Protein Kinase Cα (PKCα) Is Resistant to Long Term Desensitization/Down-regulation by Prolonged Diacylglycerol Stimulation*

Received for publication, October 1, 2015, and in revised form, January 5, 2016. Published, JBC Papers in Press, January 14, 2016, DOI 10.1074/jbc.M115.696211

Michelle A. Lum†, Carter J. Barger‡, Alice H. Hsu§, Olga V. Leontieva†, Adrian R. Black‡§ and Jennifer D. Black†‡§

From the †Eppley Institute for Research in Cancer and Allied Diseases and the Fred and Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, Nebraska 68198-5930 and the ‡Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York 14263

Sustained activation of PKCα is required for long term physiological responses, such as growth arrest and differentiation. However, studies with pharmacological agonists (e.g. phorbol 12-myristate 13-acetate (PMA)) indicate that prolonged stimulation leads to PKCα desensitization via dephosphorylation and/or degradation. The current study analyzed effects of chronic stimulation with the physiological agonist diacylglycerol. Repeated addition of 1,2-dioctanoyl-sn-glycerol (DiC₈) resulted in sustained plasma membrane association of PKCα in a pattern comparable with that induced by PMA. However, although PMA potently down-regulated PKCα, prolonged activation by DiC₈ failed to engage known desensitization mechanisms, with the enzyme remaining membrane-associated and able to support sustained downstream signaling. DiC₈-activated PKCα did not undergo dephosphorylation, ubiquitination, or internalization, early events in PKCα desensitization. Although DiC₈ efficiently down-regulated novel PKCs PKCδ and PKCe, differences in Ca²⁺ sensitivity and diacylglycerol affinity were excluded as mediators of the selective resistance of PKCα. Roles for Hsp/Hsc70 and Hsp90 were also excluded. PMA, but not DiC₈-targeted PKCα to detergent-resistant membranes, and disruption of these domains with cholesterol-binding agents demonstrated a role for differential membrane compartmentalization in selective agonist-induced degradation. Chronic DiC₈ treatment failed to desensitize PKCα in several cell types and did not affect PKCBβI; thus, conventional PKCs appear generally insensitive to desensitization by sustained diacylglycerol stimulation. Consistent with this conclusion, prolonged (several-day) membrane association/activation of PKCα is seen in self-renewing epithelium of the intestine, cervix, and skin. PKCα deficiency affects gene expression, differentiation, and tumorigenesis in these tissues, highlighting the importance of mechanisms that protect PKCα from desensitization in vivo.

Protein kinase C (PKC) α is a signaling protein that regulates important events in self-renewing tissues, including progression through the cell cycle, differentiation, and cell survival and apoptosis (1–5). The PKC family comprises three subgroups, termed conventional (cPKC), novel (nPKCs), and atypical PKCs, that have specific cofactor sensitivities as a function of differences in structure (6–8). PKCα is a member of the cPKC subgroup, which also includes PKCβII and PKCγ (6, 9). Catalytic competence of PKCα requires phosphorylation on three priming sites in the C-terminal domain (activation loop (Thr⁴⁹⁷), turn motif (Thr⁶³⁸), and hydrophobic motif (Ser⁶⁵⁷)) (10). The primary physiological activator of PKCα and other cPKCs is diacylglycerol (DAG), which binds to the conserved C1 domain in the regulatory region of catalytically competent enzyme (9). DAG is generated through cleavage of phosphatidylinositol 4,5-bisphosphate by phospholipase C (PLC) and by the actions of phospholipase D (PLD) and phosphatidic acid phosphohydrolases on phosphatidylincholine (11). Activation of PLC and PLD by cell surface receptors (e.g. G-protein-coupled receptors and receptor tyrosine kinases) leads to accumulation of DAG and recruitment of cPKCs from the cytoplasm to the plasma membrane. Membrane association of PKCs induces conformational changes that result in kinase activation: thus, membrane association has been widely recognized as an indicator of PKC activity (12). Membrane association and activation of cPKCs also require Ca²⁺-dependent binding of phosphatidylinerine to the C2 domain (13). The C2 domain of cPKCs has a low intrinsic affinity for Ca²⁺; thus, in the presence of low levels of DAG, activation of cPKCs is dependent on elevated intracellular Ca²⁺ levels. However, binding of DAG, anionic phospholipids (phosphatidylinerine and phosphatidylinositol 4,5-bisphosphate), and Ca²⁺ is cooperative; therefore, with higher membrane DAG levels, low concentrations of intracellular Ca²⁺ can support cPKC activation (14–16). A number of pharmacological PKC agonists, such as phorbol esters and bryostatins, bind the C1 domain of cPKCs with high affinity and can induce membrane translocation and activation of these

* This work was supported in part by National Institutes of Health Grants DK060632, DK054909, CA191894, CA016056, and CA036727 and T32 Award CA009476 (to C. J. B.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

† Recipient of the University of Nebraska Medical Center Dean for Graduate Studies Stipend Fellowship.

‡ To whom correspondence should be addressed: Eppley Inst. for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE 68198-5930. Tel.: 402-559-6097; Fax: 402-559-4651; E-mail: jennifer.black@unmc.edu.

§§ The abbreviations used are: cPKC, conventional PKC; nPKC, novel PKC; 17-AAG, 17-(allylamino)-17-demethoxygeldanamycin; bryostatin, bryostatin-1; CHX, cycloheximide; DAG, diacylglycerol; DiC₈, 1,2-dioctanoyl-sn-glycerol; DOPA, 1,2-dioctanoyl-sn-glycerol-3-phosphate; Hsp, heat shock protein; Id1, inhibitor of DNA binding 1; PES, 2-phenylethynesulfonamide; PLC, phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; IEC, intestinal epithelial cell; TRITC, tetramethylrhodamine isothiocyanate; DRM, detergent-resistant membrane.
PKCα Is Resistant to Diacylglycerol-induced Desensitization

isoforms independently of DAG (17). The nPKCs, PKCδ, PKCε, PKCe, PKCη, and PKCθ, are also activated by DAG and pharmacological agonists; however, the C2 domain of these enzymes lacks a Ca\textsuperscript{2+} binding motif, rendering their activation insensitive to Ca\textsuperscript{2+} (6–8). The atypical PKCs, PKCζ and PKCu/λ, lack a C2 domain and do not bind DAG or pharmacological agonists but are instead activated by protein-protein interactions (6–8).

Acute and long term mechanisms of inactivation of PKC signaling have also been characterized. DAG levels are tightly regulated in the cell; thus, a major mechanism of PKC inactivation following physiological signaling is through rapid metabolism of DAG by DAG kinases and/or DAG lipases (18). The resultant loss of lipid activator (together with the return of intracellular Ca\textsuperscript{2+} concentrations to basal levels) results in reverse translocation of cPKCs and nPKCs to the cytoplasm where they adopt an inactive conformation that is competent for reactivation upon regeneration of cofactors (19). Notably, reverse translocation of PKCs is not simply a passive diffusion from the membrane but is dependent on PKC activity and is prevented by PKC inhibitors (20). Acute termination of PKC signaling may also involve multisite dephosphorylation by cellular phosphatases (e.g. PP2A and PH domain leucine-rich repeat protein phosphatase) or oxidative mechanisms (19). Another mechanism of inactivation, engaged in response to long term stimulation by physiological activators or pharmacological agonists, is agonist-induced degradation of PKCs. For PKCα, these long term desensitization mechanisms, which appear to be triggered in a cell type- and agonist-specific manner, include (a) dephosphorylation of activation loop (Thr\textsuperscript{497}), turn motif (Thr\textsuperscript{638}), and hydrophobic motif (Ser\textsuperscript{667}) priming sites followed by proteasomal processing of the dephosphorylated species, (b) ubiquitin/proteasome-dependent degradation of the mature, fully phosphorylated enzyme at the plasma membrane, and (c) vesicle trafficking-dependent degradation in lysosomes (19, 21–23). Agonist-induced degradation of PKCs leads to loss of associated signaling and reversal of their downstream effects in the continued presence of agonists.

Consistent with these reversal mechanisms, agonist treatment often leads to transient activation of PKCs; however, it has long been recognized that many biological responses, such as activation of T cells and mitogenic responses in endothelial and vascular smooth muscle cells, require prolonged activation of PKCs (24–27). Our studies in intestinal epithelial cells (IECs) have determined that PKCα has multiple cell cycle-related effects that lead to growth arrest both in vitro and in vivo and that maintenance of these effects requires sustained activation of the enzyme (1, 2, 28–31). To further examine the basis for the sustained activation of PKCα in vivo, the current study determined the effects of prolonged activation of PKCα with DAG in IEC-18 intestinal crypt cells. Our studies demonstrate that PKCα is resistant to long term desensitization by prolonged diacylglycerol-induced activation and point to the existence of mechanisms that protect cPKCs from dephosphorylation/degradation under conditions of sustained input signaling.

