Regulation of epithelial transport and barrier function by distinct protein kinase C isoforms

JAEEKYUNG CECILIA SONG, CELINA M. HANSON, VANCE TSAI, OMID C. FAROKHZAD, MARGARET LOTZ, AND JEFFREY B. MATTHEWS

Division of General and Gastrointestinal Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215

Received 5 October 2000; accepted in final form 13 March 2001.

Song, Jaekyung Cecilia, Celina M. Hanson, Vance Tsai, Omid C. Farokhzad, Margaret Lotz, and Jeffrey B. Matthews. Regulation of epithelial transport and barrier function by distinct protein kinase C isoforms. Am J Physiol Cell Physiol 281: C649–C661, 2001.—The phorbol ester phorbol 12-myristate 13-acetate (PMA) inhibits Cl− secretion (short-circuit current, Isc) and decreases barrier function (transepithelial resistance, TER) in T84 epithelia. To elucidate the role of specific protein kinase C (PKC) isoenzymes in this response, we compared PMA with two non-phorbol activators of PKC (bryostatin-1 and carbachol) and utilized three PKC inhibitors (Go-6850, Go-6976, and rottlerin) with different isoenzyme selectivity profiles. PMA sequentially inhibited cAMP-stimulated Isc and decreased TER, as measured by voltage-current clamp. By subcellular fractionation and Western blot, PMA (100 nM) induced sequential membrane translocation of the novel PKCe followed by the conventional PKCd and activated both isozymes by in vitro kinase assay. PKCs was activated by PMA but did not translocate. By immunofluorescence, PKCe redistributed to the basolateral domain in response to PMA, whereas PKCd moved apically. Inhibition of Isc by PMA was prevented by the conventional and novel PKC inhibitor Go-6850 (5 μM) but not the conventional isoform inhibitor Go-6976 (5 μM) or the PKC inhibitor rottlerin (10 μM), implicating PKCe in inhibition of Cl− secretion. In contrast, both Go-6976 and Go-6850 prevented the decline of TER, suggesting involvement of PKCa. Bryostatin-1 (100 nM) translocated PKCd and PKCa and inhibited cAMP-elicted Isc. However, unlike PMA, bryostatin-1 down-regulated PKCδ protein, and the decrease in TER was only transient. Carbachol (100 μM) translocated only PKCe and inhibited Isc with no effect on TER. Go-6850 but not Go-6976 or rottlerin blocked bryostatin-1 and carbachol inhibition of Isc. We conclude that basolateral translocation of PKCe inhibits Cl− secretion, while apical translocation of PKCd decreases TER. These data suggest that epithelial transport and barrier function can be modulated by distinct PKC isoforms.

The PKC family of serine/threonine kinases plays a crucial role in diverse cellular responses such as membrane trafficking, cytoskeletal organization, ion transport, cell growth, and differentiation. At least 11 isoforms of PKC are known, and these are usually categorized into three distinct subtypes: conventional (cPKC) isoforms (α, βI, βII, and γ), novel (nPKC) isoforms (δ, ε, η, μ, and θ), and atypical (aPKC) isoforms (ζ and ι/λ) (23, 31). These three subtypes vary in their sensitivity to activators and cofactors: the cPKC isoforms are dependent on phosphatidylinositol and the second messengers diacylglycerol (DAG) and Ca2+, and they can also be activated by PMA. The nPKC isoforms are similar to the cPKC isoforms in sensitivity to activators, except they obtain full catalytic activity in the absence of Ca2+. The aPKC isoforms are independent of DAG or Ca2+ and, as a general rule, cannot be directly activated by PMA. The PKC isoforms are widely distributed to varying degrees in mammalian tissue- and cell-specific patterns. Moreover, PKC isoforms exhibit distinct subcellular localizations within individual cell types.

A hallmark of activation of PKC family members is translocation from one biological compartment in the inactive state (e.g., cytosol) to another in the activated state (e.g., plasma or organelar membrane). However, translocation is not an absolute requirement; examples
of changes in kinase activity without changes in subcellular localization (and vice versa) are known. Because there is considerable overlap in substrate specificity of the individual PKC isoforms, the precise subcellular localization of inactive and active forms probably confers isozyme specificity in regulating biological processes (23, 31).

