Isoenzyme-specific Translocation of Protein Kinase C (PKC)βII and not PKCβI to a Juxtanuclear Subset of Recycling Endosomes

INVolvement of phospholipase D*

Kevin P. Becker and Yusuf A. Hannun‡
From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

Elucidation of isoenzyme-specific functions of individual protein kinase C (PKC) isoenzymes has emerged as an important goal in the study of this family of kinases, but this task has been complicated by modest substrate specificity and high homology among the individual members of each PKC subfamily. The classical PKCβ and PKCβII isoenzymes provide a unique opportunity because they are the alternatively spliced products of the β gene and are 100% identical except for the last 50 of 52 amino acids. In this study, it is shown that green fluorescent protein-tagged PKCβII and not PKCβI translocates to a recently described juxtanuclear site of localization for PKCα and PKCβII isoenzymes that arises with sustained stimulation of PKC. Mechanistically, translocation of PKCβII to the juxtanuclear region required kinase activity. PKCβII, but not PKCβI, was found to activate phospholipase D within this time frame. Inhibitors of phospholipase D (1-butanol and a dominant negative construct) prevented the translocation of PKCβII to the juxtanuclear region but not to the plasma membrane, thus demonstrating a role for phospholipase D in the juxtanuclear translocation of PKCβII. Taken together, these results define specific biochemical and cellular actions of PKCβII when compared with PKCβI.

Members of the protein kinase C (PKC) family of lipid-dependent serine/threonine kinases function as integral signaling intermediates in the transmission of numerous extracellular signals. PKC currently consists of 11 closely related isoenzymes that can be grouped into 3 subfamilies (classical, novel, and atypical) on the basis of lipid cofactor requirements and structural homology (1, 2). A major form of cellular regulation of PKC involves the dynamic redistribution or “translocation” of PKC isoenzymes from cytosol to the plasma membrane. Membrane translocation of PKC is regulated through receptor-coupled lipid hydrolases that act on phosphoinositides and phosphatidylcholine to generate diacylglycerol (DAG) lipid second messengers. The recruitment and activation of PKC at membranes also link activation of the enzyme to the proximity of membrane protein substrates (3).

One of the major targets of PKC is phosphatidylcholine-specific phospholipase D (PC-PLD) (4). Activated PC-PLD hydrolyzes the phosphodiester bond of phosphatidylcholine to generate free choline and phosphatidic acid, which is converted to DAG through the actions of lipid phosphate phosphatases. DAG derived from phosphatidylcholine hydrolysis has been proposed to mediate the reciprocal regulation of PKC, but there are incomplete and conflicting data on this topic (5–7). One possible functional significance of PC-PLD-derived DAG arises from the sustained presence of DAG in the membrane for extended periods of time and in relatively high concentrations in comparison with DAG derived from phosphoinositide-specific PLC, and this phosphatidylcholine-derived DAG has been proposed to mediate long-term cellular processes that require sustained PKC activation (8).

The implication of specific PKC isoenzymes in distinct pathological processes has led to an intense effort to identify and elucidate the mechanisms that regulate isoenzyme-specific functions of PKC. To date, these efforts have been complicated by the large number of highly homologous PKC family members, expression of multiple PKC isoenzymes in each cell type, and the finding that there are only minor differences in substrate selectivity among different PKCs.

One of the best opportunities to study the mechanisms of isoenzyme specificity arises from the alternatively spliced gene products of the classical PKC (cPKC) β gene. This alternative splicing creates two proteins, PKCβI and PKCβII, which display 100% identity over their first 621 amino acids but then diverge in the last 50–52 carboxyl-terminal residues, which are encoded by two consecutive and alternatively spliced exons (9). Importantly, these divergences are 100% conserved across rat, rabbit, and human, suggesting that there are distinct functions encoded by both gene products. To that end, we previously identified residues in the carboxyl terminus of PKCβII that are homologous to actin-binding proteins (10). We demonstrated that with long-term stimulation with phorbol esters, PKCβII differentially interacts with the actin cytoskeleton, where it becomes activated and is then protected from phorbol 12-myristate 13-acetate (PMA)-induced down-regulation.

