The Role of Protein Kinase D in Neurotensin Secretion Mediated by Protein Kinase C-α/-δ and Rho/Rho Kinase*

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Jing Li‡, Kathleen L. O’Connor‡‡, Mark R. Hellmich‡‡, George H. Greeley, Jr.§, Courtney M. Townsend, Jr.‡, and B. Mark Evers‡§

From the ‡Department of Surgery and §Sealy Center for Cancer Cell Biology, The University of Texas Medical Branch, Galveston, Texas 77555-0536

Regulatory hormones, localized to specialized endocrine cells in the small bowel, control numerous physiological functions of the gastrointestinal (GI) tract including secretion, motility, and mucosal growth (1). The gut peptide neurotensin (NT), a tri-decapeptide localized to enteroendocrine cells (N cells) of the distal small bowel (2, 3), facilitates fatty acid translocation (4), affects gut motility (5), and stimulates growth of normal gut mucosa (6, 7). In addition to its trophic effects on normal GI tissues, NT stimulates proliferation of certain pancreatic, colonic, and prostatic cancers bearing NT receptors (NTR) (8). Although the mechanisms for pancreatic hormone release have been well characterized (9), the signal transduction pathways regulating stimuli-induced gut hormone secretion are not entirely understood. One reason for this paucity in our understanding is the relative lack of useful in vitro models that recapitulate in vivo properties of intestinal endocrine cells.

The BON endocrine cell line was established from a human pancreatic carcinoid tumor and characterized in our laboratory (10). These cells have served as an invaluable in vitro model for hormone secretion studies. Similar to the terminally differentiated N cell of the small bowel, BON cells express high levels of NT/neuromedin N mRNA, synthesize and secrete NT peptide, and process the NT/neuromedin N precursor protein in a fashion identical to that of the normal intestine (11). BON cells exhibit morphological and biochemical characteristics consistent with the enteroendocrine cell phenotype, including the presence of numerous dense core granules and the expression and secretion of chromogranin A and other peptides (e.g. pancreaticastatin) (10, 12, 13). Thus, the BON cell line provides an excellent model to delineate the mechanisms underlying gut peptide secretion.

We have shown that protein kinase C (PKC), particularly isomers PKC-α and -δ, plays a role in the stimulated release of NT (13–15). The PKCs comprise a family of intracellular serine/threonine-specific kinases that are, depending on the isoform, typically activated by Ca2⁺, lipid second messengers or protein activators and mediate the effects of a wide range of physiological stimuli, including growth factors, hormones, and neurotransmitters (16, 17). Protein kinase D (PKD), originally referred to as PKC-μ, is a serine/threonine protein kinase with unique structural, enzymological, and regulatory properties that are different from those of the PKC family members (18, 19). The most distinct characteristics of PKD are the presence of a catalytic domain distantly related to Ca2⁺-regulated kinases, a pleckstrin homology (PH) domain within the regulatory region, and a highly hydrophobic stretch of amino acids in its N-terminal region (18, 19). Expression of PKD has been demonstrated in some endocrine cells, including insulin- and gastrin-secreting cells (20–22). PKD activation occurs through several mediators via a PKC-dependent pathway (23–26).

Recent studies indicate a close association between PKC isoforms and members of the Rho family of small GTP-binding proteins including Rho (A, B, and C), Rac1, and Cdc42 (27, 28). The Rho family members, well-known regulators of the actin cytoskeleton and phosphoinositide metabolism (29), have been implicated in hormone secretion from endocrine cells (30–32). Rho kinases (ROK) are the first effectors of Rho to be discovered and, to date, two ROK isoforms have been identified: ROKα and ROKβ (33). There are now several examples where Rho GTPases participate in regulated secretory pathways, such as in mast cells (34), PC12 cells (35), and neurons (36).

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¶ To whom correspondence should be addressed: The University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0536. Tel.: 409-772-5612; Fax: 409-747-4819; E-mail: mevers@utmb.edu.

