLUT2 trafficking in native rat jejunal enterocytes has also been reported as PKC-β1 sensitive (14). At the molecular level, a receptor for activated PKC-β (RACK-1) has been shown to accumulate within TGN, move coordinately with PKC-β2 to the plasma membrane (24), and coimmunoprecipitate with both PKC-β1 and with the pleckstrin homology domains of phospholipid-binding proteins involved in post-TGN/plasma membrane vesicle transport (23).

On the basis of these observations and our present results, we hypothesize that PKC-β2 normally facilitates apical plasma membrane CFTR function by promoting channel movement within post-TGN stages of the constitutive biosynthetic pathway. However, during chronic DOPPA and PMA stimulation, PKC-β2 downregulation (recorded as the cytoplasmic accumulation of kinase; Figs. 1 and 4) inhibits post-TGN vesicle movement and slows the biosynthetic delivery of CFTR into the subcellular apical pole. The corresponding decrease in apical I_sc-cAMP (Figs. 1–4 in Ref. 9a) recorded under these conditions is hypothesized to reflect the continuing activity of non-PKC-dependent apical plasma membrane CFTR retrieval mechanisms [endocytosis (7)], thereby providing a cellular basis for apical plasma membrane current inhibition. The DOPPA-induced loss of CFTR in the apical pole associated with increased cytoplasmic partitioning and cellular mass of PKC-β2 occurred independently of changes in the partitioning/mass of other isoforms. This supports the hypothesis that PKC-β/e exert their effects on CFTR current generation at or close to the apical plasma membrane, whereas the effects of PKC-β2 occur more distally within the cellular biosynthetic pathway.

GRANTS

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