The role of specific PKC isoform(s) in the alteration of T84 cell epithelial transport and barrier function by PMA has not been clearly defined. While PMA can have non-PKC cellular targets (e.g., β-chimaerin), PKC-inactive phorbol esters do not affect T84 monolayer TER or Cl− secretion. Interestingly, we have found that several non-phorbol PKC agonists exert some but not all of the effects of PMA on epithelial phenotype. For example, bryostatin-1 has minimal effect on barrier function despite its ability to inhibit transepithelial Cl− secretion (7). In fact, bryostatin-1 is able to partially antagonize the effect of PMA on barrier function (7). We hypothesized that different PKC agonists are capable of activating selective subsets of PKC isoforms that differentially affect the cellular properties of transport and barrier function in T84 epithelia. To approach this hypothesis, we exploited the differential effects of three PKC agonists and three isozyme-selective PKC inhibitors to elucidate the major isoforms involved in downregulation of transport and barrier function in this model system.

METHODS

Cell culture. T84 human intestinal epithelial cells obtained from Dr. Kim Barrett (University of California, San Diego) were grown to confluence at pH 7.4 in 162-cm2 flask (Corning Costar, MA) with a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 nutrient mixture supplemented with 6% fetal bovine serum (FBS), 15 mM HEPES, 14.3 mM NaHCO3, and antibiotics/antimycotic. Flasks were passaged weekly and fed every 3 days. Cell monolayers for I-I conditions, short-circuit current (Isc) of T84 monolayers grown in confluent monolayers grown on 0.33-cm2 permeable supports were treated with PMA in medium for the specified time and washed three times with cold PBS. Cells were then fixed in 4% paraformaldehyde for 1 h at room temperature, washed with PBS twice, permeabilized with 0.1% (vol/vol) TX-100 in PBS for 7 min, and rinsed with PBS twice. Filter membranes were cut out in rectangular shapes from the Transwell plastic assembly, placed between 50 μl of blocking buffer (1% normal goat serum, 3% BSA in PBS) at both the top and bottom of the monolayers, and incubated for 30 min at room temperature. Polyclonal antibodies against either PKCα or PKCe were diluted to 10 μg/ml in the blocking buffer containing 0.1% TX-100, and 50 μl of each antibody were placed at both the top and bottom of the monolayers. After overnight incubation in a moisture chamber at 4°C, monolayers were washed in PBS three times for 10 min and incubated in rhodamine-conjugated goat anti-rabbit polyclonal IgG (1:100 dilution) for 1 h at room temperature along with FITC-phalloidin for F-actin staining. Monolayers were then washed three times in PBS and mounted on the microscope slide with Vectashield mounting medium. Confocal images were acquired using a Zeiss inverted microscope equipped with MRC-1024 and Lasersharp software (Bio-Rad).

Subcellular fractionation. T84 cells grown to confluence on collagen-coated permeable supports (4.7 cm2) were washed with ice-cold PBS three times and scraped into 400 μl of the cold homogenization buffer (HB) containing 20 mM Tris·HCl, pH 7.5, 250 mM sucrose, 4 mM EDTA, 2 mM EGTA, and Complete protease inhibitor cocktail tablets. The cells were homogenized on ice with 25 strokes of a glass tissue homogenizer. The resulting homogenate was ultracentrifuged at 86,000 g for 50 min at 4°C (TLA 45 rotor, TL-100 Ultracentrifuge, Beckman). The supernatant was designated the cytosolic fraction. The pellet was resuspended in 400 μl of the HB containing 0.5% (vol/vol) TX-100 by brief sonication and incubated in ice for 30 min. At the end of the incubation period, the samples were centrifuged at 14,000 g for 20 min at 4°C. The resulting supernatant was designated the membrane fraction.

Gel electrophoresis and Western blotting. Equal amounts (~50 μg/sample) of protein, as determined by the Bradford assay, were combined with Laemmli’s Sample Buffer containing 5% (vol/vol) β-mercaptoethanol and boiled for 5 min. Proteins were separated by electrophoresis on 7.5% SDS-PAGE gels and transblotted to nitrocellulose membranes.
The protein-bound nitrocellulose sheets were first incubated for overnight at 4°C in a blocking buffer containing 20 mM Tris, pH 7.5, 500 mM NaCl, and 5% nonfat dry milk. Nitrocellulose sheets were then incubated with the polyclonal antibodies to different PKC isoforms diluted in the blocking buffer (PKCa 1:10,000, PKCe 1:100, PKCβ 1:100, and PKCγ 1:100) for 1 h at room temperature and rinsed for 30 min with a wash buffer containing 20 mM Tris, pH 7.5, 500 mM NaCl, and 0.2% Tween 20. Finally, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (1:3,000 dilution) for 1 h at room temperature and washed for 30 min with agitation, during which the wash buffer was changed every 5 min. PKC bands were visualized with ECL (enhanced chemiluminescence) detection reagents.

