Sequential Protein Kinase C (PKC)-dependent and PKC-independent Protein Kinase D Catalytic Activation via G<sub>q</sub>-coupled Receptors

DIFFERENTIAL REGULATION OF ACTIVATION LOOP SER<sup>744</sup> AND SER<sup>748</sup> PHOSPHORYLATION*

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Protein kinase D (PKD) is a serine/threonine protein kinase rapidly activated by G protein-coupled receptor (GPCR) agonists via a protein kinase C (PKC)-dependent pathway. Recently, PKD has been implicated in the regulation of long term cellular activities, but little is known about the mechanism(s) of sustained PKD activation. Here, we show that cell treatment with the preferential PKC inhibitors GF 109203X or Go<sup>6</sup>983 blocked rapid (1–5-min) PKD activation induced by bombesin stimulation, but this inhibition was greatly diminished at later times of bombesin stimulation (e.g. 45 min). These results imply that GPCR-induced PKD activation is mediated by early PKC-dependent and late PKC-independent mechanisms. Western blot analysis with site-specific antibodies that detect the phosphorylated state of the activation loop residues Ser<sup>744</sup> and Ser<sup>748</sup> revealed striking PKD-independent phosphorylation of Ser<sup>748</sup> as well as Ser<sup>744</sup> phosphorylation that remained predominantly but not completely PKC-dependent at later times of bombesin or vasopressin stimulation (20–90 min). To determine the mechanisms involved, we examined activation loop phosphorylation in a set of PKD mutants, including kinase-deficient, constitutively activated, and PKD forms in which the activation loop residues were substituted for alanine. Our results show that PKC-dependent phosphorylation of the activation loop Ser<sup>744</sup> and Ser<sup>748</sup> is the primary mechanism involved in early phase PKD activation, whereas PKD autophosphorylation on Ser<sup>748</sup> is a major mechanism contributing to the late phase of PKD activation occurring in cells stimulated by GPCR agonists. The present studies identify a novel mechanism induced by GPCR activation that leads to late, PKC-independent PKD activation.

A rapid increase in the synthesis of lipid-derived second messengers with subsequent activation of protein phosphorylation cascades has emerged as a fundamental signal transduction mechanism triggered by multiple extracellular stimuli, including hormones, neurotransmitters, chemokines, and growth factors (1). Many of these agonists bind to G protein-coupled receptors (GPCRs), activate heterotrimeric G proteins and stimulate isoforms of the phospholipase C family, including β, γ, δ, and ε (reviewed in Refs. 1 and 2). Activated phospholipase Cs catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate mobilizes Ca<sup>2+</sup> from intracellular stores (3, 4) whereas DAG directly activates the classic (α, β, and γ) and novel (δ, ε, η, and θ) isoforms of PKC (5–7). Although it is increasingly recognized that each PKC isozyme has specific functions <i>in vivo</i> (5–8), the mechanisms by which PKC-mediated signals are propagated to critical downstream targets remain incompletely defined.

PKD, also known initially as PKCμ, (9, 10), and two recently identified serine protein kinases termed PKD2 (11) and PKC<sub>μ</sub>/PKD3 (12, 13), which are similar in overall structure and primary amino acid sequence to PKD (14), constitute a new protein kinase family within the Ca<sup>2+</sup>/calmodulin-dependent protein kinase group (11) and separate from the previously identified PKCs (11). Salient features of PKD structure include an N-terminal regulatory region containing a tandem repeat of cysteine-rich zinc finger-like motifs (termed the cysteine-rich domain) that confers high affinity binding to phorbol esters and DAG (9, 16, 17), followed by a pleckstrin homology (PH) domain that negatively regulates catalytic activity (18, 19). The C-terminal region of the PKDs contains its catalytic domain, which is distantly related to Ca<sup>2+</sup>-regulated kinases.

In unstimulated cells, PKD is in a state of low kinase catalytic activity maintained by the N-terminal domain, which represses the catalytic activity of the enzyme by autoinhibition. Consistent with this model, deletions or single amino acid substitutions in the PH domain result in constitutive kinase activity (18–20). Physiological activation of PKD within cells occurs via a phosphorylation-dependent mechanism first identified in our laboratory (21). In response to cellular stimuli, PKD is converted from a low activity form into a persistently active form that is

4 The abbreviations used are: GPCR, G protein-coupled receptor; PKC, protein kinase C; PKD, protein kinase D; DAG, diacylglycerol; PH, pleckstrin homology; GFP, green fluorescent protein; MARCKS, myristoylated alanine-rich C kinase substrate.

