Identification of Two Distinct Pathways of Protein Kinase Ca
Down-regulation in Intestinal Epithelial Cells*

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Signal transduction pathways are controlled by desensitization mechanisms, which can affect receptors and/or downstream signal transducers. It has long been recognized that members of the protein kinase C (PKC) family of signal transduction enzymes undergo down-regulation in response to activation. Previous reports have indicated that key steps in PKCα desensitization include caveolar internalization, priming site dephosphorylation, ubiquitination of the dephosphorylated protein, and degradation by the proteasome. In the current study, comparative analysis of PKCα processing induced by the PKC agonists phorbol 12-myristate 13-acetate and bryostatin 1 in IEC-18 rat intestinal epithelial cells demonstrates that: (a) at least two pathways of PKCα down-regulation can co-exist within cells, and (b) a single PKC agonist can activate both pathways at the same time. Using a combined biochemical and morphological approach, we identify a novel pathway of PKCα desensitization that involves ubiquitination of mature, fully phosphorylated activated enzyme at the plasma membrane and subsequent down-regulation by the proteasome. The phosphatase inhibitors okadaic acid and calcylina A accelerated PKCα down-regulation and inhibitors of vesicular trafficking did not prevent degradation of the protein, indicating that neither internalization nor priming site dephosphorylation are requisite intermediate steps in this ubiquitin/proteasome dependent pathway of PKCα down-regulation. Instead, caveolar trafficking and dephosphorylation are involved in a second, proteasome-independent mechanism of PKCα desensitization in this system. Our findings highlight subcellular distribution and phosphorylation state as critical determinants of PKCα desensitization pathways.

The PKCα family of phospholipid-dependent serine/threonine kinases plays a central role in signal transduction and has been implicated in regulation of a variety of fundamental cellular processes, including cell growth and cell cycle progression (1), differentiation (2), apoptosis (3), and survival (4). The tumor promoting properties of PKC α agonists such as phorbol esters, together with evidence for changes in the expression and/or activity of PKC isozymes in a variety of malignancies, point to a role for altered PKC signaling in neoplastic transformation (e.g. Ref. 5). Strict control of PKC function is achieved by two coordinated mechanisms: consecutive phosphorylation on three “priming sites” (activation loop, turn motif, and hydrophobic motif), which is required for catalytic competence (6), and binding of second messengers (diacylglycerol and phosphatidylinositol), which promotes membrane targeting required for conformational activation (7). Membrane compartmentalization of PKC is stabilized by binding of the enzyme to anchoring proteins that interact exclusively with the active kinase (8). As a consequence of such spatial control, PKC is sequestered in the vicinity of its substrates and performs its biological function(s) through substrate phosphorylation. The active species of PKC shows markedly enhanced sensitivity to priming site dephosphorylation (i.e. inactivation) by cellular phosphatases, indicating that phosphorylated residues are more exposed in the membrane-bound active conformation of the enzyme (9–11). Notably, recent studies identified a regulatory role of molecular chaperones, such as heat-shock proteins (e.g. Hsp70), in sustaining the signaling lifetime of the active kinase by allowing it to become rephosphorylated and cycle back into the pool of functional enzyme (12).

PKC signaling is also controlled by desensitization mechanisms. The transduction of signals triggered by external stimuli such as growth/differentiation factors can be terminated through desensitization of membrane receptors and/or down-regulation of downstream signal transducers. Despite their importance, however, desensitization pathways are less understood than the steps involved in signal generation and transduction. It has long been recognized that activation of PKC isozymes by physiological (e.g. hormones and growth factors (13)) or nonphysiological agonists (e.g. phorbol esters, bryostatin 1 (Bryo)) ultimately leads to enzyme inactivation and down-regulation through a post-transcriptional mechanism (14). Increasing evidence indicates that the tumor promoting properties of phorbol esters, and of the PKC enzyme system itself, are critically tied to PKC isoform desensitization. Intestinal tumorigenesis, for example, is associated with a marked reduction in levels of PKC isoform expression and activity that appears to provide a growth advantage to these cells (5, 15). Elucidation of the desensitization pathways for these molecules is, therefore, of key importance.

Although understanding of the mechanism(s) underlying inactivation of PKC signaling is limited, studies with PKCs and e have indicated that a key step in the process is dephosphorylation of the priming sites on the enzyme (16–18). The dephosphorylated species is then thought to undergo ubiquitination and proteolytic degradation by the 26 S proteasome, a large multicatalytic protease complex (17, 19–21). Studies using exogenously expressed protein have further suggested that

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down-regulation of PKCs involves internalization of the active enzyme through a caveolae-dependent mechanism, followed by multisite dephosphorylation and down-regulation in a perinuclear compartment (22). Notably, recent studies have indicated that, in contrast to PKCα and ε, PKCδ requires phosphorylation for ubiquitin/proteasome-dependent degradation in response to phorbol esters and serum in NIH 3T3 cells (23), pointing to the existence of isozyme-specific mechanisms of PKC down-regulation.

In the current study, comparison of the effects of phorbol 12-myristate 13-acetate (PMA) and Bryo on PKCα expression/activity in intestinal epithelial cells unveiled a previously undescribed mechanism of PKCα down-regulation and distinguished at least two pathways of PKCα desensitization. Our findings demonstrate, for the first time, that a major pathway for PKCα down-regulation involves ubiquitination of the fully phosphorylated, mature enzyme at the plasma membrane and subsequent degradation through a proteasome-dependent mechanism. Phosphatase inhibitors accelerated PKCα processing and inhibitors of vesicular trafficking did not prevent degradation of the enzyme; thus, neither dephosphorylation of priming sites nor internalization are required for this pathway of PKCα down-regulation. We also provide evidence for the existence of a second pathway of PKCα desensitization that involves caveolae-dependent trafficking of the active enzyme to a perinuclear compartment, removal of priming phosphates, and degradation of the phosphorylated species through a proteasome-independent mechanism. These studies indicate that PKC agonists can activate more than one pathway of PKCα down-regulation and that the localization and phosphorylation state of the enzyme induced by a particular agonist may be critical determinant(s) of PKC isozyme desensitization mechanisms.

