Dynamic Sequestration of the Recycling Compartment by Classical Protein Kinase C*

Jolanta Idkowiak-Baldys, Kevin P. Becker, Kazuyuki Kitatani, and Yusuf A. Hannun

From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

It has been previously shown that upon sustained stimulation (30–60 min) with phorbol esters, protein kinase C (PKC) α and βII become sequestered in a juxtanuclear region, the pericentrion. The activation of PKC also results in sequestration of transferrin, suggesting a role for PKC in regulating endocytosis and sequestration of recycling components. In this work we characterize the pericentrion as a PKC-dependent subset of the recycling compartment. We demonstrate that upon sustained stimulation of PKC, both protein (CD59, caveolin) and possibly also lipid (Bodipy-GM1) cargo become sequestered in a PKC-dependent manner. This sequestration displayed a strict temperature requirement and was inhibited below 32 °C. Treatment of cells with phorbol myristate acetate for 60 min led to the formation of a distinct membrane structure. PKC sequestration and pericentrion formation were blocked by hypertonic sucrose as well as by potassium depletion (inhibitors of clathrin-dependent endocytosis) but not by nystatin or filipin, which inhibit clathrin-independent pathways. Interestingly, it was also observed that some molecules that internalize through clathrin-independent pathways (CD59, Bodipy-GM1, caveolin) also sequestered to the pericentrion upon sustained PKC activation, suggesting that PKC acted distal to the site of internalization of endocytic cargo. Together these results suggest that PKC regulates sequestration of recycling molecules into this compartment, the pericentrion.

Members of the protein kinase C (PKC)2 superfamily (1) can be grouped into three families (classical, novel, and atypical) that consist of 11 isoforms that are involved in various signal transduction pathways and cellular regulatory processes. Classical isoforms of PKC (cPKC) (α, β, βII, γ) are activated by diacylglycerol (DAG) and calcium, which bind to their C1 and C2 domains, respectively. By contrast, novel PKC isoforms have a truncated C2 domain, and their activation is calcium-independent. Importantly, the phorbol ester 4β-phorbol-12-myristate-13-acetate (PMA) was shown to mimic DAG action on PKC (2) and is an established activator of PKC. The atypical PKCs are calcium- and DAG-independent as they lack a C2 domain and have a truncated C1 domain (3–5).

DAG- and calcium-activated cPKC isoforms rapidly translocate to the plasma membrane within seconds to minutes after generation of DAG or stimulation with PMA, thus accessing membrane substrates (6). It was shown previously by our laboratory that upon sustained stimulation (30–60 min) with PMA, and PKCα and βII become subsequently sequestered in a subset of recycling endosomes, the pericentrion, identified by co-localization with Rab11 (7). Moreover, sustained activation of PKC also resulted in sequestration and retention of recycling transferrin, suggesting a role for PKC in regulating endocytosis and sequestration of recycling components. Mechanistically, this sequestration was shown to be phospholipase D-dependent (8, 9) and was also selectively inhibited by a mechanism involving ceramide formed from the salvage pathway (10).

The uptake and intracellular transport of molecules involves their flow through different endosomal compartments (11). The first step of internalization may either engage clathrin-coated pits (12) or may occur in a clathrin-independent fashion (13). Once internalized, molecules traffic in early endosomes, where often dissociation of ligand from receptor takes place. Next, they are sorted within the sorting endosomes. Molecules that are targeted for degradation enter late endosomes and then the lysosomes. Other molecules are recycled back to the cell surface through the endosomal recycling compartment. By targeting molecules to the recycling compartment, cells can, thus, adjust the availability of receptors or other membrane proteins at the plasma membrane.

These considerations led us to propose that sustained activation of PKC may regulate plasma membrane function through the sequestration of recycling components. Along those lines, recent studies indicate that activation of PKC regulates the availability of the dopamine transporter on the cell surface (14, 15). Also, the norepinephrine transporter was shown to be down-regulated through its sequestration upon PKCα activation (16). Rotmann et al. (17) found that treatment of cells with PMA resulted in internalization (possibly to the pericentrion) of the human cationic amino acid transporter hCAT-1. Furthermore, synaptotagmin IX was shown to be internalized to a compartment that resembles the pericentrion in a PKCα-dependent manner (18). All these data indicate that active PKCα can indeed selectively regulate sequestration of certain molecules from the cell surface.

