Opposing effects of PKCα and PKCε on basolateral membrane dynamics in intestinal epithelia

JAEEKYUNG CECILIA SONG,1 PATANGI K. RANGACHARI,2 AND JEFFREY B. MATTHEWS1
1Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267; and 2Department of Medicine, McMaster University, Hamilton, Ontario, Canada L8S 4L8

Received 7 March 2002; accepted in final form 17 July 2002

Song, Jaekyung Cecilia, Patangi K. Rangachari, and Jeffrey B. Matthews. Opposing effects of PKCα and PKCε on basolateral membrane dynamics in intestinal epithelia. Am J Physiol Cell Physiol 283: C1548–C1556, 2002.—PKC is a critical effector of plasma membrane dynamics, yet the mechanism and isoform-specific role of PKC are poorly understood. We recently showed that the phorbol ester PMA (100 nM) induces prompt activation of the novel isoform PKCε followed by late activation of the conventional isoform PKCα in T84 intestinal epithelia. PMA also elicited biphasic effects on endocytosis, characterized by an initial stimulatory phase followed by an inhibitory phase. Activation of PKCε was shown to be responsible for stimulation of basolateral endocytosis, but the role of PKCα was not defined. Here, we used detailed time-course analysis as well as selective activators and inhibitors of PKC isoforms to infer the action of PKCα on basolateral endocytosis. Inhibition of PKCα by the selective conventional PKC inhibitor Gö-6976 (5 μM) completely blocked the late inhibitory phase and markedly prolonged the stimulatory phase of endocytosis measured by FITC-dextran uptake. The PKCε-selective agonist carbachol (100 μM) induced prolonged stimulation of endocytosis devoid of an inhibitory phase. Actin disassembly caused by PMA was completely blocked by Gö-6850 but not by Gö-6976, implicating PKCε as the key isoform responsible for actin disruption. The Ca2+ agonist thapsigargin (5 μM) induced early activation of PKCα when added simultaneously with PMA. This early activation of PKCα blocked the ability of PMA to remodel basolateral F-actin and abolished the stimulatory phase of basolateral endocytosis. Activation of PKCα stabilizes F-actin and thereby opposes the effect of PKCε on membrane remodeling in T84 cells.

proteins; cytoskeleton; gastrointestinal mucosa; calcium

PROTEIN KINASE C (PKC) is a family of at least 10 different serine/threonine isoforms (20) that have been implicated in a variety of cellular responses such as membrane trafficking, migration, ion transport, and cell differentiation. Each isoform has distinct enzymological properties, tissue distribution, and subcellular localization (22), implying that each affects a unique complement of biological functions. The basis for this functional diversity and for the selective regulation of individual isoforms is not well understood. PKC isoforms are usually subdivided into three groups, conventional (cPKC), novel (nPKC), and atypical (aPKC), on the basis of their activation requirements, which, in turn, reflect the structure of their regulatory domains (20, 21). The regulatory domain of cPKC isoforms (α, β1, β2, and γ) contains two common regions, C1 and C2. The C1 region mediates diacylglycerol (DAG) and phorbol ester binding. The presence of a C2 region makes the cPKC isoforms distinct from other subfamilies in that they require Ca2+ for activation. The nPKC isoforms (δ, ε, η, and θ) contain a C1 region, and, therefore, DAG and phorbol ester binding activate these isoforms. However, nPKC are Ca2+ independent because they lack the C2 region. The aPKC isoforms (ζ and η) are independent of DAG or Ca2+ and, as a general rule, cannot be directly activated by phorbol esters such as PMA. This structural heterogeneity implies that intracellular Ca2+ is a key determinant of the specific pattern of PKC isoform activation.

