Rapid Protein Kinase D Translocation in Response to G Protein-coupled Receptor Activation

DEPENDEON PROTEIN KINASE C*S

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Protein kinase D (PKD)/protein kinase C (PKC) μ is a serine/threonine protein kinase that can be activated by physiological stimuli like growth factors, antigen-receptor engagement and G protein-coupled receptor (GPCR) agonists via a phosphorylation-dependent mechanism that requires PKC activity. In order to investigate the dynamic mechanisms associated with GPCR signaling, the intracellular translocation of a green fluorescent protein-tagged PKD was analyzed by real-time visualization in fibroblasts and epithelial cells stimulated with bombesin, a GPCR agonist. We found that bombesin induced a rapidly reversible plasma membrane translocation of green fluorescent protein-tagged PKD, an event that can be divided into two distinct mechanistic steps. The first step, which is exclusively mediated by the cysteine-rich domain in the N terminus of PKD, involved its translocation from the cytosol to the plasma membrane. The second step, i.e. the rapid reverse translocation of PKD from the plasma membrane to the cytosol, required its catalytic domain and surprisingly PKC activity. These findings provide evidence for a novel mechanism by which PKC coordinates the translocation and activation of PKD in response to bombesin-induced GPCR activation.

Many hormonal peptides and neurotransmitters bind to G-protein coupled receptors (GPCR) (1–3) that promote G-protein-mediated activation of β isoforms of phospholipase C (4) to produce two second messengers: inositol 1,4,5-trisphosphate, which mobilizes Ca2+ from internal stores; and diacylglycerol, which activates PKC (5, 6). There are multiple related PKC isoforms (7–9), which can be classified into three distinct subgroups on the basis of structural and regulatory differences: the conventional PKCs (α, β1, β2, γ), which are regulated by calcium, diacylglycerol (DAG), and phospholipids; the novel PKCs (δ, ε, η, and θ), which are regulated by DAG and phospholipids; and the atypical PKCs (ζ and λ), regulation of which is less characterized, but that have been proposed to be regulated by D-3 phosphoinositides (10). The DAG-regulated PKC isoforms all bind phorbol esters and are major cellular targets for this class of tumor promoter (11). It is increasingly recognized that each isoform has specific functions in vivo and that the biological activity of individual PKC isoforms is intimately regulated by their subcellular localization (12). However, the mechanisms by which PKC-mediated signals in the plasma membrane are propagated to critical downstream cytosolic targets remain largely undefined.

Protein kinase D (PKD)/protein kinase Cμ is a serine/threonine protein kinase with structural, enzymological, and regulatory properties different from other PKC family members (13, 14). The most salient features of PKD structure include the presence of a catalytic domain distantly related to Ca2+-regulated kinases, a pleckstrin homology (PH) domain that regulates its enzymatic activity, and a highly hydrophobic stretch of amino acids in its N-terminal region (15–17). The N-terminal region also contains a cysteine-rich domain (CRD) comprising a tandem repeat of cysteine-rich, zinc finger-like motifs, which confers high affinity binding to phorbol esters and plays a negative role in the regulation of catalytic activity of PKD (13, 14, 18, 19). PKD can be activated in intact cells by pharmacological agents including biologically active phorbol esters and cell permeant DAGs as well as by physiological stimuli including GPCR agonists, growth factors, and antigen-receptor engagement (1–3, 20–25). In all cases, PKD activation has been shown to be mediated by a PKC-dependent signal transduction pathway that involves the phosphorylation of Ser744 and Ser748 within the activation loop of the catalytic domain of PKD (1, 23, 24, 26, 51).

In unstimulated cells, PKD has been localized to the cytosol and to several intracellular compartments including Golgi and mitochondria (20, 27–31). Recently, it has been demonstrated that treatment of COS-7 cells and lymphocytes with phorbol 12,13-dibutyrate induces a striking and persistent transloc-
tion of PKD from the cytosol to the plasma membrane. Antigen receptor engagement also caused rapid PKD translocation to the plasma membrane but in contrast to the response to phorbol 12,13-dibutyrate, this PKD redistribution was transient, with PKD returning to the cytosol within minutes of antigen engagement (20, 31). These studies demonstrated that the translocation of PKD to the plasma membrane is mediated by its CRD, but the mechanism responsible for its reverse translocation from the plasma membrane to the cytosol has not been defined. Furthermore, very little is known regarding the intracellular distribution of PKD in cells stimulated by neuropeptide agonists that act through GPCRs to induce Gαo-mediated activation of phospholipase C.