**EXPERIMENTAL PROCEDURES**

**Antibodies—**PKCα-specific antibodies were purchased from Upstate Biotechnology (UBI; mouse monoclonal anti-catalytic domain), Santa Cruz Biotechnology (H-7 and C-29) and BD Transduction Laboratories (mouse monoclonal; BD anti-PKCα antibody). Rabbit anti- phospho-Thr638 PKCα (P638) and anti-phospho-Ser657 PKCα (P657) antibodies were from Cell Signaling Technology and UBI, respectively. Mouse monoclonal anti-ubiquitin (P4D1 IgG), -agarose conjugate, anti-ubiquitin (P4D1) antibody, and anti-P2APα antibody were obtained from Santa Cruz Biotechnology and BD Transduction Laboratories, respectively. Horseradish peroxidase-conjugated goat anti-rabbit IgG and rat anti-mouse IgG antibodies were obtained from Chemicon International and Jackson Immunoresearch Labs, respectively. TRITC-conjugated donkey anti-rabbit IgG and goat anti-mouse IgG were from Jackson Immunoresearch Labs.

**Cell Culture and Drug Treatment Protocols—**The IEC-18 cell line (ATCC CRL-1589), a nontransformed intestinal crypt cell line derived from rat ileal epithelium, was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 4 mM glutamine, 5 μg/ml insulin, and 5% fetal bovine serum (Intergen) at 37 °C, 5% CO2. PKCα isoforms were activated in IEC-18 cells by treatment with 100 nM PMA (Sigma) or 100 nM Bryo (Bioul or LC Laboratories) for various times. Control cells were treated with appropriate vehicle (see below). PKCα kinase activity was inhibited using 5 μM bisindolylmaleimide I (BIM). Proteasome and/or calpain activity was inhibited with 25 μM lactacystin (Lacta; Dr. E. J. Corey, Harvard University or Bioul), 150 μM E-64 (Lea-Nle-ChO [ALLN]; Calbiochem), 50 μM E-64 (Bioul), or a combination of Lacta and E-64. Caveolar trafficking was inhibited using the cholesterol-binding drug nystatin (50 μg/ml; Calbiochem) and protein synthesis was inhibited with 200 μM cycloheximide (CHX; Sigma). With the exception of nystatin, which was added 1 h prior to PMA or Bryo, all inhibitory treatments were initiated 30 min before PKC agonist exposure. The activity of cellular phosphatases was inhibited by preincubation with various concentrations of okadaic acid, sodium salt (OA; Calbiochem or LC Laboratories) or calyculin A (Cal A; Sigma or LC Laboratories) for 1 h or 30 min, respectively.

The following solvents were used to prepare drug stock solutions: ethanol for PMA, Bryo, and CHX; Me3SO for BIM, OA, Cal A, ALLN, and E-64, and double distilled water for Lacta. Nystatin was freshly prepared as a 2.5 mg/ml suspension in double distilled water.

**Preparation of Whole Cell Lysates and Subcellular Fractions—**For preparation of whole cell lysates, cells were harvested in boiling SDS lysis buffer (10 mM Tris, pH 7.4, 1% SDS), boiled for 5 min, and sheared by 5–10 passes through a 27-gauge needle. Extracts were cleared by centrifugation (10 min; 10,000 × g, room temperature). Cytosolic and membrane subcellular fractions were prepared as we have described (24). The cytoskeletal/Triton X-100-insoluble (Triton-insoluble) fraction was isolated from the Triton-insoluble pellet as described (25). Protein concentrations were determined in quadruplicate by bicinchoninic acid protein assay (Pierce) and lysates and subcellular fractions were boiled in Laemmlly sample buffer for 5 min before being subjected to SDS-PAGE.

**Immunoprecipitation (IP)—**To immunoprecipitate PKCα cells, cells were lysed in boiling 1% SDS lysis buffer as described above. Lysates containing 500 μg of protein were diluted in IP buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 100 mM NaF, 1% Triton X-100, 0.5% Nonidet P-40, 10 mM N-ethylmaleimide and protease/prophosphatase inhibitor mixtures). A two-step IP protocol (Protocol A) using a mixture of PKCα-specific antibodies was used to maximize immunoprecipitation of PKCα species (i.e. phosphorylated, nonphosphorylated, and ubiquitinated forms of the enzyme). Step 1 involved overnight incubation of diluted lysates with 2.5 μg of BD anti-PKCα antibody, followed by a 1-h incubation with 30 μl of protein A/G-agarose beads (Santa Cruz Bio-technology). Immunocomplexes were then pelleted, washed 6 times in IP buffer and boiled in Laemmlly sample buffer. In Step 2, the remaining supernatant was further incubated (3 h) with a mixture of anti-PKCα antibodies including H-7, C-20, and P657 (2 μg each). Immunocomplexes were collected with 30 μl of protein A/G-agarose beads (2 h) and processed as described in Step 1. Immunoprecipitates from Steps 1 and 2 were combined and subjected to SDS-PAGE and anti-PKCα and anti-ubiquitin immunoblotting. To specifically immunoprecipitate phosphorylated PKCα (Protocol B), diluted lysates were incubated overnight with 3 μl of P638 and 2 μg of P657 antibodies and immunocomplexes were collected with protein A/G-agarose beads as above.

To immunoprecipitate ubiquitinated proteins, IEC-18 cell lysates containing 600 μg of protein were first precleared with 10 μl of protein A/G-agarose beads (SAG) for 10 min with 2 μg of normal mouse IgG (Santa Cruz Biotechnology), followed by addition of 30 μl of protein A/G Plus-agarose (2 h) to precipitate the pelleted beads were discarded. Precleared lysates were incubated with 20 μl of anti-ubiquitin (P4D1) IgG-agarose conjugate overnight. Immunocomplexes were pelleted, processed as described above, and subjected to immunoblotting using anti-PKCα or P657 antibodies.

**35S/Met/Lys- Metabolic Labeling of PKCα and Chase Analysis—**Cell cultures grown in 10-cm plates were incubated in Met/Cys-free Dulbecco's modified Eagle's medium (Invitrogen) containing 5% dialyzed fetal bovine serum, 4 mM glutamine, and 5 μg/ml insulin for 15 h to label 15S-met- and 15S-lys-labeled cultures were rinsed with warm PBS and 35S was chased for various intervals in label-free complete growth medium containing various drugs as indicated. Following drug treatment, cells were rinsed twice with cold PBS and lysed in boiling 1% SDS lysis buffer. Lysates were preclarsed as described above and PKCα was immunoprecipitated with BD anti-PKCα antibody and subjected to anti-PKCα immunoblotting and autoradiography; 32P was detected using a PhosphorImager and ImageQuant Software (Amersham Biosciences).