We previously showed that in model polarized T84 human intestinal epithelia, PMA induces a dramatic increase in the rate of basolateral fluid-phase endocytosis without affecting endocytosis at the apical membrane (28). Of the four PKC isoforms identified in T84 cells (α, ε, δ, and ζ), PMA induced early activation of two novel isoforms, PKCε and PKCδ, followed by late activation of the conventional isoform PKCα (27). PMA was also shown to induce biphasic effects on basolateral endocytosis characterized by an early stimulation period (stimulatory phase) followed by a later return to baseline rates (inhibitory phase) (28). Selective inhibition of PKCε completely abolished the early stimulatory phase, suggesting that PKCε is the key isoform responsible for PMA-induced stimulation of basolateral endocytosis by a mechanism that appeared to involve localized actin disassembly. The basis for the inhibitory phase was not determined, but preliminary data reported in abstract form suggested a possible role for PKCα (29). PKCδ did not appear to be involved in either the stimulatory or inhibitory phase, on the
basis of insensitivity to the PKCδ-specific inhibitor rottlerin. There have been several reports describing antagonistic effects of different PKC isoforms on the regulation of the same biological function (2, 5, 6, 12, 33). For example, in rat fibroblasts, PKCα and PKCδ were shown to have opposite effects on epidermal growth factor receptor-mediated transformation and phospholipase D activity. PKCβ1 and PKCβII had opposite roles in vascular smooth muscle cell proliferation. PKCβ and PKCζ mediated opposing effects on proximal tubule Na+/K+-ATPase activity. These considerations suggested to us the possibility that, in T84 cells, PKCα could play a counterregulatory role to PKCe in control of basolateral membrane dynamics.

**MATERIALS AND METHODS**

**Cell culture.** T84 human intestinal epithelial cells obtained from Dr. Kim Barrett (University of California, San Diego, CA) were grown to confluence as described previously (27). Small confluent T84 monolayers grown on collagen-coated permeable supports (4.7 cm², 3.0-μm pore size) was measured as described previously (27).

**Subcellular fractionation.** T84 cells grown to confluence on collagen-coated permeable supports (4.7 cm²) were fractionated into the cytosolic and membrane fractions as described previously (28). Briefly, monolayers were scraped with 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, and the homogenate was ultracentrifuged at 14,000 g for 50 min at 4°C. The supernatant was designated the membrane fraction. The pellet was resuspended in HB containing 0.5% (vol/vol) Triton X-100 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.

**In vitro kinase assay.** Confluent T84 monolayers grown on 4.7-cm² permeable supports were treated with various PKC agonists, and proteins were extracted with the lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (vol/vol) Triton X-100, 2 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na3VO4, and Complete protease inhibitor cocktail tablets (27). Polyclonal antibodies against cPKCα (2 μg), nPKCε (4 μg), or nPKCδ (2 μg) were added to each lysate for overnight rotation at 4°C. After incubation, immune complexes were precipitated with the use of protein A-agarose beads, resuspended in kinase buffer (35 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.5 mM EGTA, 10 μCi [γ-32P]ATP, 60 μM cold ATP, and 1 mM Na3VO4), and incubated with myelin basic protein (MBP) as a substrate at 30°C for 30 min. After incubation, the reaction was terminated with Laemmli sample and subjected to SDS-PAGE (15% gels). The gel was then dried and subjected to autoradiography.

**Gel electrophoresis and Western blotting.** Equal amounts of protein (~50 μg/sample) were subjected to SDS-PAGE and Western blot as described previously (27). Briefly, proteins were separated on 8% gels, transblotted to nitrocellulose membranes, and incubated with the polyclonal antibodies to different PKC isoforms for 1 h. After brief washing, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h, washed, and visualized with enhanced chemiluminescence (ECL) detection reagent.

**Immunofluorescence and microscopy.** Monolayers grown on 0.33-cm² permeable supports were treated with various agonists and prepared for confocal microscopy as described previously (27). Briefly, cells were fixed and permeabilized with 0.1% (vol/vol) Triton X-100. Cells were then incubated with the blocking buffer (1% normal goat serum, 3% BSA in PBS) followed by the primary antibody against PKCα. After overnight incubation in a moisture chamber at 4°C, monolayers were incubated in rhodamine-conjugated goat anti-rabbit polyclonal IgG along with FITC-phalloidin for F-actin staining. Confocal images were acquired with a Zeiss inverted microscope equipped with MRC-1024 and Lasersharp software (Bio-Rad).

**Materials.** Tissue culture reagents and protein A-agarose beads were purchased from Invitrogen. Gel electrophoresis and Western blotting reagents were from Bio-Rad, with the exception of ECL detection reagent, which was purchased from Amersham. Complete protease inhibitor cocktail tablets were from Roche. Anti-PKCα was obtained from Sigma and Santa Cruz Biotechnology for Western blotting and immunostaining, respectively. Anti-PKCe was purchased from Santa Cruz Biotechnology. Secondary antibodies were obtained from Bio-Rad and Jackson Laboratories for Western blotting and immunostaining, respectively. Vectashield mounting medium was from Vector Laboratories. The PKC inhibitors Gö-6976, Gö-6850, and rottlerin were obtained from Calbiochem. [γ-32P]ATP with specific activity of 3,000 Ci/mmol was purchased from NEN. All other chemicals were from Sigma.

