Bombesin, Vasopressin, Endothelin, Bradykinin, and Platelet-derived Growth Factor Rapidly Activate Protein Kinase D through a Protein Kinase C-dependent Signal Transduction Pathway*

José L. Zugaza‡, Richard T. Waldron, James Sinnett-Smith, and Enrique Rozengurt§

From the Growth Regulation Laboratory, Imperial Cancer Research Fund, P.O. Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX, United Kingdom

Protein kinase D (PKD) is a serine/threonine protein kinase that is activated by phorbol esters via protein kinase C in intact cells. To assess the physiological significance of this putative pathway, we examined the regulation of PKD in living cells by mitogenic regulatory peptides and by platelet-derived growth factors (PDGF)

Our results demonstrate that bombesin rapidly induces PKD activation in Swiss 3T3 cells, as shown by auto-phosphorylation and syntide-2 phosphorylation assays. Maximum PKD activation (14-fold above base-line levels) was obtained 90 s after bombesin stimulation. Bombesin also induced PKD activation in Rat-1 cells stably transfected with the bombesin/gastrin releasing peptide (GRP) receptor and in COS-7 cells transiently co-transfected with PKD and bombesin/GRP receptor expression constructs. No inducible kinase activity was demonstrated when COS-7 cells were transfected with a kinase-deficient PKD mutant. Bombesin-mediated PKD activation was prevented by treatment of Swiss 3T3 cells with the protein kinase C inhibitors GF 109203X and Ro 31-8220. In contrast, these compounds did not inhibit PKD activation when added directly in vitro. Vasopressin, endothelin, and bradykinin also activated PKD in Swiss 3T3 cells through a PKC-dependent pathway. Platelet-derived growth factor-stimulated PKD activation in Swiss 3T3 cells and in porcine aortic endothelial cells stably transfected with PDGF-β receptors. Treatment with GF 109203X or Ro 31-8220 inhibited PKD activation induced by PDGF. Thus, our results indicate that PKD is activated by multiple signaling peptides through a protein kinase C-dependent signal transduction pathway in a variety of cell types.

Protein kinase C (PKC), a major cellular target for the potent tumor-promoting phorbol esters (1, 2), has been implicated in both short and long term regulation of cellular responses, including ion fluxes, gene expression, cell-cell communication, cell morphology, differentiation, and proliferation (1–7). However, despite the recognized importance of this key enzyme, events that occur downstream of PKC activation remain poorly defined.

Molecular cloning has demonstrated the presence of multiple related PKC isoforms (6, 8, 9), i.e. classic PKCs (α, β1, β2, and γ), novel PKCs (δ, ε, η, and θ), and atypical PKCs (ζ and λ) all of which possess a highly conserved catalytic domain. The regulatory domain of both classic and novel PKCs has a tandem repeat of zinc finger-like cysteine-rich motifs that confers phospholipid-dependent phorbol ester and DAG binding to these PKC isoforms (10–14). In contrast, atypical PKCs contain a single cysteine-rich motif, do not bind phorbol esters, and are not regulated by DAG (10, 15–17).

The recently identified mouse serine protein kinase, named PKD, also consists of regulatory and catalytic domains (18). However, comparison of the deduced amino acid sequence of the catalytic domain of PKD with that of other protein kinases indicates that PKD is a distinct protein kinase that is distinctly related to Ca²⁺-regulated kinases but does not belong to any of the protein kinase subfamilies (19). In particular, the kinase subdomains of PKD show little similarity to the highly conserved regions of the kinase subdomains of the PKC family. Consistent with this, PKD does not phosphorylate a variety of substrates utilized by PKCs, indicating that PKD is a protein kinase with distinct substrate specificity (18, 20). The amino-terminal region of PKD contains a putative transmembrane domain, two cysteine-rich, zinc finger-like motifs, and a pleckstrin homology domain that is not found in any of the PKCs. A fusion protein containing the zinc finger-like domains of PKD-bound [³²P]PDB with high affinity (18). Furthermore, immuno-purified PKD was markedly stimulated by PDB or DAG in the presence of phosphatidylinserine (20). A human protein kinase called atypical PKCµ (21, 22) with 92% homology to PKD (extending to 98% homology in the catalytic domain) is also stimulated by phorbol esters and phospholipids (23). These in vitro results indicate that PKD/PKCµ is a novel phorbol ester/DAG-stimulated protein kinase.

Recently we reported that exposure of intact cells to biologically active phorbol esters and membrane-permeant DAG induces PKD activation via a PKC-dependent pathway (24). PKD activity recovered from phorbol ester-stimulated cells can be measured by kinase assays in the absence of lipid activators (24). These results revealed an unsuspected connection between PKCs and PKD and implied that PKD can function downstream of PKCs in a novel signal transduction pathway. To assess the physiological significance of this putative pathway, it was important to determine whether growth promoting...

© 1997 by The American Society for Biochemistry and Molecular Biology, Inc.
PKD Activation by Neuropeptides and PDGF