**Western Blot Analysis—**Whole cell lysates, subcellular fractions (20 μg of protein), and immunoprecipitates were separated using 10% SDS-polyacrylamide gels. Proteins were transferred to a cellulose membrane, which was generally blocked and incubated with primary and secondary antibodies in Tris-buffered saline containing 5% nonfat dried milk and 0.1% Tween 20. Blots were routinely stained with 0.1% Fast green (Sigma) immediately after transfer to ensure equal loading and even transfer. Immunoblotting using P638 and P657 was performed according to protocols provided by the respective manufacturer. Primary antibody dilutions were as follows: 1:3000 for PKCα and P638; 1:2000 for P657; 1:1000 for ubiquitin. Horseradish peroxidase-conjugated secondary antibodies were used at 1:2000 or 1:3000. Bound horseradish peroxidase was detected using the SuperSignal CL System (Pierce) and Kodak X-OMAT AR film.

**Fluorescence Analysis—**Cells grown on glass coverslips were treated as indicated and fixed in 2% formaldehyde/PBS (24). Following washes in PBS, cells were permeabilized in 0.2% Triton X-100 in PBS (PBS/Triton) for 10 min and incubated in C-20 anti-PKCα antibody (1.50 in PBS/Triton) for 1 h at room temperature. Cells were then...
washed for 30 min in PBS/Triton and incubated in TRITC-conjugated donkey anti-rabbit secondary antibody (1:100) for 30 min. After washing in PBS/Triton for 30 min, coverslips were mounted with Aquamount (Polysciences) and viewed with a Zeiss Axioskop epifluorescence microscope, using a ×63 Plan Apochromat (1.4 NA) objective lens. The staining patterns observed with the C-20 antibody were confirmed with BD anti-PKCα antibody. A similar protocol was used for immunofluorescence analysis of PP2A in IEC-18 cells; data obtained with monoclonal anti-PP2Ac antibody from Transduction Laboratories were confirmed with polyclonal anti-PP2Ac antibody from UBI. TRITC-conjugated goat anti-mouse secondary antibody (1:100) was used for detection of mouse primary antibody. Images were obtained with a Hamamatsu C7780 digital camera and processed for presentation using Adobe Photoshop 7.0.

RESULTS

Both Bryo and PMA Promote Down-regulation of PKCα in IEC-18 Cells; However, Only Bryo Induces Dephosphorylation of the Enzyme

To gain insight into the mechanisms underlying the degradation of activated PKC, the PKC agonists PMA and Bryo (at 100 nM) were compared for their ability to activate and down-regulate PKCα in IEC-18 intestinal epithelial cells. Translocation of the protein to the particulate subcellular fraction was used as a measure of enzyme activation (26). As shown in Fig. 1A, both agents promoted redistribution of PKCα from the cytosol to the membrane and Triton-insoluble fractions, although translocation was slightly slower in Bryo-treated cells (as seen at 15 min). Activation of PKCα by either agonist was followed by progressive disappearance of the enzyme (Fig. 1, A and B), an effect that required enzyme catalytic activity because it was blocked by the PKC inhibitor BIM (data not shown). Importantly, Bryo-induced down-regulation of PKCα was accompanied by significant accumulation of a faster migrating species of the enzyme (Fig. 1, A and B), detectable by 30–45 min of treatment (Fig. 1C). In contrast, a high mobility form of PKCα was not detected until later, if detected at all, in PMA-treated cells (Fig. 1, A and B).

λ-Phosphatase treatment of PKCα immunoprecipitated from control, Bryo-, or PMA-treated cells confirmed that the high mobility species, which accumulated in both the membrane and Triton-insoluble subcellular fractions (Fig. 1A), corresponded to the nonphosphorylated enzyme (data not shown) (6, 27). Consistent with this notion, this species was not recognized by phosphorylation site-specific antibodies (P657 and P638) (Fig. 2D). Immunoblot analysis of subcellular fractions (Fig. 1A) and whole cell lysates (Fig. 1B) revealed that disappearance of mature phosphorylated PKCα during the course of treatment with Bryo was accompanied by progressive accumulation of the nonphosphorylated enzyme, so that by 4 h of chronic exposure to this agent the ratio of the two forms was shifted toward a prevailing amount of the nonphosphorylated protein. In contrast, in PMA-treated samples, the phosphorylated species remained predominant throughout the course of treatment.

To determine whether the nonphosphorylated species of PKCα that accumulated in response to PKC agonists in IEC-18 cells was produced by dephosphorylation of the activated mature enzyme, IEC-18 cells were metabolically labeled with 35S Met/Cys-containing medium. The labeling medium was then replaced with complete medium without 35S Met/Cys and the cells were treated with Bryo or PMA in the presence or absence of BIM for 6 h. PKCα was immunoprecipitated from whole cell lysates and analyzed by autoradiography and anti-PKCα immunoblotting. As shown in Fig. 2A, only mature PKCα was labeled with 35S in control cells (C; open arrowhead). Bryo chased 35S-labeled mature PKCα into a high mobility band (open arrow) that co-migrated with the high mobility nonphosphorylated protein detected by Western blotting (closed arrow). These
data demonstrate that the faster migrating species of PKCa produced by Bryo derives from the activated mature enzyme, and confirms that Bryo induces dephosphorylation of PKCas in IEC-18 cells. The accumulation of the Bryo-induced 35S-labeled high mobility species was inhibited by BIM, indicating that PKCa activity is required for dephosphorylation of mature enzyme in Bryo-treated cells. Notably, PMA treatment did not chase 35S-labeled mature PKCa into a faster migrating species, pointing to a different source for the high mobility band sometimes detected in PMA-treated cells.