**Statistical analysis.** Data are reported as means ± SE. Data were analyzed by one-way ANOVA with Bonferroni/Dunn’s post hoc test for comparison with control.

**RESULTS**

**Activation of PKCe and PKCα are temporally associated with stimulation and inhibition of basolateral endocytosis, respectively.** Our previous results (28) suggested that PMA elicits biphasic effects on basolateral uptake of FITC-dextran in T84 monolayers. This was confirmed in repeated experiments, shown in Fig. 1A, that extended the time course of observation over 150 min. During the initial stimulatory phase, 100 nM PMA initially induced translocation of PKCe to the membrane fraction, whereas translocation of PKCα lagged 1 h behind the activation of PKCe and was temporally associated with the inhibitory phase. In vitro kinase assays for PKCe and PKCα were consistent with these
which we have previously validated the isoform selectivity profile (27). Gö-6976 is largely selective for (Ca\(^{2+}\)-dependent) cPKC isoforms in vitro, whereas Gö-6850 blocks both cPKC and nPKC (14, 34). In T84 cells, we found that Gö-6976 at 5 \(\mu\)M exerted a slight inhibitory effect on PKC\(\varepsilon\) in addition to PKC\(\alpha\), but there was no evidence of inhibition of PKC\(\varepsilon\).

We found that inhibition of PKC\(\alpha\) with pretreatment with Gö-6976 had a potentiating effect on basolateral endocytosis triggered by PMA. When Gö-6976 was added before PMA treatment, FITC-dextran uptake from the basolateral buffer was greater than when PMA was added alone (Fig. 2A). By 60 min of treatment with PMA, PKC\(\alpha\) began to appear at the mem-

translocation data (Fig. 1C) and with our earlier reported results (29).

To begin to determine whether activation of PKC\(\varepsilon\) and PKC\(\alpha\) functionally correlated with these two phases of endocytosis elicited by PMA, we used two PKC-specific inhibitors, Gö-6976 and Gö-6850, for

\[ \text{PKC} \rightarrow \text{regulation of endocytosis} \]

\[ \text{activating protein (MBP)} \]

\[ \text{PKC activation} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]

\[ \text{PKC inhibitors} \]

\[ \text{PKC isoform} \]

\[ \text{PKC activity} \]

\[ \text{PKC protein (MBP)} \]

\[ \text{PKC translocation} \]
brane fraction (Fig. 1B) and inhibition of PKCα activation by Gö-6976 caused further enhancement of endocytosis induced by PMA. Furthermore, inhibition of PKCα by Gö-6976 completely blocked the late inhibitory phase of endocytosis and markedly prolonged the stimulatory phase of endocytosis. This effect was not seen with the PKCδ-specific inhibitor rottlerin (Fig. 2B) at a concentration demonstrated by kinase assay to block PKCδ activity, excluding a role for this isozyme in the inhibitory phase. Thus the delayed activation of PKCα by PMA temporarily and functionally correlates with the decline in basolateral endocytosis to basal levels after 2 h of PMA treatment. We previously showed that inhibition of both PKCε and PKCα by Gö-6850 blocked both the stimulatory and inhibitory phase of PMA-elicited response (28).

These findings strongly suggest that PKCε stimulates, whereas PKCδ inhibits, basolateral endocytosis in T84 cells. Experiments with a second PKC agonist, carbachol (CCh), indirectly supported this concept. We previously reported that CCh, unlike PMA, activates only PKCε and not PKCα in this model (Fig. 3A) (27, 28) and that the stimulation of endocytosis by CCh was blocked by Gö-6850 but not Gö-6976 (28). However, CCh was noted to induce a more strikingly sustained stimulatory phase than PMA, as shown in Fig. 3B.

CCh-elicited stimulation of endocytosis did not diminish until 6 h after treatment (data not shown). In the case of CCh, the termination of the endocytosis response was not associated with activation of PKCα but, rather, deactivation of PKCε determined by translocation assay (Fig. 3A).

