Protein kinase D (PKD) potentiates cellular DNA synthesis in response to G protein-coupled receptor (GPCR) agonists but the mechanism(s) involved has not been elucidated. Here, we examined whether PKD overexpression in Swiss 3T3 cells regulates the activation/inactivation kinetics of the extracellular-regulated protein kinase (ERK) in response to the mitogenic GPCR agonists bombesin and vasopressin. Addition of bombesin or vasopressin to Swiss 3T3 cells overexpressing PKD induced a striking increase in the duration of MEK/ERK/RSK activation as compared with cultures of either control Swiss 3T3 cells or Swiss 3T3 cells expressing a kinase-inactive PKD mutant. In contrast, the duration of ERK activation in response to epidermal growth factor, which acts via protein kinase C/PKD-independent pathways, was not increased. Furthermore, bombesin or vasopressin promoted a striking increase in phosphorylation (at Ser-374) and accumulation of c-Fos (the c-fos proto-oncogene product) in Swiss 3T3 cells overexpressing wild-type (but not kinase-inactive) PKD. Inhibition of the sustained phase of ERK/RSK activation abrogated the increase in c-Fos accumulation and DNA synthesis induced by bombesin or vasopressin in PKD-overexpressing cells. Our results demonstrate that PKD selectively potentiates mitogenesis induced by bombesin or vasopressin in Swiss 3T3 cells by increasing the duration of MEK/ERK/RSK signaling.

Neuropeptides are implicated as growth factors in a variety of fundamental processes including development, inflammation, tissue regeneration, and neoplastic transformation (1–3). In particular, bombesin and its mammalian counterpart gastrin-releasing peptide bind to a GPCR (4, 5) that promotes Gαq-mediated activation of β isoforms of phospholipase C (6–8) to produce 2 s messengers: Ins(1,4,5)P3 that mobilizes Ca2+ from internal stores and diacylglycerol that activates conventional (α, β1, β2, and γ) and novel (δ, ε, η, and θ) PKCs (9, 10). The bombesin/gastrin-releasing peptide GPCR also interacts with members of the G13 family leading to Rhoad-dependent actin remodeling and tyrosine phosphorylation of focal adhesion proteins, including FAK (10–17). Subsequently, bombesin induces striking activation of serine phosphorylation cascades (11–14) and promotes increased expression of immediate early response genes, stimulation of DNA synthesis, and cell proliferation (15–19). The mechanism(s) linking the early signaling pathways to the subsequent stimulation of cell proliferation remains incompletely understood.

PKD (also initially known as PKCμ) is a serine/threonine protein kinase with structural, enzymology, and regulatory properties different from the PKC family members (20, 21). PKD most distinct characteristics are the presence of a catalytic domain distantly related to Ca2+-regulated kinases, a pleckstrin homology (PH) region that regulates enzyme activity and a highly hydrophobic stretch of amino acids in its N-terminal region (22–24). This N-terminal region also contains a tandem repeat of cysteine-rich, zinc finger-like motifs, which confers high affinity for phorbol esters and plays a negative role in the regulation of catalytic kinase activity (25–28). The identification of PKD-2 and PKD-3, similar in overall structure, primary amino acid sequence, and enzymology properties to PKD/PKCμ (29–32), supports the notion that PKD isoenzymes constitute a separate family of serine protein kinases.

PKD can be activated in intact cells through multiple G protein pathways, including Gq, Gi, and G12 (33–39), as well as by biologically active phorbol esters, growth factors and anti-gen-receptor engagement (36, 37, 39–45). In all these cases, rapid PKD activation is mediated by PKC-dependent phosphorylation of Ser-744 and Ser-748 within the activation loop of the catalytic domain of PKD (33, 46–48). PKD activation is associated with its translocation to the plasma membrane and subsequent transient accumulation in the nucleus (25, 28, 50, 51). These findings reveal that PKD is activated by multiple growth-promoting factors (22, 52) suggesting that it functions in mitogenic signaling. Indeed, we reported that PKD overexpression markedly potentiates DNA synthesis induced by the GPCR agonists bombesin and vasopressin in Swiss 3T3 cells (53), a cell line that has been used extensively as a model system to elucidate signal transduction pathways in the mitogenic action of GPCR agonists (1, 54, 55). These results suggest that PKD plays an important role in mediating cellular DNA synthesis in response to GPCR agonists, but the mechanism(s) involved has not been elucidated.

One of the major signaling pathways involved in the mitogenic response induced by both receptor tyrosine kinases and
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GPCRs is the ERK cascade (56, 57). The ERKs (ERK-1 and ERK-2) are directly activated by phosphorylation on specific tyrosine and threonine residues by the dual-specificity ERK kinase (or MEK). Studies on the mechanisms by which mitogenic GPCRs activate ERK revealed considerable heterogeneity, depending on receptor and cell context (58). It is increasingly recognized that the duration and intensity of ERK pathway activation is of critical importance for determining specific biological outcomes, including proliferation, differentiation, and transformation (59, 60). For example, sustained pathway activation is of critical importance for determining specifically recognized that the duration and intensity of ERK

activation of DNA replication in fibroblasts whereas transient ERK activity (20–30 min) is not sufficient to promote mitogenesis in these cells (60, 61). The protein products of immediate early genes (e.g., c-Fos) have been proposed to function as molecular sensors of ERK1/2 signal duration (62). As a first step to elucidate the mechanism(s) by which PKD facilitates GPCR-induced mitogenesis, we examined whether PKD overexpression influences the duration or intensity of ERK signaling in response to mitogenic GPCR agonists.