Previous studies have indicated that PMA treatment can cause a delay in post-translational processing of PKCa, resulting in accumulation of nonphosphorylated protein (28, 29). To investigate the contribution of de novo protein synthesis to production of the faster migrating form of PKCa by PMA, IEC-18 cells were exposed to this PKCa agonist in the absence or presence of the protein synthesis inhibitor CHX. As shown in Fig. 2B, CHX completely prevented accumulation of nonphosphorylated PKCa in PMA-treated cells. These data, together with the inability of PMA to chase 35S-labeled mature PKCa into a faster migrating species (see Fig. 2A), point to de novo protein synthesis as the only source for the nonphosphorylated PKCa observed in response to PMA treatment. Delayed maturation of newly synthesized PKCa protein also appears to contribute to the Bryo-produced high mobility form, because inhibition of protein synthesis reduced the amount of this species at each time point examined (Fig. 2C). Indeed, accumulation of the Bryo-induced faster migrating form of PKCa could only be completely abrogated by combined exposure to BIM (to inhibit dephosphorylation of active mature enzyme) and CHX (to prevent accumulation of newly synthesized protein) (Fig. 2D). Collectively, these data indicate that the nonphosphorylated species of PKCa that accumulates in Bryo-treated cells consists of dephosphorylated activated mature enzyme (see 35S data in Fig. 2A) and nonphosphorylated newly synthesized protein (Fig. 2, C and D), whereas the PMA-induced high mobility species represents only nonphosphorylated newly synthesized enzyme (Fig. 2, A and B). Thus, PMA does not promote PKCa dephosphorylation in IEC-18 cells, while still inducing down-regulation of the protein.

PKC Agonist-induced Down-regulation of PKCa Is Calpain-independent and Can Involve Proteasome- or Non-proteasome-mediated Pathways

To examine the involvement of calpain and the proteasome in Bryo- and PMA-induced down-regulation of PKCas, IEC-18 cells were treated with these agents for 4 or 16 h in the presence or absence of E-64 (calpain inhibitor), Lacta (a highly specific inhibitor of the proteasome), ALLN (which inhibits the proteasome and calpain), or Lacta + E-64. Western blot analysis revealed that at the 4-h time point, Lacta and ALLN, but not E-64, protected PKCas from Bryo- or PMA-induced down-regulation (Fig. 3A). These data excluded the involvement of calpain and implicated a proteasomal pathway in down-regulation of PKCas by PMA or Bryo in IEC-18 cells. Notably, the form of PKCa protected by proteasome inhibitors was the mature species phosphorylated on Thr638 and Ser657, as indicated by immunoblot analysis using phospho-specific antibodies (P638 and P657) (Fig. 3A). Inhibition of proteasome and/or calpain activity did not result in increased levels of the nonphosphorylated form of the enzyme (Fig. 3A). To exclude the possibility that proteasome inhibitors prevent the dephosphorylation of PKCa induced by Bryo, thus forcing accumulation of the mature species, 35S labeling experiments were performed. IEC-18 cells were metabolically labeled with [35S]Met/Cys as
described above, and then treated with Bryo in the presence or absence of ALLN in $^{35}$S-free medium for 2 h. As shown in Fig. 3B, a radiolabeled faster migrating species (arrows) accumulated in Bryo-treated cells in the presence of ALLN, indicating that inhibition of the proteasome does not block dephosphorylation of the enzyme. Together, these data demonstrate that, despite continued dephosphorylation of PKCa in Bryo-treated cells, proteasome inhibition resulted in selective protection of the mature species, pointing to this form as the substrate for proteasomal processing in IEC-18 cells. These findings further argue that PKC agonist-induced dephosphorylation and proteasomal degradation of PKCa are independent processes.

Analysis of IEC-18 cells treated with PKC agonists for 16 h in the presence or absence of proteasome and/or calpain inhibitors yielded an unexpected result (Fig. 3C). Consistent with findings at 4 h of treatment (Fig. 3A), Lacta and ALLN protected PKCa from down-regulation by PMA at 16 h. In contrast, proteasome inhibitors failed to protect PKCa from Bryo-induced down-regulation at this time point. Taken together, the data indicate that PMA induces down-regulation of PKCa mainly through a proteasome-mediated mechanism, whereas down-regulation of the enzyme by Bryo involves both proteasome-dependent and -independent pathways. The data also suggest that the mature, fully phosphorylated form of PKCa is the target for degradation by the proteasome system.

**Dephosphorylation Is Not Required for Bryo- or PMA-induced Down-regulation or Ubiquitination of PKCa in IEC-18 Cells**

**Phosphatase Inhibitors Do Not Block Bryo- or PMA-induced Down-regulation of PKCa in IEC-18 Cells**—As discussed above, the faster migrating form of PKCa that accumulates in Bryo-treated cells is produced, in part, through dephosphorylation of the activated mature protein. These data are consistent with the notion that, at least in the case of Bryo, down-regulation of activated PKCa involves dephosphorylation of the enzyme. However, the findings that: 1) down-regulation of PKCa by PMA is not accompanied by dephosphorylation of the mature protein (Fig. 2A); and 2) proteasome inhibitors protected fully phosphorylated PKCa, while not inhibiting production of the dephosphorylated protein by Bryo (Fig. 3, A and B), suggested that dephosphorylation may not be required for processing of PKCa by the ubiquitin/proteasome system. To investigate this idea further, the effects of the phosphatase inhibitors Cal A and OA (known to inhibit PKCa dephosphorylation in other cell types (11, 30)) were examined in this system. OA has been shown to selectively inhibit PP2A, but not PP1, in vivo, whereas Cal A inhibits the activity of both phosphatases (31). The ability of Cal A and OA to exert an inhibitory effect on PP2A activity in IEC-18 cells was confirmed by Western blot analysis of the levels of demethylation of Leu$^{509}$ and phosphorylation on Tyr$^{507}$ (data not shown), modifications of PP2A known to reflect inhibition of enzyme activity by these phosphatase inhibitors (31–33). Inhibition of PP1 activity by Cal A, but not OA, in these cells was indicated by down-regulation of the catalytic domain of $\alpha$PP1 (data not shown) (31). Notably, Western blot analysis revealed that neither Cal A nor OA inhibited down-regulation of PKCa by Bryo or PMA in IEC-18 cells (Fig. 4); in fact, these inhibitors consistently accelerated the disappearance of the mature phosphorylated form of the enzyme, while inhibiting the accumulation of nonphosphorylated protein in Bryo-treated cells. These data demonstrate for the first time that PKCa can be down-regulated without undergoing dephosphorylation. In addition, they implicate a Cal A/OA-sensitive phosphatase(s) in Bryo-induced dephosphorylation of PKCa in IEC-18 cells.