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental material.
Characterization of Pericentrion

A 0.01% DMSO

Caveolin-1  GFP-PKC-alpha  Overlay

100nM PMA

Caveolin-1  GFP-PKC-alpha  Overlay  Gö 6976  Gö 6976

1h pretreatment  1h posttreatment

B 0.01% DMSO

CD59  GFP-PKC-alpha  Overlay

100nM PMA

CD59  GFP-PKC-alpha  Overlay  Gö 6976  Gö 6976

1h pretreatment  1h posttreatment

C 0.01% DMSO

Flotillin-1  GFP-PKC-alpha  Overlay

100nM PMA

Flotillin-1  GFP-PKC-alpha  Overlay

D 100nM PMA  0.01% DMSO

YFP-5HT2A receptor
In this work we characterize the pericentrion as a PKC-dependent subset of the recycling compartment. Interestingly, the results show that both protein and possibly lipid cargo are sequestered in a PKC-dependent manner. This sequestration displays a strict and unusual temperature requirement and leads to the formation of a distinct membrane structure. These results also show that sequestration of PKC and formation of the pericentrion require plasma membrane integrity and are clathrin-dependent processes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Eagle’s minimal essential media and HEPES were from Invitrogen. The HEK 293 cell line was purchased from American Tissue Culture Collection (Manassas, VA). PMA and Gö 6976 were purchased from Calbiochem. Bodipy-GM1, Bodipy-SM, Bodipy LDL, DilC16 (3), and Lysotracker Red were purchased from Molecular Probes (Eugene, OR). DRAQ5 nuclear dye was from Alexis Biochemicals. Anti-CD59 antibody was a generous gift from Dr. Stephen Tomlinson (Medical University of South Carolina, Charleston, SC). Anti-caveolin-1, anti-flotillin-1, anti-Lamp1, and anti-calnexin antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-(Na+K+)-ATPase was purchased from Abcam.

**Cell Culture**—HEK 293 cells were maintained in Eagle’s minimal essential media supplemented with 10% (v/v) fetal bovine serum 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 following standard protocols. The wild type pBK-CMV-GFP-PKCα was previously described (19). PKCβ2 sequence cloned previously (19) was subcloned into pDsRed-Monomer-N1 (BD Biosciences) XhoI and KpnI sites. The wild type and dominant negative plasmid of dynamin K44A (hemagglutinin-tagged) was a generous gift from Dr. Sammanda Ramamoorthy (Medical University of South Carolina, Charleston, SC).

**Transient Transfection, Indirect Immunofluorescence, and Confocal Microscopy**—Cells were plated onto 35-mm confocal dishes (MatTek) at a density 5 × 10^5 cells/dish and grown for 24 h. Transient transfection of DNA (0.5–1 µg/dish) was performed with the use of Lipofectamine. The expression level of transfected proteins was examined by indirect immunofluorescence microscopy. Cells were fixed for 10 min with 3.7% paraformaldehyde. Samples were then incubated with appropriate primary antibodies and counterstained with TRITC-conjugated anti-mouse IgG (Invitrogen).
formed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. Transfected cells were grown for 24 h in 10% fetal bovine serum media. A transfection efficiency of 40–60% was obtained in these experiments. Cells expressing green fluorescent protein (GFP) fusion alone were viewed after a 10-min fixation with 3.7% formaldehyde. For indirect immunofluorescence, after fixation, cells were permeabilized with 0.1% TritonX-100 for 10 min. Cells were washed 3 times with phosphate-buffered saline (PBS) and blocked with 2% human serum, PBS for 1 h at room temperature. All incubations with primary antibodies were performed in 2% human serum, phosphate-buffered saline overnight at 4 °C. All confocal images were taken with a laser-scanning confocal microscope (LSM 510 Meta; Carl Zeiss, Thornwood, NY). Each microscopic image is representative of 20 fields over a minimum of three experiments, and all images were taken at the equatorial plane of the cell. Raw data images were cropped in Adobe Photoshop® 7.0 for publication.