The results presented here demonstrate that overexpression of wild-type (but not kinase-inactive) PKD dramatically increases the duration of MEK/ERK/RSK activation and the accumulation of c-Fos (the c-fos proto-oncogene product) induced by either bombesin or vasopressin in Swiss 3T3 cells. Inhibition of the sustained phase of ERK activation by treatment with either U0126 or PD98059 abrogated the accumulation of c-Fos protein and the potentiation of DNA synthesis induced by bombesin or vasopressin in Swiss 3T3 cells overexpressing PKD. Our results indicate that an increase in the duration of the ERK signal is one of the mechanisms by which PKD facilitates GPCR-induced mitogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Stock cultures of Swiss 3T3-PKD.GFP cells, which overexpress PKD, Swiss 3T3-PKD/D618N.GFP cells, which overexpress the kinase-inactive PKD mutant PKD618N, and control Swiss 3T3-GFP cells generated as previously described (53), were maintained at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum in a humidified atmosphere containing 10% CO\(_2\) and 90% air. For experimental purposes, cells were plated in 100-mm dishes at 6 × 10\(^5\) cells/dish or 35-mm dishes at 1 × 10\(^5\) cells/dish and grown in DMEM containing 10% fetal bovine serum for 7–9 days until cells became confluent and quiescent (63). Phoenix packaging cells (kindly provided by Dr. G. Nolan, Stanford University, Stanford, CA) were cultured in the same medium in a humidified atmosphere containing 5% CO\(_2\).

**Production of Retrovirus—**To generate Swiss 3T3 cells stably overexpressing PKD, cultures of these cells were transduced with retrovirus encoding either wild-type or kinase-inactive murine PKD, in which PKD and GFP were translated from the same bicistronic mRNA and expressed as two separate proteins. After transduction, cells expressing higher levels of GFP were sorted by FACS, collected and propagated for further studies.

Specifically, construction of the retroviral plasmid expressing wild-type PKD (MSCV-PKDiresGFP) has been previously described (53). To generate retroviral plasmid containing cDNA of the kinase-inactive PKD (MSCV-PKD/D618NiresGFP), the fragment of the wild-type PKD cDNA spanning from +1660 to +2910 of the published sequence and containing the K618N mutation was amplified by PCR from the previously described kinase-inactive construct (54). The 5'-PCR primer contained the endogenous HpaI restriction site. The 3’-PCR primer was designed to introduce an additional HpaI site at the 3’-end of the amplified fragment. The HpaI digest of the MSCV-PKDiresGFP construct released the +1660/+2910 fragment of the wild-type PKD. The PCR-amplified fragment of the kinase-inactive PKD (+1660 to +2910) was ligated into the HpaI sites of the MSCV-PKDiresGFP vector, thus producing MSCV-PKD/D618NiresGFP. The nucleotide sequence of the MSCV-PKD/D618NiresGFP was validated by sequencing. For retrovirus production, logarithmically growing Phoenix ecotropic cells were transfected with MSCV-PKDiresGFP, MSCV-PKD/K618NiresGFP or MSCV-iresGFP using FuGENE 6 transfection reagent per protocol of the manufacturer. Virus-containing supernatants were collected at 48 h after transfection and used immediately. At growing Swiss 3T3 cells were incubated with the virus-containing supernatants in the presence of 5 μg/mL polybrene for 5 h. Cells were collected 48–72 h later and GFP-positive fractions were FACS-sorted using a Becton Dickinson FACStar PLUS machine. GFP-positive cells were propagated, and multiple aliquots were frozen. A fresh batch of transduced cells was generated every 2 months. Following sorting, GFP-positive cells were maintained as described earlier in this section.

**Immunoblotting and Detection of MEK, ERK, Ribosomal S6 Kinases (RSK), FAK, PKD, and c-Fos—**Confluent, quiescent Swiss 3T3-GFP, Swiss 3T3-PKD.GFP and Swiss 3T3-PKD/D618N.GFP cells were lysed in 2× SDS-polyacrylamide gel electrophoresis sample buffer (20 mM Tris-HCl, pH 6.8, 6% SDS, 4% ~2-mercaptoethanol, 10% glycerol) and boiled for 10 min. After SDS-PAGE, proteins were transferred to Immobilon-P membranes. The transfer was carried out at 100 V, 0.4 A at 4 °C for 4 h using a Bio-Rad transfer apparatus. The transfer buffer consisted of 200 mM glycine, 25 mM Tris, 0.1% SDS, and 20% CH\(_3\)OH. For detection of proteins, membranes were blocked using 5% nonfat dried milk in phosphate-buffered saline (pH 7.2) and then incubated for at least 2 h with the desired antibodies diluted in phosphate-buffered saline (pH 7.2) containing 3% nonfat dried milk. Bound primary antibodies to immunoreactive bands were visualized by enhanced chemiluminescence (ECL) detection with horseradish peroxidase conjugated anti-mouse or anti-rabbit. The phosphospecific antibodies used were as follows: the phopho-ERK1/2 monoclonal antibody recognizes the ERK-1/2 only when they are phosphorylated on Thr-202 and Tyr-204 (pERK1/ERK2); the phopho-MEK1/2 polyclonal antibody recognizes MEK1/2 only when they are phosphorylated on Ser-217 and Ser-221 (pMEK1/2); the phospho-p90RSK polyclonal antibody is specific to p90RSK only when it is phosphorylated on Thr-574; the phopho-MARKS polyclonal antibody is specific to the phosphorylated state of MARKS only when it is phosphorylated on Ser-152 and Ser-156 (pMARKS); the phospho-FAK910 polyclonal antibody recognizes FAK only when it is phosphorylated on Ser-910; the phospho-FAK979 polyclonal antibody recognizes FAK only when it is phosphorylated on Tyr-979, the major autophosphorylation site of FAK; the phospho-Pyk2 polyclonal antibody recognizes FAK only when it is phosphorylated on Tyr-577; the phospho-PKD polyclonal antibody PKDS916 recognizes PKD only when it is autophosphorylated on Ser-916. The phospho-c-Fos polyclonal antibody recognizes c-Fos only when it is phosphorylated on Ser-374. The phospho-ERK1/2 antisera was determined as previously described (20). Briefly, the immune complexes were visualized by enhanced chemiluminescence (ECL) detection with horseradish peroxidase conjugated anti-mouse or anti-rabbit. The phosphospecific antibodies used were as follows: the phopho-ERK1/2 monoclonal antibody recognizes the ERK-1/2 only when they are phosphorylated on Thr-202 and Tyr-204 (pERK1/ERK2); the phopho-MEK1/2 polyclonal antibody recognizes MEK1/2 only when they are phosphorylated on Ser-217 and Ser-221 (pMEK1/2); the phospho-p90RSK polyclonal antibody is specific to p90RSK only when it is phosphorylated on Thr-574; the phospho-MARKS polyclonal antibody is specific to the phosphorylated state of MARKS only when it is phosphorylated on Ser-152 and Ser-156 (pMARKS); the phospho-FAK910 polyclonal antibody recognizes FAK only when it is phosphorylated on Ser-910; the phospho-FAK979 polyclonal antibody recognizes FAK only when it is phosphorylated on Tyr-979, the major autophosphorylation site of FAK; the phospho-Pyk2 polyclonal antibody recognizes FAK only when it is phosphorylated on Tyr-577; the phospho-PKD polyclonal antibody PKDS916 recognizes PKD only when it is autophosphorylated on Ser-916. The phospho-c-Fos polyclonal antibody recognizes c-Fos only when it is phosphorylated on Ser-374. The phospho-ERK1/2 antisera was determined as previously described (20). Briefly, the immunocomplexes were washed twice with lysis buffer A and twice with lysis buffer B (30 mM Tris-HCl, pH 7.6, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS). The lysates were clarified by centrifugation at 15,000 x g for 10 min at 4 °C. Immunoprecipitations were carried out at 4 °C for 2–4 h using a FAK C-20 polyclonal antibody for FAK and a c-Fos polyclonal antibody for c-Fos. The immune complexes were recovered using protein-A coupled to agarose and solubilized with 2× sample buffer.