**Mature Phosphorylated PKCa Is Ubiquitinated in Response to Activation by PMA or Bryo in IEC-18 Cells**—Previous studies have demonstrated that PKCa can be ubiquitinated in response to activation by PMA or Bryo (19, 20). To determine the ability of these agents to induce ubiquitination of PKCa in IEC-18 cells, reciprocal immunoprecipitation experiments were...
Phosphatase inhibitors do not block Bryo- or PMA-induced down-regulation of PKCa in IEC-18 cells. C, vehicle control. Cells were treated with Bryo (A) or PMA (B) in the presence or absence of the phosphatase inhibitors Cal A (2 nM) or OA (750 nM) for the indicated times and whole cell lysates were analyzed for PKCa expression by immunoblotting. Data in A and B are representative of at least five independent experiments.

Distinct Pathways of PKCa Down-regulation

Performed. First, the activated enzyme was immunoprecipitated from whole cell lysates using a mixture of anti-PKCa antibodies and subjected to anti-PKCa and anti-ubiquitin immunoblotting. Anti-PKCa antibody detected the 80-kDa enzyme and higher molecular weight species (Fig. 5A, i, asterisks and dots). These species were attributed to ubiquitinated PKCa, because they were also detected with anti-ubiquitin antibody (asterisks and dots indicate the same pattern of immunoprecipitated PKCa bands recognized by both anti-PKCa and anti-ubiquitin antibodies). In the reciprocal experiment (Fig. 5A, ii), immunoprecipitation of ubiquitinated proteins from PKC agonist-treated cells using anti-ubiquitin antibody, followed by anti-PKCa immunoblotting, confirmed that PKCa activation results in accumulation of ubiquitinated species of the enzyme in IEC-18 cells (see lanes 1, 4, and 5 in Fig. 5A, ii) (note the similar pattern of PKCa bands in ubiquitin and PKCa immunoprecipitates; asterisks and dots). PMA and Bryo promoted ubiquitination of PKCa with different kinetics; at 5 and 15 min, the level of Bryo-induced PKCa ubiquitination was lower than that of PMA (Fig. 5A, i). The delayed induction of PKCa ubiquitination by Bryo is consistent with the slower translocation of the enzyme in Bryo-treated cells (Fig. 1A).

To test the idea that mature phosphorylated PKCa is the substrate for ubiquitination in IEC-18 cells, phosphorylation site-specific antibodies (P638/P657) were used to immunoprecipitate PKCa molecules phosphorylated on Thr638/Ser657. As shown in Fig. 5B, i, P638/P657 antibodies immunoprecipitated ubiquitinated species of PKCa (lanes 1–3), indicating that the enzyme phosphorylated at these site(s) undergoes ubiquitination in IEC-18 cells. This finding was confirmed in reciprocal immunoprecipitation experiments that demonstrated that phosphorylated PKCa (P657) could be detected in ubiquitin immunoprecipitates (Fig. 5B, ii). Consistent with these findings, the phosphatase inhibitors OA and Cal A generally increased accumulation of ubiquitinated PKCa in PKC agonist-treated cells (Fig. 5, A, ii, lanes 6–9, and B, i, lanes 5 and 6), arguing that protection of the phospho-site(s) on the enzyme facilitates the ubiquitination process. These data are in keeping with evidence that: 1) proteasome inhibitors protect mature phosphorylated PKCa from PMA- and Bryo-induced down-regulation, but do not promote accumulation of the dephosphorylated form of the protein (Fig. 3); and 2) phosphatase inhibitors accelerate down-regulation of PKCa by both agents (Fig. 4).

Taken together, these findings point to a pathway of PKCa down-regulation induced by both PMA or Bryo that involves ubiquitination of the activated mature phosphorylated enzyme and subsequent degradation by the proteasome. Bryo treatment also identifies a second non-proteasome-mediated degra-
Fig. 6. Effects of PMA and Bryo treatment on the subcellular distribution of PKCα in IEC-18 cells. Cells grown on glass coverslips were treated with PMA (b–f), Bryo (h–l), or vehicle (a and g) for the indicated times, fixed in 2% formaldehyde, and processed for PKCα immunofluorescence. Arrows and arrowheads indicate membrane and perinuclear staining, respectively. N, nucleus. Data are representative of at least three independent experiments.

Down-regulation pathway in IEC-18 cells, which likely involves dephosphorylation of the enzyme.

**Down-regulation of Phosphorylated PKCα Does Not Require Vesicle Trafficking in IEC-18 Cells**

Previous studies have indicated that down-regulation of PKCα involves internalization of the activated enzyme through a caveolar mechanism and degradation in a perinuclear compartment (22). To determine the location of PMA-induced processing of PKCα, IEC-18 cells treated with PMA for various times were processed for immunofluorescence analysis of PKCα localization (Fig. 6). PKCα is diffusely distributed throughout the cytoplasm of untreated cells, with a concentration in the perinuclear region (Fig. 6, panels a and g). As shown in Fig. 6, b–f, PMA targets PKCα only to the plasma membrane in IEC-18 cells: considerable recruitment is seen by 1 min (not shown) and membrane staining is well defined by 5 min (Fig. 6, panel b, arrow). Although significantly reduced, membrane staining is still detectable at 2 h (Fig. 6, panel f, arrow). Whereas PKCα staining was readily seen at the plasma membrane, there was no evidence of internalized protein at any time point examined (i.e. up to 4 h, data not shown). As reported previously (22), inhibition of PKCα activity by BIM protected PKCα protein from down-regulation by PMA, with immunostaining detected only at the plasma membrane (Fig. 7A, panel b, arrow). Notably, inhibition of the proteasome by Lacta or ALLN also protected PKCα only at the plasma membrane (Fig. 7B, panels a–c, arrows) and did not block ubiquitination of the enzyme (Fig. 7C), confirming that the enzyme is targeted for proteasomal processing in this compartment. Furthermore, inhibition of caveolar trafficking with the cholesterol binding drugs nystatin (Fig. 8A, panels a–d), filipin, or methyl-β-cyclohexanextrin (not shown) did not alter the distribution of PKCα in PMA-treated IEC-18 cells and did not prevent PMA-induced PKCα down-regulation (compare panels c and d in Fig. 8A). As shown in Fig. 8B, i, this finding was confirmed by immunoblot analysis. Although the mature enzyme undergoes complete down-regulation in the presence of nystatin (not shown), a slight delay in its disappearance was noted (lanes 4 and 6, Fig. 8B, i); this delay might reflect nystatin-induced aberrations in membrane properties that impact processing of the protein. Collectively, these data indicate that PMA-induced ubiquitination of PKCα occurs at the plasma membrane and caveolar internalization is not required for down-regulation of the enzyme.