factors that elevate DAG, and thus activate PKC, also induce PKD activation in intact cells. Quiescent Swiss 3T3 fibroblasts have proved to be a useful model system for elucidating signal transduction pathways involved in cell proliferation (4) and regulatory peptides of the bombesin family have been identified as potent mitogens for these cells (25, 26). Binding of bombesin to its specific seven transmembrane domain receptor activates heterotrimeric G proteins of the Go alpha subfamily (27, 28) that stimulate the beta isoforms of phospholipase C to induce the rapid formation of the intracellular second messengers DAG and inositol 1,4,5-trisphosphate, which activate classic and novel PKCs and mobilize Ca**(2+), respectively (29–34). PDGF, which stimulates tyrosine phosphorylation and subsequent activation of phospholipase C gamma 1 (reviewed in Ref. 35), leads to a similar sequence of events. The experiments presented here were designed to determine whether bombesin and other growth factors can induce PKD activation in intact cells. Our studies demonstrate that PKD activation is a novel early event in the action of multiple signaling peptides.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 cells were maintained in DMEM supplemented with 10% FBS in a humidified atmosphere containing 10% CO2 at 37°C. For experimental purposes, cells were plated in 90-mm dishes at 6 x 10^5 cells/dish in DMEM containing 5% FBS until they became 50–70% confluent and quiescent. COS-7 cells were plated in 90-mm dishes at 9 x 10^5 cells/dish in DMEM containing 10% FBS. Rat-1 cells and Rat-1 cells stably transfected with the bombesin/GRP receptor (36) were subcultured in 90-mm dishes at 3 x 10^5 cells/dish in DMEM containing 5% FBS until they became 50–70% confluent (2–3 days) and then switched down to 0.5% FBS for 3–4 days until they were quiescent. Porcine aortic endothelial cells stably transfected with PDGF-beta receptors were maintained and propagated in Ham's F-12 medium (37). Cells were serum-starved by incubation in Ham's F-12 medium containing 1 mg/ml bovine serum albumin for 12 h.

cDNA Expression Vectors and Transfection of COS-7 Cells—The PKD cDNA fragment spanning bases 125 to 3179 was inserted into the mammalian expression vector pcDNA3, as described (20). A kinase-defective mutant (PKDK618M) was generated by site-directed mutagenesis using the Altered Sites II in vitro mutagenesis kit (Promega) and subcloned into pcDNA3 (pcDNA3-PKDK618M).

Exponentially growing COS-7 cells, 40–60% confluent, were transfected with the various plasmids using Lipofectin (Life Technologies, Inc.). Briefly, 12 μg of DNA were used for 90-mm dishes. The DNA was diluted in 1 ml with Opti-MEM I medium. Life Technologies, Inc. then mixed with Lipofectin (36 μl) diluted to 100 μl with Opti-MEM I medium. After 15 min, the DNA-Lipofectin complex was diluted to 10 μl with Opti-MEM I medium, mixed gently, and overlaid onto rinsed (1 x with Opti-MEM I) COS-7 cells. The cultures were incubated at 37°C for 6 h, and the medium was then replaced with fresh DMEM containing 10% FBS. The cells were used for experimental purposes 72 h later. In the co-transfection experiments, 6 μg of pcDNA3-PKD or pcDNA3-PKDK618M and 6 μg of BNP-PCD2 containing the cDNA encoding the bombesin/GRP receptor (38) as indicated were mixed prior to dilution with Opti-MEM I medium.

Immunoprecipitation—Cultured cells were washed three times in ice-cold phosphate-buffered saline and lysed in 50 ml Tris/HCl, pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 100 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 1% Triton X-100 (lysis buffer A). PKD was immunoprecipitated at 4°C for 2 h with the PA-1 anti-peptide antisera (1:50 dilution). PA-1 antisera was raised against the synthetic peptide EE-REMKLASSERVSL, that corresponds to the COOH-terminal region of the predicted amino acid sequence of PKD as described previously (20). The immunocomplexes were recovered using protein A coupled to agarose.

Kinase Assay of PKD—PKD autophosphorylation was determined in an in vitro kinase assay by mixing 20 μl of immunocomplexes with 20 μl of a phosphorylation mixture containing (final concentrations) 100 μM [γ-32P]ATP (specific activity, 400–600 cpm/pmol), 30 mM Tris/HCl, pH 7.4, 10 mM MgCl2, and 1 mM dithiothreitol. After 10 min of incubation at 30°C, the reactions were terminated by adding an equal volume of 2 x SDS-PAGE sample buffer (1 M Tris/HCl, pH 6.8, 0.1 M Na2SO4, 6% SDS, 0.5 mM EDTA, 4% mercaptoethanol, 10% glycerol), and analyzed by SDS-PAGE. The gels were dried and the 110-kDa radioactive protein bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and subsequent enhanced chemiluminescence detection.

Materials—[γ-32P]ATP (370 MBq/ml) was from Amersham International (UK). PDB, bombesin, vasopressin, b-endorphin, bradykinin, microcystin, and calcyculin A were obtained from Sigma Aldrich (UK). The inhibitors GF 1, Ro 31-8220, and R-59 949 were from Calbiochem (UK). Protein A-agarose and Staphylococcus aureus V8 protease were from Boehringer Mannheim (U.K.). The MEK-1 inhibitor PD 098059 was a generous gift of David H. Coy, Tulane University, New Orleans, LA. LY294002 was a generous gift from Zeneca, UK. Other items were from standard suppliers or as indicated in the text.

RESULTS

Bombesin Induces PKD Activation in Swiss 3T3 Cells—To examine whether bombesin induces PKD activation, confluent

Downloaded from http://www.jbc.org/ by guest on July 25, 2018
and quiescent Swiss 3T3 cells were exposed to a saturating concentration of this peptide (10 nM) for various periods and lysed. The extracts were immunoprecipitated with the PA-1 antiserum raised against a peptide composed of the 15 carboxy-terminal amino acids of PKD. The immunocomplexes were incubated with [γ-32P]ATP and then analyzed by SDS-PAGE and autoradiography to examine the level of autophosphorylation. Stimulation of the cells with bombesin induced a striking increase in PKD activity that was maintained during cell disruption and immunoprecipitation (Fig. 1). A marked increase of PKD activity (8-fold) was obtained after 0.5 min and reached a maximum (14-fold over base-line levels) after 1.5 min of bombesin stimulation (Fig. 1A).