Materials. Tissue culture reagents and protein A-agarose beads were purchased from Life Technologies, and gel electrophoresis and Western blotting reagents were from BioRad, with the exception of ECL detection reagent, which was purchased from Amersham. Complete protease inhibitor cocktail tablets were from Boehringer Mannheim, and FITC-phalloidin was from Molecular Probes. Anti-PKCα for Western blotting was obtained from Sigma, and anti-PKCα, anti-PKCβ, and anti-PKCδ for immunofluorescent staining and in vitro kinase assay were purchased from Santa Cruz Biotechnology. Secondary antibodies conjugated with various fluorescent dyes were from Jackson Laboratories, and Vectashield mounting medium was from Vector Laboratories. Secondary antibodies conjugated with HRP were obtained from Bio-Rad. The PKC inhibitors Gö-6976, Gö-6850, and rottlerin were obtained from Calbiochem. [γ-32P]ATP with a specific activity of 3,000 Ci/mmol was purchased from NEN. All other chemicals were from Sigma.

Statistical analysis. Data were reported as means ± SE. Data were analyzed by one-way ANOVA with Bonferroni/Dunn’s post hoc test for comparison with control. Statistical significance is indicated where P < 0.05.

RESULTS

Effect of PMA on T84 cell transport function. Our previous results (7, 8, 20, 22) and the results of others (30) indicate that treatment of confluent T84 monolayers grown on permeable supports with PMA progressively inhibits cAMP-elicited Cl− secretion. Figure 1A shows the peak \( I_{sc} \) achieved in response to stimulation by 10 μM forskolin at varying times after exposure to 100 nM PMA. Under control conditions, forskolin markedly stimulated \( I_{sc} \), reaching the peak (baseline \( I_{sc} = 4.0 \pm 0.2 \mu A/cm^2 \)), peak \( I_{sc} = 135 \pm 16 \mu A/cm^2 \), \( P < 0.05 \)) within 15 min. Inhibition of peak \( I_{sc} \) was seen well within 1 h of PMA exposure. The IC50 for \( I_{sc} \) inhibition by PMA was ~70 nM (Fig. 1B).

Effect of PMA on PKC isozymes in T84 cells. We previously showed that PMA increases total PKC activity in T84 cells (20) and translocates both cPKCa and nPKCe (35) but not nPKCδ or aPKCa. To further delineate the action of PMA on PKC isozymes in T84 cells, we performed an in vitro kinase assay. The time course for kinase activation of PKCa and PKCe by PMA closely correlated with membrane translocation of these isozymes (Figs. 2A and 6A). Unexpectedly, despite the absence of translocation in Western blot experiments, PKCδ kinase activity was increased within 30 min of exposure to PMA (Fig. 2A). Thus, PMA increases the kinase activity of PKCa, PKCe, and PKCδ in T84 cells, but translocation is only evident by our methods for PKCa and PKCe. We next examined which of these isoforms was involved in regulation of transport and barrier function.

Effect of isoform-selective PKC inhibitors on PMA inhibition of T84 transport function. To elucidate the PKC isoform involved in regulation of T84 transport function, we first examined the effect of three structurally unrelated PKC inhibitors, Gö-6976, Gö-6850, and rottlerin, which have well-established but different activity profiles against cPKCa and nPKCe isoforms. The substituted indocarbazole compound Gö-6976 has been shown to inhibit cPKCa isoforms exclusively (IC50 = 2 nM against PKCa in vitro) (19), with no demonstrable in vitro inhibitory activity against novel Ca2+-independent or aPKCa isoforms.
even at high micromolar concentrations. We examined this selectivity for T84 cells by in vitro kinase assay. As expected, 5 mM Gö-6976 had no inhibitory effect on PKCɛ in T84 cells but clearly inhibited activity of PKCα in vitro (Fig. 2B). However, Gö-6976 did show some inhibitory activity against nPKCd at the 5 mM concentration in T84 cells. The bisindolmaleimide compound Gö-6850 is known to inhibit both cPKC and nPKC isoforms (IC₅₀ = 8 nM and 132 nM in vitro for PKCα and PKCɛ, respectively) (19). In T84 cells, 5 mM Gö-6850 completely inhibited PMA activation of cPKCa and nPKCd, and nPKCe in the in vitro kinase assay as shown in Fig. 2B. At 10 μM, rottlerin is rather specific for the nPKCd isoform (IC₅₀ = 3–6 μM), weakly active against cPKC isoforms (IC₅₀ = 30 μM), and inactive against PKCe (IC₅₀ = 100 μM) (46). We also confirmed this for T84 cells. Rottlerin showed a selective inhibition of PKCd in vitro at 10 μM concentration without affecting PKCe while weakly inhibiting PKCα in T84 cells (Fig. 2B).