In the present study, we examined the temporal and spatial distribution of PKD using real-time visualization of fluorescence-tagged PKD in Swiss 3T3 fibroblasts and MDCK epithelial cells stimulated with bombesin, an agonist that activates PKD through a well characterized Gαo-coupled heptahelical receptor (32). We found that bombesin induces a striking and transient PKD translocation to the plasma membrane of fibroblasts and epithelial cells. Our results demonstrate that the intracellular redistribution of PKD in response to bombesin stimulation can be separated in two distinct mechanistic steps. The first step corresponds to the CRD-mediated plasma membrane translocation of PKD, whereas the second one corresponds to its rapid reverse translocation from the plasma membrane to the cytosol. This last step requires the catalytic domain of PKD and PKC-mediated phosphorylation of Ser744 and Ser748 within the activation loop of PKD. Thus, PKC is not only required for the phosphorylation of PKD at the activation loop Ser744 and Ser748 (see Ref. 51) but also promotes the rapid reverse translocation of PKD. These findings provide evidence for a novel mechanism coordinating the translocation and activation of PKD in response to bombesin-induced GPCR activation in fibroblasts and epithelial cells.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs**—Vectors encoding chimeric fusion proteins between green fluorescent protein (GFP) and PKD, pPKDAPH, pPKDACRD, and PKDp287G have been described previously (31). The single point mutant S916A was generated by PCR site-directed mutagenesis employing the bombesin/gastrin-releasing peptide receptor (33) and PKD wild-type, was assembled into a plasmid expression vector. Rabbit polyclonal anti-PKD/PKCα antibodies (1:1000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or primary antibodies, PKD antibodies (1:500), monoclonal mouse anti-HA (C29; 1:500), and monoclonal mouse anti-KDEL (1:500) (Research Biochemicals, Inc., Natick, MA) were used as primary antibodies. The fixed cells were first washed with 0.05% Tween 20 in PBS, 1% gelatin, and stained at 37°C in 10% buffered formalin and water immersion objectives under 488-nm argon excitation and 500-nm emission. During real-time analysis, 50 cells were analyzed for each experiment and each experiment was performed in quadruplicate. The selected single cell displayed in the appropriate figures was representative of 85% of the population of positive cells.

For video presentation, cell images were captured during 10 min (exposure time 400 ms) every 15 s as a time series of 24-bit uncompressed TIFF files. Selected images were cropped using Adobe Photoshop, version 5.5 (Adobe Systems Inc., San Jose, CA) and assembled with the public domain software GifBuilder version 0.5 (Y. Piguet), and saved as Quicktime (version 4) files (Apple Corp., Cupertino, CA).

Quantitative analysis of the relative plasma membrane fluorescence intensity was performed on images of live cells obtained with a Leica TCS-SP upright laser-scanning confocal microscope (Leica, Heidelberg, Germany) using a 63 × 1.25-NA HCX PL APO water immersion objective under 488-nm argon excitation and 500–550-nm emission. During image acquisition, a constant temperature of 37°C was maintained following the methodology described above. Sequential images before and after bombesin stimulation were captured as uncompressed 8-bit TIFF files using Leica TCS-NT software (version 1.6.587). Quantification was performed using the public domain NIH Image program, version 1.62, and Adobe Photoshop, version 5.5 (Adobe Systems Inc.). The relative change in plasma membrane fluorescence intensity in the cell midsection, before and after bombesin stimulation, was calculated for each condition in four Swiss 3T3 cells by line intensity profiles across the cell plasma membrane.