We also determined whether bombesin-mediated PKD activation could be demonstrated using an exogenous substrate. The synthetic peptide syntide-2 (39, 40) has been identified as an efficient substrate for the catalytic domain of PKD (18) and for the full-length PKD (20). Therefore, we have chosen syntide-2 as a model exogenous substrate to assay PKD activity immunoprecipitated from lysates of Swiss 3T3 cells treated with or without bombesin. As illustrated in Fig. 1B, bombesin stimulated rapid and robust PKD activation as shown by the syntide-2 phosphorylation assay. These results demonstrate that bombesin stimulation of intact Swiss 3T3 cells induces an activated state of PKD that is maintained during cell lysis and protein isolation.

Bombesin induced PKD activation in a concentration-dependent fashion with half-maximal stimulation occurring at 0.5 nM as seen by autophosphorylation and syntide-2 phosphorylation assays (Fig. 2A). The bombesin/GRP receptor antagonist [d-F5-Phe6,d-Ala11]bombesin-(6–13)-OMe (Ant), or an equivalent amount of solvent for 20 min as indicated. Cells were left unstimulated (open bars) or were stimulated for 10 min with 10 nM bombesin (Bom, closed bars) or for 10 min with 200 nM PDB (hatched bars). The cultures were lysed, and the lysates were immunoprecipitated with PA-1 antiserum and analyzed by in vitro kinase assay followed by SDS-PAGE and autoradiography. The results shown are the mean ± S.E. of three independent experiments each performed in duplicate.

in response to either bombesin or PDB showed identical patterns of phosphopeptide fragments (Fig. 3).

Bombesin Stimulates PKD Activation in Rat-1 and COS-7 Cells Expressing Bombesin/GRP Receptors—As bombesin activates PKD in Swiss 3T3 cells, we also examined whether bombesin stimulates PKD activation in other cell types. As shown in Fig. 4, bombesin stimulated a rapid and concentration-dependent activation of PKD in Rat-1 cells stably transfected with the bombesin/GRP receptor (36). Half-maximal activation occurred at 0.5 nM. The antagonist [d-F7-Phe6,d-Ala11] bombesin-(6–13)-OMe also prevented PKD activation induced by bombesin in these cells.

To confirm that the kinase activity induced by bombesin was due to activation of PKD rather than to the presence of a co-precipitating protein kinase, we examined bombesin-induced PKD autophosphorylation and syntide-2 phosphorylation in COS-7 cells co-transfected with a bombesin/GRP receptor construct (BNR-pCD2) and with either a wild-type PKD expression vector (pcDNA3-PKD) or a kinase-defective PKD mutant (pcDNA3-PKDK618M) in which lysine 618 in the ATP-binding site is substituted by a methionine. Bombesin treatment of COS-7 cells, co-transfected with pcDNA3-PKD and BNR-pCD2, resulted in activation of wild-type PKD (Fig. 5). In contrast, no inducible kinase activity was seen in COS-7 cells transfected with pcDNA3K618M despite similar PKD and PKDK618M expression levels (Fig. 5). These results demonstrate that the bombesin-induced kinase activity measured in PKD immunoprecipitates was due to the activation of PKD.

Peptide mapping with S. aureus V8 protease of PKD labeled
Confluent and quiescent Swiss 3T3 cells (10^7 cells/condition) were either left unstimulated or stimulated for 10 min with either 200 nM PDB or 10 nM bombesin as indicated, followed by two-dimensional tryptic peptide mapping on thin layer plates as described under “Experimental Procedures.” The directions of electrophoresis at pH 1.9 (i.e., toward the cathode) and ascending thin layer chromatography (TLC) are indicated by arrows to the lower left of the figure. Sample origins are indicated by small filled squares. Phosphopeptide patterns observed after autoradiography are shown. Approximately six prominent ^32^P-labeled tryptic peptide spots were found to be reproducibly induced by either phorbol ester (PDB) or bombesin (Bom) treatments. Interestingly, at least two of these prominent spots also appeared on maps generated from unstimulated cells upon long term exposure of films to thin layer plates. Approximately 1000 cpm were spotted to control plates, and approximately 5000 cpm to plates were used for maps of PKD activated within cells by PDB or bombesin treatments. The control autoradiogram shown was exposed to the thin layer plate for 6 days, and the PDB- or bombesin-stimulated autoradiograms were exposed to the respective thin layer plates for 2 days. Results shown are typical of four similar experiments.

FIG. 3. Two-dimensional tryptic phosphopeptide maps of PKD autophosphorylated in vitro and immunoprecipitated from cells stimulated with bombesin or PDB. Confluent and quiescent Swiss 3T3 cells (10^7 cells/condition) were either left unstimulated or stimulated for 10 min with either 200 nM PDB or 10 nM bombesin as indicated, followed by two-dimensional tryptic peptide mapping on thin layer plates as described under “Experimental Procedures.” The directions of electrophoresis at pH 1.9 (i.e., toward the cathode) and ascending thin layer chromatography (TLC) are indicated by arrows to the lower left of the figure. Sample origins are indicated by small filled squares. Phosphopeptide patterns observed after autoradiography are shown. Approximately six prominent ^32^P-labeled tryptic peptide spots were found to be reproducibly induced by either phorbol ester (PDB) or bombesin (Bom) treatments. Interestingly, at least two of these prominent spots also appeared on maps generated from unstimulated cells upon long term exposure of films to thin layer plates. Approximately 1000 cpm were spotted to control plates, and approximately 5000 cpm to plates were used for maps of PKD activated within cells by PDB or bombesin treatments. The control autoradiogram shown was exposed to the thin layer plate for 6 days, and the PDB- or bombesin-stimulated autoradiograms were exposed to the respective thin layer plates for 2 days. Results shown are typical of four similar experiments.