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Throughout these studies, multiple lines of evidence indicated that PKC activity is necessary for rapid PKD activation within intact cells. For example, rapid PKD activation was selectively and potently blocked by cell treatment with preferential PKC inhibitors (e.g. GF 109203x or Gö 6983) that do not directly inhibit PKD catalytic activity (21, 22), implying that PKD activation in intact cells is mediated, directly or indirectly, through PKCs. In line with this conclusion, cotransfection of potential PKC inhibitors (selectively and potently blocked by cell treatment with preferential PKC inhibitors) resulted in robust PKD activation in the absence of cell stimulation (21, 44 – 46). Many reports demonstrated the operation of a rapid PKC/PKD signaling cascade in response to multiple GPCR agonists in a broad range of cell types, including normal and cancer cells (reviewed in Ref. 14). Our previous studies identified Ser744 and Ser748 in the PKD activation loop (also referred as the activation segment or ‘T-loop’) as phosphorylation sites critical for PKC-mediated PKD activation (reviewed in Ref. 14). Collectively, these findings demonstrated the existence of rapidly activated PKC-PKD protein kinase cascade(s) and raised the possibility that some PKC-dependent biological responses involve PKD acting as a downstream effector.

PKD has been reported recently to mediate several important cellular activities and processes, including signal transduction (30, 47 – 49), chromatin modification (50), Golgi organization and function (51, 52), c-Jun function (47, 53, 54), NFκB-mediated gene expression (43, 55, 56), and cell survival, migration, and differentiation and DNA synthesis and proliferation (reviewed in Ref. 14). Thus, mounting evidence indicates that PKD has a remarkable diversity of both its signal generation and distribution and its potential for complex regulatory interactions with multiple downstream pathways, leading to multiple responses, including long term cellular events. Despite increasing recognition of its importance, very little is known about the mechanism(s) of sustained PKD activation as opposed to the well documented rapid, PKC-dependent PKD activation.

The results presented here demonstrate that prolonged GPCR-induced PKD activation is mediated by sequential PKC-dependent and PKC-independent phases of regulation. We report here, for the first time, that PKD autophosphorylation on Ser748 is a major mechanism contributing to the late phase of PKD activation occurring in cells stimulated by GPCR agonists. The present studies expand previous models of PKD regulation by identifying a novel mechanism induced by GPCR activation that leads to late, PKC-independent PKD activation.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—COS-7 cells were maintained by subculture in 10-cm tissue culture plates every 3–4 days in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 10% CO2. For experimental dishes, cells were subcultured at 6 × 10⁶ cells/ml in 6-cm (5-ml) dishes on the day prior to transfections. All transfections and cotransfections were carried out with equivalent amounts of DNA (4 μg/6-cm dish). Transfections were carried out in Opti-MEM (Invitrogen) using Lipofectamine Plus according to the manufacturer’s suggested conditions (Invitrogen). Transfected cells were incubated for 24 h before analysis.

cDNA Constructs Used in Transfections—The cloning of the cDNA encoding PKD into pDNA3 and the vector encoding the chimeric fusion protein between green fluorescent protein (GFP) and PKD, GFP-PKD, have been described previously (16, 57). BNR-pCD2 containing the cDNA encoding the bombesin/GRP receptor was kindly provided by Dr. J. F. Battey (NIDCD, National Institutes of Health, Bethesda, MD). The Arginine vasopressin receptor 1A was obtained from the University of Missouri-Rolla cDNA Resource Center. Vectors encoding fusion proteins between GFP and PKD mutants PKD-ΔPH, PKD-574A/574B, and PKD-P287G were previously described (38, 58). Chimeric fusion proteins between GFP and the PKD mutants 574A, 574B, D733A (59), and K618N (21) were generated by subcloning PKD constructs into the EcoRI site of a pEF-link2-GFP-C3 expression vector. All GFP constructs were verified by restriction enzyme analysis and sequencing as well as Western blot analysis to check their expression in transfected cells.

Immunoprecipitations—Transfected COS-7 cells were washed twice with Dulbecco’s modified Eagle’s medium and equilibrated in 5 ml of the same medium at 37 °C for 1–2 h. Some dishes were treated with various pharmacological agents during this equilibration period or with agonists for different times at the end of this period, as indicated in the corresponding figure legends. Cells were lysed in buffer A (50 mM Tris-HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 100 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride (Pefabloc), and 1% Triton X-100). GFP-PKD was immunoprecipitated at 4 °C for 3 h with GFP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), as previously described (13). PKD was immunoprecipitated with the PKD (PKD C-20) antiserum (1 μg/ml) raised against the C-terminal region of PKD (Santa Cruz Biotechnology). The immune complexes were recovered using protein A coupled to agarose.