**Indirect Immunofluorescence**—Swiss 3T3 cells cultured as described above in Lab-Tek chamber slides (Nalgene Nunc International, Naperville, IL) were fixed for 20 min in 25% cold methanol and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at 25°C. The fixed cells were first incubated during 18 h at 25°C in blocking buffer (PBS, 1% gelatin, 0.05% Tween 20) (BB) and then stained at 37°C for 60 min with a rabbit polyclonal anti-PKD/PKCα antibody diluted in BB. Subsequently, the cells were washed with PBS, 0.05% Tween 20 (WF) at 25°C
and contained at 37 °C for 60 min with a mouse monoclonal directed against a Golgi-associated protein (58-kDa Golgi protein) (34). After the cells were thoroughly washed with WF, they were stained at 37 °C for 60 min with fluorescein-conjugated goat-anti-rabbit diluted in BB, washed again with WF, and incubated at 37 °C for 60 min with Texas Red-conjugated goat anti-mouse Immunoglobulins. Finally, the cells were washed with WF and the samples mounted with a Gelvatol-glycerol solution containing 2.5% 1,4-diazobicyclo-[2.2.2]octane. The samples were examined with a Zeiss epifluorescent Axioskop with a Zeiss Plan-Apochromat 40×/1.0 or 63×/1.40 oil immersion objectives. Images were captured as uncompressed 24-bit TIFF files with a SPOT cooled (−12°C) single CCD color digital camera. Three to six coverslips per treatment were analyzed by SPOT version 2.1 software (Diagnostic Instruments, Inc.). Fluorescein or Texas Red signals were observed with HI Q filter sets for fluorescein isoiothyocyanate or rhodamine/tetramethylrhodamine B isothiocyanate, respectively (Chroma Technology). The cell images displayed in the figures are representative of more than 100 cells.

Cell Fractionation—Cytosolic and membrane fractions of Swiss 3T3 cells were prepared according to the method of McKenzie (35). Briefly, the cells were resuspended in ice-cold 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5 (TE buffer), containing protease inhibitors (leupeptin (10 μg/ml) and phenylmethylsulfonyl fluoride (5 mM)) and homogenized by 20 strokes in a glass Potter-Elvehjem type homogenizer. The homogenate was centrifuged at 500 × g for 15 min to pellet nuclei and unbroken cells, leaving a membrane-containing supernatant. Plasma membranes were purified by centrifugation of the supernatant for 30 min at 40,000 × g. The plasma membrane-containing pellet was washed with TE buffer and re-centrifuged for 30 min at 40,000 × g. Proteins in the resulting supernatant fractions were pooled and precipitated with ice-cold acetone. Pellets corresponding to the cytosolic and plasma membrane fraction were resuspended in 2× SDS-PAGE and subsequently analyzed by SDS-PAGE and Western blot analysis.

Western Blot Analysis—Protein samples resuspended in 2× SDS-PAGE sample buffer were resolved under denaturing conditions by 10% SDS-PAGE and transferred to Immobilon-P transfer membranes (Millipore Corp., Bedford, MA) using standard procedures. The membranes were incubated with primary rabbit polyclonal antibody against GFP (Santa Cruz Biotechnology, Palo Alto, CA) or a phosphospecific antiserum that recognizes PKD phosphorylated at Ser316 (36). Western blot analysis with anti-GFP antibodies verified the expression and equal loading of GFP-PKD. The results presented in Fig. 1B indicate that activation of either endogenously or exogenously expressed bombesinGPCR induces a rapid and persistent increase in the catalytic activity of GFP-PKD within fibroblasts and epithelial cells.