**RESULTS**

**PMA Induces Sequestration of Plasma Membrane Proteins to the Pericentrion**—Because sustained activation of PKCα and -βI resulted in sequestration of transferrin to a juxtanuclear location (the pericentriol) distinct from the Golgi (7), it became critical to determine whether the PKC effects are specific to transferrin or were more generalized to various recycling components. To this end we investigated the effects of sustained activation of PKC on several plasma membrane proteins including caveolin-1, CD59, and flotillin-1. Stimulation of HEK 293 cells with 100 nM PMA for 60 min resulted in concentration of caveolin-1 (a key constituent of caveolae) (21) at the pericentriol region and its colocalization with GFP-tagged PKCα (Fig. 1A). CD59 is a glycosylphosphatidylinositol-anchored protein present in lipid rafts that plays important roles in regulation of the complement system (22). Sustained stimulation of cells with PMA also resulted in accumulation of CD59 protein in the pericentriol and its colocalization with GFP-tagged PKCα (Fig. 1B). It is important to note that sequestration of CD59 was not blocked by the Golgi disrupting agent brefeldin A (data not shown). Thus, sustained activation of PKC regulates the relocalization/sequestration of several plasma membrane proteins.

Moreover, pretreatment with Gö 6976, an inhibitor specific for members of the cPKC subfamily, prevented sequestration of CD59 and caveolin (Fig. 1, A and B), suggesting that the PMA effects are mediated by cPKCs and not by other potential targets of PMA. Importantly, adding Gö 6976 after PMA treatment caused dissociation of both CD59 and caveolin from the pericentriol (Fig. 1, A and B). Therefore, the effects of cPKC on sequestration require sustained activity of PKC.

In contrast, flotillin-1, a putative marker of lipid rafts, did not seem to sequester into the pericentriol after PMA treatment (Fig. 1C). Instead, it partially colocalized with a Golgi marker (anti-giantin) (data not shown). Also, treatment of cells with PMA did not induce sequestration of overexpressed serotonin receptor in the...
absence of agonist (Fig. 1D). Thus, whereas several proteins respond to sustained stimulation of PKC, this is not a generalized nonspecific effect. Taken together these results demonstrate that long term stimulation of cPKC with PMA leads to selective sequestration of membrane proteins (including PKC) to the PKC-dependent endosomal recycling compartment.

**PMA Induces Sequestration of the Fluorescently Tagged Ganglioside Bodipy-GM1 to the Pericentrion**—Because activation of PKC by phorbol esters led to sequestration of several plasma membrane proteins, it became of great interest to determine whether this translocation was accompanied by membrane lipids or was restricted to membrane proteins. Using a fluorescent analog of the GM1 ganglioside (Bodipy-GM1) (Fig. 2A), it was observed that in the absence of PMA, Bodipy-GM1 localized to the plasma membrane, and some internalization was also observed. Interestingly, upon 1 h of stimulation with PMA, internalized Bodipy-GM1 became more clustered and localized in the pericentrion. This sequestration was also highly dependent on PKC activity as Bodipy-GM1 was dispersed with Gö 6976 treatments. By contrast, localization of fluorescent sphingomyelin was not changed after PMA treatment, revealing a perinuclear localization (Fig. 2B) (distinct from the lysosomes as shown previously (23)). This localization was not influenced significantly by PKC activation with PMA or inhibition with Gö 6976 and, thus, seems to be PKC-independent. Interestingly when Bodipy-SM was analyzed together with fluorescent PKC, it partially colocalized with sequestered PKC, indicating its PKC-independent presence in the recycling compartment. Bodipy SM was also shown to be present in the Golgi (20). We observed that after treatment with brefeldin A there was only some diffusion of internalized sphingomyelin (data not shown), suggesting that it is present in both recycling endosomes and Golgi. These results show that sustained PKC activity leads to sequestration of Bodipy-GM1 and suggest