**Immunoprecipitation and Kinase Assay of ERK1/2—**Cultures of Swiss 3T3 cells, treated as described in the individual experiments, were washed and lysed in 50 mM Tris/ HCl pH 7.6, 2 mM EDTA, 2 mM DTT, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride and 1% Triton X-100 (lysing buffer A). Cell lysates were clarified by centrifugation at 15,000 x g for 10 min at 4 °C. The cell lysate was clarified by centrifugation at 15,000 x g for 10 min at 4 °C. Immunoprecipitations were carried out at 4 °C for 2–4 h using a FAK C-20 polyclonal antibody for FAK and a c-Fos polyclonal antibody for c-Fos. The immune complexes were recovered using protein-A coupled to agarose and solubilized with 2× sample buffer.

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fected Swiss 3T3 cells were washed twice with DMEM and incubated with DMEM/Waymouth’s medium (1:1, v/v) containing [3H]thymidine (0.2 μCi/ml, 1 μCi) and various agonists as described in the figure legends. After 40 h of incubation at 37 °C, cultures were washed twice with PBS and incubated in 5% trichloroacetic acid at 4 °C for 20 min to remove acid-soluble radioactivity, washed with ethanol, and solubilized in 1 ml of 2% Na2CO3, 0.1 M NaOH. The acid-insoluble radioactivity was determined by liquid scintillation counting in 6 ml of Beckman ReadySafe.

**Materials**—pMSCVneo retroviral vector was from Clontech (Palo Alto, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). PuGENE 6 transfection reagent and protein-A agarose were obtained from Roche Applied Science. Polybrene was from Aldrich (Milwaukee, WI), U0126 was from Calbiochem (San Diego, CA). EGF, vasopressin and bombesin were obtained from Sigma. [3H]thymidine and [γ-32P]ATP (370 MBq/ml) were from Amer sham Biosciences. The following antibodies were purchased from Signaling Technology (Beverly, MA), phospho-p44/p42 MAPK (pERK1/2), phospho-MEK1/2, phospho-p90 S6K, and phospho-MAPKs. The phosphospecific polyclonal antibodies to Ser-910, Tyr-397, and Tyr-577 of FAK were obtained from BioSource International (Camarillo, CA). Anti-c-Fos polyclonal and the ERK1/2 kinase assay were from Upstate Biotechnology. Anti-ERK-2 and anti-FAK C-20 polyclonal antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA). The phosphospecific monoclonal antibody to Ser-374 of c-Fos was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Phospho-PKD916 (53) and the PA-1 (64) antisera were generated by the Antibody Core of the CURE: Digestive Diseases Research Center. All other materials were of the highest grade available.

**RESULTS AND DISCUSSION**

**Protein Kinase D Overexpression Selectively Increases the Duration of ERK Signaling Induced by Bombesin in Swiss 3T3 Cells**—In an effort to elucidate the mechanisms(s) by which PKD overexpression potentiates the mitogenic activity of GPCR agonists, we examined the intensity and duration of ERK activation in bombesin-stimulated Swiss 3T3-PKD.GFP cells, which overexpress PKD, and control Swiss 3T3-GFP cells, generated as previously described (53). We previously demonstrated that PKD overexpressed in Swiss 3T3 cells retains tight, signal-dependent, regulation of multisite phosphorylation and kinase catalytic activity (53). Quiescent monolayers of Swiss 3T3-PKD.GFP cells and control Swiss 3T3-GFP cells were challenged with 10 nM bombesin at 37 °C for various times and then lysed. The active forms of ERK-1 and ERK-2 in the extracts were detected by Western blotting using an antibody that detects the dually phosphorylated forms of these enzymes at the regulatory threonine and tyrosine residues. As shown in Fig. 1, ERK activation loop phosphorylation in both Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells increased dramatically to similar levels within 5 min of bombesin stimulation. In Swiss 3T3-GFP cells, the ERK signal was transient, i.e. ERK phosphorylation declined rapidly after 5 min, and returned to near baseline levels by 60 min. Similar kinetics were obtained in untransfected Swiss 3T3 cells (results not shown).