In Vitro Kinase Assays—Immune complexes were washed twice with lysis buffer and then twice with kinase buffer consisting of 30 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 1 mM dithiothreitol. Autophosphorylation reactions were initiated by combining 20 μl of immune complexes with 5 μl of a phosphorylation mixture containing 100 μM [γ-32P]ATP (specific activity, 6000 Ci/mmol) in kinase buffer. Following incubation at 30 °C for 10 min, the reactions were terminated by the addition of 1 ml of ice-cold kinase buffer and placed on ice. Immune complexes were recovered by centrifugation, and the proteins...
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RESULTS

In order to determine the role of PKC in PKD catalytic activation within cells stimulated for various times with a \( G_q \)-coupled receptor agonist, COS-7 cells were transiently co-transfected with expression plasmids encoding wild type GFP-PKD and the bombesin-prefering GPCR. We have previously demonstrated that the fusion of GFP to the N terminus of PKD did not produce any detectable effect on PKD basal catalytic activity, GPCR-induced activation, and intracellular distribution (38, 57, 58, 62). After 18 h, the cells were treated for 1 h with the preferential PKC inhibitor GF 109203X (also known as bisindolylmaleimide I) prior to stimulation with 10 nM bombesin for increasing times (1–45 min). GFP-PKD was immunoprecipitated from cell lysates with anti-GFP antibody, and the immune complexes were incubated with \( [\gamma-^{32}P]ATP \), subjected to SDS-PAGE, and analyzed by autoradiography to detect the prominent 140-kDa band corresponding to autophosphorylated GFP-PKD.

GFP-PKD isolated from unstimulated COS-7 cells had low catalytic activity that was markedly activated by bombesin stimulation of intact cells in a time-dependent manner (Fig. 1A, IVK). An increase in PKD catalytic activation was detected within 1 min of bombesin stimulation, and maximal increase in PKD catalytic activity was obtained after 20 min of incubation. Exposure to 3.5 \( \mu \text{M} \) GF 109203X potently blocked rapid PKD activation induced by bombesin stimulation, in agreement with previous studies in many different cellular model systems (14). Surprisingly, treatment with GF 109203X was less inhibitory at later times of bombesin stimulation (Fig. 1, A (IVK) and B). Similar results were obtained when the immunoprecipitates were generated using antibodies directed against the C-terminal region of PKD rather than against the N-terminal GFP (data not shown).

We also determined PKD catalytic activity in immune complexes by its ability to phosphorylate the synthetic peptide syntide-2, an excellent exogenous substrate for PKD (9, 16, 19, 34). As shown in Fig. 1C, treatment of COS cells with GF 109203X decreased bombesin-induced PKD activation by \( \sim 95\% \) at the early time point (2.5 min) but inhibited PKD catalytic activation by only \( \sim 30\% \) at the later time of stimulation (45 min). The results presented in Fig. 1 suggest, for the first time, that PKD is activated in response to bombesin stimulation through early PKC-dependent and late PKC-independent phases.

To verify that the inhibitory effect of GF 109203X on PKC-mediated phosphorylation of cellular substrates was maintained at all times of bombesin stimulation, we determined the phosphorylation state of MARCKS, a well established substrate of PKCs (63, 64). As shown in Fig. 1A, basal and bombesin-induced MARCKS Ser\(^{152/156} \) phosphorylation was virtually abolished by treatment with GF 109203X at all times of incubation. Thus, late-phase PKD activation in response to bombesin
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A

Time (min) 0 1 2.5 5 10 20 45

IVK

α-pS744

α-pS748

α-pMARCKS

α-GFP

GF1 + + + + + +

B

% of maximal GFP-PKD kinase activity

Time (min) 0 1 2.5 5 10 20 45

DG1

C

Syntide-2 phosphorylation (gpmol/10^6 cells)

Time (min) 0 1 2.5 5 10 20 45

GF1

FIGURE 1. Kinetics of bombesin-induced PKC-dependent and PKC-independent PKD activation. COS-7 cells were transiently co-transfected with GFP-PKD and the bombesin/GRP receptor expression constructs. After 24 h of transfection, cells were incubated in the presence (+) or in the absence (−) of 3.5 μM GF 109203X (GF1) for 1 h prior to stimulation with 10 nM bombesin for various times (0–45 min) as indicated and then lysed in ice-cold buffer. A, GFP-PKD was immunoprecipitated from lysates with an anti-GFP antibody bound to protein A-agarose and assayed for autophosphorylation or syntide-2 phosphorylation activity, as described under “Experimental Procedures.” A, autophosphorylation assay reactions were analyzed by SDS-PAGE followed by autoradiography, and a representative autoradiogram is shown (IVK). Aliquots of the cell lysates were analyzed by SDS-PAGE and Western blot using anti-pS744, anti-pS748, an antibody that detects the phosphorylated state of each of these residues (19, 61). Although the antibody referred to as PKD catalytic activation depends on its phosphorylation on the activation loop residues Ser^744 and Ser^748 (19, 42, 59, 61). Consequently, we examined phosphorylation of Ser^744 and Ser^748 in the same cell lysates produced in the experiments shown in Figs. 1 and 2. We used Western blot analysis with site-specific antibodies that detect the phosphorylated state of each of these residues (19, 61). Although the antibody referred to as PKD anti-pS744 was generated against a peptide containing phosphoserine at positions corresponding to both serine residues in the PKD activation loop, i.e. Ser^744 and Ser^748, it detects primar-
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We also determined whether PKC-dependent and PKC-independent phases of PKD activation loop phosphorylation are elicited in response to various concentrations of bombesin. COS-7 cells, transiently co-transfected with expression plasmids encoding wild type GFP-PKD and the bombesin-prefering GPCR, were treated with or without Gö 6983 and then stimulated with 0.1, 1, or 10 nM bombesin for either 2.5 or 45 min. As shown in Fig. 3A, treatment with Gö 6983 blocked PKD phosphorylation at Ser\textsuperscript{744} and Ser\textsuperscript{748} induced by stimulation with increasing concentrations of bombesin for 2.5 min. In contrast, Gö 6983 did not prevent the phosphorylation of the PKD activation loop Ser\textsuperscript{748} induced by stimulation with 0.1, 1, or 10 nM bombesin for 45 min. These results reinforce the notion that bombesin induces PKD-dependent phosphorylation of PKD on Ser\textsuperscript{748} as well as phosphorylation of Ser\textsuperscript{744} that remains predominantly, but not exclusively, PKC-dependent at later times of cell stimulation.