**Bryo Promotes Internalization of a Pool of Activated PKCα via a Caveolar Mechanism**—To investigate the basis for the observed differences in down-regulation pathways triggered by PMA and Bryo in IEC-18 cells, we examined the effects of Bryo on PKCα subcellular distribution. Consistent with the conclusion that ubiquitination of phosphorylated PKCα occurs at the plasma membrane, Bryo targeted a proportion of the enzyme to this subcellular compartment (Fig. 6, h–l, arrow). Notably, Bryo-treated cells also exhibited discrete perinuclear staining (Fig. 6, h–l, arrowheads) that increased over time (compare 5- and 30-min time points, panels h and j). Inhibition of PKCα activity by BIM resulted in increased membrane staining (Fig. 7A, compare panels c and d, arrow) and undetectable perinuclear accumulation of the enzyme in Bryo-treated cells, indicating that: (a) PKCα first translocates to the plasma membrane before accumulating in a perinuclear region, and (b) kinase activity is required for perinuclear localization of the protein, as reported previously (22). As seen with PMA, BIM protected plasma membrane-associated PKCα from down-regulation by Bryo (Fig. 7A, e and f). Consistent with the notion that ubiquitination/proteasome targeting of PKCα occurs at the cell periphery, inhibition of the proteasome by Lacta or ALLN preserved plasma membrane staining of PKCα in Bryo-treated cells (Fig. 7B, d–f). As shown in panels e and f, plasma membrane-associated PKCα fluorescence was readily seen at 2 h (arrows), a time when staining in this compartment was

Dist. Pathways of PKCα Down-regulation

5794
low-to-undetectable in the absence of proteasome inhibitors (panel d). It should be noted, however, that Lacta or ALLN did not prevent perinuclear accumulation of PKCa in IEC-18 cells (Fig. 7B, e and f, arrowheads). Taken together, the data demonstrate that Bryo promotes plasma membrane accumulation of PKCa, followed by kinase activity-dependent internalization of a proportion of the enzyme. Inhibition of proteasome function protects plasma membrane-associated PKCa from down-regulation at early times of treatment (e.g. 2 h), but does not prevent perinuclear accumulation of the protein.

To determine whether caveolar trafficking is involved in Bryo-induced internalization of PKCa, IEC-18 cells were ex-
FIG. 8. Caveolar trafficking mediates Bryo-induced perinuclear accumulation of PKCα in IEC-18 cells. A, cells grown on glass coverslips were treated with PMA (a–d) or Bryo (e–h) in the presence or absence of 50 μg/ml nystatin for the indicated times, fixed in 2% formaldehyde, and processed for immunofluorescence analysis of PKCα distribution. Nystatin blocks perinuclear accumulation of PKCα in

B. (i) 4 h

|       | C | B | P | B | P |
|-------|---|---|---|---|---|
| Nystatin | - | + | - | - | + | + |

(ii) 1 h

|       | Bryo |
|-------|------|
| Nystatin | - | + |

C. 15 min

|       | C | B | P | B | P |
|-------|---|---|---|---|---|
| Nystatin | - | + | - | + |

Further analysis revealed a significant increase in PKCα levels in the perinuclear region, indicating a regulatory role for caveolar trafficking in PKCα distribution.
posed to Bryo in the presence or absence of cholesterol-binding drugs (nystatin, filipin, or methyl-β-cyclodextran). Immunofluorescence analysis revealed that these agents (data shown for nystatin only) increased plasma membrane PKCa staining (Fig. 8A, compare e and f, arrow) and blocked perinuclear accumulation of the protein, producing a pattern of PKCa immunostaining remarkably similar to that seen with PMA (compare panels a and e). As seen with PMA, nystatin did not prevent Bryo-induced PKCa down-regulation (Fig. 8A, panels c and g). Notably, in the presence of nystatin, the pattern and kinetics of PKCa down-regulation by Bryo became similar to those observed in PMA-treated cells (Fig. 8, A, panels c and g, and B, i, lanes 5 and 6) (note that a prominent band of mature PKCa remained evident for a longer period in these cells). Immunoblot analysis further demonstrated that nystatin consistently decreased accumulation of nonphosphorylated PKCa in Bryo-treated cells (Fig. 8B, i, lanes 3 and 5, and ii) and increased Bryo-induced ubiquitination of PKCa to the level seen in response to PMA (Fig. 8C). Thus, caveolar trafficking appears to mediate Bryo-induced perinuclear accumulation as well as dephosphorylation of PKCa in IEC-18 cells. Inhibition of caveolar trafficking does not block Bryo-induced down-regulation of PKCa, but rather alters the pattern and kinetics of PKCa processing so that it more closely resembles that induced by PMA in this system.

**Kinase Activity Is Required for Bryo-induced Internalization of PKCa and for Its Dephosphorylation in the Perinuclear Region**—Taken together, the findings that: 1) nonphosphorylated PKCa appears in Bryo-treated cells by 30–45 min (Fig. 1C), coincident with a significant increase in perinuclear staining (Fig. 6, panel j); 2) BIM and nystatin block perinuclear accumulation of PKCa (Figs. 7A and S7A) and inhibit inhibition of dephosphorylated protein (Figs. 2A and S8B); and 3) proteasome inhibitors do not prevent Bryo-induced dephosphorylation of PKCa (Fig. 3B) and do not prevent perinuclear localization of the enzyme (Fig. 7B), suggest that, consistent with previous reports (22), dephosphorylated protein is located in the perinuclear region of the cell and PKCa likely undergoes dephosphorylation in this compartment. To determine whether kinase activity is required both for PKCa trafficking to the perinuclear region and for its dephosphorylation in that compartment, cells were preincubated with Bryo for 30 min to allow internalization of the enzyme (Fig. 9A, panel a), and then treatment was continued in the presence of BIM for an additional 1.5 h (Fig. 9A, panel b). As shown in Fig. 9B (lane 3), BIM prevented dephosphorylation of PKCa localized in the perinuclear region. Data shown in Fig. 9, A, panel c, and B, lane 4, which are included for comparison, confirm that inhibition of PKCa activity by BIM prior to addition of Bryo blocks internalization and dephosphorylation of the enzyme. Together, these findings support the notion that kinase activity is required both for trafficking of PKCa to the perinuclear compartment and for its dephosphorylation. Notably, immunofluorescence analysis of the subcellular distribution of PP2A placed the phosphatase in a perinuclear compartment and demonstrated a significant increase in PP2A perinuclear localization following 45 min of PKC agonist treatment (Fig. 9C).