In striking contrast, bombesin-induced ERK activation in Swiss 3T3 cells overexpressing PKD was dramatically prolonged as compared with control Swiss 3T3 cells. Indeed, ERK phosphorylation in Swiss 3T3-PKD.GFP cells was robust even after 240 min of bombesin stimulation (Fig. 1A, upper panel). These results were corroborated when ERK phosphorylation was examined in response to increasing concentrations of bombesin added for either 5 or 240 min. Bombesin stimulation for 5 min increased ERK phosphorylation in both Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells, in a concentration-dependent manner (Fig. 1A). Bombesin stimulation for 240 min induced ERK phosphorylation in Swiss 3T3-PKD.GFP cells in a concentration-dependent manner with maximal effect at 3 nM whereas no effect of bombesin at this concentration was detected in control Swiss 3T3-GFP cells and only a slight increase in ERK phosphorylation was seen in these cells at 10 nM (Fig. 1A, lower panel). These results indicate that overexpression of PKD dramatically increases the duration of ERK signaling in response to bombesin, especially in cells stimulated by low concentrations of this neuropeptide (1–3 nM).

To substantiate that overexpression of PKD promotes sustained ERK catalytic activity in response to bombesin, the ERKs were immunoprecipitated from lysates of either Swiss 3T3-PKD.GFP cells or Swiss 3T3-GFP cells treated with or without 10 nM bombesin for 5 min or 240 min and the resulting immunocomplexes were subjected to in vitro kinase assays using MBP, as a substrate. As shown in Fig. 1B, bombesin stimulation for 5 min induced a striking increase (≈8-fold) in ERK kinase activity in both Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells. After 240 min of incubation with bombesin, ERK catalytic activity from Swiss 3T3-PKD.GFP cells was markedly increased as compared with that immunoprecipitated from Swiss 3T3-GFP cells. Thus, the duration of ERK phosphorylation and catalytic activation in response to bombesin is markedly increased in cells overexpressing PKD.

The binding of bombesin to its heptahelical GPCR in Swiss 3T3 cells induces a complex array of signaling events, including PKC-dependent phosphorylation of MARCKS (65, 66) and rapid tyrosine phosphorylation of the non-receptor tyrosine kinase FAK, via a Rho-dependent but PKC-independent pathway (3, 55). As shown in Fig. 1C, PKD overexpression did not increase the intensity or duration of either MARCKS phosphorylation at Ser-152/Ser-156 or FAK tyrosine phosphorylation in response to bombesin. These results indicate that PKD overexpression does not have a generalized effect on the kinetics of bombesin receptor signaling but selectively increases the duration of ERK activation.

**PKD Overexpression Selectively Increases the Duration of ERK Signaling Induced by Vasopressin in Swiss 3T3 Cells**—Vasopressin, which acts in Swiss 3T3 cells via an endogenously expressed Gq-coupled V1 receptor subtype (67, 68), also induces a marked increase in PKD activity (36, 53). In contrast, EGF does not induce any significant increase in the phosphorylation or catalytic activity of PKD (53). To examine the notion that PKD overexpression selectively increases the duration of ERK signaling by Gq-coupled agonists that act via PKD, we determined the kinetics of ERK phosphorylation and catalytic activation in response to either vasopressin or EGF in Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells. As shown in Fig. 2A, ERK phosphorylation in both Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells increased within 5 min of vasopressin stimulation. In Swiss 3T3-GFP cells, ERK phosphorylation declined to near baseline levels by 30 min. In striking contrast, vasopressin-induced ERK phosphorylation in Swiss 3T3 cells overexpressing PKD was strikingly prolonged and it was still evident even after 240 min of vasopressin stimulation (Fig. 2A, upper panel). Similarly, vasopressin stimulation for 5 min induced a striking increase in ERK kinase activity in both Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells (Fig. 2B). After 240 min of incubation with vasopressin, ERK activity from Swiss 3T3-PKD.GFP cells was markedly increased as compared with that from Swiss 3T3-GFP cells, which was virtually identical to the activity of unstimulated cells. These results indicate that the duration of ERK phosphorylation and catalytic activation in response to vasopressin are markedly increased in cells overexpressing PKD. In sharp contrast, the kinetics of ERK phosphorylation (Fig. 2A, lower panel) and activation (Fig. 2B) induced by EGF in Swiss 3T3-PKD.GFP cells was identical to that obtained in Swiss 3T3-GFP cells.
We verified that PKD overexpression did not increase the duration of either MARCKS phosphorylation or FAK tyrosine phosphorylation in response to either vasopressin or EGF (Fig. 2C). The results presented in Fig. 2 are consistent with the hypothesis that PKD overexpression selectively increases the duration ERK signaling in Swiss 3T3 cells stimulated by Gq-coupled receptor agonists.

The Increase in the Duration of ERK Signaling Induced by Bombesin and Vasopressin in PKD-overexpressing Cells Requires the Catalytic Activity of PKD—Next, we examined whether the PKD-induced increase in the duration of ERK signaling in response to GPCR stimulation requires the catalytic activity of PKD. Previously, we reported that mutation of Lys916 in the catalytic domain of PKD renders this enzyme non-functional (40). To generate Swiss 3T3 cells stably overexpressing kinase-inactive PKD, cultures of these cells were infected with retrovirus encoding PKD-K618N and GFP expressed as two separate proteins translated from the same bicistronic mRNA. After infection, cells expressing higher levels of GFP (termed Swiss 3T3-PKDK618N.GFP cells) were sorted by FACS, collected and propagated for further studies.