We also determined whether biphasic PKD activation is induced via stimulation of a different G\textsubscript{q}-coupled heptahelical receptor. In these experiments, COS-7 cells were transiently co-transfected with expression plasmids encoding GFP-PKD and the type 1 A vasopressin GPCR. As shown in Fig. 3B, cell stimulation with vasopressin induced a rapid increase in the phosphorylation of the activation loop residues Ser\textsuperscript{744} and Ser\textsuperscript{748} of GFP-PKD. Treatment with 10 nM bombesin for 2.5 min did not prevent the phosphorylation of Ser\textsuperscript{748} and partially inhibited phosphorylation of Ser\textsuperscript{744} induced by vasopressin stimulation for 45–90 min. The results

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Effect of GF 109203X or Gö 6983 on the phosphorylation of the activation loop Ser\textsuperscript{744} and Ser\textsuperscript{748} of PKD induced by bombesin stimulation.}
\end{figure}

ily the phosphorylated state of Ser\textsuperscript{744}, as previously noted by us (61) and further confirmed in this study (see below). In order to detect the phosphorylated state of Ser\textsuperscript{748}, we used a different phosphoantibody, referred to as PKD anti-pS748, generated originally by us (61) and now commercially available (see “Experimental Procedures”). Equivalent loading of the gel was verified using Western blot analysis with an antibody directed against GFP (Fig. 1) or the C-terminal region of PKD (Fig. 2).

Cell stimulation with bombesin rapidly induced phosphorylation of the activation loop residues Ser\textsuperscript{744} and Ser\textsuperscript{748} of either GFP-PKD (Fig. 1A) or PKD (Fig. 2A). As expected, treatment with the PKC inhibitors GF 109203X or Gö 6983 blocked PKD phosphorylation at Ser\textsuperscript{744} and Ser\textsuperscript{748} induced by bombesin stimulation for 1–5 min. These results are entirely consistent with previous results indicating that bombesin induces PKD activation through PKC-mediated activation loop phosphorylation of Ser\textsuperscript{744} and Ser\textsuperscript{748}.

The striking feature of the results presented in Fig. 1A is that treatment with GF 109203X did not prevent the phosphorylation of the PKD activation loop residue Ser\textsuperscript{748} induced by bombesin stimulation for 20–45 min. Furthermore, the phosphorylation of PKD on Ser\textsuperscript{748} was also partially restored in the presence of GF 109203X at later times of bombesin stimulation (i.e. 20–45 min). PKC-independent Ser\textsuperscript{748} phosphorylation obtained with GFP-PKD in response to bombesin stimulation in the presence of GF 109203X was corroborated in the experiments presented in Fig. 2 using PKD (instead of GFP-PKD) and Gö 6983 (instead of GF 109203X). The results presented in Figs. 1 and 2, showing striking PKC-independent phosphorylation of Ser\textsuperscript{748} and partial PKC-dependent phosphorylation of Ser\textsuperscript{744}, demonstrate a novel, time-dependent mechanism of PKD regulation in GPCR-stimulated cells.

FIGURE 2. Effect of GF 109203X or Gö 6983 on the phosphorylation of the activation loop Ser\textsuperscript{744} and Ser\textsuperscript{748} of PKD induced by bombesin stimulation.