The abbreviations used are: NT, neurotensin; PKC, protein kinase C; PKD, protein kinase D; HA, hemagglutinin; BBS, bombesin; PMA, phorbol 12-myristate 13-acetate; GST, glutathione S-transferase; RIA, radioimmunoassay; GFP, green fluorescent protein; siRNA, small interfering RNA; GRP, gastrin releasing peptide; WT, wild type.
The potential role of Rho family members in gut peptide secretion is not known. The purpose of our present study was 2-fold: (i) to determine whether PKD is involved in NT secretion, and (ii) to delineate upstream regulators of PKD in the BON endocrine cell line. We found that the novel PKD protein mediates phorbol ester- or bombesin (BBS)-stimulated NT secretion and is regulated by PKC-α, -δ, and the Rho/ROK pathway. Importantly, these findings describe a novel signaling mechanism for gut hormone release from specialized endocrine cells.

EXPERIMENTAL PROCEDURES

Expression Constructs and Small Interfering RNA (siRNA)—The GFP-tagged PKD expression plasmids, wild-type (PKDwt) and PKDΔN (a constitutively active PKD with PH domain deleted) were provided by Dr. Franz-Josef Johannes (The Fraunhofer Institute for Interfacial Engineering) (37). The pSUPER PKD siRNA vector and the control vector pSUPER were provided by Dr. Alex Toker (Harvard Medical School, Boston, MA) (38). The PKC-α and -δ expression plasmids (pTB701- HA-PKC-α and pTB701-HA-PKC-δ) and the control plasmid (pTB701-HA) were provided by Dr. Yoshitaka Ono (Kobe University) (39). The HA-tagged ROKα expression plasmids, including the full-length (ROKαN), the deletion mutant (ROKαN-c) and pX40 control plasmid, were provided by Dr. Thomas Leung (National University of Singapore, Singapore) (40). The vectors pCEFLAU5 encoding wild-type RhoA (RhoAwt), RhoA(Q63L) (constitutively active mutant), and RhoA(N19) (dominant negative mutant) were provided by Dr. J. Silvio Gutkind (National Institutes of Health, Bethesda, MD) (41). The GFP-tagged PKD, were cultured in 25-mm round coverslips in 6-well plates and imaged in real time before and after PMA treatment. Cells were placed inside a prewarmed (37 °C) chamber on the stage of an LSM 510 META confocal system configured with an Axiovert 200M inverted microscope (Zeiss, Jena, Germany). GFP fluorescence images were acquired using a plan-apochromat 63×, 1.4 NA oil immersion objective and the 488 nm line of an argon ion laser for excitation. The image acquisition and processing was carried out using the Zeiss LSM510 workstation (v 3.0) and the Zeiss Image Browser (v3.1) software.

Immunoprecipitation and in Vitro Kinase Assays—Immunoprecipitation and in vitro kinase assays were performed as described previously (14). In brief, equal amounts of protein were resolved on Novex Tris-glycine or NuPAGE Bis-Tris gels (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membranes; the membranes were incubated with primary antibodies overnight at 4 °C followed by secondary antibodies conjugated with hors eradisher peroxidase. Membranes were developed using the ECL detection system.

RESULTS

siRNA Directed Against PKD Inhibits NT Secretion and PKD Phosphorylation in BON Cells—Based on findings that both PMA and bradykinin1 induce the activation of endogenous PKD in BON cells (14), we determined whether PKD is involved in PMA-mediated NT secretion. We took advantage of the RNA interference (RNAi) to selectively reduce PKD expression (Fig. 1A). BON cells were transfected with the PKD siRNA vector (pSUPER.PKD siRNA) and the control vector (pSUPER). Cells were collected at 48 or 96 h after transfection, and lysates immunoblotted to assess expression of endogenous PKD (Fig. 1A, 6, A-C, and 8C) or a one-factor experiment (Fig. 6D). Fisher’s least significant difference procedure was used for multiple comparisons with Geisser-Greenhouse adjustment for the number of comparisons. A p value < 0.05 was considered significant.

NT Radioimmunoassay (RIA)—Parental BON cells were treated with PMA in secretion medium for 30 min. The BON/GPR/GFP-GFP cells were treated with the GPR ligand, BBS, in Krebs-Henseleit Buffer, containing 0.2% FCS, 1% of Cat-I, 5.1×105 IU of HEPES, 0.1% bovine serum albumin (pH 7.4). For inhibitor treatments, cells were pretreated with inhibitors for 30 min, followed by combined treatments with PMA (10 nM) and inhibitors for another 10 min. Medium was collected and stored at –80 °C until RIA for NT. RIA for NT was performed in duplicate samples as described previously (42, 43).