Intracellular Distribution of Fluorescence-tagged and Endogenous PKD in Swiss 3T3 Cells—Previous reports indicated that PKD partially localizes to the Golgi compartment as well as to the cytosol, mitochondria and plasma membrane in different cell types (20, 27–31). We also detected that in Swiss 3T3, in addition to its cytosolic localization, some GFP-PKD fluorescence was more pronounced at the perinuclear area, consistent with a partial localization of this protein to the Golgi compartment. Interestingly, we also found that when Swiss 3T3 cells were shifted from 37 °C to 20 °C, GFP-PKD accumulated within 5 min in a single area adjacent to the nucleus (Fig. 2, A–C), consistent with Golgi localization. Similarly, when Swiss 3T3 expressing GFP-PKD were preincubated at 20 °C and then shifted to 37 °C, the fluorescent signal previously detected at one prominent spot in the perinuclear area redistributed to the cytosol within 7 min (Fig. 2, D and E). These results indicate that GFP-PKD can associate with the Golgi and imply that its dissociation from this compartment is a temperature-sensitive process. We did not detect any substantial redistribution of GFP-PKD to the Golgi compartment after bombesin stimulation in Swiss 3T3. These findings, in addition to those already published (20, 27–31), suggest that the preferential localization of PKD to different cellular compartments may be dependent on the cell type and experimental conditions.

To rule out possible interference with the normal intracellular distribution of PKD due to the presence of the fluorescent tag at its N terminus, we analyzed the localization of a chimeric protein between the RFP from Discosoma sp. fused to the C terminus of PKD. No difference was detected at 37 °C in the distribution of PKD-RFP compared with GFP-PKD in Swiss 3T3 cells (Fig. 2F). Further support for these observations was obtained by indirect immunofluorescence analysis of the distribution of endogenous PKD and the Golgi-associated 58-kDa protein in Swiss 3T3 cells. As illustrated by representative cells displayed in Fig. 2 (G and I), the distribution of endogenous PKD at 37 °C was very similar to that detected with either GFP-PKD or PKD-RFP at the same temperature (Fig. 2, A, E, and F). The inclusion of the immunizing peptide encompassing the C terminus of PKD completely prevented the staining of the endogenous PKD (data not shown). We detected, in ~20% of the analyzed cells, an increased PKD signal in a single area adjacent to the nucleus which colocalized with the signal corresponding to the Golgi-associated 58-kDa protein, an specific Golgi marker (34, 38). However, the amount and distribution of PKD in the remaining cells was very homogenous. Further support that the distribution of the fluorescence-tagged PKDs was comparable to that of native PKD was seen by colocalization with specific GFP-PKD (Fig. 2, compare I and J with G and H). Subsequent experiments examining agonist induced GFP-PKD re-
Distribution were carried out at 37 °C.

Activation of Bombesin-GPCR Induces Transient Translocation of GFP-PKD from the Cytosol to the Plasma Membrane of Fibroblasts and Epithelial Cells—Next, we analyzed the effect of bombesin stimulation on the intracellular distribution of GFP-PKD expressed in either Swiss 3T3 cells or MDCK cells. As illustrated by the image presented in Fig. 3, GFP-PKD expressed in unstimulated Swiss 3T3 cells was distributed throughout the cytosol and excluded from the nucleus with very little fluorescent signal localized to the plasma membrane.

Real time imaging revealed that bombesin stimulation of Swiss 3T3 cells induced a rapid translocation of GFP-PKD to the plasma membrane, causing a localized fluorescence in the plasma membrane at the cell periphery and partially masking the cell nuclei (Fig. 3A). Translocation of GFP-PKD occurred within 2 min and reached a maximum between 3 and 5 min.
The association of GFP-PKD with the plasma membrane was transient. The reverse translocation of GFP-PKD from the plasma membrane to the cytosol was virtually complete within 10 min of bombesin stimulation (Fig. 3A). A similar time course of GFP-PKD translocation to the plasma membrane and reverse translocation to the cytosol in bombesin-stimulated Swiss 3T3 fibroblasts was also observed and quantified by laser confocal microscopy (Fig. 3B) and subsequently confirmed by biochemical fractionation, as described below (Fig. 9).