A

| PKD | Time (min) |
|-----|-----------|
| IVK |          |
| α-pS744 |          |
| α-pS748 |          |
| α-PKD C-20 |          |
| GF1 | - + + + + |

B

| GFP-PKD | Time (min) |
|---------|------------|
| IVK     |            |
| α-pS744 |            |
| α-pS748 |            |
| α-GFP   |            |
| Gö 6983 | - + + + + |

C

![Graph showing phosphorylation of PKD and GFP-PKD](image)

The phosphorylation of PKD on Ser\textsuperscript{748} was also partially restored in the presence of Gö 6983 (instead of GF 109203X). The results presented in Figs. 1 and 2, showing striking PKC-independent phosphorylation of Ser\textsuperscript{748} and partial PKC-dependent phosphorylation of Ser\textsuperscript{744}, demonstrate a novel, time-dependent mechanism of PKD regulation in GPCR-stimulated cells.

We also determined whether PKC-dependent and PKC-independent phases of PKD activation loop phosphorylation are elicited in response to various concentrations of bombesin. COS-7 cells, transiently co-transfected with expression plasmids encoding wild type GFP-PKD and the bombesin-prefering GPCR, were treated with or without Gö 6983 and then stimulated with 0.1, 1, or 10 nM bombesin for either 2.5 or 45 min. As shown in Fig. 3A, treatment with Gö 6983 blocked PKD phosphorylation at Ser\textsuperscript{744} and Ser\textsuperscript{748} induced by stimulation with increasing concentrations of bombesin for 2.5 min. In contrast, Gö 6983 did not prevent the phosphorylation of the PKD activation loop Ser\textsuperscript{748} induced by stimulation with 0.1, 1, or 10 nM bombesin for 45 min. These results reinforce the notion that bombesin induces PKD-dependent phosphorylation of PKD on Ser\textsuperscript{748} as well as phosphorylation of Ser\textsuperscript{744} that remains predominantly, but not exclusively, PKC-dependent at later times of cell stimulation.

We also determined whether biphasic PKD activation is induced via stimulation of a different G\textsubscript{q}-coupled heptahelical receptor. In these experiments, COS-7 cells were transiently co-transfected with expression plasmids encoding GFP-PKD and the type 1 A vasopressin GPCR. As shown in Fig. 3B, cell stimulation with vasopressin induced a rapid increase in the phosphorylation of the activation loop residues Ser\textsuperscript{744} and Ser\textsuperscript{748} of GFP-PKD. Treatment with 10 nM bombesin for 2.5 min did not prevent the phosphorylation of Ser\textsuperscript{748} and partially inhibited phosphorylation of Ser\textsuperscript{744} induced by vasopressin stimulation for 45–90 min. The results...
induced PKD activation. We previously showed that mutation of Lys$^{618}$ in the catalytic domain of PKD renders this enzyme kinase-deficient (16). Previous studies also demonstrated that this PKD mutant translocates to the plasma membrane (58) and undergoes rapid phosphorylation at Ser$^{744}$ and Ser$^{748}$ in response to phorbol ester stimulation (19, 61). We verified that bombesin induced rapid translocation of kinase-deficient PKD (GFP-PKD(K618N)) from the cytosol to the plasma membrane (results not shown). As shown in Fig. 4, the level of Ser$^{744}$ phosphorylation of GFP-PKD(K618N) induced by bombesin stimulation for 2.5 min or 45 min in cells pretreated with or without the PKC inhibitor Gö 6983 was comparable with that of wild type PKD. These results imply that PKD phosphorylation on Ser$^{744}$ is mediated by PKC transphosphorylation, although a low level of a PKC-independent pathway could be detected at the longer time of bombesin stimulation in both wild type GFP-PKD and GFP-PKD(K618N).

In striking contrast to the results obtained with Ser$^{744}$ phosphorylation, the level of Ser$^{748}$ phosphorylation of GFP-PKD(K618N) induced by bombesin stimulation was markedly decreased as compared with wild type PKD (see bars in Fig. 4). Furthermore, the low level of Ser$^{748}$ phosphorylation of GFP-PKD(K618N) induced by bombesin stimulation for either 2.5 or 45 min was completely blocked by cell treatment with 2.5 μM Gö 6983. In parallel cultures of COS cells transfected with GFP-PKD, we verified that treatment with 2.5 μM Gö 6983 prevented Ser$^{748}$ phosphorylation of GFP-PKD induced by bombesin stimulation for 2.5 min but attenuated only slightly the phosphorylation of this residue in cells treated with this agonist for 45 min, in line with previous results shown in this study. In all cases, the protein expression levels of the transfected PKD mutants were comparable with that of wild type GFP-PKD, as shown by Western blot analysis with an antibody directed against GFP. Taken together, these results indicate that bombesin GPCR stimulation induces late Ser$^{748}$ via a PKC-independent pathway that requires PKD catalytic activity, and therefore it is likely to be mediated by autophosphorylation.

We obtained similar results with a second kinase-deficient PKD mutant, in which the functionally critical aspartate residue (Asp$^{733}$ in PKD) in the DFG triplet in subdomain VII is mutated to Ala (59). These results with PKD mutated at Lys$^{618}$ or Asp$^{733}$ indicate that the catalytic activity of PKD is necessary for activation loop phosphorylation on Ser$^{748}$ during the PKC-independent phase of GPCR-induced PKD activation.