Real Time Confocal Microscopy—BON cells, transiently expressing GFP-tagged PKD, were cultured in 25-mm round coverslips in 6-well plates and imaged in real time before and after PMA treatment. Cells were placed inside a prewarmed (37 °C) chamber on the stage of an LSM 510 META confocal system configured with an Axiovert 200M inverted microscope (Zeiss, Jena, Germany). GFP fluorescence images were acquired using a plan-apochromat 63×, 1.4 NA oil immersion objective and the 488 nm line of an argon ion laser for excitation. The image acquisition and processing was carried out using the Zeiss LSM510 workstation (v 3.0) and the Zeiss Image Browser (v3.1) software.

Protein Preparation and Western Blotting—Protein preparation and Western blotting were performed as described previously (14). In brief, equal amounts of protein were resolved on Novex Tris-glycine or NuPAGE Bis-Tris gels (Invitrogen) and electrophoretically transferred to polyvinylidene difluoride membranes; the membranes were incubated with primary antibodies overnight at 4 °C followed by secondary antibodies conjugated with horseradish peroxidase. Membranes were developed using the ECL detection system.

Immunoprecipitation and in Vitro Kinase Assays—Immunoprecipitation and in vitro kinase assays were performed as described previously (14). In brief, proteins (50 μg) were incubated with PKD or GFP antibodies (1:50) on a shaker for 2 h at 4 °C followed by another 2 h incubation with 30 μl of protein A-Sepharose beads at 4 °C. The immunocomplexes were suspended in 20 μl of kinase buffer and kinase reaction, with the exception of subtilisin-2 as a substrate, was started by adding 5 μCi of [γ-32P]ATP and incubated for 10 min at 30 °C. Reactions were stopped by the addition of 2× Tris-glycine sample buffer. Samples were denatured by boiling for 5 min and separated by NuPAGE 4–12% Bis-Tris gels. Gels were incubated in Gel-Dry drying solution (Invitrogen) for 5 min and dried at 60 °C for 60 min followed by exposure to x-ray film.

RhoA Activity Assay—RhoA activity was assessed using the Rho binding domain of Rhotekin as described (44, 45). In brief, cells (3 × 106) were transfected with 50 μg of GST-C3 or GST protein and plated onto 60-mm dishes in growth medium, allowed to adhere and then serum-starved in secretion medium overnight. Cells were treated with PMA (10 nM) for 10 min and then extracted with a radioimmunoprecipitation assay buffer. After centrifugation at 14,000 × g for 2 min, the extracts were incubated for 30 min at 4 °C with glutathione beads (Amersham Biosciences) coupled with bacterially expressed GST-RBD (Rho binding domain of Rhotekin) fusion protein (44), and then washed three times. The RhoA content was determined by immunoblotting samples using rabbit anti-RhoA antibody.

Statistical Analysis—All experiments were repeated at least two times and data are reported as means ± S.E. Data were analyzed using analysis of variance for a two-factor factorial experiment (Figs. 1B, 2A, 6, A-C, and 8C) or a one-factor experiment (Fig. 6D). Fisher’s least significant difference procedure was used for multiple comparisons with Geisser-Greenhouse adjustment for the number of comparisons. A p value < 0.05 was considered significant.
NT secretion from BON cells compared with the control vector. Taken together, our results, using complementary techniques, strongly demonstrate an important role for PKD in NT secretion from BON cells.

Overexpression of Wild-type and Constitutively Active PKD Increases PMA-mediated NT Secretion—To further confirm the regulation of PKD on NT secretion, BON cells were transiently transfected with PKD<sub>WT</sub>, or the constitutively active PKD<sub>ΔPH</sub>, both linked to GFP, or the empty vector (pEGFP-N1), as a control (Fig. 2). 24 h after, the cells were serum-starved, then treated with vehicle (Me<sub>2</sub>SOr) or PMA (10 nM) for 30 min, and the medium collected for measurement of NT by RIA (Fig. 2A).