Experimental Procedures

Cell Culture and Drug Treatments—IEC-18 rat intestinal crypt-like cells (ATCC CRL-1589) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals), 4 mM l-glutamine, and 5 μg/ml insulin. HEC-1-A human endometrial cancer cells (ATCC HTB-112) were grown in McCoy’s 5A medium supplemented with 10% FBS and 1% penicillin/streptomycin. SNG-M human endometrial cancer cells (Japanese Collection of Research Bioresources JCRB0179) were grown in F-12 Ham’s medium supplemented with 1.0 mM l-glutamine, 10% FBS, and 1% penicillin/streptomycin. All incubations were performed in a humidified incubator at 37 °C in the presence of 5% CO\textsubscript{2}. PKC isoforms were activated in cells by treatment with 100 nM phorbol 12-myristate 13-acetate (PMA; Biomol), 100 nM bryostatin-1 (bryostatin; Sigma-Aldrich), 20 μg/ml 1,2-diacytanoil-sn-glycerol (DiC\textsubscript{12}; Enzo Life Sciences and Cayman Chemical), or 25 μg/ml 1,2-dioleoyl-sn-glycerol (Sigma). Where indicated, cycloheximide, 2-phenylethynylsulfonamide (PES; Sigma-Aldrich), 17-((allylamo)-17-demethoxyglandanamycin (17-AAG; A. G. Scientific), bisindolylmaleimide I (Calbiochem), nystatin, filipin, methyl-β-cyclodextrin (Sigma-Aldrich), 1,2-dioctanoyl-sn-glycero-3-phosphate (DOPA; Avanti Polar Lipids), A23187 (Sigma), and/or MG132 (Calbiochem) was added 30 min prior to agonist treatment. Drugs were dissolved in dimethyl sulfoxide except for bryostatin and PMA, which were dissolved in ethanol or acetonitrile; nystatin, which was prepared as a 50 mM suspension in ethanol and diluted 1:2 in dimethyl sulfoxide before treatments; DiC\textsubscript{12} from Cayman Chemical, which was dissolved in acetonitrile; and DOPA, which was dissolved in methanol. Equal volumes of solvent were used as vehicle controls (concentrations of solvents were below 0.2% (v/v) and did not affect the intracellular location, levels, or agonist-induced down-regulation of PKC isoforms).

Plasmids and Transfections—The HA-tagged wild type PKCα expression vector (in pGL2) was a gift from Drs. Irwin Gelman and Li-Wu Guo (Roswell Park Cancer Institute, Buffalo, NY). The Y123W mutation was introduced using the QuikChange site-directed mutagenesis kit (Agilent Technologies) using primers ATCAGTGTGGTCAGCTCGGG-GACCTATCATCAAGG and CCTTGATGGATAAGTC-CCAGAGCAGTGACCCACAGTGAT, and mutagenesis was confirmed by sequencing. Transfections were performed using a 3:1 ratio of X-tremeGENE 9 (Roche Applied Science) to plasmid DNA according to the manufacturer’s instructions, and agonist treatments were performed 48 h after transfection.

Antibodies—Primary antibodies for immunofluorescence, Western blotting, and immunohistochemistry were as follows: rabbit anti-C-terminal PKCα (Abcam, ab32376), mouse anti-PKCα (Upstate Biotechnology, Inc.; mouse monoclonal anti-catalytic domain), rabbit anti-inhibitor of DNA binding 1 (Id1) (Biocheck, 195-14), rabbit anti-4E-BP1 (Millipore, 07-1416), rabbit anti-β-actin (Sigma-Aldrich, A2066), rabbit anti-ERK 1/2 (Cell Signaling Technology, 9102), mouse anti-HA (Covance, 16B12), rabbit anti-PKCβ1 (Santa Cruz Biotechnology, sc-209), rabbit anti-PKCδ (Santa Cruz Biotechnology, sc-213), and rabbit anti-PKCε (Santa Cruz Biotechnology, sc-214). Sec-
ordinary antibodies were as follows: TRITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories), Alexa Fluor 488-conjugated donkey anti-rabbit (Invitrogen), horse-
radish peroxidase-conjugated goat anti-mouse IgM (Santa Cruz Biotechnology), horseradish peroxidase-conjugated goat anti-rabbit IgG (Millipore), and biotinylated anti-rabbit (Vector Laboratories). Immunoprecipitation of ubiquiti-
nated proteins used P4D1 anti-ubiquitin antibody (Santa Cruz Biotechnology).

**Immunofluorescence Analysis**—Cells grown on glass coverslips were treated as indicated before fixation in 2% formalde-
hyde, PBS for 15 min at room temperature and processing for immuno-
fluorescence microscopy as we have described previously using 0.2% saponin or 0.05% Triton X-100 for permeabi-
lation (23). Primary antibody dilutions were as follows: rabbit anti-C-terminal PKCα at 1:500 and TRITC-conjugated anti-
rabbit secondary antibody at 1:100 or Alexa Fluor 488-conju-
gated donkey anti-rabbit secondary antibody at 1:800. In experi-
ments involving Triton X-100 extraction, nuclei were stained with Hoechst 33258. Images were obtained using a Zeiss Axios-
kop epifluorescence microscope with a Hamamatsu C7780 dig-
ital camera or a Leica DM5500B epifluorescence microscope with a Leica DFC450 digital camera.

**Western Blotting Analysis**—Cells were lysed in 1% SDS, 10 mM Tris, and protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Western blotting analysis of samples loaded based on equal protein content was performed using standard procedures as we have described previously (23, 32). Nitrocellulose membranes were routinely stained with 0.1% Fast Green (Sigma-Aldrich) to con-
firm equal loading and even transfer. Antibody dilutions were as follows: anti-PKCα, 1:20,000 (Abcam) and 1:1000 (Upstate Biotechnology, Inc.), anti-PKCβI, 1:1000; anti-PKCβII, 1:3000; anti-PKCε, 1:3000; anti-ERK 1/2, 1:2000; anti-Id1, 1:2000; anti-
HA, 1:1000; anti-4E-BP1, 1:2000; anti-actin, 1:10,000; horse-
radish peroxidase-conjugated goat anti-mouse IgM, 1:2000; horseradish peroxidase-conjugated goat anti-rabbit IgG, 1:2000; and biotinylated anti-rabbit antibody, 1:250.

**Immunoprecipitation**—Cell lysis in 1% SDS, 10 mM Tris; neu-
tralization of SDS with 2× immunoprecipitation buffer (300 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA, 200 mM NaF, 1% Triton X-100, 1% Nonidet P-40); preclearing of the extracts; and immunoprecipitation with P4D1 anti-ubiquitin antibody were performed as we have described (23).

**Imaging of Ca2+-dependent Fura-2 AM Fluorescence**—IEC-18 cells grown on glass coverslips were treated with A23187 for 30 min prior to addition of 5 μM Fura-2 AM (eBioscience). Cells were incubated with A23187 + Fura-2 AM for an additional 30 min, then rinsed in PBS, and mounted on glass slides. The Fura-2 AM fluorescence was visualized by epifluores-
cence microscopy in live cells within 10 min of mounting the coverslips onto slides.

**Analysis of Detergent-resistant Membranes**—Separation of detergent-resistant membranes (DRMs) on sucrose gradients was essentially as described (33). Briefly, cells grown in two 15-cm plates were treated with vehicle (acetonitrile), 20 μM DiC8, or 100 nM PMA for 15 min; rinsed two times with ice-cold PBS; and extracted for 30 min on ice in 1 ml of 1% Triton extraction buffer (1% Triton-X-100, 50 mM Tris-HCl, 150 mM NaCl, 100 μM CaCl2, Phosphatase Inhibitor Mixtures 1 and 2 (Sigma), Protease Inhibitor Mixture without metal chelating reagents (Sigma)) containing vehicle, DiC8, or PMA as appro-
riate. Extracts were diluted with an equal volume of 80% (w/v) sucrose in 50 mM Tris-HCl, 150 mM NaCl, 100 μM CaCl2, and overlaid with 2 ml of 30% sucrose and 1 ml of 5% sucrose in the same buffer. Following centrifugation at 250,000 × g for 18 h at 4 °C in a Sorval AH-650 rotor, equal fractions were collected from the top of the gradient using an Auto Densi-Flow II (Haake Buchler Instruments Inc.). 20 μl of each fraction was subjected to Western blotting for PKCα, and the relative levels of PKCα were quantified using NIH ImageJ software as we have described (22).

Analysis of PKCα association with DRMs by immunofluores-
cence was adapted (34, 35). Cells grown on coverslips were treated with vehicle, DiC8, or PMA as above prior to extraction for 30 min at 4 °C in 150 μl of 1% Triton extraction buffer containing the corresponding vehicle or PKC agonist. Cells were then immediately fixed with 2% formaldehyde and pro-
cessed for PKCα immunofluorescence (see above). Images of cells from the different treatments were taken at the same mag-
nification and exposure and processed identically using Adobe Photoshop software. Quantification of immunofluorescence was performed on unprocessed images using NIH ImageJ soft-
ware and normalized to the number of cells in each field.

**Immunohistochemistry**—Antigen retrieval with Dako target retrieval at 90 °C, inactivation of endogenous peroxidase activ-
ity, and immunohistochemical detection using Vectastain Elite ABC reagent (Vector Laboratories) and Dako 3,3′-diaminoben-
zidine chromogen solution (K3466) were as we have described previously (30). Counterstaining was performed with hematoy-
lin. Antibody dilutions were 1:2000–1:5000 for anti-PKCα (Abcam) and 1:250 for the biotinylated anti-rabbit antibody.

**Results**

**Activation and Reverse Translocation of PKCα by DiC8 in IEC-18 Cells**—Analysis of the effects of DAG on PKCα in IEC-18 cells utilized the synthetic DAG DiC8. This short chain DAG was chosen because its use as a mimic of endogenous DAG signaling is long established and is supported by evidence that it (a) rapidly incorporates into the plasma membrane and activates PKCs, (b) is a substrate for DAG-metabolizing enzymes, (c) has essentially the same binding affinities for C1 domains of PKCs as long chain DAGs, and (d) elicits the same physiological responses as phorbol esters (e.g. Refs. 36–40). Importantly, DiC8 (a) activates PKCα in IEC-18 cells, (b) has the same physiological effects as PMA in these cells, and (c) induces downstream responses in these cells that are consistent with the effects of PKCα manipulation in vivo (e.g. Refs. 2, 28–31, 41, and 42).