Phosphorylation of Ser$^{748}$ in a Constitutively Activated Form of PKD—Autoinhibition is a central feature of the regulation of protein kinase catalytic activity. Previous results from this laboratory indicated that PKD mutants lacking the PH domain (i.e. amino acids 429–557 of PKD) were highly active in the absence of cell stimulation (18, 19). These results suggest that in the intact kinase, the PH domain contributes to maintain PKD in an inactive, autoinhibited state, and consequently, removal of this domain facilitates activation. In view of the results presented in Fig. 4, suggesting that PKD autophosphorylates at Ser$^{748}$, we hypothesized that a constitutively active PKD, lacking the PH domain, is also constitutively phosphorylated on this residue.

To test this hypothesis, we analyzed activation loop phosphorylation in cell lysates from cells transfected with wild type GFP-PKD or with a GFP-PKD mutant lacking the PH domain.
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FIGURE 4. PKD catalytic activity is required for PKD-Ser748 phosphorylation in a PKC-independent manner. COS-7 cells were transiently co-transfected with the bombesin/GPR receptor and GFP-PKD, GFP-PKD(K618N), or GFP-PKD(D733A) expression constructs. After 24 h of transfection, cells were incubated in the presence (+) or in the absence (−) of 2.5 μM Gö 6983 for 1 h prior to stimulation of the cells with 10 nM bombesin for various times as indicated (in min) and then lysed with 2× SDS-PAGE sample buffer as indicated under “Experimental Procedures.” Cell lysates were analyzed by SDS-PAGE and Western blot using anti-pS744, anti-pS748, and anti-GFP antibodies. Western blots are representative of three independent experiments. Bottom panels, Band intensities from the autoradiograms were quantified by densitometric scanning. The results shown are the mean ± S.E. of three independent experiments and are expressed as a percentage of the maximum increase in PKD Ser748 (left) or PKD Ser744 (right) induced by treatment with bombesin in cells transfected with GFP-PKD (gray bars) or GFP-PKD(K618N) (black bars) preincubated in the presence (+) or the absence (−) of Gö 6983.

PKD Mutation of P287G in cys2 Prevents Early and Late-phase PKD Activation—The N-terminal regulatory region of PKD binds DAG and phorbol esters in a phospholipid-dependent manner via tandemly repeated cysteine-rich, zinc finger-like motifs (known as cys1 and cys2) that comprise the CRD. A PKD mutant containing a substitution in a highly conserved proline in cys2, PKD P287G, displays reduced affinity for phorbol ester/DAG binding (67), decreased translocation from the cytosol to the plasma membrane, and impaired catalytic activation in response to bombesin (68). In order to determine whether translocation of PKD to the plasma membrane is necessary for both early and late phases of PKD activation, we examined the extent of PKD P287G activation loop phosphorylation during treatment with a bombesin at either 0.3 or 10 nM for various times. As shown in Fig. 5B, Ser744 and Ser748 phosphorylation of GFP-PKD(P287G) induced by bombesin (0.3 or 10 nM) stimulation for 2.5 min was markedly decreased as compared with wild type PKD, in line with the requirement of PKD translocation from the cytosol to the plasma membrane for its PKC-mediated activation loop phosphorylation. Although phosphorylation of GFP-PKD(P287G) on Ser744 and Ser748 induced by stimulation with 10 nM bombesin for 45 min was partially restored, the phosphorylation of both residues was completely blocked by cell treatment with 2.5 μM Gö 6983 (Fig. 5B). In parallel cultures of COS cells transfected with GFP-PKD, we verified that treatment with 2.5 μM Gö 6983 prevented Ser744 and Ser748 phosphorylation of GFP-PKD induced by bombesin stimulation for 2.5 min but attenuated only slightly the phosphorylation of Ser748 in cells treated with this agonist for 45 min (Fig. 5B). In all cases, the protein expression levels of the transfected PKD mutants were comparable with that of wild type PKD, as shown by Western blot analysis with an antibody directed against GFP. These results show that mutation of PKD at Pro287 prevented the development of a PKC-independent phase of PKD activation loop phosphorylation and imply that correct subcellular localization of PKD is necessary not only for the early PKC-dependent but also for the late PKC-independent phase of PKD phosphorylation in response to GPCR agonists.