In the absence of PMA treatment, overexpression of PKD<sub>ΔPH</sub> increased NT release compared with the empty vector (pEGFP-N1) and PKD<sub>WT</sub>. The PH domain exerts an inhibitory effect on the catalytic domain of PKD (46); therefore, the increase in basal NT release suggests that the PH domain plays an inhibitory role in the regulation of its enzymatic activity. NT secretion was significantly increased by PMA treatment of BON cells transfected with the empty vector. Importantly, PMA treatment of cells transfected with either PKD<sub>WT</sub> or PKD<sub>ΔPH</sub> resulted in a significantly enhanced NT secretion compared with PMA treatment of BON cells transfected with the empty vector, but the increase in secretion from BON cells transfected
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FIG. 3. PMA induces translocation of wild-type PKD and constitutively active PKD in BON cells. BON cells were transiently transfected with GFP-tagged wild-type PKD, constitutively active PKD or the empty vector and allowed to recover for 24 h. Cells were then serum-starved in secretion medium overnight. BON cells were incubated in PBS, pH 7.4, and placed inside a prewarmed (37 °C) chamber and imaged in real time as described under “Experimental Procedures.” Top, PKDWT in quiescent BON cells was distributed evenly throughout the cytosol of these cells with no apparent association with specific intracellular compartments. PKDAPH, rapidly translocated to the plasma membrane when PMA was added. Middle, the constitutively active PKDAPH localized to the cytosol as well as the nucleus. Moreover, PKDAPH was noted to translocate from the cytosol to the membrane by PMA stimulation. The PKDAPH in the nucleus failed to translocate to the membrane. Bottom, compared with wild-type PKD, the GFP control vector was noted throughout the cytosol and the nucleus. Translocation was not found in the cells transfected with the empty vector after PMA stimulation. A representative result from three experiments is shown.

FIG. 4. PKM induces PKD activation through a PKC and ROCK-dependent pathway. Cells were serum-starved in secretion medium overnight and pretreated with vehicle (0.1% MeSO) or inhibitors for 30 min and then treated with 10 nM PMA in combination with inhibitors for another 10 min. A, PKD activation was determined by Western blot analysis using the phospho-PKD antibodies, including Ser744/748 (upper panel) and Ser916 (middle panel). PKD was examined as a loading control (bottom panel). B, PKD activation was determined by in vitro kinase assays using syntide-2 as substrate. A representative experiment from five experiments is shown.

with PKDAPH was lower than NT secretion noted from PKDWT transfected BON cells.

Overexpression of PKD plasmids was confirmed by immunoblot analysis for GFP tag (Fig. 2B). GFP-tagged PKD was evenly distributed in cells transfected with PKDWT or PKDAPH, either in the presence or absence of PMA, but not in the control vector (upper panel). The membrane was reprobed with an antibody that recognizes phosphorylated serine 744/748 (middle panel). Phosphorylation of exogenous PKD was not detected in BON cells transfected with the PKDWT, in the absence of PMA, but was induced in BON cells transfected with either the PKDWT and PKDAPH treated with PMA. These data indicate that the response of exogenous PKD to PMA is similar to endogenous PKD. A weak signal was detected in BON cells transfected with PKDAPH, in the absence of PMA treatment, demonstrating the constitutively active status of PKD with the PH domain deleted. This observation is in agreement with the increase of NT secretion found in BON cells transfected with PKDAPH without PMA stimulation (Fig. 2A). Similar levels of endogenous (endo) phosphorylated PKD (∼115 kDa) were detected in all BON cells treated with PMA, showing that stimulation of endogenous PKD by PMA was not affected by exogenous PKD. Total endogenous PKD was reprobed as a loading control (Fig. 2B, bottom panel). Taken together, these results further support the findings that activation of overexpressed PKD enhances PMA-mediated NT secretion.