**PMA Induces Selective Sequestration of Recycling Components**—The above results along with the previous results showing PKC-dependent sequestration of transferrin (7) suggested that
PKC exerted an effect on recycling components. At least two “independent” recycling systems have been identified. Transferrin is a known marker of recycling endosomes, whereas LDL is transported and degraded in the lysosomal pathway. We, therefore, employed fluorescent LDL from human plasma, Bodipy-LDL, as a marker of the degradative pathway. Sustained stimulation of HEK 293 cells did not result in accumulation of Bodipy-LDL (Fig. 3A) in the pericentrion, suggesting a lack of sequestration of lysosomally targeted endosomes by PKC. In addition to LDL as a well studied cargo for the endolysosomal system, the fluorescent lipid analog DiIC₁₆ (3) has been shown by Mukherjee et al. (24) to be targeted to the late endosomes/lysosomes. The results showed that DiIC₁₆ (3) did not colocalize with GFP-tagged PKCa after PMA treatment (Fig. 3B). Additionally, no colocalization of sequestered PKC with the lysosomal protein Lamp1 was seen (Fig. 3C). Taken together these data demonstrate that sustained stimulation of cPKC leads to selective sequestration of membrane recycling components and not endolysosomal components.

Detection of PKC Translocation by Cell Fractionation and Relationship to the Rab11 Compartment—Because PKC translocated to a compartment different from the plasma membrane, we wondered whether this could be detected biochemically. Therefore, translocation of PKC to the pericentrion was also analyzed using cellular fractionation (Fig. 4A) using OptiPrep density gradient and following a protocol that was used previously to detect recycling compartment fractions (25). In control cells, PKC was present throughout the gradient. Treatment of cells with PMA led to the formation of a distinct peak (fractions 19–23) of PKCa that was absent in cells treated with vehicle only. Interestingly this peak was formed within fractions that are enriched in Rab11 protein. These recycling endosomal fractions were separated from lysosomal fractions (Lamp1 positive), and they also separated form the endoplasmic reticulum using calnexin as a marker.

Importantly, when cells were treated with Gö 6976, the PKC-enriched fractions 19–23 disappeared. Instead, PKC became enriched in plasma membrane fractions 3–5 (enriched in (Na+ K+) -ATPase) as well as fractions 25–35 that probably represent dispersed vesicular structures observed under confocal microscopy after Gö 6976 posttreatment (see Fig. 1).

Interestingly, the location of Rab11 as detected by confocal microscopy was not affected significantly by PMA (Fig. 4B). In both vehicle- and PMA-treated cells, Rab11 displayed a juxtanuclear location consistent with previous studies (26). Interestingly Gö 6976 posttreatment led to slight dispersal of this compartment, which could correspond to changes in distribution in the density gradient.

To further analyze relationship between the pericentrion and the Rab11 compartment, colocalization studies were performed. As analyzed by confocal microscopy (Fig. 4C), sequestered PKC colocalized but also extended beyond the Rab11 compartment. Taken together, these data indicate that there are indeed changes in cellular localization (distribution) of PKCa–GFP after PMA treatment and that the pericentrion becomes associated with the Rab11-positive compartment.