In another line of investigation, we have recently reported that sustained stimulation (45–60 min) of PKC with DAG-mimicking phorbol and non-phorbol PKC agonists can induce the translocation of PKCo and PKCβII to a subcomponent of the endosomal recycling compartment concentrated in a juxtanuclear location around the microtubule-organizing center/
that this is required for the mechanism of translocation to the juxtanuclear compartment, PKCβII did not. Further analysis showed that PKCβII translocation to the compartment required kinase activity, implicating a substrate-dependent interaction. The specificity for PKCβII versus PKCβI and the requirement for kinase activity raised the possibility that PC-PLD may be involved in the process. Evidence is provided to implicate PLD in the selective translocation of PKCβII to the juxtanuclear compartment. These results define a novel pathway of translocation of PKCβII requiring differential activation of PLD. These data suggest that PKCβII differentially activates PLD and that this is required for the mechanism of translocation to the subset of juxtanuclear recycling endosomes. The implications of these results are discussed.

EXPERIMENTAL PROCEDURES

Materials—Eagle’s minimal essential medium, Dulbecco’s modified Eagle’s medium, and HEPES were from Invitrogen (Gaithersburg, MD). HeLa cells were kindly provided by Dr. Dennis Watson (Medical University of South Carolina, and the HEK 293 cell line was purchased from American Type Culture Collection (Manassas, VA). Palmitic acid was from PerkinElmer Life Sciences. PMA was purchased from Calbiochem. Mouse monoclonal anti-hemagglutinin (HA) antibody was from Covance (Oakland, CA). Phospholipid standards were from Avanti Polar Lipids, Inc. (Alabaster, AL). Anti-mouse TRITC secondary antibodies were from Molecular Probes, Inc. (Eugene, OR). Whatman Silica Gel 60 TLC plates were from Fisher. All other chemicals were from Sigma.

Cell Culture—HeLa cells were maintained in Dulbecco’s modified Eagle’s medium, and HEK 293 cells were maintained in Eagle’s minimal essential medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a 5% CO2 incubator at 37 °C. Cells were passaged every 3–4 days to maintain cells in logarithmic growth.

Plasmid Construction—All recombinant DNA procedures were carried out according to following standard protocols. The wild type pBK-CMV-GFP-PKC-βII and kinase-defective pBK-CMV-GFP-K371R-βII ePKC constructs have been described previously (12). HA-tagged KR-PLD1 or GFP-PKC βII/βI or HA-tagged KR-PLD1 and GFP-PKCβI or GFP-PKCβII using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s recommendations. Twelve h after transfection, cells were treated with either ME soo or 100 nM PMA for 1 h. GFP-tagged cPKC isoenzymes displayed a diffuse cytoplasmic localization with exclusion from the nucleus (Fig. 1, A).

Transphosphatidylation Assay—The cellular activity of PLD was assessed with a modified transphosphatidylation assay (13). Twelve h after transfection, HEK 293 cells were labeled overnight (12–16 h) in 35-mm dishes with 3.0 μCi/ml [3H]palmitic acid in minimal essential medium supplemented with 10% FBS and 0.1% delipidated bovine serum albumin. Cells were then washed three times with PBS, and serum-free minimal essential medium/0.1% bovine serum albumin medium was added. Cells were allowed to recover in serum-free media at 37 °C for 30 min before stimulation. Ten min before stimulation, 0.4% 1-butanol was added to the cells. Cells were stimulated with 100 nM PMA for the specified times and at the noted concentrations. All incubations were performed at 37 °C. After stimulation, the culture media were removed, and the cells were washed rapidly three times with 1 ml of ice-cold PBS. Total cellular membranes were extracted via the method of Bligh and Dyer (14). Lipids were dried down and resuspended in 75 μl of chloroform:methanol (2:1). Fifty μl of the 75-μl volume was loaded per lane. The TLC solvent system consisted of ethyl acetate:iso-octane:acetic acid (9:5:2). A phosphatidyl-butanol standard (Avanti Polar Lipids, Inc.) was included in parallel to confirm lipid species. The plate was sprayed with ENHANCE Spray (PerkinElmer Life Sciences) to amplify the tritium signal and exposed for autoradiography for 24 h. The phosphatidyl-butanol band and the total remaining lipids were scraped and counted separately. The [3H]phosphatidyl-choline band was compared with total labeled lipids to generate the percentage of increase over total labeled lipid. Each experiment was repeated three to five times.