To confirm the above results of PKD phosphorylation, we directly assayed the activity of PKD using the same cell lysates as above by in vitro kinase assays of GFP immunoprecipitates (Fig. 2C). PKD activity in immunocomplexes was determined by phosphorylation of syntide-2, a synthetic peptide reported to be an excellent substrate for PKD (47) (lower bands), as well as by autophosphorylation (upper bands). Extracts from BON cells transfected with the empty vector showed a basal level of syntide-2 (with or without stimulation); PKDWT activity was increased in the absence of PMA compared with the basal level of syntide-2, suggesting the existence of weak kinase activity in the wild-type PKD even without PMA stimulation. The phosphorylation of syntide-2 was enhanced by PMA in BON cells transfected with PKDWT, further demonstrating the stimulation of PKD by PMA. BON cells expressing constitutively active PKD exhibited a similar level of syntide-2 phosphorylation as
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Fig. 6. Rho/ROK pathway alters PMA-mediated NT secretion. A, BON cells were transfected with GST-C3 toxin (100 μg) or GST and cultured overnight. Cells were incubated in serum-free medium for 1 h and treated with 10 nM PMA for 30 min. Medium was collected, and the level of NT secreted into the medium was measured by RIA using duplicate samples. Results are expressed as means ± S.E. (n = 6); *, p < 0.05 versus GST; †, p < 0.05 versus PMA and GST. B, top, 48 h after transfection, BON cells were incubated in secretion medium for 30 min and treated with PMA (10 nM) for another 30 min. Medium was collected for NT RIA. Results are expressed as means ± S.E. (n = 6); *, p < 0.05 versus control siRNA; †, p < 0.05 versus PMA and control siRNA. Bottom, cells were lysed, and Western blotting analysis was performed to detect the inhibition of ROKα expression by ROKα siRNA using anti-ROKα antibody (upper panel). Actin was reprobed for the loading control (lower panel). C, BON cells were transiently transfected with the empty vector (pXJ40), the corresponding full-length ROKα or constitutively active ROKα (1–543) vectors for 24 h. Cells were incubated with secretion medium overnight and incubated with vehicle or PMA (10 nM) for 30 min. Top, overexpression of ROKα was demonstrated by blotting with anti-HA antibody. Bottom, NT secretion was measured by RIA as described above. Results are expressed as means ± S.E. (n = 6); *, p < 0.05 versus empty vector (pXJ40); †, p < 0.05 versus PMA and empty vector. D, BON cells were incubated with secretion medium overnight and then preincubated with vehicle or the ROK inhibitors, Y27632 or HA1077, for 30 min before addition of PMA (10 nM). After another 30 min, the medium was collected and the level of NT secreted into the medium was measured by RIA using duplicate samples. Experiments were performed in triplicate; results are expressed as means ± S.E. (n = 6); *, p < 0.05 versus control (vehicle treatment); †, p < 0.05 versus 10 nM PMA. Experiments were performed in triplicate.

noted with PKDWT. With PMA stimulation, the activity of both PKDWT and PKDΔPH was increased with PKDWT being more pronounced. These results are consistent with the findings of NT release following transfection with these constructs (Fig. 2A). Increased phosphorylation of syntide-2 was not noted in BON cells transfected with PKDΔPH compared with BON cells transfected with PKDWT in the presence of PMA. This is consistent with the result in Fig. 2A in which NT secretion is lower in BON cells transfected with PKDΔPH than BON cells transfected with PKDWT in the presence of PMA but inconsistent with the result in Fig. 2B in which PKD phosphorylation is much higher in BON cells transfected with PKDΔPH than BON cells transfected with PKDWT. These results may be explained by different reaction conditions in the kinase assay in vitro compared with the in vivo conditions.

PMA Induces Translocation of PKDWT and PKDΔPH in BON Cells—Translocation is considered to be a marker of PKC activation (48). To further confirm the role of PKD on NT secretion, translocation of PKD tagged with GFP was visualized in BON cells using time-lapse confocal microscopy (Fig. 3). Intense fluorescence was observed in BON cells transfected with PKDWT (top panel). The addition of PMA (100 nM) induced a rapid translocation of PKDWT from the cytosol to the plasma membrane within 1 min after stimulation. These results are consistent with our report that endogenous PKD translocated to the membrane from the cytosol after PMA stimulation in fixed cells (14). In quiescent BON cells transfected with PKDΔPH, the fluorescence was present in the cytosol with intense fluorescence in the nuclei (middle panel). The addition of PMA induced a rapid translocation of PKDΔPH from the cytosol to the plasma membrane; the intense fluorescence noted in the nuclei persisted after PMA treatment. In addition, vesicle-like structures were observed close to the membrane in BON cells transfected with either PKDWT or PKDΔPH (Supplementary video of Fig. 3, middle panel) after addition of PMA. As a control, fluorescence in BON cells transfected with empty vector was observed before and after the addition of PMA (bottom panel); no changes in GFP localization were noted after PMA treatment. These results demonstrate the activation of PKD by PMA and further support the role of PKD in NT secretion.
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**Classic or Novel PKC and Rho Kinase Inhibitors Attenuate PMA-mediated PKD Activation**—PKD is activated in a PKC-dependent fashion in some cell types (23–26). To determine whether PKC isoforms are involved in PMA-induced PKD activation in BON cells, we examined the effect of three PKC inhibitors, Gö6983, GFX, and Ro31–8220, which inhibit activity of classic and novel PKC isoforms but not PKD (49, 50) (Fig. 4). BON cells were pretreated with these inhibitors (1 μM for each) for 30 min prior to a 10-min exposure to PMA (10 nM). As shown in Fig. 4A, Gö6983, GFX, and Ro31–8220 completely blocked PKD transphosphorylation at Ser744/748 (upper panel) and significantly attenuated PKD autophosphorylation at Ser916 (middle panel), suggesting the requirement of upstream PKC isoforms in the activation of PKD. We examined whether ROK inhibitors, Y27632 and HA1077 (both at a concentration of 15 μM), affect the activation of PKD. Both inhibitors blocked significantly attenuated PKD phosphorylation with the most pronounced inhibition noted with the Y27632 compound. These findings demonstrate the involvement of upstream ROK in the activation of PKD. Total PKD was probed to assess loading equality (bottom panel).