Translocation of PKCa and Formation of the Pericentrion Exhibit a Strict Temperature Requirement—While examining translocation of proteins and lipids to the pericentrion, we found that proper incubation temperature during treatments was crucial. Stimulation with PMA for 1 h at 37 °C resulted in the translocation of GFP-PKC α to the pericentrion (Fig. 5A); however, at lower temperatures (4–30 °C), this translocation was not observed (Fig. 5A). Importantly, translocation of PKC at 37 °C was fully reversed with subsequent incubation at 4 °C. Moreover, re-incubation at 37 °C restored translocation of PKC α (Fig. 5A). Notably, at all temperatures, PMA was fully capable of causing translocation of PKC to the plasma membrane, suggesting that it is the sequestration step that displays the strict temperature requirement (Fig. 5A). Temperature also exerted a similar effect on the PMA-induced and PKC-mediated sequestration of the CD59 protein, such that no sequestration was observed at 4 °C. This sequestration also displayed dynamic reversibility such that it was reversed by incubation at 4 °C.
after the initial sequestration at 37 °C and then restored by re-incubating at 37 °C (Fig. 5B). As expected, 30 °C did not affect endocytosis in general, since uptake of transferrin was comparable with cells incubated at 37 °C (Fig. 5C). It should be noted that some sequestration at 30 °C was observed only when cells were incubated with PMA for extended periods of time (data not shows), suggesting that the temperature effect may be a kinetic one affecting the rates of sequestration. These data emphasize the exquisite dependence of translocation of PKC and the sequestration of its targets to the pericentriolar region, which require physiologic temperature.

PKC-dependent Sequestration of Recycling Components Coincides with the Formation of a Distinct Membrane Structure—The active translocation of PKC, Bodipy-GM1, and proteins to the pericentriol suggested that a distinct compartment containing endosomes and membrane structures may exist around the centrosome in a PKC-dependent manner. To visualize this compartment, we employed three-dimensional reconstruction.

Reconstruction of a cell image in three-dimensions using Volocity 3 (Improvision) software showed that the pericentriol is formed by concentration of recycling endosomes in close proximity to the nucleus (Fig. 6B), and this was distinct from the lysosomal compartment (Fig. 6A). These data indicate PKC-dependent structural reorganization in the perinuclear region.

PKC-dependent Sequestration of Molecules to the Pericentriol Is Clathrin-dependent—Next, we investigated which endocytic pathway (clathrin versus caveolar) is involved in the PKC-dependent sequestration. We employed various methods to further analyze this requirement.

When clathrin-dependent endocytosis was blocked by depleting cells of potassium or incubating in hypertonic sucrose, transferrin was not internalized, GFP-PKCa remained on the plasma membrane, and no pericentriol formation was observed (Fig. 7A). Also CD59, sequestration to the pericentriol was inhibited (Fig. 7B). Moreover, we observed inhibition of PKCa sequestration (observed only in about 5% of transfected cells) and pericentriol formation in cells transfected with a dominant negative mutant of dynamin K44A (Fig. 8) that is known to inhibit constriction and budding of clathrin coated pits (27). Thus, the clathrin-dependent pathway appears to be required for the PKCa-dependent formation of the pericentriol irrespective of whether the target cargo utilize clathrin-dependent (e.g. transferrin) or clathrin-independent (e.g. CD59) pathways of internalization.

To inhibit clathrin-independent endocytosis, cells were pre-treated with nystatin or filipin before PMA treatment, and then sequestration of GFP-PKCa, Alexa Fluor transferrin, and CD59 was examined. It was observed that inhibition of clathrin-independent endocytosis did not prevent sequestration of GFP-PKCa or transferrin to the pericentriol (Fig. 7A) (sequestration to pericentriol was still observed in ~60% of cells, a proportion similar to control PMA-treated cells). Thus, the actions of PKC do not require the clathrin-independent pathway. Interestingly, even though both nystatin and filipin were able to inhibit basal internalization of CD59 in untreated cells (data not shown), they were not able to prevent PMA-stimulated sequestration of CD59 (Fig. 7B).