Having confirmed the selectivity profile for Gö-6850, Gö-6976, and rottlerin in T84 cells, we proceeded to test whether these inhibitors attenuated the effect of PMA on Iₛₑ. Figure 3A shows that pretreatment of T84 monolayers with the cPKCa and nPKCd inhibitor Gö-6976 (5 mM) for 1 h had no effect on the PMA-induced inhibition of peak Iₛₑ. However, pretreatment with the cPKC and nPKC inhibitor Gö-6850 (5 μM) prevented the inhibition of Iₛₑ by PMA with an IC₅₀ value of ~1–2 μM in vivo as shown in Fig. 3B. These differential effects by Gö-6976 and Gö-6850 could be accounted for by the ability of Gö-6850 to inhibit PKCe, unlike Gö-6976. Rottlerin had no effect on Iₛₑ at 10 μM (Fig. 3A). Because cPKCa, nPKCe, and nPKCd are the three isoforms activated in response to PMA within the sensitivity of the antibodies we used, these inhibitor studies imply that PKCe may be the key PKC isoform responsible for inhibition of Cl⁻ secretion.
Effect of PMA and isozyme-selective inhibitors on T84 cell-barrier function. Figure 4A shows that treatment of T84 monolayers with 100 nM PMA results in a decrease in TER to 28 ± 6% of control within 4 h (baseline TER 1,142 ± 21 V cm² vs. 4-h PMA TER 318 ± 32 V cm²). However, closer examination of the time course of this change indicates that the earliest evidence of a change in basal TER occurs well after the observed inhibition of cAMP-stimulated $I_{sc}$, which was prominently evident within 1 h. The IC$_{50}$ of PMA for inhibition of TER after 2 h exposure was 300–500 nM as shown in Fig. 4B. In contrast to results with cAMP-stimulated $I_{sc}$, both Go-6976 and Go-6850 at 5 μM inhibited the PMA-induced decline of the TER (Fig. 5A). IC$_{50}$ values for Go-6976 and Go-6850 in vivo were ~2 and ~1 μM, respectively (Fig. 5B). Rottlerin (10 μM) partially reversed the effect of PMA. This partial inhibition could be due to the ability of rottlerin to weakly inhibit PKCα at 10 μM (Fig. 2C). Rottlerin at a higher concentration inhibited both cPKCα and nPKCɛ (data not shown) and thus is not a valid tool for the inhibitor study. The strong sensitivity to Go-6976 and partial inhibition by rottlerin suggest that activation of the conventional isoform PKCα is associated with downregulation of junctional integrity.

Spatiotemporal characteristics of PKCɛ and PKCα translocation in response to PMA. Given the finding from the inhibitors study that nPKCɛ and cPKCα are the major isoforms responsible for regulation of transport and barrier function in T84 cells, we further characterized the time course for their membrane translocation upon PMA addition. With Western blot analysis, both PKCɛ and PKCα were found predominantly associated with the cytosolic fraction in control monolayers (Fig. 6A). Upon PMA addition, PKCɛ promptly translocated to the membrane as early as 15 min after PMA addition. Translocation of PKCα, however, did not begin to become evident until 60 min after treatment. Activation of both PKCɛ and PKCα continued for at least 4 h without significant degradation. The sequential activation of PKCɛ followed by PKCα is consistent with our functional data obtained using selective PKC isoform inhibitors that correlate early activation of PKCɛ with $I_{sc}$ inhibition (Fig. 1A) and later activation of PKCα with inhibition of TER (Fig. 4A).
If this hypothesis were correct, we might expect PKC to inactivate PKC inhibition of transepithelial secretion could be due to IC50 values for the effects of Go\textsuperscript{6976} and Go\textsuperscript{6850} on PMA-inhibited