To further confirm these results, in vitro kinase assays were performed (Fig. 4B). The same cell extracts above were immuno-precipitated with PKD antibody, and the catalytic activity was then assayed with syntide-2 as an exogenous substrate (47). PKD activity was increased by PMA stimulation compared with the activity without PMA treatment. Activation of PKD was attenuated by all three PKC inhibitors as well as the two ROK inhibitors, particularly the Y27632 compound, which significantly attenuated PKD phosphorylation (Fig. 4C). Western blotting using anti-PKC-α/H9251 control siRNA; cells were lysed at 48 h after transfection. As shown in Fig. 4A, Gö6983, GFX, and Ro31–8220 completely blocked PKD transphosphorylation at Ser744/748 (upper panel) and significantly attenuated PKD autophosphorylation at Ser916 (middle panel), suggesting the requirement of upstream PKC isoforms in the activation of PKD. We examined whether ROK inhibitors, Y27632 and HA1077 (both at a concentration of 15 μM), affect the activation of PKD. Both inhibitors blocked significantly attenuated PKD phosphorylation with the most pronounced inhibition noted with the Y27632 compound. These findings demonstrate the involvement of upstream ROK in the activation of PKD. Total PKD was probed to assess loading equality (bottom panel).

**PKD Activation Is PKC-α- and -δ-dependent**—We have reported that PMA-mediated translocation of PKC-α and -δ from the cytosol to the membrane associated with PMA-mediated NT secretion (14), suggesting that PKC-α and -δ might act as upstream kinases for PKD. We first examined whether PKC-α contributed to PMA-induced PKD activation by overexpression of wild-type PKC-α (Fig. 5A). Phosphorylation of PKD was up-regulated in BON cells overexpressing wild-type PKC-α compared with the control vector in the presence of PMA (top). The membrane was stripped and reprobed with PKD antibody to monitor the loading equality (middle). The membrane was reprobed with anti-HA antibody to show the overexpression of PKC-α (bottom). Regulation of PKC-α-mediated PKD phosphorylation was further confirmed using PKC-α siRNA (Fig. 5B). BON cells were transfected with the PKC-α siRNA and the control siRNA; cells were lysed at 48 h after transfection. Western blotting using anti-PKC-α antibody was performed to confirm PKC-α inhibition (top). The blot was reprobed with anti-PKD (Ser744/748) antibody; PKD phosphorylation was markedly reduced by PKC-α siRNA (middle). The membrane was stripped and reprobed with PKD antibody to monitor the loading equality (bottom).

**Similar experiments were performed to determine the regulation of PKC-δ on PKD phosphorylation (Fig. 5C).** Overexpression of wild-type PKC-δ dramatically up-regulated PKD phosphorylation compared with the control vector in the presence of PMA (top). The loading equality was controlled by probing for total PKD expression using the same membrane (middle). The membrane was reprobed with anti-HA antibody to confirm overexpression of PKC-δ (bottom). Treatment with PKC-δ siRNA, as shown by Fig. 5D, blocked PKC-δ expression (top) as well as PKD phosphorylation (middle). Total PKD expression was assessed as the loading control (bottom). This result further confirmed the upstream regulation of PKC-δ on PKD activity.