Bombedin Induces PKD Phosphorylation in Intact Swiss 3T3 Cells—The preceding experiments demonstrated that treatment with bombesin markedly increased the level of PKD autophosphorylation in in vitro kinase assays. We next examined whether bombesin induces PKD phosphorylation in intact cells. Confluent and quiescent cultures of Swiss 3T3 cells metabolically labeled with ^32^P_i in COS-7 cells either transfected with a wild-type PKD expression construct and stimulated with PDB or cotransfected with the wild-type PKD expression construct and the bombesin/GRP receptor expression construct and stimulated with bombesin showed identical phosphopeptide fragments (results not shown).

Inhibitors of PKC Prevent PKD Activation Induced by Bombesin—Recent results indicated that phorbol esters induce PKD activation via a PKC-dependent pathway (24). Consequently, we examined whether PKC was required for PKD activation in response to bombesin. As shown in Fig. 7, treatment of intact Swiss 3T3 cells with various concentrations of the PKC inhibitors GF I (43) and Ro 31-8220 (44) inhibited PKD activation induced by subsequent addition of bombesin in a concentration-dependent manner. In striking contrast, GF I or Ro 31-8220 added directly to the in vitro kinase assay at identical concentrations to those used previously in intact cells did not inhibit PKD activity stimulated by bombesin in intact cells (Fig. 7). Similar results were obtained when the effects of GF I and Ro 31-8220 on bombesin-induced PKD activation were examined in Rat-1 cells stably transfected with the bombesin/GRP receptor (Results not shown). Thus, GF I and Ro 31-8220 did not inhibit PKD directly but interfered with bombesin-mediated PKD activation in intact cells by blocking PKC.

Next, we examined whether other signaling pathways contribute to bombesin-mediated PKD activation in Swiss 3T3 cells. To determine the role of Ca^2+ mobilization in PKD activation by bombesin, quiescent Swiss 3T3 cells were treated with the tumor promoter thapsigargin. This agent specifically inhibits the endoplasmic reticulum Ca^2+ ATPase and thereby depletes Ca^2+ from intracellular stores (45). Treatment with 30 nM thapsigargin for 30 min abolished the increase in cytosolic Ca^2+ induced by subsequently added bombesin (results not shown) but did not prevent PKD activation induced by bombesin. Similarly, chelation of extracellular Ca^2+ with EGTA to prevent Ca^2+ influx did not affect PKD activation. Furthermore, a combination of thapsigargin and EGTA did not inhibit bombesin-induced PKD activation (Fig. 8A). Thus inhibition of...
PKD Activation by Neuropeptides and PDGF

**FIG. 5.** Bombesin stimulates PKD activation in COS-7 cells co-transfected with PKD and bombesin/GRP receptor expression constructs. Upper, exponentially growing COS-7 cells (40–60% confluent) were co-transfected with either pcDNA3-PKD or pcDNA3-PKD618M and BNR-pCD2 containing the cDNA encoding the bombesin/GRP receptor, as indicated. 72 h post-transfection, the cultures were treated for 10 min with either 10 nM bombesin (+, Bom) or with an equivalent amount of solvent (−). The cultures were then lysed, and the lysates were immunoprecipitated with PA-1 antiserum and further analyzed by an in vitro kinase assay (IVK) prior to SDS-PAGE and autoradiography. In addition, aliquots of the lysates were analyzed by Western blotting for the expression of PKD (W. Blot) in each case. Similar results were obtained in three independent experiments. Lower, parallel cultures of co-transfected COS-7 cells were treated for 10 min with either 10 nM bombesin (+) or with an equivalent amount of solvent (−). The cultures were then lysed, and the lysates were immunoprecipitated with PA-1 antiserum. PKD activity was measured by syntide-2 phosphorylation as described under “Experimental Procedures.” The results are expressed as the mean ± S.E. (n = 6).

Ca\(^{2+}\) influx or mobilization from intracellular stores did not affect bombesin-mediated PKD activation.

Bombesin stimulates activation of p42\(^{mapk}\)/p44\(^{mapk}\) (46, 47) and p70\(^{euk}\) (48) and tyrosine phosphorylation of p125\(^{fak}\) and other substrates (49–51) in Swiss 3T3 cells. Inhibition of p70\(^{euk}\) activation with rapamycin, of p42\(^{mapk}\) and p44\(^{mapk}\) with the selective MEK-1 inhibitor PD 098059, and of tyrosine phosphorylation of p125\(^{fak}\) with either cytochalasin D or genistein did not affect PKD activation in response to bombesin (Fig. 8B). In contrast, pretreatment with GF I or Ro 31-8220 markedly reduced subsequent PKD activation induced by bombesin in parallel cultures of Swiss 3T3 cells (Fig. 8B).

The compound R 59 494, a selective inhibitor of DAG kinase, enhances DAG accumulation in a variety of cell types including Swiss 3T3 cells (52, 53). As shown in Fig. 8C, R59 494 potentiated the ability of a submaximal concentration of bombesin (0.4 nM) to stimulate PKD activation in these cells. The results presented in Figs. 7 and 8 suggest that bombesin-induced DAG accumulation stimulates PKD activation via PKC.

**FIG. 6.** Bombesin induces PKD phosphorylation in vivo in a time- and dose-dependent manner. Confuent and quiescent Swiss 3T3 cells were incubated for 12 h with carrier-free \(^{32}P\) in phosphate-free DMEM and then stimulated with different concentrations of bombesin for 10 min (upper autoradiogram). In parallel cultures, \(^{32}P\)-labeled cells were stimulated with 10 nM bombesin for different times (graph and inset). Cells were lysed, immunoprecipitated with PA-1 antiserum, and the samples analyzed by SDS-PAGE and autoradiography. Results are representative of two independent experiments.