In our previous report, we observed that PMA selectively enhanced basolateral membrane endocytosis by a mechanism involving PKCe and postulated that PMA inhibition of transepithelial secretion could be due to PKCe action at the basolateral membrane (35). If this hypothesis were correct, we might expect PMA to induce translocation of PKCe to the basolateral domain. Moreover, since the functional data and Western blot experiments described above suggest that PKCe and PKCa differentially affect epithelial transport and barrier function, we might further anticipate that PKCa and PKCe would localize to different subcellular regions upon activation by PMA. Results of immunostaining of PKCa and PKCe in the polarized T84 mono-

layers were consistent with these concepts. Under control conditions, PKCe was distributed in a punctate pattern diffusely throughout the cytoplasm (Fig. 7A, PKCe stained in red). As early as 15 min after PMA addition, PKCe was cleared from the cytoplasm and moved toward the cell periphery, as indicated by the enlargement of the unstained black center in Fig. 7B. As time elapsed, red staining became more sharply defined along the cell boundary and less prominent at the subapical region. By 1 h of treatment with PMA, PKCe was mostly associated with the basolateral membrane and sharply outlined the individual cells (Fig. 7, C and D). In contrast, PKCa was initially localized at the basal cytoplasm under control conditions (Fig. 8A). However, 30 min after exposure to PMA, PKCa began to redistribute toward the apical region (Fig. 8C) in many cells, and after 60 min, it became clearly localized to the apical membrane and subapical cytoplasmic domain. In some cells, less pronounced localization of PKCa along the basolateral membrane was occasionally detected (Fig. 8D). The basis for this cell-to-cell variability is uncertain.

Effect of bryostatin-1 and carbachol on T84 cell transport and barrier function. Like PMA, the non-phorbol ester PKC agonist bryostatin-1 rapidly translocated PKCe to the membrane fraction of T84 cells (Fig. 9A). PKCe in this fraction was sustained for at least 4 h after bryostatin-1 addition without any evidence of downregulation (degradation) of total PKCe protein (Fig. 9B). We also confirmed similar activation of PKCe by the in vitro kinase assay (data not shown). In parallel experiments, the peak $I_{sc}$ elicited by forskolin was significantly inhibited by 100 nM bryostatin-1 (e.g., 25 ± 1% control at 2.5 h, Fig. 9C), similar to our earlier reported findings (7). Inhibition of $I_{sc}$ was prevented by 5 μM Go\textsuperscript{6850} but not Go\textsuperscript{6976} or 10 μM rottlerin, consistent with the notion that PKCe is the key isoform involved in inhibition of epithelial Cl\textsuperscript{−}secretion. Membrane translocation of PKCe, on the other hand, occurred substantially later with bryostatin-1 than with PMA (Fig. 10A) and, also in contrast to PMA, the total level of both cytosolic and membrane PKCa reduced to 39 ± 10% control, suggestive of PKCa downregulation or degradation. Barrier function was only transiently (and relatively minimally) affected (Fig. 10B). The basal $I_{sc}$ was 94 ± 5% control at 4 h after bryostatin-1 addition. The acetylcholine analog carbachol (CCh) is known to induce phospholipid turnover and generate DAG, thereby activating PKC. CCh, unlike PMA or bryostatin-1, induces a transient activation of Cl\textsuperscript{−}secretion associated with a transient fall in $I_{sc}$, but both $I_{sc}$ and TER return to baseline levels within ~10 min. In our previous study, we showed that CCh activates PKCe but not PKCa during the initial 30 min (35). In the present study we have further characterized the PKC isoform response to 100 μM CCh. PKCe rapidly translocated to the membrane and continued to be active for at least 2 h (Fig. 11A). After 4 h, however, PKCe began to return to the cytosol, and by 12 h, PKCe was mostly associated with the cytosolic fraction. This sustained
activation and the later deactivation of PKCe was paralleled by a significant inhibition of the peak I_{sc} response to forskolin (IC_{50} = 2 nM) that was followed by subsequent recovery (Fig. 11B). As expected, the inhibitory effect of carbachol on forskolin-stimulated I_{sc} was prevented by Gö-6850 but not Gö-6976 (Fig. 11C), consistent with the concept that PKCe negatively regulates Cl\(^{-}\) secretion in these cells. CCh had no effect on PKCa at any time point examined (Fig. 12A). Consistent with the postulated role of PKCe in regulation of barrier function, no effect of CCh on TER could be detected when measured at time points after termination of the early transient (<10 min) drop in TER associated with transient stimulation of I_{sc} by CCh (Fig. 12B). CCh had no effect on the activity of nPKC\(\varepsilon\) when examined using the in vitro kinase assay (data not shown).