An antiserum specifically recognizing the phosphorylated form of a PKD C-terminal residue, Ser-916, has been used to detect in vivo autophosphorylation at this site by active PKD (69). Thus, this antibody detects the conversion of PKD from an inactive state to an active form within intact cells. Here, lysates from Swiss 3T3-PKD.GFP, Swiss 3T3-PKDK618N.GFP and Swiss 3T3-PKD.GFP cells stimulated with bombesin, vasopressin or EGF for 5 min or 240 min were analyzed by SDS-PAGE followed by Western blot analysis using the pS916 antiserum. Bombesin or vasopressin stimulation for either 5 min or 4 h induced a dramatic increase in the immunoreactivity of the wild-type PKD band indicative of phosphorylation at Ser 916 (Fig. 3A). In contrast, EGF did not produce any increase in PKD immunoreactivity, either at early or late times of exposure, consistent with the notion that this tyrosine kinase receptor agonist does not induce any significant activation of PKD. As expected, we only detected faint bands in lysates from Swiss 3T3-PKDK618N.GFP cells, confirming that the mutation K618N renders PKD inactive in intact cells. We verified that wild-type and kinase-deficient PKD were expressed at similar levels in Swiss 3T3 cells (Fig. 3B).
PKD Overexpression Increases the Duration of MEK Activation

Our next objective was to determine whether the catalytic activity of PKD is necessary for prolonging the duration of ERK signaling in response to GPCR agonists. We analyzed ERK1/2 phosphorylation and catalytic activity in lysates from Swiss 3T3-PKD.GFP, Swiss 3T3-PKD618N.GFP and Swiss 3T3-PKD.GFP cells stimulated with bombesin, vasopressin or EGF for 5 or 240 min. As shown in Fig. 3 (C and D), the increase in the duration of ERK phosphorylation and catalytic activity in response to GPCR agonists requires the catalytic activity of PKD because it was seen in Swiss 3T3-PKD.GFP but not in Swiss 3T3 cells overexpressing a kinase-inactive PKD.

PKD Overexpression Increases the Duration of MEK Activation Induced by Bombesin in Swiss 3T3 Cells—Subsequently, we examined whether PKD overexpression also prolongs the activation of MEK induced by bombesin. Activation of MEK1 and MEK2 occurs through phosphorylation of Ser-217 and Ser-221, located in the kinase activation loop (70). Consequently, MEK activation in response to bombesin (10 nM) added for various times (from 2.5 to 120 min) was evaluated by Western blotting using an antibody that detects the active forms of MEK.

As shown in Fig. 4A, bombesin stimulation for 2.5 min induced a striking increase in MEK phosphorylation in Swiss 3T3-PKD.GFP, Swiss 3T3-PKD618N.GFP, and Swiss 3T3-PKD.GFP cells. After 60 and 120 min of incubation with bombesin, MEK from Swiss 3T3-PKD.GFP cells was markedly phosphorylated at the activation loop as compared with that from Swiss 3T3-GFP cells or Swiss 3T3-PKD618N.GFP cells, indicating that the duration of MEK activation in response to bombesin is also increased in cells overexpressing PKD.

To test whether the increase in the duration of ERK activation requires functional MEK, cultures of Swiss 3T3-PKD.GFP and Swiss 3T3-PKD.GFP cells were treated with the selective inhibitors of MEK activation U0126 (71) and PD98059 (72). In agreement with the previous results, bombesin and vasopressin induced sustained ERK activation in cells overexpressing PKD. Treatment with the MEK inhibitors prevented ERK-1 and ERK-2 activation in response to bombesin or vasopressin treatment for either 5 min or 240 min (Fig. 4, B and C). We verified that treatment with U0126 did not exert any detectable effect on PKD activation, as shown by immunoblotting with antibodies that detect the phosphorylated state of PKD at
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PKD Overexpression Selectively Increases the Duration of FAK Phosphorylation at Ser-910 Induced by Bombesin or Vasopressin in Swiss 3T3 Cells—If PKD overexpression increases the duration of ERK pathway activation within cells in response to GPCR agonists, we would expect a selective prolongation of the activation/phosphorylation of downstream targets of ERK. An analysis of the individual phosphorylation sites of FAK provides an attractive approach to test this possibility because GPCR agonists, including bombesin, stimulate FAK phosphorylation not only at multiple tyrosines but also at Ser-910, a direct target of ERK (73). Specifically, we determined here whether PKD overexpression differentially prolongs the kinetics of FAK phosphorylation at Ser-910 in bombesin-treated Swiss 3T3 cells. Cultures of Swiss 3T3-PKD.GFP cells, Swiss 3T3-GFP cells or Swiss 3T3-PKDK618N.GFP cells were stimulated with bombesin for 60 or 240 min and lysed. The extracts were analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of FAK at Ser-910 (pS-910). As shown in Fig. 5A, bombesin-induced FAK phosphorylation at Ser-910 was strikingly enhanced in PKD-overexpressing cells as compared with control cells or cells overexpressing the PKDK618N kinase-deficient mutant.

To examine the specificity of the enhancement of FAK phosphorylation at Ser-910 in response to bombesin in Swiss 3T3-PKD.GFP cells, we also determined autophosphorylation of FAK at Tyr-397 (pY-397) and Src-mediated phosphorylation of FAK at Tyr-577 (pY-577), located in the activation loop of the kinase domain (74, 75). We demonstrated previously that these residues are phosphorylated in response to bombesin through an ERK-independent pathway (76). As seen in Fig. 5B, bombesin-induced phosphorylation of FAK at either Tyr-397 or Tyr-577 was not altered by PKD overexpression, confirming that the enhancement of FAK phosphorylation is restricted to Ser-910, a residue directly targeted by ERK. We again verified that PKD overexpression increases the duration of ERK signaling in response to bombesin by immunoblotting the same membranes with the antibodies that detect the doubly phosphorylated state of ERK (Fig. 5A).

In accord with our previous results, PKD overexpression also enhanced FAK phosphorylation at Ser-910 in response to cell stimulation with vasopressin but not with EGF (Fig. 5B). Furthermore, the increase in FAK phosphorylation at Ser-910 induced by bombesin in PKD-overexpressing cells was abrogated by treatment with the MEK inhibitor U0126 (Fig. 5C). Collectively, the results shown in Fig. 5 substantiate the notion that overexpression of catalytically competent PKD increases the duration of ERK signaling in Swiss 3T3 cells.