RESULTS

Isoenzyme-specific Translocation of PKCβII to a Subset of Recycling Endosomes—To investigate the isoenzyme specificity of PKCβ translocation to the subset of recycling endosomes centered around the microtubule-organizing center/centrosome described previously (Ref. 11), HeLa cells were transiently transfected with a GFP-tagged PKCβI or PKCβII construct and stimulated with PMA for 1 h. Confocal microscopic imaging revealed that the absence of phorbol ester stimulation, both GFP-tagged cPKC isoenzymes displayed a diffuse cytoplasmic localization with exclusion from the nucleus (Fig. 1, A and B, centrosome (11). Kinetic studies with a fluorescence-conjugated transferrin ligand as a marker of membrane trafficking demonstrated that the cPKC-positive compartment functions to sequester membrane recycling components.
Addition of 100 nM PMA for 1 h induced translocation of GFP-PKCβII to both the plasma membrane and the juxtanuclear location (Fig. 1A, panel 2). In contrast, stimulation of GFP-PKCβ with PMA for 1 h resulted in the translocation of PKCβ to the plasma membrane only, and no further translocation was evident (Fig. 1B, panel 2). These results suggest that amino acid residues encoded within the carboxyl terminus of PKCβ and PKCβII determine the specificity for translocation of PKCβII to this subset of recycling endosomes.

To control for the possibility that the specificity of translocation for the two cPKC isoenzymes was related to different rates of translocation, a time course experiment was performed at 0, 1, 3, and 6 h. No juxtanuclear translocation of PKCβ was evident at any time point, suggesting that the differences between translocation were not related to rates of the process (data not shown).

Of note, in a previous study, Blobe et al. (10) stated that PKCβII contained actin binding sequences within its carboxyl terminus not found in PKCβ. Given the above-mentioned results, we examined the cellular actin pattern during translocation of PKCβII to the juxtanuclear compartment. It was observed that upon stimulation with PMA for 1 h, there was a concentration of actin at the juxtanuclear location and that this overlapped with translocated GFP-PKCβII (Fig. 2, A and B). These results reveal a significant effect of PMA on the reorganization of the actin cytoskeleton, and they demonstrate the co-localization of PKCβII but not PKCβ with the reorganized actin. Moreover, treatment of cells with cytochalasin D, which depolymerizes actin, caused partial unraveling of the pericentriolar actin. Additionally, treatment of cells with cytochalasin D, which depolymerizes actin, caused partial unraveling of the pericentriolar actin. 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blocked by preincubation with 3 μM of the selective cPKC inhibitor Gö 6976 (Fig. 4A). Interestingly, this time frame of PLD activation correlated closely with the translocation of PKCβII to the juxtanuclear compartment.

To determine whether PKCβ and PKCβII regulated PLD differentially, HEK 293 cells were transiently transfected with each PKC isoenzyme, and the transphosphatidylation reaction was assayed. For each experiment, the efficiency of transfection was similar for both isoenzymes and ranged between 40% and 50% of total cell population. Interestingly, in cells that were transfected with PKCβII, there was a higher basal level of PLD activity in the absence of phorbol stimulation when compared with PKCβI. Upon stimulation with PMA, cells that expressed PKCβII demonstrated a 50% increase in phosphatidyl-butanol accumulation (Fig. 4B). In contrast, there was no further stimulation of PLD in cells overexpressing PKCβI. These data disclose significant isoenzyme specificity of activation of PLD by cPKC, and they also suggest the possibility that the isoenzyme specificity in translocation of PKCβ isoenzymes to the juxtanuclear compartment may be related to the differential activation of PLD.