**DISCUSSION**

Activation of PKC by phorbol esters exerts complex and time-dependent effects on transepithelial Cl\(^{-}\) secretion. In dog trachea, for example, PMA induces a transient activation of I_{sc} within minutes of exposure, followed by a progressively profound inhibition of cAMP-dependent electrogenic ion transport (2). In native tissue models, it is difficult to ascertain whether the effect of PMA (and, by extension, PKC) is exerted directly at the level of the Cl\(^{-}\) secretory epithelial cell or on altered neurohormonal regulatory input. However, PKC has been shown to acutely regulate several ion transporters and channels involved in Cl\(^{-}\) secretion. PKC has been shown to activate CFTR Cl\(^{-}\) channels at low intracellular Ca\(^{2+}\) concentrations (15), perhaps by facilitating protein kinase A-dependent phosphorylation of CFTR (12, 48). Liedtke and coworkers (16–18) showed that Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC1) in airway epithelia is acutely activated by PKC\(\varepsilon\). The use of cultured epithelial cell lines as models of electrogenic Cl\(^{-}\) secretion has facilitated detailed mechanistic analysis of the effects of PKC on specific elements of the secretory apparatus. In certain intestinal lines (e.g., HT29cl.19A cells) (1, 42, 43), but not in others (T84 cells) (2, 20), PMA induces a transient, albeit small, increase in I_{sc}. However, the dominant effect of PMA appears to be inhibitory. Warhurst et al. (45) noted that phorbol ester markedly inhibited the T84 cell Cl\(^{-}\) secretory response to prostaglandin E\(_2\) by a mechanism that involved receptor desensitization (45), although a variety of later studies indicated a profound inhibition of I_{sc} responses to all cAMP-mediated stimuli, including permeant analog (20, 22, 30). Initially, the mechanism of inhibition of Cl\(^{-}\) secretion was thought to involve downregulation of CFTR gene expression (39). However, later studies indicated that the inhibitory effect of PMA on Cl\(^{-}\) secretion in fact preceded effects at the apical membrane by at least several hours (20, 30). Rather, inhibition of transepithelial Cl\(^{-}\) secretion by PMA correlated most closely with inhibition of several basolateral membrane transport sites including K\(^{+}\) channels (1, 30) and NKCC1 (8, 20). However, the basis for PMA inhibition of multiple independent transport pathways at the basolateral membrane by PMA was not established. As one possible unifying mechanism, we postulated that PMA promoted the endocytic retrieval (and thereby reduced surface expression) of multiple transport pathways at the basolateral membrane (35).

In the current study, we have provided several lines of evidence linking activation of PKCe to the progressive inhibition of cAMP-dependent Cl\(^{-}\) secretion by PMA in the T84 cell model. First, inhibition of I_{sc} by PMA was prevented by the cPKC and nPKC isoform...
Fig. 7. PMA translocates PKCe to the basolateral membrane. T84 monolayers were treated with 100 nM PMA and then fixed, permeabilized, and incubated with anti-PKCe, followed by incubation with rhodamine-conjugated secondary antibody and FITC-phalloidin. The representative vertical (x-z) sections of each monolayer obtained by confocal microscopy are shown. AP, apical membrane; BA, basal membrane; LA, lateral membrane (open arrowhead). Red staining represents PKCe and green staining represents F-actin. The control monolayer (A) shows a diffuse distribution of PKCe throughout the cytoplasm (arrows). As early as 15 min after PMA addition, PKCe clears from the cytoplasm and moves toward the cell periphery (B, arrows). At 30 min after PMA addition (C), red staining becomes more sharply defined along the cell boundary (LA) and less prominent at the subapical region. By 1 h of treatment with PMA (D), PKCe mostly associates with the basolateral membrane and sharply outlines the individual cells (arrows).

Fig. 8. PMA translocates PKCa to the apical membrane. Monolayers were incubated with 100 nM PMA for the time indicated, fixed, permeabilized, and fluorescently labeled with anti-PKCa (red) and FITC-phalloidin (green). Confocal images of the vertical sections of each monolayer are shown. The control monolayer (A) shows the immunolocalization of PKCa at the basal cytoplasm (arrows). PKCa mostly remains at the basal cytoplasm (B, arrow) even after 15-min exposure to PMA. However, after 30 min (C), PKCa begins to redistribute toward the apical region in many cells (arrows), and after 60 min (D) PKCa becomes clearly localized to the apical membrane and subapical cytoplasmic domain (arrow). In some cells, less pronounced localization of PKCa along the basolateral membrane was occasionally detected.
inhibitor Go-6850 but not the PKCα-selective inhibitor Go-6976 or the PKCδ-specific inhibitor rottlerin. The specificity of these inhibitors was confirmed by in vitro kinase assay. Second, early inhibition of forskolin-stimulated \( I_{sc} \) temporally correlates with immediate activation of PKCε as shown by in vitro kinase assay as well as early translocation of PKCε, which occurred within 15 min after PMA addition. Moreover, bryostatin-1 and CCh both inhibited \( I_{sc} \) and translocated PKCε, and in the case of CCh, PKCε is the only PKC isoform noted to be translocated and activated during this time period. Finally, immunolocalization studies showed that in response to PMA, PKCε takes on a distribution associated with the basolateral membrane.