Because the cell surface distribution of membrane proteins in fibroblasts and epithelial cells can be different, we also analyzed the translocation of GFP-PKD from the plasma membrane to the cytosol in MDCK epithelial cells cotransfected with plasmids encoding GFP-PKD and bombesin receptor. MDCK cells are one of the best studied epithelial cell model systems (39). The distribution of GFP-PKD in unstimulated MDCK cells was almost identical to that in Swiss 3T3 cells. Specifically, GFP-PKD was homogenously distributed throughout the cytosol and excluded from the nucleus in the majority of the MDCK cells (Fig. 3A). Stimulation of MDCK cells with 10 nM bombesin promoted a striking translocation of GFP-PKD to the plasma membrane detected as a localized fluorescence at the cell periphery, which was evident within 2 min and reached a maximum between 3 and 5 min (Fig. 3A). Redistribution of GFP-PKD to the plasma membrane was also evident from the partial masking of the cell nuclei by the diffuse fluorescence signal at the cell surface. A prominent localization of GFP-PKD was also detected at intercellular plasma membrane junctions.

Similar to the data obtained with Swiss 3T3 cells, bombesin-induced GFP-PKD translocation to the plasma membrane of MDCK cells was transient. The fluorescent signal previously detected at the plasma membrane returned to the cytosol within 10 min of bombesin stimulation (Fig. 3A). The remarkable dynamics of the transient translocation of GFP-PKD in MDCK cells in response to activation of the bombesin GPCR can be observed as a movie (see panel a of the supplemental materials, available in the on-line version of the journal). In contrast, the distribution of GFP (detected in the nuclei as well as throughout the cytosol of both cell types) was not affected by bombesin stimulation in either Swiss 3T3 fibroblasts or MDCK cells (Fig. 3A). The precise timing of GFP-PKD translocation varied from cell to cell, but consistent maximal plasma membrane localization of GFP-PKD was detected within 3–5 min after bombesin stimulation. GFP-PKD then dissociated from the plasma membrane, returning to the cytosol completely within 8–10 min of bombesin stimulation. The morphology of non-transfected Swiss 3T3 or MDCK cells was indistinguishable from GFP- or GFP-PKD-transfected ones (data not shown).
The results presented in Fig. 3 demonstrate that agonist stimulation of the bombesin GPCR induces a striking and transient translocation of GFP-PKD to the plasma membrane in both Swiss 3T3 fibroblasts and MDCK epithelial cells.

The Cysteine-rich Domain of PKD Is Sufficient to Mediate the Plasma Membrane Translocation of GFP-PKD in Response to GPCR Activation—In order to identify the domain(s) of PKD responsible for its translocation to the plasma membrane in response to GPCR activation, we examined the redistribution of a set of GFP-PKD mutants (see Fig. 1A). The N-terminal regulatory region of PKD contains a DAG-binding CRD comprising a tandem repeat of cysteine-rich motifs and a PH domain (13). To determine the contribution of the CRD and the PH domain to bombesin-induced PKD translocation, different GFP-tagged PKD mutants were expressed transiently in either Swiss 3T3 or MDCK cells, and the translocation of these molecules was monitored by real-time imaging of live cells after bombesin stimulation.

The GFP-PKD-ΔCRD mutant contains a deletion of the entire CRD domain, whereas the GFP-PKD-P287G mutant contains a proline to glycine substitution within the second cysteine-rich motif of the CRD. In both cases, binding of phorbol esters/DAG to PKD is prevented (19). As shown in Figs. 4 and 5, the deletion of the entire catalytic domain of PKD (GFP-PKD-Cat) and GFP and the CRD of PKD (GFP-PKD-CRD) in unstimulated and bombesin-stimulated cells. As illustrated in Figs. 4 and 5, the deletion of the entire catalytic domain of PKD did not interfere with bombesin-induced plasma membrane translocation of the resulting fusion protein GFP-PKD-ΔCat, despite its partial nuclear localization. The results in Figs. 4 and 5 also show that bombesin stimulation promoted the translocation to the plasma membrane of GFP-PKD-CRD in both Swiss 3T3 and MDCK cells, demonstrating that the CRD is necessary and sufficient to mediate the translocation of PKD to the plasma membrane in response to GPCR activation. A summary of the translocation properties of the different mutants analyzed is shown in Fig. 1A.