In addition to nystatin and filipin, we employed methyl-β-cyclodextrin (Fig. 7C), which is known to form soluble inclusion complexes with cholesterol (28) and to extract cholesterol from cells in culture (29, 30) and which has been often employed as an inhibitor of caveolae-dependent endocytosis. Pretreatment for 60 min with methyl-β-cyclodextrin (10 mM) completely inhibited PMA-induced translocation of PKC to the pericentriol but not to the plasma membrane. Interestingly when cells were first treated with PMA for 1 h and then treated with meth-
Characterization of Pericentrion

DISCUSSION

In the present work we characterize a PKCα-dependent subset of recycling endosomes, the pericentrion. It is shown that both proteins and possibly also lipids are selectively sequestered to the juxtanuclear region when PKCα is continuously active. Interestingly, the formation of the pericentrion and sequestration of proteins and lipids to this compartment displayed a strict temperature requirement, occurring at 37 °C. This active translocation of PKCα and other membrane constituents appears to lead to the formation of a membranous structure in the perinuclear region. The present data also indicate involvement of clathrin-dependent endocytosis in PKCα sequestration to the pericentrion, although proteins that are internalized through different pathways can be also delivered to this compartment. Although the data present in this manuscript describe changes upon PMA treatment, these results raise a number of important implications as to the regulation and function of the PKC-dependent sequestration of membrane proteins and lipids with physiological agonists.

An interesting and critical observation came with the realization of the strict and unusual temperature requirement for pericentrion formation. Thus, pericentrion formation did not occur at temperatures below 32 °C, emphasizing the dependence of PKCα translocation and sequestration of PKC targets on physiological temperature. It also distinguishes this process from general endocytosis, which is only blocked below 17 °C (37). This strict temperature dependence may explain the delayed appreciation of the perinuclear localization of PKC-dependent sequestration despite previous appreciation of PKC-dependent internalization of target molecules (see below). Although the mechanism of this strict temperature dependence is not known, it is nonetheless a critical observation that needs to be considered in investigations on internalization and recycling.

**FIGURE 7.** Effects of inhibitors of endocytosis on pericentrion formation. HEK 293 cells transiently transfected with GFP-PKCa for 24 h were pretreated with nystatin, filipin, and hypertonic sucrose or depleted of potassium and then treated with 100 nM PMA for 1 h. A, cells were treated in the presence of 10 μg/ml Alexa Fluor transferrin. B, after PMA treatment, cells were fixed, and immunofluorescence for CD59 protein was performed. C, HEK 293 cells pre- or posttreated with 10 nM methyl-β-cyclodextrin (MBCD) and treated with 0.01% Me2SO (DMSO) or 100 nM PMA. The bar represents 10 μm.
The current findings, that not only certain plasma membrane proteins but possibly also specific plasma membrane lipids (such as GM1, based on results with Bodipy-GM1) are enriched in the PKC-dependent pericentrion, have implications as to the structure of the pericentrion as well as to the recycling processes affected by PKC stimulation. This ability of PKC to induce internalization and sequestration of plasma membrane lipids suggested that the site of concentration of the sequestered cargo should show membrane-related structures.

Importantly, this ability of sustained stimulation of PKC to cause sequestration of both proteins and lipids clearly indicates a generalized effect of PKC on the dynamics of recycling rather than specific effects on individual proteins. For example, PKC activation has been implicated in acute receptor desensitization due to direct phosphorylation of these receptors. However, the current results show that the sustained activation of PKC affects the sequestration of glycosylphosphatidylinositol-linked proteins (CD59) as well as lipids, negating a role for direct phosphorylation of the target molecules and suggesting effects on lipid- and protein-containing endosomes. Therefore, these sustained effects (minutes to hours) of PKC on sequestration of endosomes should be distinguished from acute effects (seconds to minutes) on receptor desensitization.

The nature of the cargo subject to PKC-dependent sequestration also begins to illuminate the specific process affected by PKC. Thus, sustained PKC stimulation resulted in sequestration of several recycling membrane proteins (CD59, transferrin receptor, and caveolin) but not molecules destined for the lysosomes (LDL, DilC<sub>16</sub> (3)) or other membrane proteins, such as flotillin, a proposed lipid raft marker that has also been seen to accumulate in maturing phagosomes (38) and in lysosomes and Golgi (39). Thus, this newly recognized subset of PKC-influenced cargo appears to be composed of proteins and lipids that are...
known to be continuously recycled in the cell and that form at least a subset of recycling endosomes.