The roles of PKCε in cell function, particularly in epithelial cells, remain poorly understood. A role in cytoskeletal organization was suggested on the basis of findings that F-actin can serve as an isozyme-selective RACK (receptor for activated C kinase) for PKCε (29) and that PKCε is a MARCKS (myristoylated alanine-rich C kinase substrate) kinase (3). In cardiac myocytes, PKCε appears to play a major role in ischemic preconditioning and functions within the context of p42/p44 mitogen-activated kinase pathway in response to diverse cellular growth factors and forms of cell...
stress (27, 28). PKCe has been shown to associate with caveolae in cardiac myocytes (33), suggesting that it may play a role in membrane traffic and in coordinating integrated signaling responses within these specialized membrane microdomains. Weller et al. (47) recently showed that activation of PKCe in colon cancer cells may be a trigger for proliferative responses to PMA, and, indeed, overexpression of PKCe promotes tumorigenicity (26). In T84 cells, Chow et al. (4) showed that PKCe is activated in response to epidermal growth factor (EGF) and may participate in the negative regulation of Ca\(^{2+}\)-dependent Cl\(-\) secretion by EGF and CCh.

In addition to vectorial transport, epithelial cells also possess the property of barrier function. PKC appears to play an important role in junction formation after epithelial disassembly, such as in the Ca\(^{2+}\) “switch” model, although the precise mechanism whereby PKC regulates this process remains to be established (37).

PKC-dependent junction assembly is initiated at the level of E-cadherin and the zonula adherens, rather than at the tight junctions, and thus this process may not necessarily be mediated by the same PKC isoenzyme(s) that influence paracellular permeability (which is largely determined at the level of the zonula occludens). PKC-dependent junctional hyperpermeability in confluent T84 monolayers is known to be induced simply by an elevation of cell Ca\(^{2+}\) (38), although whether the Ca\(^{2+}\) dependence of junctional permeability reflects a specific role for a cPKC is uncertain.

The present experiments closely link extended activation of PKCa to impaired barrier function in model T84 epithelia. First, we observed that TER remains relatively constant after PMA treatment (and PKCe translocation) until a time that follows the later translocation of PKCa to the membrane fraction. Second, the PMA-associated fall in TER was prevented by Gö-

---

Fig. 12. CCh does not activate PKCa and has no effect on basal TER. A: up to 4 h after addition of 100 \(\mu\)M CCh, PKCa remained associated with the cytosolic fraction as shown by both Western blot (top) and densitometric analysis (bottom). B: CCh had no effect on basal TER (see text). TER remained unaltered up to 4 h after CCh addition.

---

Fig. 11. CCh inhibits cAMP-elicited \(I_{sc}\) via activation of PKCe. A: monolayers were treated with 100 \(\mu\)M CCh for the time indicated, and translocation of PKCe was examined. PKCe rapidly translocated to the membrane in response to CCh and remained active for at least 4 h. At 4 h, however, PKCe began to return to the cytosol, and by 12 h, PKCe was mostly associated with the cytosolic fraction. Densitometric analysis of PKCe distribution (bar graph) is shown as a percentage of total PKCe found in membrane fractions. \(^*P < 0.05\). B: monolayers were incubated with 100 \(\mu\)M CCh, and the changes in peak cAMP-elicited \(I_{sc}\) were examined for 4 h. CCh caused a significant inhibition of cAMP-elicited \(I_{sc}\), followed by a subsequent recovery. \(^*P < 0.05\). C: representative inhibition by 100 \(\mu\)M CCh at 2.5 h is shown (\(n = 3\) for each condition). Inhibition of \(I_{sc}\) was prevented by pretreatment with 5 \(\mu\)M Gö-6850 but not with Gö-6976 or rottlerin. \(^*P < 0.05\).
PKC AND EPITHELIAL FUNCTION

6976, a PKC inhibitor that is highly selective for Ca\(^{2+}\)-dependent cPKC isoforms. Third, in response to PMA, PKC\(\alpha\) translocated from the basal cytoplasm to the apical zone of T84 monolayers, in the vicinity of the junctional complexes and perijunctional actomyosin ring known to affect junctional integrity. Comparison of the effect of PMA with that of other PKC agonists provides indirect support for the hypothesis that PKC\(\alpha\) is the key isoform involved in junctional regulation. For example, CCh, unlike PMA, had no effect on TER and did not alter PKC\(\alpha\) subcellular distribution at any point time.