**Distinct Pathways of PKCa Down-regulation**

Previous studies have led to a model of PKCa desensitization involving: 1) caveolar-mediated trafficking of the enzyme to a perinuclear compartment (22); 2) dephosphorylation of the priming sites in the enzyme (i.e. Thr497, Thr638, and Ser657) (16–18); and 3) ubiquitin conjugation of the dephosphorylated protein and degradation through a proteasomal pathway (19). Both internalization and down-regulation were shown to be dependent on PKCa activity. In this report, we demonstrate, for the first time, that at least two pathways of PKCa down-regulation can co-exist within cells and that a single PKC agonist can activate both pathways at the same time (Fig. 10). Our data further demonstrate that neither caveolae-dependent trafficking nor dephosphorylation are required for PKCa degradation, and that the enzyme can be processed through proteasome-dependent and -independent pathways.

**Neither Internalization nor Priming Site Dephosphorylation Is Required for PKC Agonist-induced Ubiquitin/Proteasome-mediated Down-regulation of PKCa in IEC-18 Cells**—Analysis of the mechanisms underlying PMA-induced down-regulation of endogenous PKCa in intestinal epithelial cells revealed that a major pathway of PKCa desensitization involves: (a) targeting of the enzyme to the plasma membrane; (b) ubiquitination of fully phosphorylated PKCa at the cell periphery; and (c) degradation of the protein through a proteasome-dependent mechanism (Fig. 10A). This process, which requires PKC activity, appears to be the only pathway of PKCa down-regulation triggered by PMA in IEC-18 cells. That internalization is not required for PKCa ubiquitination or down-regulation through this pathway is indicated by: 1) immunofluorescence data demonstrating that PMA targets PKCa exclusively to the plasma membrane in IEC-18 cells (Fig. 6); 2) evidence that inhibitors of caveolar trafficking (e.g. nystatin) do not prevent PKCa ubiquitination or down-regulation by this agonist (Fig. 8); and 3) morphological and biochemical findings showing that proteasome inhibitors preserve PKCa immunostaining at the plasma membrane, while not preventing ubiquitination of the enzyme (Fig. 7). Several lines of evidence further indicate that priming site dephosphorylation does not play a requisite role in PMA-induced degradation of PKCa through the proteasome: 1) PMA does not promote dephosphorylation of the protein in IEC-18 cells (Fig. 2); 2) the phosphatase inhibitors Cal A and OA are unable to inhibit down-regulation of PKCa by PMA in these cells and, in fact, consistently accelerate the disappearance of the mature phosphorylated protein (Fig. 4); 5) phospho-tyrosine site-specific antibodies (P638 and P657) immunoprecipitate ubiquitinated PKCa from PMA-treated cells, and phosphorylated PKCa is detected in ubiquitin immunoprecipitates (Fig. 5); 4) phosphatase inhibitors facilitate the ubiquiti- nation process (Fig. 5); and 5) proteasome inhibitors protect the mature phosphorylated (on Thr497 and on Ser657) form of the enzyme from PKC agonist-induced down-regulation (Fig. 3). Taken together, these findings strongly support the notion that neither internalization nor dephosphorylation are required for PMA-induced down-regulation of PKCa in IEC-18 cells. Nota- bly, phosphorylation was recently shown to be required for down-regulation of PKCβ (23), indicating that processing of the fully phosphorylated enzyme may be a common mechanism for...
Desensitization of several members of the PKC family. As seen here with PKCα, the phosphatase inhibitor Cal A also accelerated PMA-induce down-regulation of PKCδ in NIH 3T3 cells (23).

Although Bryo was shown to promote caveolae-dependent internalization and priming site dephosphorylation of PKCα in IEC-18 cells, our studies demonstrate that this agent also targets a proportion of PKCα protein for degradation through the ubiquitin/proteasome mechanism that is triggered by PMA in these cells (Fig. 10B). Immunoprecipitation of PKCα with phosphosite-specific antibodies confirmed that, like PMA, Bryo induces ubiquitination of mature, phosphorylated PKCα in this system (Fig. 5). Furthermore, phosphatase inhibitors increased the levels of Bryo-induced PKCα ubiquitination (Fig. 5) and accelerated down-regulation of the enzyme while inhibiting the accumulation of dephosphorylated protein (Fig. 4). Importantly, Bryo-induced down-regulation of PKCα was not prevented by caveolae-disrupting drugs, despite the inhibitory effects of these agents on PKCα internalization and dephosphorylation (Fig. 8). Finally, at least at early times of treatment (e.g., 4 h), Bryo-induced PKCα down-regulation could be significantly inhibited by proteasome inhibitors (Fig. 3). Consistent with these findings, Bryo targets PKCα to the plasma membrane in IEC-18 cells, and proteasome inhibitors protect plasma membrane-associated protein from down-regulation (Figs. 6 and 7). Thus, both Bryo and PMA can promote PKCα down-regulation through a mechanism that does not require enzyme dephosphorylation or internalization. This notion is further supported by previous studies in renal epithelial cells (19) and fibroblasts (17) demonstrating that Laacta preserved active, mature PKCα from Bryo-induced down-regulation, whereas it did not seem to protect the nonphosphorylated enzyme. Furthermore, treatment with Bryo in the presence or absence of Laacta was shown to produce >80 kDa, apparently ubiquitinated, species of PKCα that were labeled with [32P] (19). Notably, the phosphorylation site mutant T638A, which is hypersensitive to 12-O-tetradecanoylphorbol-13-acetate-induced dephosphorylation, was shown to be down-regulated slightly more slowly than the wild-type protein (11).

An increasing number of proteins, including cell cycle regulatory molecules, transcription factors, and cell surface receptors, have been shown to be regulated through phosphorylation and selective degradation through the ubiquitin/proteasome pathway. Phosphorylation at specific site(s) can provide the signal for recognition by the ubiquitin/proteasome system (34). Whether phosphorylation of a specific site(s) on the PKCα molecule is involved in targeting the enzyme for degradation via this pathway (e.g., by inducing conformational changes that unmask ubiquitin recognition signals) will be determined in future studies.