The ability of naturally occurring and synthetic DAGs to activate PKCα is limited by their rapid metabolism in cell mem-
branes (43, 44), which leads to depletion of the agonist and reverse translocation of the enzyme to the cytoplasm (20). This process was characterized in IEC-18 cells by administrating a single treatment with a 20 μg/ml concentration of the short chain DAG DiC8 and analyzing PKCα localization by immuno-

MARCH 18, 2016 • VOLUME 291 • NUMBER 12
PKCα Is Resistant to Diacylglycerol-induced Desensitization

fluorescence microscopy. This was the method of choice for evaluating PKCα membrane association because the endogenous protein is fixed in situ for analysis. Subcellular fractionation is highly sensitive to extraction conditions and has resulted in both under- and overestimation of PKCα membrane association in response to DAG stimulation (e.g. Refs. 45 and 46). Multiple studies have determined that fluorescence imaging provides a more accurate depiction of the dynamics of PKC signaling molecules (47). As shown in Fig. 1A, i, DiC8 induced rapid translocation of PKCα to the plasma membrane in IEC-18 cells accompanied by clearance of the enzyme from the cytoplasm within 15 min. This effect was indistinguishable from that elicited by the potent PKC agonist PMA and was also seen with 1,2-sn-dioleoylglycerol (Fig. 1B), indicating that DAG can efficiently translocate PKCα to the plasma membrane in IEC-18 cells. As expected, the association of PKCα with the membrane was transient following a single DiC8 treatment with the enzyme disappearing from the membrane and gradually accumulating in the cytoplasm 1–3 h following addition of the agonist (Fig. 1A, ii). The effect can be linked to depletion of the short chain DAG from the membrane because reaccumulation of PKCα in the cytoplasm was accelerated when DiC8 was removed from the cells by washing (with complete reversal evident by 1 h; Fig. 1C, panel h). Reverse translocation was also seen following washout of PMA, although cytosolic accumulation was slower than seen with DiC8 (Fig. 1C, panel i), likely reflecting the slower metabolism of PMA. The appearance of PKCα in the cytoplasm following removal of agonist was not primarily the result of accumulation of newly synthesized PKCα because it was not affected when new protein synthesis was inhibited with cycloheximide (Fig. 1C, panels k and l).

In previous studies, we have determined that some PKC agonists, such as bryostatin, can induce lipid raft-dependent endocytosis of membrane-associated PKCα in IEC-18 cells (22), and DAG has been shown to be internalized to the endoplasmic reticulum for triglyceride synthesis in some systems (48). To differentiate between endocytic internalization and reverse translocation, cells were treated with the cholesterol-seques-tering agent nystatin, which inhibits lipid raft-dependent endocytosis (22, 49, 50). Nystatin effectively blocked the internalization of PKCα elicited by bryostatin (Fig. 2A, compare panels c and d) but had no effect on the cytosolic reaccumulation of the enzyme following a single DiC8 treatment (Fig. 2A, panels g and h). Thus, the return of PKCα to the cytoplasm following DiC8 treatment can be attributed to reverse translocation as a result of metabolism of DiC8 at the plasma membrane rather than endocytic trafficking.

Because reverse translocation of PKCs is dependent on their kinase activity (20), reaccumulation of PKCα in the cytoplas following DiC8 treatment is indicative of enzyme activation. To confirm that this is the case in IEC-18 cells, the effect of the PKC inhibitor bisindolylmaleimide I was investigated. As shown in Fig. 2B, bisindolylmaleimide I blocked the return of PKCα to the cytosol (compare panels g and h), establishing that DiC8 treatment promotes both membrane translocation and activation of the enzyme in IEC-18 cells. This finding is supported by the ability of a single DiC8 treatment to elicit PKCα-dependent downstream effects in these cells, including activation of 4E-BP1, stimulation of ERK, and down-regulation of cyclin D1, as we have reported previously (31, 41, 42).

To determine whether DAG-induced PKCα activity can be maintained for prolonged periods in IEC-18 cells, cells were subjected to repeated stimulation with DiC8 at intervals shorter than the time required for reverse translocation to occur (i.e. 10 or 60 min). As long as DiC8 was replenished before reverse translocation had taken place, repeated additions were able to sustain activation of PKCα as reflected in maintenance of the enzyme at the plasma membrane over the full 12-h duration of the treatments (Fig. 3, A and B). Sustained activity was further confirmed by the ability of the enzyme to undergo activation-dependent reverse translocation upon removal of DiC8 by washing at any time during the treatment (data not shown).

Collectively, these data demonstrate that a single DiC8 treatment leads to rapid membrane association and activation of PKCα but that the enzyme reverts to the inactive state between 1 and 3 h of treatment as a result of loss of agonist in the membrane and reverse translocation. Repeated additions of DiC8, however, are able to maintain PKCα in the active state for prolonged periods of time.

Chronic DiC8 Stimulation Has Sustained Effects on Downstream Targets of PKCα Signaling—Based on the finding that the repeated administration of DiC8 over a 12-h period results in sustained membrane association of PKCα (Fig. 3B), we asked whether this effect was accompanied by prolonged downstream signaling. We have previously demonstrated that treatment of IEC-18 cells with PKC agonists results in PKCα-dependent cell cycle-repressive effects (1, 51), including rapid down-regulation of the transcriptional regulator Id1 and dephosphorylation/activation of the translational repressor 4E-BP1 (30, 42). These effects are transient following PMA or bryostatin treatment with reversal coinciding with agonist-induced degradation of the enzyme (30, 42). However, as shown in Fig. 4, the sustained membrane association of PKCα induced by repeated addition of DiC8 was associated with sustained down-regulation of Id1 and dephosphorylation of 4E-BP1 (as indicated by the appearance of more rapidly migrating bands on Western blots; Fig. 4, arrows).

Chronic DiC8 Stimulation Does Not Result in Down-regulation of PKCα—The ability of DiC8 to support sustained activation of PKCα was surprising because it is well established that prolonged stimulation of PKC isozymes can lead to long term desensitization via degradation of the activated kinase (19, 21, 22). This down-regulation can be readily seen in PMA- and bryostatin-treated IEC-18 cells: as shown in Fig. 5A, i, PMA- or bryostatin-induced down-regulation of PKCα is evident as early as 30 min of treatment and is almost complete by 2 h. However, sustained activation of PKCα by repeated addition of DiC8 failed to down-regulate PKCα (Fig. 5A, ii). No down-regulation was seen even when DiC8 was added as frequently as every 10 min or when activation was extended as long as 12 h (Fig. 5, B and C). Importantly, the failure of DiC8 to affect PKCα levels did not reflect a general inability of the agonist to induce down-regulation of PKCs because the other two DAG-responsive PKCs in IEC-18 cells, novel PKCδ and PKCε, were potently down-regulated by DiC8 treatment (Fig. 5A, ii).
A role for compensatory increases in protein synthesis in the failure of DiC₈ to down-regulate PKCα/H₉₂₅₁ in IEC-18 cells was excluded by addition of the protein synthesis inhibitor cycloheximide (CHX): as shown in Fig. 5D, levels of PKCα remained unchanged following a 6-h incubation with DiC₈ in the presence or absence of CHX (compare the first two lanes on the left with the last two lanes on the right). This finding pointed to an inability of DiC₈ to promote degradation of the enzyme. Ago-

**FIGURE 1.** Activation and reverse translocation of PKCα by DiC₈. A, i, panels a–c, and ii, panels a and b, IEC-18 cells grown on coverslips were given a single treatment with vehicle (Control), 20 μg/ml DiC₈, or 100 nM PMA for the indicated times prior to fixation and processing for immunofluorescence detection of PKCα. The closed arrow in A, ii, panel a, points to membrane association of PKCα, whereas the open arrow in A, ii, panel b, indicates reverse translocation of the enzyme into the cytoplasm. B, panels a and b, as in A except that cells were treated with vehicle or 25 μg/ml 1,2-dioleoyl-sn-glycerol (1,2-DOG) for 15 min. C, panels a–l, cells were treated with vehicle (Control), DiC₈, or PMA in the absence or presence of 200 μM CHX. After 15 min, cells were either fixed and processed for PKCα immunofluorescence (panels a–f) or washed twice with PBS and incubated for a further 1 h in fresh medium containing vehicle or CHX before fixation and processing (panels g–l). Open arrows in panels h, i, k, and l indicate cytoplasmic PKCα after reverse translocation. Scale bars, 10 μm. Images are representative of at least three independent experiments.
nist-induced down-regulation of PKCα can involve ubiquitination of the enzyme and degradation by the proteasome or lipid raft-dependent intracellular trafficking and lysosomal processing (19, 21, 22). Involvement of lysosomal degradation can be excluded because repeated addition of DiC₈ does not induce internalization of the protein (Fig. 3). Furthermore, relocation of PKCα to the cytoplasm following a single DiC₈ treatment does not involve an endocytic pathway because it was not affected by nystatin (Fig. 2 A). Induction of proteasomal degradation by DiC₈ was excluded by the failure of the proteasome inhibitor MG132 to affect levels of PKCα in DiC₈-treated cells while strongly protecting PKCδ and PKCe from degradation induced by the short chain DAG (Fig. 5D). To determine whether DAG induces ubiquitination of PKCα, ubiquitinated proteins were immunoprecipitated from lysates of DiC₈- or PMA-treated cells with antiubiquitin antibody P4D1 and probed for PKCα. Although PMA treatment produced a smear of high molecular weight PKCα species characteristic of the polyubiquitinated enzyme (23, 52), DiC₈-stimulated PKCα showed no mono- or polyubiquitination (Fig. 6 A). Thus, DiC₈ does not target PKCα for ubiquitination. This does not reflect an inherent inability of DAG to promote ubiquitination/proteasomal degradation of PKCs because the degradation of PKCδ and PKCe was blocked by the proteasome inhibitor MG132 (Fig. 5D).