**PMA induces actin rearrangement via activation of PKCε not PKCα.** We previously showed that PMA increases basolateral endocytosis via disruption of actin cytoskeleton (28). As shown in Fig. 4, PMA induced significant remodeling of basolateral F-actin and condensation of staining around the cell periphery. We examined sensitivity of PMA-elicited cytoskeletal remodeling to isoform-selective PKC inhibitors. Pretreatment with Gö-6850 but not Gö-6976 attenuated this actin remodeling, suggesting that PKCε is the isoform responsible for actin disassembly caused by PMA. Inhibition of PKCδ by Gö-6976 did not attenuate, and instead appeared to qualitatively exacerbate, the degree of disruption of the actin cytoskeleton.

Thapsigargin prevents stimulation of basolateral endocytosis by PMA. We wondered whether the combination of PMA plus the Ca^{2+}-ATPase inhibitor thapsigargin would accelerate activation of PKCα and allow us to more directly address the potential opposing actions of PKCα and PKCε. Indeed, this proved to be the case. Thapsigargin alone had no effect on PKCα or PKCε. However, the combined addition of PMA and thapsigargin accelerated activation of PKCα. As shown in Fig. 5A, PKCα had already translocated to the membrane by 30 min. Activation of PKCε by PMA, in contrast, was not affected by thapsigargin. Moreover, the combined addition of thapsigargin and PMA failed to stimulate basolateral endocytosis (Fig. 5B); stated differently, thapsigargin blocked the ability of PMA to stimulate endocytosis. The simultaneous activation of PKCα and PKCε by costimulation with thapsigargin and PMA led to inhibition of the early stimulatory effects of PMA on basolateral endocytosis. In contrast to thapsigargin, the Ca^{2+} agonist CCh, which does not induce early activation of PKCε either alone or in combination with PMA, did not antagonize the effects of PMA on basolateral endocytosis. In fact, combined addition of CCh and PMA exaggerated the initial stimulatory phase (data not shown). Although CCh may have other effects that may interfere with PMA’s ability to induce basolateral endocytosis, these data further strengthen the possibility that PKCα may be involved in inhibition of endocytosis.

To further address the mechanism whereby thapsigargin blocks PMA-elicited endocytosis, we examined the role of Ca^{2+} entry pathways. To do so, we removed extracellular Ca^{2+} from either the apical or basolateral buffer during the combined treatment with PMA and thapsigargin. As shown in Fig. 6A, removal of apical Ca^{2+} did not alter the translocation of PKCα by PMA and thapsigargin. However, removal of basolateral Ca^{2+} completely blocked translocation of PKCα, suggesting that basolateral Ca^{2+} entry is required for PKCα activation. Removal of basolateral Ca^{2+} also prevented the ability of thapsigargin to inhibit PMA-
stimulated endocytosis (Fig. 6B). Because thapsigargin is known to activate store-operated Ca$_{2+}$/H$_{11001}$ channels (SOCs) (24) that are thought to be restricted to the basolateral membrane of T84 cells (11), we examined whether the SOC inhibitor La$_{3+}$/H$_{11001}$ (1) would affect membrane translocation of PKC$_{a}$. As shown in Fig. 6C, membrane translocation of PKC$_{a}$ by thapsigargin and PMA was completely abolished by the presence of the SOC-specific inhibitor La$_{3+}$, and, as evidenced by data not shown, thapsigargin did not block PMA-stimulated endocytosis in the presence of basolateral La$_{3+}$.

PKC$_{a}$ rapidly translocates to the basal membrane upon addition of thapsigargin with PMA. We previously showed that inactive PKC$_{a}$ is localized in the basal cytoplasm of T84 cells (27). Vertical images captured from confocal microscopy revealed that PKC$_{a}$ is found in basal zone of the cell in a diffuse cytoplasmic pattern in control monolayers (Fig. 7A). When PMA was added alone, PKC$_{a}$ moved apically and was found in the apical domain (Fig. 7B). In striking contrast, with PMA plus thapsigargin, PKC$_{a}$ rapidly cleared from the basal cytoplasm and relocated to the basal membrane (Fig. 7D), suggesting that Ca$_{2+}$ influx in the basal region could redirect the subcellular localization of PKC$_{a}$ from the apical domain (without thapsigargin) to the basal membrane (with thapsigargin). Thapsigargin alone without PMA-induced elevation of DAG had no effect on PKC$_{a}$ distribution (Fig. 7C).