Identification of a Non-proteasomal Pathway of PKCα Down-regulation Involving Priming Site Dephosphorylation of the Enzyme—Analysis of the effects of Bryo on PKCα subcellular distribution, phosphorylation state, and expression levels in IEC-18 cells further demonstrated that this agent can promote degradation of the enzyme through a second pathway, involv-
FIG. 10. Pathways of PKC agonist-induced down-regulation of PKCa in intestinal epithelial cells. A. Upon activation by PMA, mature phosphorylated PKCa is localized only at the plasma membrane, where it undergoes ubiquitination followed by degradation by the proteasome (priming phosphorylation sites (P) on PKCa are indicated in green, purple, and blue; potential activation-induced phosphorylation is indicated in orange). B. Bryo triggers two pathways of PKCa down-regulation in intestinal epithelial cells. Upon activation by Bryo, PKCa is first recruited to the plasma membrane, where it is localized in caveolae and possibly in other membrane domains. 1) A portion of activated enzyme undergoes ubiquitination at the plasma membrane and is targeted for degradation by the proteasome. 2) A pool of caveolae-associated activated PKCa is transported to the perinuclear region, where it undergoes complete dephosphorylation and eventual degradation via a proteasome-independent mechanism.
ning enzyme internalization and dephosphorylation (Fig. 10B). The existence of this pathway is supported by evidence that, in contrast to PMA, Bryo promotes rapid caveolae-dependent perinuclear accumulation of a proportion of PKCa in IEC-18 cells and induces priming site dephosphorylation of the enzyme in this compartment (Figs. 2, 8, and 9). Treatment with BIM blocked Bryo-induced internalization of PKCa while increasing the level of plasma membrane-associated protein (Fig. 7), indicating that, as previously reported (22): 1) the isozyme first translocates to the plasma membrane, before being transported to the perinuclear region; 2) kinase activity is required for perinuclear localization of the protein, but not for its association with the plasma membrane; and 3) PKCα is internalized as an active enzyme. The following findings indicate that, as reported in other systems (22), PKCα dephosphorylation occurs in the perinuclear compartment: 1) appearance of nonphosphorylated PKCα at 30–45 min of Bryo treatment coincides with a significant increase in perinuclear PKCα immunostaining (Figs. 1 and 6); 2) nystatin blocks internalization of PKCα and inhibits production of dephosphorylated protein (Fig. 8); 3) proteasome inhibitors do not prevent perinuclear localization of PKCα and do not prevent Bryo-induced dephosphorylation of the enzyme (Figs. 3 and 7); and 4) inhibition of PKC activity by BIM after the enzyme has localized to the perinuclear compartment (e.g. 30 min of Bryo treatment) prevents dephosphorylation of the protein (Fig. 9). The latter studies also demonstrated that kinase activity is not only required for trafficking of PKCα to the perinuclear region, but also for its dephosphorylation in that compartment. In agreement with reports from other laboratories (11, 35), our findings implicate PP1 and/or PP2A (which accumulates in the perinuclear region of IEC-18 cells in response to PKC agonist treatment), as phosphatase(s) mediating Bryo-induced dephosphorylation of PKCα in this system (Figs. 4 and 9).

Processing of dephosphorylated PKCα in the perinuclear compartment of IEC-18 cells appears to be independent of the proteasome because (a) inhibition of proteasome activity does not result in increased accumulation of dephosphorylated protein, despite evidence that proteasome inhibitors do not prevent internalization or priming site dephosphorylation of the enzyme (Figs. 3 and 7), and (b) in contrast to findings with PMA, proteasome inhibitors do not offer protection from Bryo-induced PKCα down-regulation following long-term PKC agonist treatment (i.e. 16 h, Fig. 3). Thus, in IEC-18 cells Bryo appears to trigger both proteasome-dependent processing of fully phosphorylated enzyme at the cell periphery, in combination with non-proteasomal degradation of dephosphorylated protein in a perinuclear compartment (Fig. 10B). If the proteasomal pathway is blocked by proteasome inhibitors, the enzyme can still be processed, albeit more slowly, through the caveolar-mediated pathway (Fig. 3). Conversely, inhibition of caveolar trafficking significantly alters the pattern and kinetics of Bryo-induced PKCα down-regulation, so that they become similar to those induced by PMA (i.e. the mature, phosphorylated form of the enzyme is retained for a longer period in the cells), indicating that the ubiquitin/proteasome-dependent mechanism becomes the major pathway of PKCα desensitization under these conditions (Fig. 8).

The ability of Bryo to trigger two distinct pathways of PKCα down-regulation, i.e. dephosphorylation (which inactivates the enzyme) and proteasome-mediated proteolysis, may explain why this agent is a more potent desensitizer of PKCα signaling than PMA in IEC-18 cells and other cell types (16, 36, 37). This property may also provide a basis for the well known ability of Bryo to induce only a subset of responses provoked by phorbol ester in some systems, and to antagonize that which does not itself produce (e.g. Ref. 38).

**PKC Agonist-induced Accumulation of Non-phosphorylated PKCa Can Involve Multiple Mechanisms**—The studies presented here highlight the notion that PKC agonist-induced accumulation of nonphosphorylated PKCa does not necessarily reflect dephosphorylation of the activated enzyme. Inhibition of Bryo-induced PKCα dephosphorylation by BIM and of cellular protein synthesis by CHX revealed that delayed maturation of PKCα contributes to the production of this species by Bryo, and that newly synthesized PKCα is the sole source of high mobility PKCα in PMA-treated cells. Delayed maturation of PKCα by phorbol ester treatment has been noted in human breast cancer and glioblastoma cells (28, 29) and may reflect increased PKCα phosphatase activity in PKC agonist-treated cells (39).

**Conclusion**—Data presented in this report: 1) provide evidence for a major pathway of PKCα down-regulation that involves ubiquitination of activated mature phosphorylated enzyme, and its subsequent degradation through the proteasome; and 2) demonstrate for the first time that, in contrast to previous reports, neither internalization nor dephosphorylation are requisite intermediate steps in the ubiquitin/proteasome pathway of PKCα down-regulation. Rather, caveolar trafficking and dephosphorylation are implicated in a second, proteasome-independent pathway of PKCα desensitization. Combined activation of at least two pathways of PKCα desensitization by Bryo in IEC-18 cells provides a basis for the higher potency of this agent compared with PMA in inactivating PKCα signaling in this system. Our findings highlight the importance of both subcellular compartmentalization and phosphorylation state in determining pathways of desensitization of PKCα signaling.

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Distinct Pathways of PKCα Down-regulation

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