Chronic DiC₈ Stimulation Fails to Trigger Early Events That Target PKCα for Degradation—Having determined that prolonged activation of PKCα by DiC₈ does not result in degradation of the enzyme, studies were conducted to determine the specific stage(s) along the degradation pathways at which this failure occurred. Two mechanisms have been described for agonist-induced ubiquitination/proteasomal degradation of PKCα (19, 21, 23). One pathway involves activation-dependent priming site dephosphorylation, which both inactivates the kinase and targets it for ubiquitin-dependent proteasomal degradation (9, 19). A second pathway involves direct ubiquitination of the active, fully phosphorylated kinase at the plasma membrane (21, 23). To determine whether DAG induces ubiquitination of PKCα, ubiquitinated proteins were immunoprecipitated from lysates of DiC₈- or PMA-treated cells with antiubiquitin antibody P4D1 and probed for PKCα. Although PMA treatment produced a smear of high molecular weight PKCα species characteristic of the polyubiquitinated enzyme (23, 52), DiC₈-stimulated PKCα showed no mono- or polyubiquitination (Fig. 6 A). Thus, DiC₈ does not target PKCα for ubiquitination. This does not reflect an inherent inability of DAG to promote ubiquitination/proteasomal degradation of PKCs because the degradation of PKCδ and PKCe was blocked by the proteasome inhibitor MG132 (Fig. 5D).

Priming phosphorylation of PKCα at the activation loop (Thr⁴⁹⁷), turn motif (Thr⁶₃₈), and hydrophobic motif (Ser⁶₅₇) sites leads to characteristic changes in electrophoretic mobility on SDS-polyacrylamide gels as previously determined using phosphosite-specific antibodies (see Ref. 21). The fully dephosphorylated species can be readily detected on Western blots by its more rapid migration at ~76 kDa compared with 80 kDa for the mature/phosphorylated enzyme as can be seen in bryosta-
Notably, faster migrating species were never detected following DiC8 treatment (Fig. 6B) even after prolonged treatment (e.g. 12 h; see Figs. 4 and 5, A and B). Although addition of the proteasome inhibitor MG132 clearly protected these unstable species in bryostatin-treated cells (Fig. 6B), no dephosphorylation of PKCα/H9251 was observed following either 2- or 6-h treatment with DiC8 even in the presence of proteasomal inhibition (Figs. 5D and 6B). Thus, DAG does not appear to induce dephosphorylation of PKCα/H9251 in IEC-18 cells.

Previous studies from our laboratory and others have demonstrated that heat shock proteins (Hsps) play important roles in regulating the phosphorylation and stability of PKCα following activation by pharmacological agonists, such as phorbol esters or bryostatin (21, 53–55). Hsp90 protects the activated enzyme from dephosphorylation and proteasomal degradation. Hsp70/Hsc70 also protects activated PKCα from dephosphorylation while facilitating PMA- and bryostatin-induced proteasomal degradation of the fully phosphorylated, mature enzyme. Based on these findings, we examined the effect of the Hsp90 inhibitor 17-AAG (56) and the Hsp70 inhibitor PES (57) on PKCα stability during chronic DiC8 treatment. As shown in Fig. 6C, although inhibition of Hsp90 with 17-AAG accelerated the degradation of PKCα in bryostatin-treated cells, it had no effect on the stability of the enzyme in DiC8-treated cells. When MG132 was included in the treatment to facilitate detection of unstable dephosphorylated forms of PKCα, the ability of 17-AAG to enhance PKCα dephosphorylation in response to bryostatin was apparent (Fig. 6C, open arrow); however, no accumulation of dephosphorylated species of PKCα was seen in DiC8-treated cells in the absence or presence of 17-AAG even when MG132 was added. Similarly, PES treatment enhanced
PKCα Is Resistant to Diacylglycerol-induced Desensitization

![Image of Western Blot](image)

**FIGURE 4.** Repeated addition of DiC8 maintains prolonged downstream signaling from PKCα. IEC-18 cells were treated with vehicle (C) or 20 μg/ml DiC8 (D) in fresh medium every 1 h for the indicated times prior to processing for Western blotting detection of the indicated proteins. Arrows indicate the migration of dephosphorylated/active forms of 4E-BP1. Data are representative of three independent experiments.

the dephosphorylation of PKCα in response to bryostatin but not in response to DiC8 (Fig. 6D). (Note that PES stabilizes the mature form of PKCα (arrowhead) in bryostatin-treated cells, reflecting the Hsp70 dependence of proteasomal degradation of this species (21).) Collectively, these findings show that the inability of chronic activation by DAG to elicit dephosphorylation/degradation of PKCα in IEC-18 cells does not reflect protective effects of the chaperones Hsp90 or Hsp70. Together with the finding that DiC8 does not induce endocytosis trafficking of PKCα to the lysosome (see above), these data indicate that DAG-induced activation of PKCα fails to trigger early steps in known desensitization/down-regulation pathways for PKCα.

**Insufficient Intracellular Calcium Does Not Account for the Failure of DiC8 to Induce PKCα Degradation—**Our findings demonstrated that DiC8 does not regulate the stability of PKCα in IEC-18 cells while promoting degradation of the nPKCs PKCβ and PKCε. A major difference between PKCα and the nPKC isozymes is that DAG stimulation of PKCα is sensitive to intracellular Ca2+ levels (58). To test whether low intracellular Ca2+ could account for the lack of DiC8-induced PKCα degradation, IEC-18 cells were pretreated with the calcium ionophore A23187 (20), which promotes influx of Ca2+ from the medium (medium Ca2+ concentration, 1.8 mM). Calcium levels were monitored using the calcium-binding dye Fura-2 AM, which fluoresces only when bound to calcium ions. Although the Fura-2 AM signal was nearly undetectable in dimethyl sulfoxide-treated control cells (Fig. 7A, panel a), it was significantly enhanced with increasing concentrations of A23187, confirming that A23187 treatment led to elevated intracellular Ca2+ levels in IEC-18 cells (Fig. 7A, panels b–e). Cells pretreated with A23187 were stimulated with DiC8 every hour to induce PKC activation. As shown in Fig. 7B, no down-regulation of PKCα was observed in response to DiC8 at any of the concentrations of calcium ionophore tested. The failure of DiC8 to promote PKCα degradation cannot, therefore, be attributed to insufficient levels of calcium for optimal translocation/activation of the enzyme.

**Affinity for DAG Does Not Account for the Resistance of PKCα to Down-regulation by DiC8—**PKCβ and PKCε have a higher resting affinity for DAG than PKCα and other calcium-dependent PKC isozymes due to an invariant tryptophan at position 22 in the C1b domain that is invariant as tyrosine in cPKCs (60). Mutation of this tyrosine to tryptophan in the cPKCs converts the C1b domain from a low affinity to a high affinity DAG-binding module (60). Thus, to determine whether the difference in DAG affinity accounted for the differential ability of DiC8 to down-regulate PKCβ and PKCα in IEC-18 cells, the tyrosine in the C1b domain of PKCα (residue 123) was mutated to tryptophan. Wild-type and mutant PKCα expression constructs were transfected into IEC-18 cells, and the ability of PMA and DiC8 to down-regulate the respective proteins was determined (cycloheximide was included in the treatments to avoid complications arising from the ability of PKC agonists to activate the CMV promoter of the expression constructs). As expected, both wild-type and tyrosine-to-tryptophan C1b mutant PKCα (Y123W) were down-regulated by PMA (Fig. 7C) as was endogenous PKCβ. However, the tyrosine-to-tryptophan C1b mutant PKCα showed the same resistance to down-regulation by DiC8 as the wild-type protein. Thus, low inherent affinity for DAG does not account for the inability of chronic DiC8 stimulation to elicit down-regulation of PKCα in IEC-18 cells.

**Phosphatidic Acid and Other Metabolites of DiC8 Do Not Affect Agonist-induced Down-regulation of PKCα—**Because DAG is known to be rapidly metabolized in cells, we investigated whether metabolites of DiC8 may affect degradation of PKCα. The ability of DAG kinase inhibitors to prolong PKCα activation in intestinal epithelial cells (61) indicates that generation of phosphatidic acid through phosphorylation is a major metabolic pathway for down-regulation of DAG-mediated signaling in this cell type. Therefore, the effects of the short chain phosphatidic acid DOPA on the ability of DiC8 and PMA to induce PKCα down-regulation were tested. Addition of either 20 or 40 μM DOPA had no discernible effect on the levels of PKCα in control or DiC8-treated cells and did not affect the down-regulation of the enzyme following PMA treatment (Fig. 8A). To assess the impact of other possible metabolites of DiC8 on agonist-induced PKCα down-regulation, the effect of incubating cells with DiC8 and PMA in combination was determined. As with DOPA, the presence of DiC8 did not prevent PMA-induced down-regulation of PKCα (Fig. 8B), confirming that interference by metabolites of DAG is not responsible for the inability of DiC8 to induce down-regulation of the enzyme.

**PMA-induced Down-regulation of PKCα Involves Targeting of the Enzyme to Detergent-resistant, Cholesterol-sensitive Membrane Domains—**It has long been recognized that a portion of activated PKCα can be targeted to specialized membrane domains, including DRMs or lipid rafts (e.g. Refs. 33 and 62–64). The fact that bryostatin targets PKCα for internalization from the plasma membrane in IEC-18 cells whereas PMA does not (23) indicates that agonists differ in their ability to promote association of the enzyme with functionally distinct
membrane domains. Therefore, two approaches were used to determine the ability of DiC₈ and PMA to localize PKCα in DRM. The first involved sucrose gradient centrifugation of cells extracted with cold Triton X-100. Because membrane binding of classical PKCs is Ca²⁺-dependent, 100 μM CaCl₂ was included in the extraction buffer and gradients. DiC₈ or PMA was added to the extraction buffers as appropriate to control for any potential metabolism of DiC₈ during processing. Following centrifugation, proteins contained within the more buoyant DRM fractions collect at the 30-5% sucrose interface near the top of the gradient (fractions 2 and 3), whereas membrane proteins that are extracted by the detergent are found in lower fractions (65).