PMA-mediated actin rearrangement is prevented by thapsigargin. Because thapsigargin prevented the PMA-elicited increase in basolateral endocytosis, we anticipated that thapsigargin would also inhibit PMA-induced actin disassembly. Indeed, this was the case, as shown in Fig. 8D. Pretreatment with the PKC$_{a}$ inhibitor Gö-6976 abolished this effect of thapsigargin (Fig. 8E). This finding strongly suggests that thapsigargin-induced translocation and/or activation of PKC$_{a}$ to the basal membrane (in the context of PMA treatment) inhibits PKC$_{a}$-mediated actin disassembly as well as stimulation of basolateral endocytosis.

DISCUSSION

In our earlier report, we found that a variety of physiological and pharmacological activators of the novel Ca$_{2+}$-independent PKCe isoform rapidly induced actin remodeling and enhanced endocytosis at the basolateral aspect of polarized T84 monolayers (27). In the present study, we developed evidence to suggest that activation of the conventional Ca$_{2+}$-dependent PKC$_{a}$ isoform opposes this action of PKCe. Treatment of T84 monolayers with PMA was observed to sequentially activate PKCe and PKC$_{a}$ in
PKC REGULATION OF ENDOCYTOSIS

account for the different time courses of their effects on basolateral endocytosis. CCh induced an extended stimulation of fluid-phase tracer uptake without the subsequent Go-6976-sensitive inhibitory phase observed with PMA attributable to PKCo.

The subcellular localization of inactive PKC isoforms and their dynamic translocation to other subcellular compartments upon activation likely accounts for specificity in regulation of various biological processes (3, 8, 15). Spatial constraints may therefore determine the relative availability or accessibility of key activating cofactors to the PKC isozymes. Thus the location of

temporal association with an early stimulatory and a later inhibitory phase of basolateral endocytosis. The inhibitory phase was eliminated by concurrent treatment with Go-6976, a selective Ca\textsuperscript{2+}-dependent PKC inhibitor that has been validated in our system, at the concentration used, to be largely selective for PKCa. Cytoskeletal remodeling induced by PMA was prevented by the PKCe/PKCa inhibitor Go-6850 but was exacerbated by Go-6976. Although the Ca\textsuperscript{2+}-ATPase inhibitor thapsigargin did not by itself affect PKC isoform activity, addition of thapsigargin plus PMA induced simultaneous rather than sequential activation of PKCe and PKCa. Moreover, thapsigargin prevented PMA-elicted stimulation of basolateral endocytosis and actin remodeling. Interestingly, this action of thapsigargin depended on the presence of Ca\textsuperscript{2+} in the basolateral bath and was abrogated by the SOC inhibitor La\textsuperscript{3+}, suggesting that basolaterally restricted capacitive Ca\textsuperscript{2+} entry may play a key strategic role in activation of PKCa and in regulation of dynamic basolateral membrane/cytoskeletal remodeling. This concept was further supported by the observation that CCh, in contrast to PMA, activates only PKCe and not PKCa. The different pattern of isoform activation between PMA and CCh appears to

Fig. 5. Simultaneous activation of PKCa with PKCe prevents increase in basolateral endocytosis by PMA. A: PMA and thapsigargin (Tg) were added either separately or simultaneously to T84 monolayers, and translocation of PKC isoforms was examined. As shown previously, PKCe remained inactive after 30 min of PMA. Elevation of intracellular Ca\textsuperscript{2+} by Tg had no effect on PKCa when added alone. However, when PMA and Tg were added simultaneously, PKCa translocated to the membrane as early as 30 min after treatment. Translocation of PKCe was unaffected by Tg. Representative blots of 3 separate experiments are shown. B: Tg alone had no effect on basolateral uptake of FITC-dextran. However, when Tg was added together with PMA, the ability of PMA to increase basolateral endocytosis was completely abolished, suggesting that activation of PKCa inhibits the effect of PKCe to stimulate basolateral endocytosis. *P < 0.05 compared with control. †P < 0.05 compared with PMA alone.