Protein Kinase D Overexpression Selectively Increases the Duration of RSK Signaling Induced by Bombesin and Vasopressin—In accord with our previous results, PKD overexpression prolongs ERK1/2 activation by bombesin and vasopressin in Swiss 3T3 cells. A, PKD autophosphorylation is markedly increased in Swiss PKD.GFP cells. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+), Swiss 3T3-GFP cells (−) and Swiss 3T3-PKDK618N.GFP cells (N) were washed and incubated at 37 °C in 2 ml of DMEM containing either 10 or 1 nM Bom, 50 nM VP, or 5 ng/ml EGF for either 5 or 240 min as indicated and then lysed with 2× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of PKD at Ser-916 (p-PKDS916). Shown here are representative autoradiograms, similar results were obtained in five independent experiments. B, overexpression of wild-type PKD and kinase inactive PKD (PKD618N) protein levels are equivalent. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+), Swiss 3T3-GFP cells (−) and Swiss 3T3-PKDK618N.GFP cells (N) were washed and incubated at 37 °C in 2 ml of DMEM containing 10 nM bombesin for either 5 or 240 min as indicated and then lysed with 2× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotted with PKD C-20 antibody (PKD C-20). Shown here are representative autoradiograms, similar results were obtained in seven independent experiments. C, PKD kinase activity is necessary to prolong ERK1/2 activation by bombesin and vasopressin. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+), Swiss 3T3-GFP cells (−) and Swiss 3T3-PKDK618N.GFP cells (N) were washed and incubated at 37 °C in 2 ml DMEM medium containing 10 or 1 nM Bom, 50 nM VP, or 5 ng/ml EGF for either 5 or 240 min as indicated and then lysed with 2× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotted with the phospho-ERK1/2 antibody. Shown here are representative autoradiograms; similar results were obtained in eight independent experiments. D, PKD overexpression prolongs ERK1/2 kinase catalytic activity. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (solid bars), Swiss 3T3-GFP cells (open bars), or Swiss 3T3-PKDK618N.GFP cells (hatched bars) were washed and incubated at 37 °C in 2 ml of DMEM containing either 10 nM Bom, 50 nM VP or 5 ng/ml EGF for 5 or 240 min as indicated. The cells were washed twice with cold PBS and lysed in ice-cold buffer A as described under "Experimental Procedures." ERK1/2 were immunoprecipitated using an anti-ERK1/2 antibody bound to protein A-agarose, and subsequently a kinase assay was performed using MBP as a substrate with the protein A-ERK1/2 immunocomplex as described under "Experimental Procedures." The results shown here are the mean cpm of 3P incorporated into MBP from three separate dishes ± S.E. Shown here is a representative plot from one experiment. Similar results were obtained in three independent experiments.
PKD overexpression increases the duration of agonist-induced MEK and ERK1/2 activation through a MEK-dependent pathway. A, PKD overexpression prolongs MEK activation induced by bombesin. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+), Swiss 3T3-GFP cells (−) and Swiss 3T3-PKDK618N.GFP cells (N) were washed and incubated at 37°C in 2 ml DMEM containing 10 nM bombesin for various times as indicated and then lysed with 2× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of MEK1/2 only when they are phosphorylated on serines 217 and 221 (p-MEK). The membranes were then stripped further analyzed by Western blotting using MEK antibody (MEK1/2) to verify equal loading. Shown here are representative autoluminograms; similar results were obtained in three independent experiments. B, bombesin-induced ERK1/2 activation is inhibited by the MEK inhibitors U1026 and PD98059. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37°C in 2 ml of DMEM either in the absence (−) or presence (+) of either 10 μM U1026 or 10 μM PD98059 for 1 h. The cultures were then stimulated with 10 nM bombesin for either 5 or 240 min as indicated. The cultures were then lysed with 2× SDS-PAGE sample buffer and the samples analyzed by SDS-PAGE and immunoblotted with phospho-ERK1/2 antibody (p-ERK1/2). Shown here are representative autoluminograms; similar results were obtained in four independent experiments. C, vasopressin-induced ERK1/2 activation is inhibited by the MEK inhibitor U1026. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37°C in 2 ml of DMEM either in the absence (−) or presence (+) of 10 μM U1026 for 1 h. The cultures were then stimulated with 50 nM vasopressin for either 5 or 240 min as indicated. The cultures were then lysed with 2× SDS-PAGE sample buffer and the samples analyzed by SDS-PAGE and immunoblotted with phospho-ERK1/2 antibody (p-ERK1/2). Shown here are representative autoluminograms; similar results were obtained in three independent experiments. D, MEK inhibitor U1026 does not inhibit PKD activation. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37°C in 2 ml of DMEM either in the absence (−) or presence (+) of 10 μM U1026 for 1 h. The cultures were then stimulated with 10 nM bombesin for either 10 min or 240 min as indicated. The cultures were then lysed with 2× SDS-PAGE sample buffer and the samples analyzed by SDS-PAGE and immunoblotted with phospho-PKD Ser-916 (pPKD S916). Shown here are representative autoluminograms; similar results were obtained in three independent experiments.

PKD overexpression increases the duration of GPCR agonist-induced phosphorylation of FAK at Ser-910. A, PKD overexpression prolongs FAK phosphorylation at Ser-910 induced by bombesin. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+), Swiss 3T3-GFP cells (−) and Swiss 3T3-PKDK618N.GFP cells (N) were washed and incubated at 37°C in 2 ml of DMEM containing 10 nM bombesin for 60 or 240 min as indicated and then lysed with 2× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotted with antibodies that detect FAK phosphorylated at Ser-910 (pS-910), Tyr-397 (pY-397), or Tyr-577 (pY-577). The membranes were also immunoblotted with phospho-ERK antibody (pERK1/2) and then stripped and further analyzed by Western blotting using ERK2 antibody (ERK2). Shown here are representative autoluminograms; similar results were obtained in five independent experiments. B, PKD overexpression prolongs FAK phosphorylation at Ser-910 induced by vasopressin. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+), Swiss 3T3-GFP cells (−), and Swiss 3T3-PKDK618N.GFP cells (N) were washed and incubated at 37°C in 2 ml of DMEM containing either 50 nM vasopressin or 50 ng/ml EGF for 60 or 240 min as indicated and then lysed with 2× SDS-PAGE sample buffer. The samples were analyzed by SDS-PAGE and immunoblotted with an antibody that detects FAK phosphorylated at Ser-910 (pS-910). Shown here are representative autoluminograms; similar results were obtained in three independent experiments. C, FAK phosphorylation at Ser-910 is inhibited by prior treatment with the MEK inhibitor U1026. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37°C in 2 ml of DMEM either in the absence (−) or presence (+) of 10 μM U1026 for 1 h. The cultures were then stimulated with 10 nM bombesin for either 10 or 240 min as indicated. The cultures were then lysed with 2× SDS-PAGE sample buffer and the samples analyzed by SDS-PAGE and immunoblotted with phospho-Ser-910 FAK antibody (pS-910). Shown here are representative autoluminograms; similar results were obtained in four independent experiments.