In untreated cells, minimal levels of PKCα were found in the DRM-containing fractions 2 and 3; however, as seen by others, PMA treatment led to appearance of the enzyme in these fractions with a corresponding loss of the enzyme from the lower non-DRM fractions (Fig. 9A). Notably, DiC₈ promoted only a slight increase in DRM-associated PKCα (Fig. 9A). To address the potential concern that the lower levels of DRM-associated PKCα in DiC₈-treated cells reflected metabolism of DiC₈ during the long centrifugation (18 h) involved in the fractionation protocol, association of PKCα with DRM was also analyzed by immunofluorescence. This second approach allowed for immediate fixation of the cells following a short (30-min) Triton X-100 extraction on ice. To preserve membrane associations during extraction, buffers contained Ca²⁺ and the appropriate PKC agonist. Consistent with results from density gradient fractionation, very little PKCα staining was evident in DiC₈-treated cells following PMA treatment (Fig. 9B). Quantification of the

---

**FIGURE 5.** Effects of chronic treatment with PKC agonists on PKCα levels in IEC-18 cells. **A, i and ii,** IEC-18 cells were given a single treatment with 100 nM bryostatin (Bryo) or PMA or were treated every 1 h with 20 μg/ml DiC₈ in fresh medium. After the indicated times, cells were processed for Western blotting detection of the indicated proteins. For the 0-h time point, cells were treated with vehicle for 2 (A) or 1 h (B, C, D) in fresh medium every 10 min (B) or every 1 h (C) prior to processing for Western blotting analysis at the indicated times. D, as in C except that 20 μM MG132 (MG) or 200 μM CHX was included in the medium as indicated. All Western blot panels show data from a single blot with dotted lines indicating where lanes have been rearranged for clarity. Data are representative of at least three independent experiments.
PKCα Is Resistant to Diacylglycerol-induced Desensitization

**FIGURE 6.** Prolonged activation by DiC8 fails to induce ubiquitination or dephosphorylation of PKCα. A, IEC-18 cells were treated with vehicle (C), 20 μg/ml DiC8 (D), or 100 nM PMA (P) for 1 h before extraction and immunoprecipitation (IP) using the P4D1 antibody, which recognizes total ubiquitin. Immunoprecipitates were then analyzed for the presence of ubiquitinated PKCα by Western blotting (WB). B, C, and D, cells were treated with vehicle (C), 20 μg/ml DiC8 (D), 100 nm brystostatin (B), 20 μM MG132, 1 μM 17-AAG, and/or 20 μM PES as indicated for 2 h or 4 h (C and D) and analyzed for expression of PKCα and β-actin by Western blotting. For control and DiC8-treated cells, drugs were readministered in fresh medium every 1 h. Arrowhead, mature, fully phosphorylated PKCα; arrow, faster migrating, non-phosphorylated PKCα. All Western blot panels show data from a single blot; dotted lines in B indicate where lanes have been rearranged for clarity. Data are representative of at least three independent experiments.

**FIGURE 7.** Low intracellular Ca2⁺ or lower affinity for DAG do not account for the insensitivity of PKCα to down-regulation following activation by DiC8. A, panels a–e, IEC-18 cells were treated with vehicle (Control) or the indicated concentration of A23187 calcium ionophore. After 30 min, 5 μM Fura-2 AM dye was added to all the cells. After an additional 30 min, cells were washed with PBS, and fluorescence due to Ca2⁺-bound Fura-2 AM was visualized with an epifluorescence microscope. Scale bars, 10 μm. B, cells were treated every hour with vehicle (C), 20 μg/ml DiC8 (D), and the indicated concentrations of A23187 in fresh medium for 3 h before analysis of PKCα and β-actin expression by Western blotting. C, IEC-18 cells transfected with a pGL2 vector expressing either HA-tagged wild-type PKCα (w.t.) or HA-tagged Y123W mutant PKCα (Y123W) were treated every 1 h with vehicle (C), 100 nm PMA (P), or 20 μg/ml DiC8 (D) in fresh medium. After 4 h, cells were harvested and analyzed by immunoblotting for expression of HA-tagged PKCα proteins using anti-HA antibody as well as for PKCδ and β-actin. Data are representative of two (A) or three independent experiments (B and C).

**FIGURE 8.** Effect of DAG metabolites on agonist-induced down-regulation of PKCα. A, IEC-18 cells were treated with 20 μg/ml DiC8 (D), 100 nM PMA (P), or vehicle (C) in the presence or absence of the indicated concentrations of DOPA (or corresponding vehicle) in fresh medium replenished every 1 h. After 3 h, cells were processed for immunoblotting analysis of PKCα and β-actin levels. B, as in A except that cells were treated with DiC8 (D), PMA (P), or DiC8 and PMA in combination (D/P). Control cells were treated with equivalent volumes of vehicle (acetonitrile). Data are representative of three independent experiments.

fluorescence signal indicated that detergent-resistant staining of PKCα represented about 10–25% of that seen in untreated cells, confirming that PMA targets an appreciable portion of cellular PKCα to DRMs.
Having established that PMA and DiC₈ differentially target PKCᵦ to DRMs/lipid rafts, the role of DRM domains in PKCᵦ down-regulation was examined. Cholesterol is a major component of DRM/lipid rafts, and agents that interfere with membrane cholesterol disrupt raft function (66). Thus, the role of DRM domains was studied using the cholesterol-binding agents methyl-β-cyclodextrin, nystatin, and filipin (67). These agents affect membrane cholesterol in different ways: methyl-β-cyclodextrin binds cholesterol in its hydrophobic pocket and extracts it from the membrane, whereas nystatin and filipin sequester cholesterol within the membrane (68). Importantly, cholesterol-disruptive agents do not affect the membrane recruitment of PKCᵦ by PMA (23). However, as can be seen in Fig. 9C, all three agents were able to inhibit PMA mediated down-regulation of PKCᵦ, pointing to lipid rafts as the site for degradation. Thus, the observed differences in targeting to DRMs provide a mechanistic explanation for the inability of DiC₈ to trigger agonist-induced down-regulation of PKCᵦ.

Partitioning of PKCᵦ into lipid rafts may position the enzyme to interact with protein(s) that mediates its long term desensitization. A potential candidate is PLD. PLD has been shown to partition into DRMs, is activated by PMA treatment, and can interact directly with PKCᵦ (11, 69–72). Therefore, a role for PLD in mediating PMA-induced PKCᵦ down-regulation was explored using 30 mM 1-butanol, which inhibits the phospholipase activity of PLD and blocks its physical association with PKCᵦ (69). As shown in Fig. 9D, 1-butanol minimally affected PKCᵦ down-regulation by PMA, indicating that PLD is

4 O. V. Leontieva and J. D. Black, unpublished data.
PKCα Is Resistant to Diacylglycerol-induced Desensitization

DiC₈ Fails to Down-regulate cPKCs in Endometrial Cancer Cells—To determine whether the inability of DiC₈ to induce down-regulation of PKCα was restricted to intestinal epithelial cells, the effect of PKC agonists was tested in HEC-1-A and SNG-M endometrial cancer cells. As in IEC-18 cells, PKCα was readily down-regulated by PMA and bryostatin in endometrial cancer cells (Fig. 10 and data not shown), but was resistant to down-regulation by repeated (every 30 min) DiC₈ treatment (Fig. 10). Thus, PKCα is insensitive to DAG-induced down-regulation in multiple cell types. Although PKCα is the only cPKC expressed in IEC-18 cells, HEC-1-A and SNG-M cells also express PKCβ1. Interestingly, this isozyme was also resistant to down-regulation by DiC₈ but not by PMA (Fig. 10). Thus, selective resistance to DAG-induced down-regulation extends to multiple members of the cPKC subfamily.

Sustained Activation of PKCα Is Observed in Vivo—Our analysis of PKCα in the self-renewing intestinal epithelium revealed that, although PKCα is cytosolic in proliferating intestinal crypt cells, it is membrane-associated/activated in all postmitotic cells of the intestinal villus (Fig. 11A) that reside in this compartment for 2–3 days before being extruded into the intestinal lumen. Notably, we have previously demonstrated that loss of the enzyme in these cells in PKCα knock-out animals is associated with increased levels of Id1, consistent with a sustained repressive effect of PKCα signaling in these postmitotic cells. A similar pattern of sustained PKCα membrane association/activation is seen in other self-renewing epithelial tissues, including skin (3) and the stratified squamous epithelium of the cervix (Fig. 11B). Thus, consistent with our findings using DiC₈ in vitro, PKCα appears to be resistant to activation-induced down-regulation in response to natural stimuli in vivo.

Discussion

Many of the actions of PKCα require prolonged signaling despite well-established mechanisms for desensitization of the enzyme following sustained activation (19, 21, 22). Here we show that DAG-stimulated PKCα is resistant to known desensitization mechanisms that are triggered by prolonged activation of the enzyme by pharmacological agonists. Sustained membrane association for as long as 12 h fails to target PKCα for dephosphorylation/proteolytic processing in DiC₈-treated cells. This resistance cannot be attributed to an inability of DiC₈ to activate the kinase or to sustain kinase activation for prolonged periods because (a) the extent of membrane translocation of PKCα induced by DiC₈ in IEC-18 cells was indistinguishable from that seen with the potent agonist PMA (Fig. 1), (b) in the absence of repeated stimulation with DiC₈, PKCα undergoes activity-dependent reverse translocation that is blocked by pharmacological inhibitors of the enzyme (Fig. 2), and (c) PKCα-specific downstream effects (e.g. Id1 down-regulation and 4E-BP1 activation) are sustained for at least 12 h in cells when DiC₈ is added repeatedly to maintain PKCα mem-

![FIGURE 10. Effects of PKC agonists on PKCα and PKCβ1 in endometrial cancer cells. HEC-1-A and SNG-M cells were treated with vehicle (C), 100 nM PMA (P), or 20 μg/ml DiC₈ (D) in fresh medium every 30 min for 6 h prior to harvesting and analysis for expression of the indicated proteins by Western blotting. Data are representative of at least three independent experiments.](image)

![FIGURE 11. Prolonged plasma membrane association of PKCα in self-renewing epithelial tissues in vivo. Immunohistochemical analysis of PKCα expression and subcellular distribution in the epithelial lining of the intestine (A, i and ii) and in the stratified squamous epithelium of the cervix (B) of the mouse is shown. PKCα is localized in the cytoplasm of proliferating cells of intestinal crypts (C) and in mitotic cells of the basal/parabasal cell layers of the stratified squamous epithelium of the cervix (D). Postmitotic, mature cells of the intestinal villus (V) and of the suprabasal layers of the cervical epithelium show membrane-associated enzyme (arrowheads). The arrow in A, ii, indicates the crypt-villus junction where cells cease dividing and commit to differentiation. Mature cells of the villus migrate toward the villus tip and are extruded into the lumen after a transit time of 2–3 days.](image)
PKCα Is Resistant to Diacylglycerol-induced Desensitization

brane association/activity (Figs. 3 and 4). The efficacy of DiC₈ was further confirmed by its ability to down-regulate PKCβ and PKCe in the same cells. The inability of DiC₈ to down-regulate PKCa is also not due to effects of a metabolite(s) of this short chain DAG because neither addition of DOPA nor co-incubation with DiC₈ affected the ability of PMA to induce loss of the enzyme (Fig. 8). Finally, the effect does not reflect a general insensitivity of PKCa to activation-induced dephosphorylation/down-regulation in the cells used in this study because bryostatin promotes PKCa dephosphorylation by 1 h of treatment, and both PMA and bryostatin induce degradation of the enzyme by 2 h (Fig. 5 and Refs. 21 and 23).