Fig. 6. Basolateral Ca\textsuperscript{2+} entry via store-operated Ca\textsuperscript{2+} channels (SOCs) is required for activation of PKCa by PMA and Tg. A: PKC translocation by PMA and Tg was examined after removal of extracellular Ca\textsuperscript{2+} either from the apical or basolateral buffer. The absence of apical Ca\textsuperscript{2+} had no effect on membrane translocation of PKCe or PKCa induced by PMA and Tg. However, removal of basolateral Ca\textsuperscript{2+} completely blocked translocation of PKCa, suggesting that basolateral Ca\textsuperscript{2+} is critical for its activation. Translocation of PKCe was unaffected by removal of basolateral Ca\textsuperscript{2+}. B: removal of basolateral Ca\textsuperscript{2+} prevented the ability of Tg to inhibit PMA-stimulated increase in basolateral endocytosis. In fact, endocytosis by PMA and Tg was further stimulated in the absence of basolateral Ca\textsuperscript{2+}. Removal of apical Ca\textsuperscript{2+} had no effect. *P < 0.05 compared with control. †P < 0.05 compared with PMA alone. C: the SOC-specific inhibitor La\textsuperscript{3+} (5 µM) was added 15 min before addition of Tg and PMA, and PKC translocation was examined. Inhibition of basolateral Ca\textsuperscript{2+} entry via SOCs completely inhibited translocation of PKCa by PMA and Tg, suggesting that elevation of local Ca\textsuperscript{2+} at the basolateral domain is important for translocating PKCa. All experiments were performed in triplicate.
inactive cPKC or nPKC isoforms in reference to sites of DAG generation in response to phospholipase-coupled membrane receptors could determine their degree of activation. In our initial report (28), we found that the DAG mimetic PMA sequentially activated PKC\(\alpha\)/H9280 and then PKC\(\alpha\)/H9251. CCh, an acetylcholine analog that induces DAG generation via basolaterally located M3 muscarinic receptors, was also shown to activate PKC\(\alpha\)/H9280; however, there was no evidence of activation of PKC\(\alpha\)/H9251 by CCh (27). We were puzzled by this observation, given the known ability of CCh to generate DAG and to increase cytosolic Ca\(^{2+}\)/H11001 from phosphoinositol-sensitive stores. One possible explanation is that DAG production in response to CCh occurs only in the limited vicinity of the basolateral M3 receptor and is available only to PKC\(\alpha\) within this microdomain, whereas PMA from the bulk solution can diffuse throughout the cytoplasm in essentially unlimited capacity and can reach both PKCe and PKC\(\alpha\). Alternatively, the increase in cytosolic Ca\(^{2+}\)/H11001 elicited by CCh could be spatially restricted to a subcellular localization in which either DAG is not available or inactive PKC\(\alpha\) is scarce.

Intracellular Ca\(^{2+}\)/H11001 concentration ([Ca\(^{2+}\)/H11001]) plays a central signaling role for a variety of cellular functions. In polarized epithelial cells such as pancreatic acinar cells, Ca\(^{2+}\) signaling has been shown to occur in a highly compartmentalized fashion (35). Inositol 1,4,5-trisphosphate (IP\(_3\))-elicited [Ca\(^{2+}\)/H11001] release begins in an apical “trigger zone” (9, 10, 32), where the vast majority of IP\(_3\) receptors are localized (13, 19, 36). Spreading of Ca\(^{2+}\)/H11001 through the cytoplasm is modulated by activation of ryanodine receptors localized at the basal pole of acinar cells (30). Ca\(^{2+}\) spreading is also dependent on agonist concentration (31). When cells are stimulated