PKC Kinase Activity Inhibition Prevents the Rapid Reverse Translocation of GFP-PKD from the Plasma Membrane to the Cytosol after GPCR Activation—The analysis of the distribution of the PKD fusion proteins lacking catalytic domains revealed an important difference between these proteins and GFP-PKD. In contrast to GFP-PKD, GFP-PKD-ΔCat and GFP-PKD-CRD remained associated with the plasma membrane for 25–30 min after bombesin stimulation (data not shown), suggesting that the rapid reverse translocation of the fluorescence-labeled PKD required its catalytic domain. We hypothesize that the activation of the catalytic domain of PKD plays a major role in promoting its rapid reverse translocation from the plasma membrane to the cytosol. We examined this hypoth-

**Fig. 4.** Plasma membrane translocation of GFP-PKD mutants in Swiss 3T3 cells in response to bombesin stimulation. Swiss 3T3 cells were transfected with constructs encoding a set of different PKD mutants fused to GFP and imaged in real time 18 h after transfection, at 37 °C. Cells expressing the GFP proteins were visualized with an epifluorescence microscope and representative images captured immediately before and at the indicated times after 10 nM bombesin stimulation. Bar, 10 μm.
esis using pharmacological and mutational approaches that prevent PKD activation in response to bombesin GPCR stimulation.

Bombesin- and G\textsubscript{q}-induced PKD activation is mediated by a PKC-dependent signal transduction pathway (1, 23, 24, 26, 32). These studies have placed PKD downstream of classical/novel PKC enzymes in a hierarchical signaling cascade regulated by DAG. To examine the role of PKCs in the regulation of GFP-PKD translocation by the bombesin GPCR, Swiss 3T3 and MDCK cells were preincubated with either Ro 31-8220 or GF I, which inhibit PKCs and prevent PKD activation (1), and then the distribution of GFP-PKD in these cells was monitored after bombesin stimulation. Treatment with either Ro 31-8220 or GF I did not affect the plasma membrane translocation of GFP-PKD, indicating that classical/novel PKCs are not necessary for PKD translocation. This conclusion is consistent with the notion that PKD translocation is mediated by the CRD. However, the inhibitors of PKC activity dramatically delayed the plasma membrane dissociation of GFP-PKD (Fig. 6), indicating that the kinase activity of PKC is necessary for the rapid reverse translocation of GFP-PKD. These results were confirmed by biochemical fractionation as described below (Fig. 9).

Mutations of Ser\textsuperscript{744} and Ser\textsuperscript{748}, within the Activation Loop of PKD, Prevent the Rapid Reverse Translocation of GFP-PKD from the Plasma Membrane to the Cytosol after GPCR Activation—As shown in the accompanying paper (51), bombesin stimulation of either Swiss 3T3 or MDCK cells induces rapid phosphorylation of Ser\textsuperscript{744} and Ser\textsuperscript{748}, within the activation loop of PKD (1, 23, 24, 26). Here we examined whether the activation loop phosphorylation is also required for rapid PKD translocation. If phosphorylation of Ser\textsuperscript{744} and Ser\textsuperscript{748} were necessary for the plasma membrane dissociation of PKD, their substitution by non-phosphorylatable amino acids should impair the dissociation of the mutated protein. In order to test this hypothesis, we replaced Ser\textsuperscript{744} and Ser\textsuperscript{748} with alanine and examined the distribution properties of the resulting fusion protein GFP-PKD-S744A/S748A in Swiss 3T3 and MDCK cells after bombesin stimulation.

The distribution of GFP-PKD-S744A/S748A in non-stimulated cells was identical to that of GFP-PKD. After bombesin stimulation, the translocation and association of GFP-PKD-S744A/S748A to the plasma membrane of both cell types did not show any significant difference, as compared with GFP-PKD. Thus, substitution of Ser\textsuperscript{744} and Ser\textsuperscript{748} for alanine did not prevent the plasma membrane translocation of GFP-PKD-S744A/S748A (Figs. 7 and 8). In addition, mutation of Ser\textsuperscript{916}, an autophosphorylation site in the C terminus of PKD (36), did not prevent the plasma membrane translocation of GFP-PKD-S916A (Figs. 7 and 8). In addition, simultaneous substitution in PKD of the serine residues 744, 748, and 916 for alanine had no effect on the plasma membrane translocation or GFP-PKD-S744A/S748A/S916A after bombesin stimulation of Swiss 3T3 and MDCK cells (data not shown).