pressin in Swiss 3T3 Cells—The 90 kDa RSKs are serine/threonine protein kinases that are activated via ERK-mediated phosphorylation (77, 78). If PKD overexpression increases the duration of ERK catalytic activity within cells in response to GPCR agonists, we would also expect a prolongation of the activation/phosphorylation of RSK, a well defined downstream
target of ERK. To test this possibility, we determined the effect of bombesin, vasopressin, and EGF on RSK activation in Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells, as revealed by immunoblotting with an antibody that detects phosphorylated Thr-574, a site directly phosphorylated by ERK and RSK (79).

As shown in Fig. 6A, stimulation for 5 min with bombesin, vasopressin or EGF induced a striking increase in RSK phosphorylation in both Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells, in agreement with the ability of these agonists to induce rapid ERK activation in these cells. After 240 min of treatment with bombesin or vasopressin (but not with EGF), the phosphorylation of RSK isolated from Swiss 3T3-PKD.GFP cells was markedly increased as compared with that from Swiss 3T3-GFP cells, which is entirely consistent with the increased duration of ERK signaling induced by Gq-coupled receptor agonists in PKD-overexpressing cells. Furthermore, the increased phosphorylation of RSK induced by bombesin and vasopressin was abrogated by treatment with the MEK inhibitor U0126 (Fig. 6A).

PKD Overexpression Induces Striking Accumulation of c-Fos Protein in Response to Bombesin or Vasopressin—Immediate early gene products, including members of the c-fos proto-oncogene family, function as cellular sensors of ERK1/2 signal duration (62). When ERK activation is transient, its activity declines before the c-Fos protein accumulates, and c-Fos is degraded rapidly. However, when ERK signaling is sustained, c-Fos is phosphorylated by ERK and RSK at serines 374 and 381, respectively (80, 81). As shown in Fig. 6, panel B, PKD overexpression markedly increased the phosphorylation of c-Fos, as judged by immunoblotting of c-Fos immunoprecipitates with an antibody that detects the phosphorylated state of c-Fos at Thr-574 (p90RSK). Shown here are representative autoluminograms; similar results were obtained in three independent experiments.

By contrast, the transient increase induced by bombesin in Swiss 3T3-GFP cells was markedly increased as compared with that from Swiss 3T3-PKD.GFP cells and Swiss 3T3-GFP cells, as revealed by immunoblotting with an antibody that detects the phosphorylated state of c-Fos at Ser-374. Shown here is a representative autoluminogram, similar results were obtained in three independent experiments. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37 °C for 2 ml of DMEM either in the absence or presence of 10 nM bombesin for 120 min. The cultures were then lysed with ice-cold buffer A and c-Fos was immunoprecipitated with an anti-c-Fos polyclonal antibody. The samples were then analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of c-Fos at Ser-374. Shown here are representative autoluminograms; similar results were obtained in six independent experiments. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37 °C in 2 ml of DMEM either in the absence or presence of 10 nM bombesin for 120 min.

PKD overexpression induces the phosphorylation of c-Fos on Ser-374. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37 °C in 2 ml of DMEM either in the absence or presence of 10 nM bombesin for 120 min. The cultures were then lysed with ice-cold buffer A and c-Fos was immunoprecipitated with an anti-c-Fos polyclonal antibody. The samples were then analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of c-Fos at Ser-374. Shown here are representative autoluminograms; similar results were obtained in three independent experiments. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37 °C in 2 ml of DMEM either in the absence or presence of 10 nM bombesin for 120 min. The cultures were then lysed with ice-cold buffer A and c-Fos was immunoprecipitated with an anti-c-Fos polyclonal antibody. The samples were then analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of c-Fos at Ser-374. Shown here are representative autoluminograms; similar results were obtained in three independent experiments. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37 °C in 2 ml of DMEM either in the absence or presence of 10 nM bombesin for 120 min. The cultures were then lysed with ice-cold buffer A and c-Fos was immunoprecipitated with an anti-c-Fos polyclonal antibody. The samples were then analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of c-Fos at Ser-374. Shown here are representative autoluminograms; similar results were obtained in three independent experiments. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37 °C in 2 ml of DMEM either in the absence or presence of 10 nM bombesin for 120 min. The cultures were then lysed with ice-cold buffer A and c-Fos was immunoprecipitated with an anti-c-Fos polyclonal antibody. The samples were then analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of c-Fos at Ser-374. Shown here are representative autoluminograms; similar results were obtained in three independent experiments. Confluent and quiescent cultures of Swiss 3T3-PKD.GFP cells (+) and Swiss 3T3-GFP cells (−) were washed and incubated at 37 °C in 2 ml of DMEM either in the absence or presence of 10 nM bombesin for 120 min. The cultures were then lysed with ice-cold buffer A and c-Fos was immunoprecipitated with an anti-c-Fos polyclonal antibody. The samples were then analyzed by SDS-PAGE and immunoblotted with an antibody that detects the phosphorylated state of c-Fos at Ser-374. Shown here are representative autoluminograms; similar results were obtained in three independent experiments.
PKD (Fig. 6C). These results are consistent with the hypothesis that sustained bombesin-induced ERK1/2 and RSK signaling in PKD-overexpressing Swiss 3T3 cells leads to c-Fos protein accumulation in these cells.