The observations that sustained activation of PKC results in close association with the Rab11 recycling compartment raises an important question on the relationship of the Rab11-positive endosome to the pericentrion. In fact, these results show that the PKC-dependent sequestration is not synonymous with the Rab11 compartment; that is, the rab11 compartment exists before and independent of PKC stimulation. Rather, PKC causes a relocation and sequestration of recycling endosomes to the Rab11 compartment. Thus, a functional definition of the pericentrion posits is as a dynamic structure whose formation from recycling endosomes is dependent on continued PKC activity. These recycling endosomes are then sequestered to the Rab11 positive compartment after sustained PKC activity.

Based on the present data, we propose (Fig. 9) that PKC regulates the formation of the pericentrion and sequestration of molecules into the pericentrion by clustering of recycling endosomes in the perinuclear region. This regulation does not seem to be at the level of internalization since (a) both molecules that internalize through clathrin-dependent (transferrin, PKCα) and clathrin-independent (caveolin, CD59, Bodipy-GM1) pathways were subject to PKC-dependent sequestration and (b) sustained activation of PKC did not induce sequestration of proteins and lipids destined to the lysosomes (such as LDL and DilC₁₆ (31)). Because it has been proposed that the sorting of cargo between recycling endosomes and endolysosomes occurs after internalization, it becomes further unlikely that PKC acts at a more proximal step in this process of sequestration.

We would also propose that the PKC-induced sequestration arises from effects of PKC on the kinetics of movement of recycling endosomes rather than causing a “terminal” event in the life cycle of recycling endosomes. This is supported by the multiple data that show the reversibility of the process and the requirement for ongoing activation of PKC. Thus, formation of the pericentrion was reversed when cells were taken from 37 to 30 °C (or 4 °C) and became reconstituted once the temperature was restored to 37 °C. Moreover, the process was reversed when PKC inhibitors were added after formation of the pericentrion, demonstrating not only the reversibility of the process but also the strict requirement for continued activity of PKC. Finally, the ability of methyl cyclodextrin to undo PKC-dependent translocation and sequestration after formation of the pericentrion suggests that the effects at the plasma membrane affect the ability of PKC to maintain the formation of the pericentrion. This further supports the conjecture that PKC action is to affect the dynamics of recycling with a resulting profound slowing that appears as “trapping” of recycling endosomes at the pericentrion (Fig. 9). At this point, we are unable to distinguish whether the action of PKC is to accelerate the afferent limb or inhibit the efferent limb of recycling (Fig. 9).

The observation that internalization of PKCα and formation of pericentrion after PMA treatment was clathrin-dependent may appear contradictory to previous work (40) showing that PKCα was localized to caveolae. Interestingly, however, Smart et al. (41) observed that PKC can be displaced from caveoli after treatment with phorbol esters (41). Therefore, it is possible that treatment with PMA leads to a relocalization of PKC to the clathrin-dependent pathway followed by internalization and sequestration in a caveoli-independent manner.

The results from this study coupled with other published studies begin to suggest important roles for the PKC-dependent sequestration of membrane proteins and lipids. Indeed, there are several examples in the literature showing PKC-dependent sequestration of molecules such as the dopamine transporter (14, 15), dopamine receptor (42), parathyroid hormone receptor (43), norepinephrine transporter (16), human cationic amino acid transporter (17), and synaptotagmin IX (18) to a compartment that resembles the pericentrion. Although those studies focused on specific PKC/target interaction, our results would suggest that the effects of PKC in those cases may well be part of the more generalized effects on recycling endosomes described in this study. Thus, such sequestration would provide important regulating events that depend on the presence of these proteins on the cell surface. It is also possible that this mechanism may provide for redirecting the action of some of these targets from the plasma membrane to the perinuclear region.

In conclusion these studies demonstrate the existence of a PKC-dependent sequestration of recycling proteins and possibly also lipids to the pericentrion. Formation of the pericentrion is clathrin-dependent, has strict temperature requirements, and coincides with the formation of membranous structure in a perinuclear region. Moreover, these findings provide novel insights into previously observed PKC-dependent sequestration of molecules.

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