The salient feature of the results shown in Figs. 7 and 8 is...
that GFP-PKD-S744A/S748A did not return from the plasma membrane to the cytosol even after 10 min of bombesin stimulation. This persistent residence of GFP-PKD-S744A/S748A in the plasma membrane over time can also be observed as a movie (see panel b of the supplemental materials, available on-line). Furthermore, quantitative analysis by laser confocal microscopy of over time relative fluorescence intensity in the plasma membrane of Swiss 3T3 cells expressing GFP-PKD-S744A/S748A also showed that this protein did not return to the cytosol after 10 min of bombesin stimulation (Fig. 7B). A significant reverse translocation of GFP-PKD-S744A/S748A was seen 25–30 min later than GFP-PKD (data not shown). In contrast, GFP-PKD-S916A translocated to the plasma membrane and reverse translocated to the cytosol as rapidly as GFP-PKD following bombesin stimulation (Figs. 7 and 8). Thus, either pharmacological inhibition of PKC or mutation of Ser744 and Ser748 to alanine strikingly prevented the rapid reverse translocation of fluorescence-tagged PKD after bombesin stimulation in both Swiss 3T3 fibroblasts and MDCK epithelial cells.

Biochemical fractionation of Swiss 3T3 expressing GFP-PKD or GFP-PKD-S744A/S748A after bombesin stimulation was employed to further confirm a role of PKC and Ser744 and Ser748 in the reverse translocation of PKD. Western blot analysis of the soluble (cytosolic) and particulate (membrane) fractions, using anti-GFP antibody, showed a significant increase of GFP-PKD in the particulate fraction within 1 min of bombesin stimulation (Fig. 9). After 5 min of bombesin stimulation, the distribution of GFP-PKD was indistinguishable from that in the unstimulated cultures. Thus, biochemical fractionation confirmed our evidence from real-time imaging that bombesin stimulation induces transient redistribution of GFP-PKD in Swiss 3T3 cells.

Pretreatment of the cells with the PKC inhibitor Ro 31-8220...
FIG. 8. Plasma membrane association of GFP-PKD mutants in MDCK cells in response to bombesin stimulation. MDCK cells were transfected with constructs encoding different PKD mutants fused to GFP and imaged in real time, 18 h after transfection, at 37°C. Cells expressing the GFP fusion proteins were visualized with an epifluorescence microscope and representative images captured immediately before and at the indicated times after 10 nM bombesin stimulation. Bar, 10 μm. Selected cell regions were magnified for comparison (insets).

FIG. 9. Biochemical fractionation analysis: Effect of PKC inhibition or mutations in the activation loop of PKD on the plasma membrane association of GFP-PKD fusion proteins after bombesin stimulation. Swiss 3T3 cells transfected with constructs encoding GFP-PKD and GFP-PKD-S744A/S748A were fractionated into soluble (cytosolic) (S) and particulate (membrane) (P) fractions (see "Experimental Procedures").

GFP-PKD did not affect the membrane translocation of GFP-PKD after bombesin stimulation, as revealed by its detection in the particulate fraction (Fig. 9). However, preincubation of Swiss 3T3 cells with Ro 31-8220 impaired the reverse translocation of GFP-PKD from the particulate to the soluble fraction (Fig. 9). A similar impairment in reverse translocation was observed with GFP-PKD-S744A/S748A. This fusion protein translocated from the soluble to the particulate fraction of bombesin-stimulated Swiss 3T3 within 3 min of stimulation. However, GFP-PKD-S744A/S748A, in contrast to GFP-PKD, was impaired in its reverse translocation to the cytosol after bombesin stimulation (Fig. 9).

Although we cannot rule out that other post-translational modifications of PKD may also be involved in its plasma membrane dissociation, our results demonstrate, for the first time, that the kinase activity of PKC and the phosphorylation of Ser744 and Ser749 are key events in the rapid intracellular translocation of PKD.