---

**Fig. 7.** PKC\(\alpha\) translocates to basal membrane upon addition of Tg with PMA. T84 monolayers grown on 0.33-cm\(^2\) permeable supports were treated with PMA and Tg, and translocation of PKC\(\alpha\) was visualized by immunolabeling and confocal microscopy. PKC\(\alpha\) was stained red by addition of anti-PKC\(\alpha\) with the rhodamine-conjugated secondary antibody. Red bars on the right side of each image denote regions of PKC\(\alpha\) localization. F-actin was stained green by FITC-phalloidin to outline the cell boundary. Both the apical and basal boundaries of the monolayers are indicated by blue arrows (base line). Three representative images are shown for each treatment condition. A: in control monolayer, PKC\(\alpha\) was dispersedly localized at the basal cytoplasm in a diffuse cytoplasmic pattern. PKC\(\alpha\) was mostly localized above the basal boundary formed by F-actin staining. B: after 1 h of treatment with PMA, PKC\(\alpha\) became clearly localized to the apical membrane and subapical cytoplasmic domain. C: Ca\(^{2+}\)/H11001 influx by Tg alone did not affect distribution of PKC\(\alpha\). D: simultaneous addition of PMA with Tg cleared PKC\(\alpha\) from the basal cytoplasm. Staining was restricted to the basal line of F-actin, implicating association of PKC\(\alpha\) with the basal membrane.
RACKs are thought to increase PKC phosphorylating isoforms involves association with anchoring proteins physiological roles (17). Targeting of activated PKC represents one mechanism for determining their unique intracellular structures following activation likely represents a sensitive pathway.

Ca\(^{2+}\) entry via a La\(^{3+}\)-6976 and that appears to require basolateral Ca\(^{2+}\) stores and thereby activates SOCs. We have found that, in T84 cells, the SOCs activated by thapsigargin are functionally restricted to the basolateral membrane domain (26). Although CCh is known to activate SOCs in other cell systems, we have found no evidence of sustained activation of basolateral membrane SOCs by CCh in T84 cells (Ref. 26 and unpublished data). Thus, whereas both thapsigargin and CCh induce an increase in \([\text{Ca}^{2+}]_{i}\), only thapsigargin is associated with sustained activation of basolateral SOCs. In the present study, we were able to use the Ca\(^{2+}\)-ATPase inhibitor thapsigargin to selectively manipulate the timing of activation of PKC\(\alpha\) after PMA stimulation. We found that thapsigargin prevented PMA-stimulated endocytosis, an effect that was inhibited by the PKC\(\alpha\)-selective inhibitor G\(_6\)-6976 and that appeared to require basolateral Ca\(^{2+}\) entry via a La\(^{3+}\)-sensitive pathway.

Translocation of specific PKC isoforms to distinct intracellular structures following activation likely represents one mechanism for determining their unique physiological roles (17). Targeting of activated PKC isoforms involves association with anchoring proteins such as receptors for activated C-kinase (RACKs). RACKs are thought to increase PKC phosphorylating efficiency by stabilizing the active kinase near its target substrate (25). RACKs specific for different PKC isoforms have been identified (4, 18). Both PMA and CCh appear to induce PKC translocation specifically to the basolateral domain. Although the specific RACK(s) governing this response remains uncertain, F-actin has been shown to represent a PKC-specific RACK (23); in addition, \(\beta\)\(^{-}\)-COP, which has been associated with caveolae, may also be a RACK for PKC\(\epsilon\) (4).

We previously showed that stimulation of basolateral endocytosis by PMA and CCh involved remodeling of the F-actin cytoskeleton via PKC\(\epsilon\) and the actin cross-linker MARCKS (myristoylated alanine-rich C kinase substrate) (28). In the present study, we found that thapsigargin prevents PMA-induced actin remodeling and stimulation of endocytosis in association with activation of PKC\(\alpha\), an effect blocked by G\(_6\)-6976. The detailed basis for PKC\(\alpha\) antagonism of PKC\(\epsilon\)-induced actin disassembly is unclear. The small GTPase protein RhoA, a well-known regulator of stress fiber formation in many cell types, is a candidate target for PKC\(\alpha\) (7, 16). One possible scenario, therefore, is that thapsigargin, by inducing basolateral Ca\(^{2+}\) influx via SOCs, allows PMA to activate PKC\(\alpha\) and, in turn, RhoA, which then prevents remodeling of F-actin cytoskeleton by PKC\(\epsilon\).

In summary, we have found that activation of PKC\(\alpha\), whether by PMA alone or in conjunction with thapsigargin, antagonizes the ability of PKCe to disassemble basolateral F-actin and stimulate basolateral membrane endocytosis in a model intestinal epithelium. Our data suggest that cytoskeletal and membrane structure may be dynamically modulated by a balance between nPKCe and cPKC\(\alpha\) that appears to depend on subtleties in agonist-regulated subcellular redistribu-
tion of the isoenzymes and the microorganization of Ca^{2+} signaling.

REFERENCES