If the dramatic increase in c-Fos accumulation induced by bombesin in Swiss 3T3.PKD-GFP cells is caused by sustained ERK signaling, inhibition of this pathway should abrogate c-Fos protein accumulation. Exposure to either U0126 (Fig. 6D) or PD98059 (results not shown) prior to bombesin or vasopressin stimulation completely blocked the persistent increase in the level of c-Fos protein in Swiss 3T3.PKD-GFP cells.

ERK1/2 are known to mediate rapid activation of c-fos gene transcription (83, 84), which is completed within 30–45 min of stimulation (85). Therefore, the inhibition of c-Fos protein accumulation by U0126 could be attributed to inhibition of c-fos gene transcription in response to bombesin rather than to stabilization of the c-Fos protein. To determine whether sustained ERK signaling is responsible for c-Fos accumulation in response to bombesin in Swiss 3T3.PKD-GFP cells, we also added U0126 2 h after bombesin stimulation; i.e. well after bombesin-induced transcriptional induction of c-fos is completed. As shown in Fig. 6D, the addition of U0126 to cells 2 h after bombesin stimulation also suppressed the accumulation of c-Fos protein measured 4 h after bombesin stimulation. Similar results were obtained with vasopressin (Fig. 6D). These results indicate that the dramatic accumulation of c-Fos protein observed in Swiss 3T3.PKD-GFP cells in response to bombesin or vasopressin requires sustained ERK signaling.

Increased Duration of ERK Signaling Mediates the Potentiation of Mitogenic Activity of Bombesin and Vasopressin in PKD-overexpressing Cells—Recently, we reported that PKD overexpression markedly potentiates the induction of DNA synthesis induced by the Gq-coupled receptor agonists bombesin and vasopressin in Swiss 3T3 cells, but the mechanism(s) involved was not defined. To determine whether the increase in the mitogenic activity of these agonists in PKD-overexpressing cells is mediated by an increase in the duration of ERK signaling, cultures of Swiss 3T3-PKD.GFP and Swiss 3T3-GFP cells were treated with U0126. As illustrated by Fig. 7 and in agreement with our previous results, stimulation with either bombesin or vasopressin of Swiss 3T3 cells overexpressing PKD induced a striking increase in the level of [3H]thymidine incorporation into DNA as compared with that produced by these stimuli in cultures of Swiss 3T3-GFP cells. Addition of U0126 together with bombesin or vasopressin completely blocked the enhancement of DNA synthesis seen in Swiss 3T3-PKD.GFP cells stimulated with these agonists.

To demonstrate that the sustained phase of ERK activation was required to mediate the enhancement of agonist-induced DNA synthesis in PKD-overexpressing cells, ERK activity was inhibited by adding U0126 to cultures that have been stimulated with either bombesin or vasopressin for 3 h. As shown in Fig. 7, addition of U0126 after bombesin or vasopressin stimulation also prevented the increase in DNA synthesis produced in cells overexpressing PKD. Similar results were obtained when the MEK inhibitor PD98059 was used instead of U0126 (Fig. 7, inset). These results strongly suggest that the sustained phase of ERK signaling is critical for the enhancement of agonist-induced DNA synthesis in PKD-overexpressing cells.

Concluding Remarks—A substantial body of evidence indicates that Gq-coupled receptor activation leads to the stimulation of the ERKs which are implicated in the regulation of such fundamental cellular processes as proliferation, differentiation and apoptosis (56, 57). It is increasingly recognized that the activation/inactivation kinetics of the ERK pathway is critical for determining specific biological outcomes (62). As a first step to elucidate the mechanism(s) by which PKD potentiates GPCR-induced mitogenesis and to identify signaling pathways that regulate the kinetics of ERK activation, we examined whether PKD overexpression influences the intensity or duration of GPCR-promoted ERK activation.

In agreement with many other previous studies, our results show that the duration of ERK activation induced by the GPCR agonists bombesin or vasopressin is short-lived in Swiss 3T3 cells. In striking contrast, we demonstrate, for the first time, that MEK/ERK/RSK activation induced by these agonists is dramatically prolonged in Swiss 3T3 cells overexpressing wild-type PKD. PKD overexpression did not increase the duration of either MARCKS phosphorylation (Ser-152 and Ser-156) or FAK tyrosine phosphorylation (Tyr-397 and Tyr-577) in re-
response to bombesin or vasopressin, indicating that PKD overexpression did not have a generalized effect on the kinetics of bombesin or vasopressin receptor signaling. In contrast, PKD overexpression prolonged bombesin-induced phosphorylation of FAK at Ser-910, a direct target of ERK. These results indicate that PKD selectively prolongs ERK signaling in response to G_{q}-coupled receptor agonists in Swiss 3T3 cells.

EGF does not induce any significant increase in the phosphorylation or catalytic activity of PKD in Swiss 3T3 cells (53), but stimulates ERK1/2 activation through a Ras-dependent, PKC-independent pathway in these cells (86). Here, we demonstrate that the duration of ERK/RSK activation induced by EGF in Swiss 3T3-PKD.GFP cells was identical to that generated by this growth factor in Swiss 3T3-GFP cells. These results indicate that PKD overexpression selectively increased the duration of ERK activation.

In conclusion, our results identify PKD as an important element in the control of the duration of the MEK/ERK/RSK pathway activation in response to GPCR agonists, a parameter of critical importance in defining the biological outcomes of ERK activation. Our study supports the hypothesis that an increase in the duration of the ERK signaling leading to accumulation of immediate gene products is, at least, one of the mechanisms by which PKD overexpression selectively enhances re-initiation of DNA synthesis by G_{q}-coupled receptor activation. Interestingly, three independent laboratories, including our own, have demonstrated attenuation of pro-apoptotic JNK signaling by PKD (49, 90–92). For example, induced expression of an activated mutant of PKD was sufficient to suppress EGF-stimulated c-Jun phosphorylation at Ser-63, a natural substrate of JNK (91). In view of the opposite effects exerted by PKD on the activity and duration of the ERK and JNK pathways, PKD emerges as a critical element in regulating such fundamental cellular functions as cell fate and proliferation.

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