Role of the Activation Loop Phosphorylation in the Late Phase of PKD Activation—Having established that biphasic PKD activation in response to bombesin is associated with changes in Ser744 and Ser748 phosphorylation, we next examined whether the phosphorylation of these residues is necessary for the late phase of PKD catalytic activation. We showed previously that PKD with both Ser744 and Ser748 altered to nonphosphorylatable alanines is not activated by rapid GPCR stimulation (33, 34, 41, 59). If these residues are also critical target sites for activating phosphorylation events during the PKC-independent phase of GPCR-induced PKD activation, their conversion...
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A

**FIGURE 5.** Effect of deletion of the N-terminal region of PKD and mutation of proline 287 to glycine on the late phase of PKD activation in response to GPCR agonists. COS-7 cells were transiently co-transfected with the bombesin/GRP receptor and GFP-PKD, GFP-PKD(ΔPH) (A), or GFP-PKD(P287G) (B) expression constructs. After 24 h of transfection, the cells were incubated in the presence (+) or in the absence (−) of 2.5 μM Gö 6983 for 1 h prior stimulation of the cells with 10 nM bombesin (A) or with either 0.3 or 10 nM bombesin (B) for various times as indicated (in min) and lysed with 2× SDS-PAGE sample buffer as indicated under “Experimental Procedures.” Cell lysates were analyzed by SDS-PAGE and Western blot using anti-pS744, anti-pS748, and anti-GFP antibodies. Shown Western blots are representative of at least two independent experiments. **Bottom panels,** band intensities from the autoradiograms shown in B were quantified by densitometric scanning. The results shown are the mean of two independent experiments and are expressed as a percentage of the maximum increase in PKD Ser744 (left) or PKD Ser748 (right) induced by treatment with 10 nM bombesin in cells transfected with GFP-PKD (white bars) or GFP-PKD(P287G) (black bars) preincubated in the presence (+) or the absence (−) of Gö 6983.

to Ala should reduce or eliminate bombesin-induced late-phase PKD activation.

To test this possibility, we initially determined whether a PKD mutant with double substitution of these residues (i.e. PKD-S744A/S748A) can be activated by treatment with bombesin for 2.5 or 45 min. As shown in Fig. 6, mutation of Ser744 and Ser748 to nonphosphorylatable Ala prevented both early (2.5 min) and late (45 min) PKD activation, indicating that the phosphorylation of these activation loop residues plays a critical role in the stimulation of both phases of PKD activation.

In view of the differential regulation of Ser744 or Ser748 revealed by the previous results presented in this study, we also examined the effect of preventing the phosphorylation of each of these residues. Consequently, we determined the phosphorylation and catalytic activity of PKD mutants with substitution in either Ser744 or Ser748 (i.e. GFP-PKD(S744A) and GFP-PKD(S748A)). Single substitution of Ser748 for Ala resulted in a PKD mutant that displayed strikingly reduced activation after bombesin stimulation for 2.5 or 45 min, as shown by in vitro kinase autophosphorylation assays (Fig. 6B, IVK) or by syntide-2 phosphorylation assays (Fig. 6C). Results in Fig. 6 also show that mutation of Ser748 to Ala did not interfere with Ser744 phosphorylation but completely eliminated immunoreactive signal obtained with the anti-pS748 antibody, providing a convenient check on the specificity of this antibody. In all cases, the protein expression levels of the transfected PKD mutants were comparable with that of wild type PKD, as shown by Western blot analysis.

Mutation of Ser748 to Ala also prevented the late phase of PKC-independent activation, since exposure to Gö 6983 blocked the diminished catalytic activation of this mutant at both early (2.5 min) and late (45 min) times of bombesin stimulation. These results substantiate a critical role of Ser748 phosphorylation in mediating PKC-independent PKD activation.

Single substitution of Ser744 for Ala resulted in a PKD mutant that displayed strikingly reduced basal activity and bombesin-induced activation after stimulation for 2.5 or 45 min, as shown in Fig. 6B (IVK) and Fig. 6C. Interestingly, this mutation also attenuated the development of PKC-independent PKD activation, in line with the notion that the late phase of PKD activation requires robust PKD catalytic activity to mediate autophosphorylation.

We conclude that substitution of Ser744 and Ser748 in the activation loop of PKD by nonphosphorylatable residues prevents the activation of this enzyme by cell stimulation with...
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FIGURE 6. Role of the activation loop phosphorylation in the late phase of PKD activation. COS-7 cells were transiently co-transfected with expression constructs encoding the bombesin/GRP receptor and GFP-PKD or the mutants GFP-PKD(S744A/S748A), GFP-PKD(S744A), or GFP-PKD(S748A). After 24 h of transfection, the cells were incubated in the presence (+) or in the absence (−) of 2.5 μM Gö 6983 for 1 h prior to stimulation of the cells with 10 nM bombesin for various times as indicated (in min) and then lysed in ice-cold buffer A. The values shown (cpm) are the mean ± S.E. of two independent experiments.

A

B

bombesin for 2.5 min or 45 min. The analysis of PKD mutants with single substitution of Ser744 and Ser748 for Ala is consistent with the notion that sustained PKD activation requires autophosphorylation on Ser748.