Bryostatin-1, compared with PMA, induced a delayed membrane translocation of PKC\(\alpha\) that was associated with a smaller and transient fall in TER. The return of TER toward control levels with extended bryostatin-1 treatment may reflect accelerated degradation (downregulation) of PKC\(\alpha\). Indeed, bryostatin-1 has been shown to induce proteosome-mediated degradation of PKC\(\alpha\) through enhanced ubiquitinization (14). Bryostatin-1, which shares with PMA an affinity for the DAG binding site of PKC, is known to induce a subset of the cellular responses evoked by PMA and, interestingly, to antagonize many of the responses it does not share with PMA (11). Thus our earlier finding that bryostatin-1 is able to partially antagonize the effect of PMA on TER (7) is likely to reflect the ability of bryostatin-1 to downregulate PKC\(\alpha\). We speculate that bryostatin-1-activated PKC\(\alpha\) can induce only minimal effects on TER before it is depleted and that the early downregulation of PKC\(\alpha\) by bryostatin-1 prevents extended PMA activation of PKC\(\alpha\) and thereby attenuates the fall in TER.

A role for PKC\(\alpha\) in junctional regulation in epithelia has previously been postulated (32). Notably, Mullin et al. (24) showed that overexpression of wild-type PKC\(\alpha\) in LLC-PK\(\alpha\) cells renders the cells more sensitive to PMA-induced junctional disruption, whereas expression of a dominant-negative PKC\(\alpha\) construct renders them resistant. Other PKC isoforms may also influence junctional structure and permeability under certain conditions. For example, junctional permeability is increased by overexpression of PKC\(\delta\) in cultured renal epithelial cells (24), and in Madin-Darby canine kidney and Caco-2 epithelial cells, the aPKC\(\zeta\) is the only isoform that specifically localizes near the tight junctional complex (6). Because the PMA-induced decrease in TER was partially inhibited by rottlerin, a role for PKC\(\delta\) cannot be entirely excluded. However, at the concentration used, rottlerin also partially inhibited PKC\(\delta\). It is unknown, and our studies could not address, whether PMA can alter PKC\(\zeta\) activity, and thus a role for this aPKC also cannot be excluded.

Our localization data indicate that PMA induces an intracellular redistribution of PKC\(\alpha\) toward the apical zone of the cell, where it may potentially interact directly or indirectly with various components of the tight junction. The target of PKC\(\alpha\) that leads to altered junctional permeability remains to be elucidated but is likely to involve the cytoskeleton. Hecht et al. (10) showed that disruption of T84 monolayer integrity by PMA is associated with disruption of perijunctional F-actin (10). The permeability characteristics of tight junctions are known to be modulated by the tension of the perijunctional actin-myosin ring, which, in turn, is mediated by myosin light chain kinase (MLCK) (41). PKC is known to itself alter phosphorylation of both MLC and MLCK, although whether this mechanism can account for the observed effects of PMA in T84 cells remains speculative. Conflicting reports have appeared regarding this concept. Turner et al. (40) showed in a Caco-2 subclone that PMA acutely increased MLCK phosphorylation and decreased MLC phosphorylation; this response was associated with an acute increase in TER, presumably due to relaxation of the perijunctional actin-myosin ring. Other Caco-2 clones, however, have been shown to behave similarly to T84 cells with a progressive decrease in TER in response to PMA (36). Enhanced actin-myosin contractile activity through PKC-mediated regulation of MLCK also has been reported (25), but in one instance, enhanced junctional permeability due to phorbol ester was shown to be independent of MLCK (34). Other potential targets of PKC\(\alpha\) must also be considered. Interestingly, phorbol ester-induced barrier dysfunction in endothelial cells appears to involve extracellular signal-regulated kinase (ERK1/2) signaling via Ras (44).

In summary, by using multiple agonists and isozyme selective inhibitors, we have been able to dissociate PKC actions on transport function and barrier function in T84 model epithelia. Activation of PKC\(\alpha\) appears to inhibit electrogenic Cl\(^{-}\) secretion, whereas extended activation of PKC\(\alpha\) decreases TER. The present studies demonstrate that PKC-dependent stimuli can elicit divergent effects on epithelial cell function through differential activation of distinct PKC isoenzymes, which in turn act at distinct subcellular localizations. It thus may prove possible to selectively target specific PKC isoenzymes for activation, inhibition, or downregulation in the context of antidiarrheal and anticancer drug development.

This work was presented in part at the American Gastroenterological Association Annual Meeting, May 2000, San Diego, CA. This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-48010 and DK-51630 (J. B. Mathews).

REFERENCES