Vasopressin, Endothelin, Bradykinin, and PDGF also Induce PKD Activation in Swiss 3T3 Cells—To investigate whether bombesin was unique in its ability to activate PKD within Swiss 3T3 cells or whether agonist-mediated activation of other G\(_{\text{q}}\)-coupled receptors would also lead to PKD activation, we also tested a variety of other biologically active peptides. Vasopressin, like bombesin, also induced striking and rapid increases in PKD activity (Fig. 9A). Maximal stimulation (15-fold over base-line values) of PKD activation by vasopressin occurred within 2 min of exposure, and this stimulation was again concentration-dependent, with half-maximal activation occurring at 0.6 nM (Fig. 9B). Similarly, endothelin also induced a time- and concentration-dependent activation of PKD in Swiss 3T3 cells (Fig. 9, C and D) with half-maximal activation achieved at 3 nM. Interestingly, whereas stimulation of PKD activity by vasopressin peaked rapidly and then declined to somewhat lower constant values, endothelin treatment of Swiss 3T3 cells induced stimulation of PKD activity somewhat more gradually, such that maximal stimulation (8-fold over base-line values) consistently occurred after approximately 5 min of exposure to this agonist (Fig. 9C). Endothelin also induced PKD activation in Rat-1 cells which also express receptors for this agonist (results not shown).

Bradykinin is known to induce rapid but transient stimulation of diacylglycerol production and PKC stimulation in Swiss 3T3 cells (30). As shown in Fig. 10C, bradykinin also induced PKD activation in Swiss 3T3 cells although it was consistently less effective in promoting PKD activation than bombesin, vasopressin, or endothelin.

To examine the role of PKC in PKD activation induced by vasopressin, endothelin, and bradykinin, we tested the effect of GF I and Ro 31-8220 on the increase in PKD activity induced by these agonists. As shown in Fig. 10, treatment of Swiss 3T3 cells with either 3.5 \(\mu\)M GF I or 3.5 \(\mu\)M Ro 31-8220 prevented PKD activation induced by subsequent exposure to vasopressin, endothelin, and bradykinin.

To determine whether other growth factors can also induce PKD activation, the effect of exposure of cells to either insulin or PDGF was examined. Saturating doses of insulin (1 \(\mu\)g/ml), which synergistically stimulate DNA synthesis in Swiss 3T3 cells but are unable to activate PKC (54), did not induce PKD activation when measured 10 min after treatment (results not shown). In contrast, exposure of the cells to increasing concentrations of PDGF caused a dose-dependent increase in PKD activity (Fig. 11A). Half-maximal and maximal (15-fold over base-line values) effects were achieved at 3 and 10 ng/ml, respectively. PKD activation in response to PDGF could also be demonstrated in porcine aortic endothelial cells (37) stably transfected with the PDGF-\(\beta\) receptor.

PDGF-induced PKD activation in Swiss 3T3 cells was prevented by treatment with 50 \(\mu\)M genistein, but it was not affected by inhibition of phosphatidylinositol 3-kinase activity with either 100 nM wortmannin or 20 \(\mu\)M Ly294002 (Fig. 11B). To examine whether PKC also mediated PKD activation induced by PDGF, we tested the effect of GF I or Ro 31-8220 on the activation of PKD induced by PDGF. Treatment of Swiss
PKD Activation by Neuropeptides and PDGF

DISCUSSION

Recently, we reported that treatment of intact cells with biologically active phorbol esters and cell-permeant diacylglycerols induces PKD activation through a PKC-dependent signal transduction pathway (24). These findings revealed a novel connection between PKCs and PKD and have important implications for the understanding of signal transduction pathways mediating the action of the second messenger DAG. In the present study we determined whether physiological activation of PKC via occupancy of specific membrane receptors for mitogenic neuropeptides and growth factors can also increase the activity of PKD in intact cells.

Our results demonstrate, for the first time, that stimulation of Swiss 3T3 cells with bombesin induces a striking activation of PKD. The concentration dependence and sensitivity of this response to a specific receptor antagonist indicates that the effects of bombesin are mediated through the same receptors that elicit other molecular responses and stimulate DNA synthesis in these cells. PKD recovered by immunoprecipitation from bombesin-stimulated cells is fully active in the absence of lipid effectors (i.e. phosphatidylserine and PDB) as shown by autophosphorylation assays as well as by phosphorylation of the exogenous substrate syntide-2. The conversion of PKD into this activated state that persists during cell disruption and protein isolation occurs within seconds of bombesin stimulation of Swiss 3T3 cells and thus is one of the early events induced by this neuropeptide agonist in these cells.

The results of transfection experiments reveal that bombesin-mediated activation of PKD is not restricted to the Swiss 3T3 cell line. Thus, stable expression of bombesin/GRP receptor in Rat-1 cells or transient co-expression of PKD and bombesin/GRP receptors in COS-7 cells allowed rapamycin activation of PKD via bombesin stimulation of cells. The low endogenous levels of PKD expression in COS-7 cells was also advantageous in that this made it possible to verify that the inducible kinase activity in PKD immunoprecipitates was indeed due to PKD activation rather than to the stimulation of a co-precipitating kinase. Hence, bombesin did not stimulate kinase activity in COS-7 cells co-transfected with bombesin/GRP receptor and a PKD kinase-deficient mutant.

3T3 fibroblasts or endothelial cells with these PKC inhibitors markedly inhibited PKD activation in response to PDGF (Fig. 11C).

of Swiss 3T3 cells and thus is one of the early events induced by this neuropeptide agonist in these cells.

of Swiss 3T3 cells and thus is one of the early events induced by this neuropeptide agonist in these cells.