DISCUSSION

Many previous studies demonstrated that PKD is rapidly activated in intact cells by GPCR agonists through PKC-dependent pathways. More recently, PKD has been implicated in the regulation of long term cellular activities, including gene expression, chromatin structure, and proliferation, but little is known about the mechanism(s) of sustained PKD activation. Here, we examined the role of PKC as upstream kinase in PKD activation in cells stimulated through heptahelical Gq-coupled receptor for increasing periods of time. Our results demonstrate that PKD activation in response to bombesin or vasopressin proceeds through a sequential mechanism, consisting of an early PKC-dependent and a late PKC-independent phase of regulation.

For many protein kinases, catalytic activity is dependent on phosphorylation within the activation loop, a regulatory element located between two highly conserved motifs, DFG in kinase subdomain VII and APE in kinase subdomain VIII of the kinase catalytic domain. Results presented here with PKD mutants in which Ser744 and/or Ser748 were altered to nonphosphorylatable alanines indicate that the phosphorylation of both residues in the activation loop of PKD is necessary for its maximal catalytic activity during early and late phases induced by GPCR agonists. At least two mechanisms, involving autophosphorylation or transphosphorylation, mediate the phosphorylation of one or more residues within the activation loop, leading to stabilization of an active conformation of the catalytic residues (66, 69). Many protein kinases that participate in signal transduction pathways, including those in mitogen-activated protein kinase cascades (70–72), are regulated by transphosphorylation of the activation loop mediated by a different upstream kinase. For example, Raf, the earliest identified effector of Ras, transphosphorylates MEK on two key residues in its activation loop, Ser217 and Ser221, and thereby stimulates MEK activation (73). It is also recognized that a substantial number of regulatory protein kinases from different families mediate their own activation loop phosphorylation, promoting their catalytic activation. Examples of serine/threonine protein kinases that mediate autoactivation include Aurora A (74), Aurora B (75), Ca2+/calmodulin-dependent protein kinase II (76), Chk2 (77), DNA-dependent protein kinase (78), DYRK (79), GSK-3 (80), JNK2 (81), Mps1 (82, 83), MTK1/MEKK4 (84), and PAK (85). In many cases, autophosphorylation occurs in trans (86).

Using site-specific antibodies that detect the phosphorylation state of either Ser744 or Ser748, our results show that the phosphorylation of these two key residues of the PKD activation loop is regulated by different mechanisms in a time-dependent manner in response to GPCR stimulation. During the early phase, the primary mechanism mediating the phosphorylation of Ser744 and Ser748 activation requires PKC activity. Unexpectedly, during the late phase of PKD activation, Ser748 phosphorylation is mediated by a PKC-independent mechanism.

In order to determine whether transphosphorylation or autophosphorylation mechanisms are responsible for PKC-independent PKD Ser748 phosphorylation in response to GPCR agonists, we examined GPCR-induced Ser748 phosphorylation in a set of PKD mutants. Our results indicate that PKD autophosphorylation on Ser748 is a major mechanism contributing to late PKD activation loop phosphorylation occurring in cells stimulated by GPCR agonists. This conclusion is supported by several lines of evidence. 1) Catalytic inactivation of PKD by...
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mutation of either Lys618 or Asp733 produced PKD forms in which the late-phase phosphorylation of Ser748 was eliminated by treatment with PKC inhibitors, demonstrating that PKC-independent Ser748 phosphorylation requires PKD catalytic activity. 2) Constitutively active PKD generated by deletion of the PH domain displayed a high level of Ser748 (but not of Ser744) phosphorylation in unstimulated cells. 3) The PKC-independent phase of PKD activation induced by bombesin was completely blocked by substitution of Ser748 for Ala. PKC-independent PKD phosphorylation on Ser748 is likely to require the recruitment of PKD to DAG-rich microenvironments, as judged by the fact that this phosphorylation is prevented in a PKD mutant (i.e. PKDP287G) with impaired ability to bind DAG and translocate to the plasma membrane. Collectively, our results identify a novel mechanism induced by GPCR activation that leads to PKC-independent PKD activation loop autophosphorylation.

In view of the new results presented here, PKD seems to be the only example so far of a protein kinase in which the phosphorylation of the key serines in its activation loop, Ser744 and Ser748, is regulated by both transphosphorylation and autophosphorylation mechanisms. Specifically, transphosphorylation by PKC is a major mechanism targeting Ser744, and auto-phosphorylation is a predominant mechanism for Ser748, especially at relatively late times of stimulation. In this context, it is important to emphasize that the pathways leading to the phosphorylation of these residues depend on the time of GPCR stimulation. For example, although PKD phosphorylation on Ser744 is mediated entirely by PKC transphosphorylation at early times of bombesin stimulation, a low level of PKC-independent phosphorylation of this residue could be detected consistently at longer times of bombesin stimulation. These findings suggest that, in addition to PKC, another, as yet unidentified, upstream protein kinase (insensitive to GF

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