Analysis of the effects of DiC₈ indicated that DAG fails to trigger early events that target PKCa for degradation. Prolonged DiC₈ treatment did not result in endocytic trafficking of PKCa, which directs the protein to lysosomes for processing following activation by bryostatin (22). Similarly, sustained activation by DiC₈ did not lead to dephosphorylation or ubiquitination of PKCa, events that target the enzyme for proteasomal degradation in response to pharmacological agonists (19, 21, 23). The lack of dephosphorylation in response to DAG is in keeping with the low levels of dephosphorylation of endogenous PKCa induced by PMA or bryostatin in IEC-18 cells (21, 23). Previous studies from our laboratory and others have determined that Hsp90 and Hsp70 act to protect activated PKCa from dephosphorylation and/or degradation in response to PMA or bryostatin treatment (9, 21). However, no protective role of these Hsps was seen for DiC₈-activated PKCa (Fig. 6), indicating that the inability of DAG to induce degradation of PKCa is due to a failure to trigger event(s) upstream of the effects of these chaperones.

Although prolonged DiC₈ treatment failed to affect PKCa levels, it readily down-regulated PKCβ and PKCe in IEC-18 cells. Based on this finding, we explored unique aspects of the regulation of these isozymes that may underlie the differential effects of prolonged DiC₈ treatment. A major difference between the nPKCs and cPKCs, including PKCa, lies in the regulation of binding to DAG and anionic lipids. The C1 domains of cPKCs and nPKCs have two DAG binding sites, C1a and C1b. The C1b domain of cPKCs has a 2 orders of magnitude lower affinity for DAG than the C1b domain of PKCβ and PKCe and cannot mediate kinase activation (60). Although the C1a domains of cPKCs and nPKCs have high affinity for DAG, the C1a domain of unstimulated PKCa is masked by interaction with the C2 and catalytic domains and is only released for DAG binding following Ca²⁺-dependent interaction of the C2 domain with anionic lipids in the plasma membrane (13, 73, 74). Thus, activation of PKCa is a two-step process in which the enzyme is first recruited to the membrane by the C2 domain, leading to release of the C1a domain for DAG binding. The C2 domain has a low intrinsic affinity for Ca²⁺, but Ca²⁺ binding is enhanced by DAG, phosphatidylserine, and phosphatidylinositol 4,5-bisphosphate in the membrane, allowing activation by DAG even at subphysiological intracellular Ca²⁺ levels (14–16, 74). Nonetheless, elevated intracellular Ca²⁺ concentrations increase the rate of PKCa activation in the presence of DAG (74). In contrast to PKCe, interaction of nPKCs with the plasma membrane is Ca²⁺-independent, suggesting that Ca²⁺ levels may be limiting for optimal activation and eventual desensitization of PKCa by DiC₈ in IEC-18 cells. However, our analysis excluded insufficient Ca²⁺ as the basis for the inability of DAG to induce PKCa dephosphorylation or degradation because these effects were still not seen when intracellular Ca²⁺ levels were increased using the calcium ionophore A23187 (Fig. 7).

The low DAG binding affinity of the C1b domain in cPKCs reflects the presence of an invariant tyrosine in this domain (at residue 123 in PKCa) that is a tryptophan in the high affinity domain of nPKCs (60). However, differences in the DAG binding affinity of the C1b domain were also excluded as an explanation for the differential effects of DAG because DiC₈ failed to down-regulate a PKCa mutant in which tyrosine 123 was replaced by tryptophan (Fig. 7) to convert its C1b domain to a high affinity DAG-binding module resembling that of PKCδ and PKCe (60).

Our data indicate that association with distinct membrane subdomains plays an important role in the ability of agonists to induce down-regulation of PKCa. PMA targets a proportion of PKCa to DRMs, whereas at minimal DRM association was seen after stimulation with DiC₈ (Fig. 9). A role for DRM domain(s) in PKCa down-regulation was confirmed by the ability of cholesterol-disrupting agents to inhibit PMA-induced down-regulation of the enzyme (Fig. 9). Differential targeting of PKCa to distinct membrane domains is in keeping with studies using rat basophilic leukemia cells that demonstrated that the C1a domain of the cPKC PKCγ is nearly immobilized in the membrane following recruitment by PMA but is diffusible in the membrane when bound to DiC₈ or DAG derived from membrane lipids (75). The selective ability of PMA to target PKCa to specific membrane domains likely reflects recruitment of the agonist-bound enzyme rather than partitioning of this agonist into these domains because (a) PMA appears to preferentially target PKCα over PKCβ and PKCe to DRMs in T lymphocytes (76) and (b) partitioning experiments did not detect PKCe in DRMs in PMA-treated IEC-18 cells.⁵

The demonstration that both PKCα and PKCβI are resistant to desensitization by chronic DiC₈ treatment (Fig. 10) indicates that the resistance to DAG-induced down-regulation is a common property of cPKCs. Although the structural basis for this resistance remains to be determined, evidence points to a role for the agonist-binding C1 domain. As demonstrated from analysis using fragments of PKCγ, DAG and PMA induce different membrane interactions of the C1a domain of cPKCs (75). The C1 domain of cPKCs is highly conserved but differs in sequence and location from that of nPKCs (6). Notably, structural analysis has indicated that DiC₈ and PMA induce different conformational changes and membrane associations of the C1 domain of PKCα (77). Thus, this domain is likely to support distinct interactions dependent on both the bound agonist and PKC class involved. Ongoing studies are directed at determining how structural differences affect membrane compartmentalization and protein-protein interactions that may regulate desensitization of PKC signaling (e.g. binding to receptors for activated C-kinase (RACKs), syndecan-4, and Pin1 (78–81)).

⁵ A. R. Black, M. A. Lum, and J. D. Black, unpublished data.
Collectively, our findings indicate that the cPKCs have unique properties that protect them from dephosphorylation/degradation under conditions of sustained input signaling. The physiological relevance of this finding is highlighted by in vivo studies in self-renewing epithelial tissues. Analysis of intestinal, epidermal, and stratified squamous epithelial systems indicates that activated, membrane-associated PKCα is also protected from down-regulation by natural agonists in vivo. In the mouse intestinal epithelium, PKCα is cytosolic/inactive in proliferating cells of the crypt and is activated coincident with growth arrest at the crypt/villus junction as indicated by a clearing of the enzyme from the cytoplasm and its appearance at the plasma membrane (Fig. 11 and Ref. 30). Notably, this pattern is maintained along the entire length of the villus, indicating that the enzyme remains activated in the face of ongoing signals without any sign of down-regulation for the 2–3 days required for migration of postmitotic cells to the villus tip. Activation of PKCα triggers a program of cell cycle withdrawal in intestinal cells that includes rapid down-regulation of Id1 (2, 30): that PKCα is active in villus cells is indicated by the finding that PKCα deficiency results in aberrant expression of Id1 in this compartment (30). Studies by Rozeugt and co-workers (82, 83) provide additional evidence for the failure of physiological agonists to induce long term desensitization of PKCα in intestinal epithelial cells. Treatment of IEC-18 cells with the Gα coupled receptor agonist angiotensin II promoted PKCα activity-dependent feedback inhibition of angiotensin II-induced EGF receptor transactivation (82). Notably, prolonged exposure of IEC-18 cells to angiotensin II for as long as 24 h failed to affect PKCα levels or phosphorylation in this system (83). A similar scenario is seen in the epidermis with PKCα becoming plasma membrane-associated/activated in the lower spinous layers coincident with growth arrest/terminal differentiation and remaining exclusively localized at the membrane during the several days it takes for cells to transit through this layer (3). Evidence for PKCα activity in this compartment was provided by knockdown experiments in an in vitro organotypic epidermis model that led to increased basal and suprabasal proliferation marker expression, decreased differentiation, and reduced epidermal stratification (3). Notably, epidermal PKCα is down-regulated within 3 h of topical administration of PMA (e.g. Refs. 84 and 85); thus, the persistence of activated PKCα in this tissue reflects selective insensitivity to down-regulation by natural agonists and is not due to rapid replacement of down-regulated enzyme. The fact that PKCα expression is lost in human colon and basal cell carcinomas (86, 87) together with the finding that PKCα knock-out enhances tumorigenesis in the mouse intestine and epidermis (59, 88) emphasizes the importance of maintaining PKCα signaling for tissue homeostasis. Future studies will address the specific mechanisms involved in protecting DAG-activated PKCα from desensitization in tissues.

Acknowledgment—We thank Kathryn J. Curry for expert technical assistance.