DISCUSSION

In the present study, we used real-time visualization as well as biochemical fractionation of GFP-PKD chimeras to demonstrate that agonist activation of the bombesin GPCR induces a redistribution of PKD to the plasma membrane of fibroblast and epithelial cells. PKD reverse translocated from the plasma membrane to the cytosol within minutes of bombesin stimulation. In order to elucidate the mechanism(s) involved in bombesin-induced PKD translocation, we analyzed the redistribution of a variety of PKD mutants. This analysis revealed that PKD translocation could be divided into at least two entirely different mechanistic steps: 1) PKD translocation from the cytosol to the plasma membrane and 2) reverse translocation from the plasma membrane to the cytosol.

The first step, bombesin-stimulated plasma membrane PKD translocation, is completely prevented by deletion of the CRD and severely impaired by a single amino acid substitution in the second cysteine-rich motif of the CRD (P287G), in agreement with results indicating that this motif is responsible for mediating DAG binding to PKD. Furthermore, a fusion protein consisting of GFP and the CRD of PKD rapidly translocated to the plasma membrane in response to bombesin stimulation in both fibroblasts and epithelial cells. We conclude that the CRD is both necessary and sufficient to mediate the first phase of PKD translocation in response to GPCR activation. These results are consistent with previous findings using GFP-PKD in phorbol ester-stimulated COS-7 cells and antigen-stimulated T and B lymphocytes.

The mechanism(s) underlying the second step of PKD trafficking, namely PKD reverse translocation, has not been examined in previous studies. It is known that bombesin binding to its cognate GPCR promotes Goα-mediated activation of β isoforms of phospholipase C to produce the second messengers inositol 1,4,5-trisphosphate and DAG, which can also be gen-
The rapid reverse translocation of PKD is coupled to PKC-mediated PKD activation. Specifically, pharmacological inhibition of PKC or mutation to alanine of Ser744 and Ser748, which are located in the activation loop of PKD, strikingly retarded the rapid reverse translocation of PKD after bombesin stimulation in both Swiss 3T3 and MDCK cells. The results presented in the accompanying paper (51) demonstrate that bombesin induces rapid phosphorylation of Ser744 and Ser748 in both Swiss 3T3 fibroblasts and MDCK epithelial cells, as indicated by using novel antibodies directed against the phosphorylated state of these residues. We conclude that PKCs not only play a critical role in PKD activation but also are required to trigger the rapid reverse translocation of PKD.

Recent studies demonstrated that translocation of Aplysia PKC as well as vertebrate PKCδII is regulated by autophosphorylation (40, 45). Although these studies also concluded that phosphorylation promotes plasma membrane dissociation of these kinases, there are important differences between our results with PKD and those findings with PKCs. Although PKC autophosphorylation is thought to occur during protein maturation (46), PKD phosphorylation is acutely induced through a PKC-dependent pathway (1, 23, 24). Furthermore, phosphorylation-mediated plasma membrane dissociation of PKCs has been proposed to act as a feedback mechanism that leads to signal termination (40), whereas PKD phosphorylation leads to its activation (26).

An important role of PKCs in PKD activation has been documented in a variety of cell types stimulated by many stimuli including neuroptides, bioactive lipids, growth factors, antigens, constitutively activated Goq, and oxidative stress (1–3, 20–25, 32, 36, 50). In all these cases, the activity of PKD is no longer dependent on membrane-delimited second messengers; therefore, PKD can be active in the cytosol. Indeed, our own results indicate that GAPK-PKD remains active long after it returned to the cytosol in bombesin-stimulated fibroblasts and epithelial cells (see Fig. 1B). An attractive hypothesis is that PKCs coordinate PKD activation with rapid reverse translocation thus providing a novel mechanism that ensures that activated PKD moves efficiently away from the plasma membrane into the interior of the cell where it propagates DAG-PKC signals initiated at the cell surface.

Acknowledgments—We thank R. Waldron, J. Sinnett-Smith, and C. Hurd for helpful discussions.

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