FIG. 7. Bombesin induces PKD activation through a PKC-dependent pathway. Confluent and quiescent Swiss 3T3 were incubated for 1 h with different concentrations of the PKC inhibitors GF I (A and filled circles) and Ro 31-8220 (C and open circles). Control PKD immunoprecipitates received an equivalent amount of solvent (-). The cultures were subsequently stimulated for 10 min with 10 nM bombesin (+, Bom). Cells were lysed, and the lysates were immunoprecipitated with PA-1 antiserum, and PKD activity was determined by an in vitro kinase assay as described under “Experimental Procedures,” followed by SDS-PAGE and autoradiography. Parallel cultures were treated with 10 nM bombesin for 10 min (B and D, closed and open triangles), lysed, and PKD immunoprecipitated with PA-1 antiserum. PKD activity was determined by an in vitro kinase assay in the absence (-) or in the presence of the indicated concentrations of either GF I or Ro 31-8220 added directly to the incubation mixture. Control PKD immunoprecipitates received an equivalent amount of solvent (-). The reactions were analyzed by SDS-PAGE and autoradiography. The results shown in the graphs are the mean ± S.E. of three independent experiments. Values are expressed as percentages of the maximum response after substration of the control. The autoradiograms are from a representative experiment; two additional experiments gave similar results.

FIG. 8. Specificity of GF I and Ro 31-8220 inhibition of bombesin-induced PKD activation. A, quiescent and confluent Swiss 3T3 cells were incubated for 30 min with 3 mM EGTA, 30 nM thapsigargin (TG), a combination of 3 mM EGTA and 30 nM thapsigargin or an equivalent amount of solvent, as indicated. Cells were then left unstimulated (open bars) or were stimulated for 10 min with 10 nM bombesin (Bom) (closed bars). B, quiescent and confluent Swiss 3T3 cells were incubated for 1 h with either 3.5 μM GF I, 3.5 μM Ro 31-8220 (Ro), 20 nM rapamycin (Rap), 100 nM wortmannin (Wor), 20 μM Ly 294002 (Ly), 60 μM H-89; 10 μM PD098059 (PD), 50 μM genistein (Gen), or for 2 h with either 2.5 μM cytochalasin D (Cyt D) or an equivalent amount of solvent (-). Cells were subsequently challenged for 10 min with 10 nM bombesin (closed bars), except for control cultures (open bars). C, quiescent and confluent Swiss 3T3 cells were incubated for 10 min with either 10 μM of the selective DAG kinase inhibitor R59 949 or an equivalent amount of solvent. Cells were then left unstimulated (open bars) or were stimulated for 10 min with 0.4 nM bombesin. In all cases (A, B, and C), the cultures were lysed, and the lysates were immunoprecipitated with PA-1 antiserum and analyzed by an in vitro kinase assay as described under “Experimental Procedures,” followed by SDS-PAGE and autoradiography. The labeled 110-kDa band corresponding to PKD was quantified by scanning densitometry. Values shown are the mean ± S.E. of three independent experiments.
PKD Activation by Neuropeptides and PDGF

A prominent early event induced by bombesin is the rapid generation of DAG and consequent activation of PKC (31–33, 55). Since direct PKC stimulation by phorbol esters has been shown to activate PKD in intact cells, bombesin could induce PKD activation through a PKC-dependent pathway. Consistent with this possibility, a DAG kinase inhibitor potentiates PKD activation induced by submaximal concentrations of bombesin, and PKD peptide maps give rise to identical patterns whether PKC activity was stimulated through bombesin-mediated generation of DAG or directly through PDB. Furthermore, pretreatment of either Swiss 3T3 cells or Rat-1 cells transfected with the bombesin/GRP receptor with the inhibitors of PKC, GF I, and Ro 31-8220 before stimulation with bombesin strikingly prevents PKD activation. Crucially, neither GF I nor Ro 31-8220 inhibits PKD activity when added directly in vitro, even at the concentrations used in intact cells to prevent bombesin-induced PKD activation. In addition, inhibition of many other signaling events induced by bombesin, including Ca\(^{2+}\) influx and/or mobilization, MEK-1-mediated mitogen-activated protein kinase activation, p70\(^{60k}\) activation, and p125\(^{65k}\) tyrosine phosphorylation, does not interfere with bombesin-induced PKD activation. Taken together these results strongly suggest that persistent PKD activation induced by bombesin is mediated by PKC.

PKC consists of multiple isozymes with possibly different biological functions. Conventional PKC isozymes (\(\alpha, \beta\), and \(\gamma\)) are Ca\(^{2+}\)-dependent, whereas novel (\(\delta, \epsilon, \eta, \) and \(\theta\)) and atypical (\(\zeta\) and \(\lambda\)) isozymes do not require Ca\(^{2+}\) for their activation (6–9).

Recent studies demonstrated that co-transfection of PKC\(\alpha\) and -\(\eta\) strongly induced PKD activation in COS-7 cells (24). The dissociation of PKD activation from Ca\(^{2+}\) influxes in bombesin-treated Swiss 3T3 cells shown in the present study is consistent with a role for Ca\(^{2+}\)-insensitive isoforms of PKC in mediating PKD activation in these cells. Further experimental work will be required to elucidate whether PKCs directly phosphorylate and activate PKD or stimulate an intermediary kinase(s) that leads to activation of PKD.

To substantiate the results obtained with bombesin, we also examined the effect of agonist activation of other seven transmembrane domain receptors that stimulate phospholipase C\(\gamma\), through Go\(_q\). Vasopressin, endothelin, and bradykinin bind to receptors that rapidly promote hydrolysis of inositol phospholipids, Ca\(^{2+}\) mobilization, and PKC activation in Swiss 3T3 cells (29–31, 56–58). In the present study, we demonstrate that these agonists also induce PKD activation in these cells via a PKC-dependent pathway.

The binding of PDGF to individual receptor chains stimulates their dimerization and subsequent transphosphorylation (59). Phospholipase C\(\gamma\) and phosphatidylinositol 3-kinase, as other cytoplasmic effector proteins, associate with specific phosphorylated tyrosine residues on the receptor and are phosphorylated on tyrosine by the intrinsic tyrosine kinase activity.