**Rho/ROK Pathway Contributes to PMA-mediated NT Secretion**—Based on the finding that PKD activation is decreased by ROK-specific inhibitors, we examined whether Rho/ROK pathway is involved in PMA-mediated NT secretion. BON cells were transfected with Clostridium botulinum C3 toxin, which specifically ADP-ribosylates Rho and impairs its function (51), and NT secretion was measured by RIA. C3 toxin significantly decreased PMA-stimulated NT secretion compared with control GST in the presence of PMA (Fig. 6A). To further confirm the involvement of Rho/ROK pathway, the role of ROKα (down-stream effector of Rho proteins) on NT secretion was examined using ROKα siRNA (Fig. 6B). PMA-mediated NT secretion was decreased in BON cells transfected with ROKα siRNA compared with the control siRNA transfected BON cells. To extend these findings on the role of ROKα in PMA-mediated NT secretion, HA-tagged ROKα, including full-length (ROKα1–543), constitutively active (ROKα1–543G12V), and control vector (pXJ40) were transfected into BON cells (Fig. 6C). The expression level was first examined by Western blotting using anti-HA antibody (top). Expression of ROKα1–543 (180 kDa) and ROKα1–543G12V (60 kDa) was detected in BON cells. As expected, no signal was detected in BON cells transfected with the control vector. NT secretion was measured in BON cells transfected with ROKα1–543, ROKα1–543G12V, and the control vector pXJ40 (bottom). Overexpression of ROKα1–543, but not ROKα1–543G12V, significantly increased NT secretion compared with the control vector in the presence of 10 nM of PMA. The role of ROKα in PMA-stimulated NT secretion was further supported using ROK inhibitors (Fig. 6D). BON cells were pretreated with varying dosages of Y27632 and HA1077 for 30 min and then treated with PMA (10 nM). Treatment with Y27632 decreased PMA-mediated NT secretion in a dose-dependent fashion. Compared with Y27632, HA1077 was less effective in blocking PMA-mediated NT secretion; significant inhibition was noted only at a concentration of 15 μM which correlates with the effects on PKD phosphorylation as shown in Fig. 4A. These results strongly support a role for Rho/ROK in PMA-mediated NT secretion in BON cells.

**Rho Proteins Regulate PKD Phosphorylation**—To determine whether PKD activation is induced by signaling pathway(s) initiated by Rho proteins, BON cells were transfected with C3
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Fig. 8. PKD siRNA inhibited BBS-mediated NT secretion from BON/GRPR cells. A, BON cells stably expressing the human GRPR tagged with GFP (BON/GRPR-GFP) were generated and expression of the GRPR was determined by fluorescent microscopy. B, BON/GRPR-GFP cells were transfected with control siRNA or PKD siRNA (from Dharmacon). 48 h later, cells were treated with 0.1% MeSO (0) or BBS at the indicated doses for 15 min. Western blots were performed using anti-PKD antibody to monitor the inhibition of PKD expression by PKD siRNA (upper panel). PKD siRNA inhibition of BBS-induced PKD activation was examined using phospho-PKD (Ser\(^{744,748}\)) antibody (middle panel). β-Actin was determined as a loading control (lower panel). C, NT release was measured by RIA. Results are expressed as means ± S.E. (n = 6). * and †, p < 0.05 versus vehicle (0.1% MeSO) of control siRNA. ‡, p < 0.05 versus 10 or 100 nM of BBS and control siRNA.

The novel PKD protein is expressed in certain endocrine cells (14, 20–22); however, to date, there has been little evidence to suggest a role for PKD in hormone secretion. The findings in our present study are the first to identify a role for PKD in stimulated gut peptide release. Either PMA or bryostatin1, which are potent activators of PKC, resulted in the activation and cellular redistribution of PKD. Silencing PKD expression by RNA interference resulted in decreased basal and BBS-stimulated NT secretion. Overexpression of both wild-type and constitutively active PKD increased PMA-mediated NT secretion from BON cells. In addition, GRPR-expressing BON cells demonstrated increased PKD activation and NT secretion in response to the ligand BBS, thus further supporting a role for PKD in the physiological release of NT. Therefore, our current study, utilizing a combinatorial approach (i.e., specific inhibition and overexpression of PKD) demonstrates a critical role for PKD in the signal transduction pathway leading to NT peptide release.