References

1. Black, J. (2010) PKC and control of the cell cycle, in Protein Kinase C in Cancer Signaling and Therapy (Kazanietz, M. G., ed) pp. 155–188, Springer Science, New York
2. Frey, M. R., Clark, J. A., Leontieva, O., Uronis, J. M., Black, A. R., and Black, J. D. (2000) Protein kinase C signaling mediates a program of cell cycle withdrawal in the intestinal epithelium. J. Cell Biol. 151, 763–778
3. Jerome-Morais, A., Rahn, H. R., Tribudan, S. S., and Denning, M. F. (2009) Role for Protein kinase C-α in keratinocyte growth arrest. J. Invest. Dermatol. 129, 2365–2375
4. Kazanietz, M. G. (ed) (2010) Protein Kinase C in Cancer Signaling and Therapy, Springer Science, New York
5. Black, A. R., and Black, J. D. (2012) Protein kinase C signaling and cell cycle regulation. Front. Immunol. 3, 423
6. Steinberg, S. F. (2008) Structural basis of protein kinase C isoform function. Physiol. Rev. 88, 1341–1378
7. Parker, P. J., and Murray-Rust, J. (2004) PKC at a glance. J. Cell Sci. 117, 131–132
8. Antal, C. E., and Newton, A. C. (2014) Tuning the signalling output of protein kinase C. Biochem. Soc. Trans. 42, 1477–1483
9. Newton, A. C. (2010) Regulation of conventional and novel protein kinase C isoforms by phosphorylation and lipids, in Protein Kinase C in Cancer Signaling and Therapy (Kazanietz, M. G., ed) pp. 9–23, Springer Science, New York
10. Newton, A. C. (2010) Protein kinase C: poised to signal. An. J. Physiol. Endocrinol. Metab. 298, E395–E402
11. Becker, K. P., and Hannun, Y. A. (2005) Protein kinase C and phospholipase D: intimate interactions in intracellular signaling. Cell. Mol. Life Sci. 62, 1448–1461
12. Kraff, A. S., and Anderson, W. B. (1983) Phorbol esters increase the amount of Ca2+, phospholipid-dependent protein kinase associated with plasma membrane. Nature 301, 621–623
13. Farah, C., and Sossin, W. (2012) The role of C2 domains in PKC signaling, in Calcium Signaling (Islam, M. S., ed.) pp. 663–683, Springer, Dordrecht, Netherlands
14. Egea-Jiménez, A. L., Pérez-Lara, A., Corbalán-García, S., and Gómez-Fernández, I. C. (2013) Phosphatidylinositol 4,5-bisphosphate decreases the concentration of Ca2+ and phosphatidylyserine and diacylglycerol required for protein kinase C α to reach maximum activity. PLoS One 8, e69041
15. Antal, C. E., Violin, J. D., Kunkel, M. T., Skovsø, S., and Newton, A. C. (2014) Intramolecular conformational changes optimize protein kinase C signaling. Chem. Biol. 21, 459–469
16. Igumenova, T. I. (2015) Dynamics and membrane interactions of protein kinase C. Biochemistry 54, 4953–4968
17. Szallasi, Z., Smith, C. B., Pettit, G. R., and Blumberg, P. M. (1994) Differential regulation of protein kinase C isoforms by bryostatin 1 and phorbol 12-myristate 13-acetate in NIH 3T3 fibroblasts. J. Biol. Chem. 269, 2118–2124
18. Etchmann, T. O., and Lass, A. (2015) DAG tales: the multiple faces of diacylglycerol—stereochemistry, metabolism, and signaling. Cell. Mol. Life Sci. 72, 3931–3952
19. Gould, C. M., and Newton, A. C. (2008) The life and death of protein kinase C. Curr. Drug Targets 9, 614–625
20. Feng, X., Becker, K. P., Stribling, S. D., Peters, K. G., and Hannun, Y. A. (2000) Regulation of receptor-mediated protein kinase C membrane trafficking by autophosphorylation. J. Biol. Chem. 275, 17024–17034
21. Lum, M. A., Balabuski, G. M., Murphy, M. E., Black, A. R., and Black, J. D. (2013) Heat shock proteins regulate activation-induced proteasomal degradation of the mature phosphorylated form of protein kinase C. J. Biol. Chem. 288, 27112–27127
22. Lum, M. A., Pandit, K. P., Paluch, B. E., Black, A. R., and Black, J. D. (2013) Agonist-induced down-regulation of endogenous protein kinase Ca through an endolysosomal mechanism. J. Biol. Chem. 288, 13093–13109

Author Contributions—M. A. L., C. J. B., A. H. H., O. V. L., and A. R. B. performed experiments and acquired data. C. J. B. and A. R. B. designed and generated constructs. M. A. L., A. R. B., and J. D. B. designed experiments, interpreted data, and wrote the manuscript.
PKCα Is Resistant to Diacylglycerol-induced Desensitization

23. Leontieva, O. V., and Black, J. D. (2004) Identification of two distinct pathways of protein kinase Ca down-regulation in intestinal epithelial cells. J. Biol. Chem. 279, 5788–5801
24. Presta, M., Tiberio, L., Rusnati, M., Dell’Era, P., and Ragnotti, G. (1991) Basic fibroblast growth factor requires a long-lasting activation of protein kinase C to induce cell proliferation in transformed fetal bovine aortic endothelial cells. Cell Regul. 2, 719–726
25. Assender, J. W., Irenius, E., and Fredholm, B. B. (1996) Endothelin-1 causes a prolonged protein kinase C activation and acts as a co-mitogen in vascular smooth muscle cells. Acta Physiol. Scand. 157, 451–460
26. Berry, N., Ase, K., Kishimoto, A., and Nishizuka, Y. (1990) Activation of resting human T cells requires prolonged stimulation of protein kinase C. Proc. Natl. Acad. Sci. U.S.A. 87, 2294–2298
27. Nishizuka, Y. (1995) Protein kinase C and lipid signaling for sustained cellular responses. FASEB J. 9, 484–496
28. Pysz, M. A., Hao, F., Hizi, A. A., Lum, M. A., Swetzig, W. M., Black, A. R., and Black, J. D. (2014) Differential regulation of cyclin D1 expression by protein kinase Ca and signaling in intestinal epithelial cells. J. Biol. Chem. 289, 22268–22283
29. Frey, M. R., Saxon, M. L., Zhao, X., Rollins, A., Evans, S. S., and Black, J. D. (1997) Protein kinase C isoform-mediated cell cycle arrest involves induction of p21WAF1/Cip1 and p27Kip1 and hypophosphorylation of the retinoblastoma protein in intestinal epithelial cells. J. Biol. Chem. 272, 9424–9435
30. Hoo, F., Pysz, M. A., Curry, K. J., Haas, K. N., Seedhouse, S. J., Black, A. R., and Black, J. D. (2011) Protein kinase Ca signaling regulates inhibitor of DNA binding 1 in the intestinal epithelium. J. Biol. Chem. 286, 18104–18117
31. Clark, J. A., Black, A. R., Leontieva, O. V., Frey, M. R., Pysz, M. A., Kunneva, L., Wolozynska-Read, A., Roy, D., and Black, J. D. (2004) Involvement of the ERK signaling cascade in protein kinase C-mediated cell cycle arrest in intestinal epithelial cells. J. Biol. Chem. 279, 9233–9247
32. Pysz, M. A., Leontieva, O. V., Bateman, N. W., Uronis, J. M., Curry, K. J., Threadgill, D. W., Jansen, K. P., Robine, S., Velchic, A., Augustin, L. H., Black, A. R., and Black, J. D. (2009) PKCα tumor suppression in the intestine is associated with transcriptional and translational inhibition of cyclin D1. Exp. Cell Res. 315, 1415–1428
33. Botto, L., Masserini, M., and Palestini, P. (2007) Changes in the composition of detergent-resistant membrane domains of cultured neurons following protein kinase Ca activation. J. Neurosci. Res. 85, 443–450
34. Mayor, S., and Maxfield, F. R. (1995) Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol. Biol. Cell 6, 929–944
35. Kozak, S. L., Heard, J. M., and Kabat, D. (2002) Segregation of CD4 and CD8 in detergent-resistant membrane domains of cultured neurons following protein kinase Ca signaling. J. Neurosci. Res. 85, 443–450
36. Howard, S., Heard, J. M., and Kabat, D. (2000) Protein kinase Cα mediated regulation of the cell cycle. J. Biol. Chem. 275, 22945–22951
37. Weintraub, B., and Bell, R. M. (1995) Insolubility and redistribution of protein kinase Cα and Black, J. D. (1991) Protein kinase Cα signaling inhibits cyclin D1 translation in intestinal epithelial cells. J. Biol. Chem. 266, 6528–6539
38. Guan, L., Song, K., Pysz, M. A., Curry, K. J., Hizi, A. A., Danielpour, D., Black, A. R., and Black, J. D. (2007) Protein kinase Cα-mediated down-regulation of cyclin D1 involves activation of the translational repressor 4E-BP1 via a phosphoinositide 3-kinase/Akt-independent, protein phosphatase 2A-dependent mechanism in intestinal epithelial cells. J. Biol. Chem. 282, 14213–14225
39. Bell, R. M. (1986) Protein kinase C activation by diacylglycerol second messengers. Cell 45, 631–632
40. Florin-Christensen, J., Florin-Christensen, M., Delfino, J. M., Stegmann, T., and Rasmussen, H. (1992) Metabolic fate of plasma membrane diacylglycerols in NIH 3T3 fibroblasts. J. Biol. Chem. 267, 14783–14789
41. Nowicki, S., Kruse, M. S., Brismar, H., and Aperia, A. (2000) Dopamine-induced translocation of protein kinase C isoforms visualized in renal epithelial cells. Am. J. Physiol. Cell Physiol. 279, C1812–C1818
42. Khalil, R. A., Lajoie, C., and Morgan, K. G. (1994) In situ determination of [Ca2+]i threshold for translocation of the α-protein kinase C isomorph. Am. J. Physiol. Cell Physiol. 266, C1544–C1551
43. Lenz, J. C., Reusch, H. P., Albrecht, N., Schultz, G., and Schaefer, M. (2002) Ca2+-controlled competitive diacylglycerol binding of protein kinase Cα isoenzymes in living cells. J. Cell Biol. 159, 291–302
44. Yen, C.-L. E., Nelson, D. W., and Yen, M.-I. (2015) Intestinal triacylglycerol synthesis in fat absorption and systemic energy metabolism. J. Lipid Res. 56, 489–501
45. Rohrberg, K. G., Heuser, J. E., Donzell, W. C., Ying, Y.-S., Glenney, J. R., and Anderson, R. G. (1992) Caveolin, a protein component of caveole membrane coats. Cell 68, 673–682
46. Parton, R. G., and Richards, A. A. (2003) Lipid rafts and caveolae as portals for endocytosis: new insights and common mechanisms. Traffic 4, 724–738
47. Black, J. D. (2000) Protein kinase C-mediated regulation of the cell cycle. Front. Biosci. 5, D406–D423
48. Lee, H. W., Smith, L., Pettit, G. R., Vinitsky, A., and Smith, J. B. (1996) Ubiquitination of protein kinase Cα and degradation by the proteasome. J. Biol. Chem. 271, 20973–20976
49. Gao, T., and Newton, A. C. (2002) The turn motif is a phosphorylation switch that regulates the binding of Hsp70 to protein kinase C. J. Biol. Chem. 277, 31585–31592
50. Gao, T., and Newton, A. C. (2006) Invariant Leu preceding turn motif phosphorylation site controls the interaction of protein kinase C with Hsp70. J. Biol. Chem. 281, 32461–32468
51. Gould, C. M., Kannan, N., Taylor, S. S., and Newton, A. C. (2009) The chaperones Hsp90 and Cdc37 mediate the maturation and stabilization of protein kinase Cα through a conserved PXXP motif in the C-terminal tail. J. Biol. Chem. 284, 4921–4935
52. Choisis, G., Huezo, H., Rosen, N., Mimnaugh, E., Whitesell, L., and Neckers, L. (2003) 17AAG: low target binding affinity and potent cell activity—finding an explanation1. Mol. Cancer Ther. 2, 123–129
53. Leu, J. I., Pimkina, J., Frank, A., Murphy, M. E., and George, D. L. (2009) A small molecule inhibitor of inducible heat shock protein 70. Mol. Cell 36, 15–27
54. Newton, A. C. (2009) Lipid activation of protein kinases. J. Lipid Res. 50, S266–S271
55. Oster, H., and Leitges, M. (2006) Protein kinase Cα but not PKCζ suppresses intestinal tumor formation in ApcMin/+ mice. Cancer Res. 66, 6955–6963
56. Dries, D. R., Gallegos, L. L., and Newton, A. C. (2007) A single residue in the C1 domain sensitizes novel protein kinase C isoforms to cellular diacylglycerol production. J. Biol. Chem. 282, 826–830
57. Stenson, W. F., Easom, R. A., Riehl, T. E., and Turk, J. (1993) Regulation of paracellular permeability in Caco-2 cell monolayers by protein kinase C. Am. J. Physiol. Gastrointest. Liver Physiol. 265, G955–G962
58. Hope, H. R., and Pike, L. J. (1996) Phosphoinositides and phosphoinositide-utilizing enzymes in detergent-insoluble lipid domains. Mol. Biol. Cell 7, 843–851
59. Monastyrskaya, K., Hostettler, A., Buergi, S., and Draeger, A. (2005) The NK1 receptor localizes to the plasma membrane microdomains, and its activation is dependent on lipid raft integrity. J. Biol. Chem. 280, 7135–7146
60. Rybin, V. O., Xu, X., and Steinberg, S. F. (1999) Activated protein kinase C isoforms target to cardiomycocyte caveolae: stimulation of local protein phosphorylation. Circ. Res. 84, 980–988
PKCα Is Resistant to Diacylglycerol-induced Desensitization