FIG. 9. Vasopressin and endothelin activate PKD in Swiss 3T3 cells in a time- and dose-dependent manner. A and C, confluent and quiescent cultures of Swiss 3T3 cells were treated for various times with 20 nM vasopressin (A) or with 50 nM endothelin (C) as indicated and lysed. The lysates were immunoprecipitated with PA-1 antiserum. PKD activity was determined by an in vitro kinase assay followed by SDS-PAGE and autoradiography. The results shown are the mean \(\pm\) S.E. of three independent experiments. The autoradiograms shown are from a representative experiment; two additional experiments gave similar results.

FIG. 10. Vasopressin, endothelin, and bradykinin induce PKD activation through a PKC-dependent pathway. Confluent and quiescent Swiss 3T3 cells were incubated for 1 h with either 3.5 \(\mu\)M GF I, 3.5 \(\mu\)M Ro 31-8220 (Ro), or an equivalent amount of solvent (–). Cells were subsequently stimulated for 10 min with either 20 nM vasopressin (VP, A) or 50 nM endothelin (End, B), and for 1 min with 50 nM bradykinin (BK, C). Cells were lysed, and the lysates were immunoprecipitated with PA-1 antiserum, and PKD activity was determined by an in vitro kinase assay as described under “Experimental Procedures,” followed by SDS-PAGE and autoradiography. The results shown are the mean \(\pm\) S.E. of three independent experiments.
PKD Activation by Neuropeptides and PDGF

PKD activation by neuropeptides and PDGF stimulates polyphosphoinositide hydrolysis leading to PKC activation, whereas the putative second messengers generated by phosphatidylinositol 3-kinase, phosphatidylinositol (3,4)-bisphosphate, and phosphatidylinositol (3,4,5)-trisphosphate have been proposed to activate novel isoforms of PKC (61, 62) and the protein kinase encoded by the akt proto-oncogene (63). Here we demonstrate that PDGF stimulates PKD activation in either Swiss 3T3 cells or in porcine aortic endothelial cells stably transfected with the PDGF-β receptor. Given that the potent phosphatidylinositol 3-kinase inhibitors wortmannin and Ly 294002 did not interfere with PKD activation induced by PDGF, it is unlikely that either phosphatidylinositol 3-kinase or Akt initiates a major pathway leading to PKD activation. In contrast, treatment of these cells with either GF-1 or Ro 31-8220 before stimulation with PDGF prevented PKD activation suggesting that PKC activation is once again a critical step.

In conclusion, our findings demonstrate that PKD can be rapidly activated in response to multiple signaling peptides in a variety of cell types. The results indicate that vasopressin, vasoressin, endothelin, bradykinin, and PDGF induce PKD activation via PKC. We conclude that PKD activation is a novel early event in the action of multiple regulatory peptides and growth factors.

REFERENCES
1. Weinstein, I. B. (1988) Cancer Res. 48, 4135–4143
2. Nishizuka, Y. (1989) Cancer (Philad.) 63, 1892–1903
3. Kikkawa, U., and Nishizuka, Y. (1986) Annu. Rev. Cell Biol. 2, 149–154
4. Rozengurt, E. (1986) Science 234, 161–166
5. Herschmann, H. R. (1991) J. Biol. Chem. 266, 28330–28338
6. Nishizuka, Y. (1992) Science 258, 607–614
7. Nishizuka, Y. (1995) FASEB J. 9, 484–496
8. Hug, H., and Sarr, T. F. (1993) Biochem. J. 291, 329–343
9. Kasper, L. V., and Parker, P. J. (1994) Trends Biochem. Sci. 19, 73–77
10. Ono, Y., Fujii, T., Orita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3099–3103
11. Burns, D. J., and Bell, R. M. (1991) J. Biol. Chem. 266, 18330–18338
12. Hubbard, S. R., Bishop, W. R., Kirschmeier, P., George, S. J., Cramer, S. P., and Hendrickson, W. A. (1991) Science 254, 1776–1779
13. Quest, A. F. G., Barde, E. S. G., and Bell, R. M. (1994) J. Biol. Chem. 269, 2953–2960
14. Zhang, G., Kazaniotis, M. G., Blumberg, P. M., and Hurley, J. H. (1995) Cell 81, 917–924
15. Nakashima, H., and Extow, J. H. (1992) J. Biol. Chem. 267, 16347–16354
16. Ways, D. K., Cook, P. P., Webster, C., and Parker, P. J. (1992) J. Biol. Chem. 267, 4789–4805
17. Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y., and Biden, T. J. (1993) J. Biol. Chem. 268, 24296–24302
18. Valverde, A. M., Sinnett-Smith, J., Van Lint, J., and Rozengurt, E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8572–8576
19. Rozengurt, E., Sinnett-Smith, J., Van Lint, J., and Valverde, A. M. (1995) Mutat. Res. 333, 153–160
20. Van Lint, J., Sinnett-Smith, J., and Rozengurt, E. (1995) J. Biol. Chem. 270, 1455–1461
21. Muller, A., Khorasani, Z., and Rozengurt, E. (1992) J. Biol. Chem. 267, 2812–2819
22. Offermanns, S., Heiler, E., Spicher, K., and Rozengurt, E. (1993) EMBO J. 12, 153–160
23. Dieterich, S., Herget, T., Link, G., Bottinger, H., Pfizenmaier, K., and Bucher, H. (1993) EMBO J. 12, 6220–6230
24. Rozengurt, E., and Sinnett-Smith, J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2096–2100
25. Zachary, I., and Rozengurt, E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7616–7620
26. Efremov, S., Heiler, E., Spicher, K., and Schultz, G. (1994) FEBS Lett. 349, 201–204
27. Heilmich, M., Battey, J., and Northrup, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 751–756
28. Heskell, J. P., Blakely, D. M., Brown, K. D., Irvine, R. F., and Berridge, M. J. (1986) Cell 47, 703–709
29. Issandou, M., and Rozengurt, E. (1990) J. Biol. Chem. 265, 11890–11896
30. Lasfargues, A., and Rozengurt, E. (1988) EMBO J. 7, 2741–2747
31. Staddon, J. M., Barker, C. J., Murphy, A. C., Chanter, N., Lax, A. J., Michell, R. H., and Rozengurt, E. (1991) J. Biol. Chem. 266, 4840–4847
32. Zachary, I., Sinnett-Smith, J., and Rozengurt, E. (1986) J. Cell. Biol. 102, 2211–2222
33. Berridge, M. J. (1993) Nature 361, 315–325
34. Extow, J. (1997) Eur. J. Biochem. 23, 10–20
35. Charleworth, A., Broad, S., and Rozengurt, E. (1996) Oncogene 12, 1337–1345
36. Rankin, S., Hoshmand-Rad, R., Claesson-Welsh, L., and Rozengurt, E. (1996) J. Biol. Chem. 271, 7829–7834
37. Battey, J. F., Way, J. M., Corjay, M. H., Shapiro, H., Kusano, K., Harkins, R., Wu, J. M., Slattery, T., Senden, E., and Feldman, R. I. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 395–399
38. Lora, T., Cruzallegui, F. H., Fesquet, D., Cavadore, J. C., Mery, J., Heans, A., and Doree, M. (1993) Nature 368, 270–273
39. Mochizuki, H., Ito, T., and Hidaka, H. (1993) J. Biol. Chem. 268, 9143–9147
40. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) Methods Enzymol. 201, 110–119
41. Coy, D., Mungan, Z., Rossowski, W., Cheng, B., Lin, J. T., Mrzienski, J., and Jensen, R. (1992) Peptides (Elmsford) 13, 775–781
42. Teulier, D., Planett, P., Coste, H., Bellevueger, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boisson, P., Boursier, E., Lorilie, F., Duhamel, L., Charron, D., and Kirilovsky, J. (1991) J. Biol. Chem. 266, 15771–15781
43. Yeo, E.-J., and Extow, J. H. (1995) J. Biol. Chem. 270, 3890–3898
44. Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P.
PKD Activation by Neuropeptides and PDGF