**Translocation of PKCβII to the Juxtanuclear Compartment Is Dependent upon PLD Activity**—To investigate whether PLD activity is required for translocation of PKC to the juxtanuclear compartment, 1-butanol was utilized as an inhibitor of PLD activity. HEK 293 cells were preincubated with 0.4% 1-butanol for 10 min before a 1-h stimulation with PMA. Preincubation with 1-butanol had no effect on the translocation of PKCβIII to the plasma membrane, but there was a complete inhibition of the translocation of PKCβII to the juxtanuclear location (Fig. 5A, panels 1 and 2). This inhibition was specific for the primary alcohol because the secondary alcohol 2-butanol did not block translocation (data not shown).

To confirm this pharmacological inhibition, a catalytically deficient mutant PLD, KR-PLD1, was cotransfected along with GFP-PKCβII into HEK 293 cells, and trafficking of PKCβII to the juxtanuclear compartment was evaluated. When GFP-PKCβII was stimulated with PMA for 1 h, the co-expression of KR-PLD1 did not inhibit the translocation of PKCβII to the plasma membrane, but there was no translocation to the juxtanuclear compartment (Fig. 5B). The ability of the KR-PLD1 mutant to inhibit PLD was evaluated in a transphosphatidylation assay that was performed in parallel with the confocal studies. Transient transfection of KR-PLD1 reduced endogenous PLD activity by ~50% (Fig. 5C), thus confirming that KR-PLD1 inhibited cellular PLD activity. These data demonstrate that PLD activity is involved in the translocation of cPKC to a subset of recycling endosomes.

**DISCUSSION**

In the present study, we have identified a role for the carboxyl-terminal V5 variable region in the differential subcellular localization of PKCβ isoenzymes to a recently defined novel intracellular target of translocation of PKCβ that co-localizes with the microtubule-organizing center/centrosome (11). Investigation into the mechanism revealed that PKCβII translocation to this juxtanuclear compartment, unlike translocation to the plasma membrane, required kinase activity of PKC. The results also revealed differential activation of PLD by PKCβII and not PKCβI. Finally, the results disclosed a critical role for PLD in the differential juxtanuclear translocation of PKCβII. Thus, these data define an isoenzyme-specific function for cPKC in the regulation of PLD and, in turn, the reciprocal regulation of PKC by PLD through the modulation of PKC subcellular localization.
Isoenzyme-specific differences in the subcellular localization and function of PKCβI and PKCβII have been reported previously. Using indirect immunofluorescence and monoclonal antibodies, several groups have observed an isoenzyme-specific association of PKCβI with the actin cytoskeleton (17, 18). In addition, Blobe et al. (10) identified an actin-binding sequence (ABS-1) located within the V5 region of PKCβII that was absent from PKCβI. This sequence was found to be necessary for the phorbol ester-induced translocation of PKCβII to the actin cytoskeleton and for isoenzyme-specific functions associated with this translocation, including protection from down-regulation. Furthermore, Yamamoto et al. (19) identified specific and opposing functional roles for the PKCβ isoenzymes in A10 vascular smooth muscle cells. They reported that upon overexpression of PKCβI, there was a stimulation of cell proliferation, whereas PKCβII overexpression was inhibitory to cell proliferation. This is supported by other data wherein the selective regulation of the expression of PKCβ isoenzymes occurs through differential splicing in lymphoid cells (20, 21). In addition, these studies are also a number of other studies that have proposed specific subcellular localization and/or functions for either one or the other isoenzyme, but in most cases there was no direct comparison between PKCβI and PKCβII, and it is not known whether the proposed functions are isoenzyme-specific (22). The results from this study define a very clear difference in the translocation of PKCβI and PKCβII such that only PKCβII translocates to the juxtanuclear region. PC-PLD is a major downstream target for PKC. Data from overexpression studies and in vitro experiments with purified or recombinant protein indicate that it is the calcium-dependent cPKC isoform that is the primary PKC regulator of PLD in the cell (23–26). Additionally, there are some data to suggest possible isoenzyme-specific differences in the phosphorylation of PKCβ and PKCβII. For instance, a role for PKCβII in PLD-dependent translocation of PKCβ has been suggested through the examination of PLD activity in cells that stably overexpressed PKCβII and displayed an enhanced formation of DAG in response to phorbol ester treatment (27). These results were further supported with studies that used purified PKC and a cell-free system to demonstrate a rank order of potency for PLD activation with “PKC beta 1 > alpha > gamma and beta 2” showing little or no activity (25). Interestingly, these studies are in apparent conflict with the current study. However, while researching the literature, it was discovered that there had been an inconsistency in the nomenclature that was applied initially to the PKCβ isoenzymes. Whereas Ono et al. (29) proposed to name the 673-amino acid splice product, PKCβIII, and the 671-amino acid protein PKCβI, Coussens et al. (30) referred to the 673-amino acid splice product as “PKC beta I” and the shorter, 671-amino acid splice product as “PKC beta II.” Currently, the accepted nomenclature is that the longer gene product is PKCβII and the shorter one, PKCβI (26).