65. Magee, A. I., and Parmryd, I. (2003) Detergent-resistant membranes and the protein composition of lipid rafts. *Genome Biol.* 4, 234

66. Wang, X.-Q., and Paller, A. S. (2006) Lipid rafts: membrane triage centers. *J. Invest. Dermatol.* 126, 951–953

67. Foster, L. J., De Hoog, C. L., and Mann, M. (2003) Unbiased quantitative proteomics of lipid rafts reveals high specificity for signaling factors. *Proc. Natl. Acad. Sci. U.S.A.* 100, 5813–5818

68. Zidovetzki, R., and Levitan, I. (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. *Biochim. Biophys. Acta* 1768, 1311–1324

69. Hu, T., and Exton, J. H. (2005) 1-Butanol interferes with phospholipase D1 activity. *Biochem. Biophys. Res. Commun.* 327, 1047–1051

70. Diaz, O., Mébarek-Azzam, S., Benzaria, A., Dubois, M., Lagarde, M., Némoz, G., and Prigent, A.-F. (2005) Disruption of lipid rafts stimulates phospholipase D activity in human lymphocytes: implication in the regulation of immune function. *J. Immunol.* 175, 8077–8086

71. Mateos, M. V., Salvador, G. A., and Giusto, N. M. (2010) Selective localization of phosphatidylincholine-derived signaling in detergent-resistant membranes from synaptic endings. *Biochim. Biophys. Acta* 1798, 624–636

72. Foster, D. A., and Xu, L. (2003) Phospholipid D in cell proliferation and cancer. *Mol. Cancer Res.* 1, 789–800

73. Slater, S. J., Seiz, J. L., Cook, A. C., Buzas, C. J., Malinowski, S. A., Kershner, J. L., Stagliano, B. A., and Stubbs, C. D. (2002) Regulation of PKCα activity by C1-C2 domain interactions. *J. Biol. Chem.* 277, 15277–15285

74. Stahelin, R. V., Wang, J., Blatner, N. R., Rafter, J. D., Murray, D., and Cho, W. (2005) The origin of C1A-C2 interdomain interactions in protein kinase Ca. *J. Biol. Chem.* 280, 36452–36463

75. Oancea, E., Teruel, M. N., Quest, A. F., and Meyer, T. (1998) Green fluorescent protein (GFP)-tagged cysteine-rich domains from protein kinase Ca as fluorescent indicators for diacylglycerol signaling in living cells. *J. Cell Biol.* 140, 485–498

76. Parolini, I., Topa, S., Sorice, M., Pace, A., Ceddar, P., Montesoro, E., Pavan, A., Lisanti, M. P., Peschle, C., and Sargiacomo, M. (1999) Phorbol ester-induced disruption of the CD4-Lck complex occurs within a detergent-resistant microdomain of the plasma membrane: involvement of the translocation of activated protein kinase C isoforms. *J. Biol. Chem.* 274, 14176–14187

77. Li, J., Ziemba, B. P., Falke, J. J., and Voth, G. A. (2014) Interactions of protein kinase C-α C1A and C1B domains with membranes: a combined computational and experimental study. *J. Am. Chem. Soc.* 136, 11757–11766

78. Poole, A. W., Pula, G., Hers, I., Crosby, D., and Jones, M. L. (2004) PKC-interacting proteins: from function to pharmacology. *Trends Pharmacol. Sci.* 25, 528–535

79. Keum, E., Kim, Y., Kim, J., Kwon, S., Lim, Y., Han, I., and Oh, E. S. (2004) Syndecan-4 regulates localization, activity and stability of protein kinase C-α. *Biochem. J.* 378, 1007–1014

80. Abrahamsen, H., O’Neill, A. K., Kannan, N., Kruse, N., Taylor, S. S., Jennings, P. A., and Newton, A. C. (2012) Peptidyl-prolyl isomerase Pin1 controls down-regulation of conventional protein kinase C isoforms. *J. Biol. Chem.* 287, 13262–13278

81. Cheng, W., Wang, L., Zhang, R., Du, P., Yang, B., Zhuang, H., Tang, B., Yao, C., Yu, M., Wang, Y., Zhang, J., Yin, W., Li, J., Zheng, W., Lu, M., and Hua, Z. (2012) Regulation of protein kinase C inactivation by Fas-associated protein with death domain. *J. Biol. Chem.* 287, 26126–26135

82. Santikulvong, C., and Rozengurt, E. (2007) Protein kinase Ca mediates feedback inhibition of EGF receptor transactivation induced by Gq-coupled receptor agonists. *Cell. Signal.* 19, 1348–1357

83. Chiu, T., Santikulvong, C., and Rozengurt, E. (2003) ANG II stimulates PKC-dependent ERK activation, DNA synthesis, and cell division in intestinal epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 285, G1–G11

84. Goodell, A. L., Oh, H.-S., Meyer, S. A., and Smart, R. C. (1996) Epidermal protein kinase C-β2 is highly sensitive to downregulation and is exclusively expressed in Langerhans cells: downregulation is associated with attenuated contact hypersensitivity. *J. Invest. Dermatol.* 107, 354–359

85. Manzow, S., Richter, K. H., Stempka, L., Fürstenberger, G., and Marks, F. (2000) Evidence against a role of general protein kinase C downregulation in skin tumor promotion. *Int. J. Cancer* 85, 503–507

86. Black, J. D. (2001) Protein kinase C isoforms in colon carcinogenesis: guilt by omission. *Gastroenterology* 120, 1868–1872

87. Neill, G. W., Ghali, L. R., Green, J. L., Ikram, M. S., Philpott, M. P., and Quinn, A. G. (2003) Loss of protein kinase Ca expression may enhance the tumorigenic potential of Gli1 in basal cell carcinoma. *Cancer Res.* 63, 4692–4697

88. Hara, T., Saito, Y., Hirai, T., Nakamura, K., Nakao, K., Chida, K. (2005) Deficiency of protein kinase Ca in mice results in impairment of epidermal hyperplasia and enhancement of tumor formation in two-stage skin carcinogenesis. *Cancer Res.* 65, 7356–7362