(1990) Proc. Natl. Acad. Sci. U. S. A. 87, 2466–2470
46. Pang, L., Decker, S. J., and Saltiel, A. R. (1993) Biochem. J. 289, 283–287
47. Seufferlein, T., Withers, D. J., Mann, D., and Rozengurt, E. (1996) Mol. Biol. Cell 7, 1865–1875
48. Withers, D. J., Seufferlein, T., Mann, D., Garcia, B., Jones, N., and Rozengurt, E. (1997) J. Biol. Chem. 272, 2509–2514
49. Rozengurt, E. (1991) Eur. J. Clin. Invest. 21, 123–134
50. Sinnett-Smith, J., Zachary, I., Valverde, A. M., and Rozengurt, E. (1993) J. Biol. Chem. 268, 14261–14268
51. Zachary, I., Sinnett-Smith, J., Turner, C. E., and Rozengurt, E. (1993) J. Biol. Chem. 268, 22060–22065
52. de Courcelles de Chaffoy, D., Roevens, P., Van Belle, H., Kennis, L., Somers, Y., and De Klerk, F. (1989) J. Biol. Chem. 264, 3274–3285
53. Morris, C., and Rozengurt, E. (1988) FEBS Lett. 231, 311–316
54. Rozengurt, E., and Sinnett-Smith, J. (1988) Prog. Nucleic Acid Res. Mol. Biol. 35, 261–295
55. Issandou, M., and Rozengurt, E. (1989) Biochem. Biophys. Res. Commun. 163, 201–208
56. Mendoza, S. A., Schneider, J. A., Lopez-Rivas, A., Sinnett-Smith, J., and Rozengurt, E. (1986) J. Cell Biol. 102, 2223–2333
57. Takuwa, N., Takuwa, Y., Bollag, W. E., and Rasmussen, H. (1987) J. Biol. Chem. 262, 182–188
58. Lopez-Rivas, A., Mendoza, S. A., Nanberg, E., Sinnett-Smith, J., and Rozengurt, E. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 5768–5772
59. Heldin, C. (1995) Cell 80, 213–223
60. Kapeller, R., and Cantley, L. C. (1994) BioEssays 16, 565–576
61. Toker, A., Meyer, M., Reddy, K. K., Palek, J. R., Aneja, B., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 32358–32367
62. Moriya, S., Kazlauskas, A., Akimoto, K., Hirai, S., Mizuno, K., Takenawa, T., Fukui, Y., Watanabe, Y., Ozaki, S., and Ohno, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 181–185
63. Franke, T., Yang, S., Chang, T., Datta, K., Kazlauskas, A., Morrison, D., Kaplan, D., and Tsichlis, P. (1995) Cell 81, 727–736
Bombesin, Vasopressin, Endothelin, Bradykinin, and Platelet-derived Growth Factor Rapidly Activate Protein Kinase D through a Protein Kinase C-dependent Signal Transduction Pathway
José L. Zugaza, Richard T. Waldron, James Sinnett-Smith and Enrique Rozengurt

J. Biol. Chem. 1997, 272:23952-23960.
doi: 10.1074/jbc.272.38.23952

Access the most updated version of this article at http://www.jbc.org/content/272/38/23952

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 63 references, 37 of which can be accessed free at http://www.jbc.org/content/272/38/23952.full.html#ref-list-1