PKD has been implicated in the organization of the Golgi apparatus, regulating the fission of vesicles from the trans-Golgi network (53). PKD is a resident protein of the Golgi compartment in certain cell types (22, 53, 54) where it is involved in constitutive transport processes. In our present study, we found that PKD\(^{WT}\)-GFP was evenly distributed in the cytosol of untreated BON cells with no discernable Golgi localization. In the presence of PMA, translocation of PKD\(^{WT}\)-GFP was noted to occur from the cytosol to the membrane. Consistent with our findings, Matthews et al. (55) noted that PKD was localized to membranes surrounding cytoplasmic granules and not in the Golgi of RBL 2H3 mast cells after treatment with phorbol ester. This report, in combination with our present study, suggests a role for PKD in stimulated peptide secretion that is distinct from its role in Golgi transport processes.

PKD activation can be mediated by a PKC-dependent signal transduction pathway that involves the phosphorylation of Ser\(^{744,748}\) of PKD within the activation loop of the catalytic...
PKD Regulates NT Release

...domain of PKD and occurs by diverse stimuli including neu-ropetides and growth factors (56) in non-endocrine cells. For example, activation of PKD can occur through activation of PKC-e in HEK393 cells (22), PKC-eta in COS-7 cells (26), and PKC-theta in T cells (25). We demonstrate PKD phosphorylation was inhibited in the novel human endocrine cell line BON by pretreatment with PKC inhibitors and by PKC-alpha and -delta siRNA and enhanced by overexpression of wild-type PKC-alpha and -delta. Therefore, our findings indicate that downstream activation of PKD is PKC-dependent, particularly PKC-alpha and -delta, in the BON endocrine cell line. Similar to our results, Tan et al. (23) demonstrated PKD-mediated PKD activation was stimulated by thrombin in aortic smooth muscle cells. Recently, Storz et al. (57) reported that PKC-delta participates in the activation of PKD in HeLa cells exposed to oxidative stress. To date, it has been shown that PKD2 is activated via PKC-alpha through the CCK2/gastrin receptor in human gastric cancer cells (58), no direct interaction between PKC-alpha and PKD has been reported. PKD appears to be a scaffold protein and is an important regulator of diverse intracellular signaling pathways (19); therefore, it is possible that PKD forms a complex with PKC-alpha or PKC-delta or other proteins mediating NT secretion in BON cells. However, the exact relationship between PKC-alpha and PKC-delta with PKD will require further investigation.

The Rho family and their upstream or downstream factors have been implicated in various cellular functions such as regulation of actin filament reorganization and exocytosis (27, 28). In mast and chromaffin cells, one or more members of the Rho family play an active role in the exocytic process (27, 28). In intestinal epithelial cells, treatment with C3 toxin prevented PKC translocation (64). In epithelial and endothelial cells, treatment with C3 toxin prevented PKC translocation and activation (65). Recently, Slater et al. (27, 28) demonstrated that Rho-GTP potently stimulates PKC-alpha activity in vitro. In other studies, PKC has been shown to activate Rho (66, 67). We found that treatment of BON cells with PMA stimulated PKC and Rho/ROK pathways, leading to PKD-regulated NT secretion. Rho has been predominantly described in the regulation of actin filament reorganization (27, 28), and PKCs are also known to be important regulators of the cytoskeleton (68). Therefore, it is possible that PKC-alpha and PKC-delta and Rho/ROK-mediated PKD activation leads to NT secretion through the reorganization of actin filaments. The precise sequence of events culminating in stimulated NT release remains to be fully elucidated.

In conclusion, the results of this study identify the novel PKD protein as a critical regulator of PKC- or BBS-mediated NT secretion from the BON or BON/GRPR cell lines, respectively. We demonstrate that PKD phosphorylation is regulated by upstream PKC-alpha and -delta, and the Rho/ROK pathway (Fig. 9 summarizes our proposed model for PKD-mediated NT secretion). Our findings identify novel signaling pathways contributing to stimulated peptide secretion from specialized endocrine cells of the GI tract. The coordinated release of intestinal peptides in response to extracellular mediators is essential for the regulation of intestinal digestion, secretion and motility; therefore, our results delineate critical signaling molecules that contribute to this important physiologic process. Interestingly, prominent regulators of PKD and NT secretion, namely PKA-sensitive PKCs (69) and RhoA (70) contribute to tumor progression. Since NT can stimulate proliferation of NTR positive tumors, it is interesting to speculate that pathways, such as RhoA and PKCs, may promote tumorigenesis through the dysregulated secretion of trophic factors such as NT.

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