Importantly, a very recent report by Hu and Exton (16) identified residues in the carboxyl terminus of PKCα as critical to the activation of PLD by PKC. It is noteworthy that the carboxyl domain of PKCα shows higher homology to the carboxyl domain of PKCβII than PKCβI. These results underscore the importance of the variable region in the PKC-PLD relationship. Coupled with the results from this study implicating PLD in the juxtanuclear translocation of PKCβII, the differential activation of PLD by PKCβII and PKCβI provides a mechanistic explanation for the selective translocation of PKCβII to the juxtanuclear region. In addition, these results raise the possibility that sustained activation (30–60 min) of DAG/PLA2-responsive PKC may displace novel isoenzyme-specific functions arising from differential subcellular localization. This could potentially lead to disparate isoenzyme effects secondary to differential down-
regulation and the differential access to compartmentalized substrates. It is interesting that we found the compartment to overlap with actin because this is predicted from the study of Blobe et al. (10), who demonstrated biochemically that the differential interaction of PKC/βII with actin prevented PMA-induced down-regulation.

In our previous study, we showed that the juxtanuclear translocation of PKC/II and PKCα regulates the kinetics of the recycling endosomes such that a component of those endosomes becomes sequestered in this juxtanuclear region. The findings of isoenzyme specificity of translocation and interaction with PLD and the role of PLD in the juxtanuclear translocation of PKC/βII therefore suggest specific roles for the PKCα/PKC/βII-PLD pathway in regulation of membrane recycling.

One interesting question raised by the current findings is the mechanism by which PLD participates in regulating juxtanuclear translocation of PKC. The results with the K/R mutant of PLD, which appears to function as a dominant negative, establish the role of PLD in this process. The observation that overexpression of the KR-PLD1 reduces cellular PLD activity by 50% but completely blocks PKC translocation to the juxtanuclear compartment suggests that there is a requirement for a specific subcellular pool of PLD activity in the translocation event. That is, KR-PLD1, which in our system localizes in a pattern similar to wild-type PLD1 (Fig. 5; data not shown), may act to specifically inhibit the PLD1 isoform in that specific compartment but may not inhibit PLD2. Moreover, the results with 1-butanol demonstrate a specific requirement for PLD activity and, presumably, the production of phosphatidic acid. It would be of interest to determine the mechanism by which phosphatidic acid regulates this translocation. (Whereas the current results do not distinguish whether it is phosphatidic acid or a subsequent metabolism to DAG that is required for this translocation, it is presumed that PMA would circumvent the requirement for DAG, thus pointing to a role for phosphatidic acid itself.)

In conclusion, these studies identify specific differences in the function of PKC/β and PKC/βII relating to activation of PLD and juxtanuclear translocation. Moreover, understanding this juxtanuclear translocation promises novel insight into a novel paradigm of PKC function whereby sustained activation results in selective translocation to a novel compartment with selective functions.

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