Neurotensin Induces Protein Kinase C-dependent Protein Kinase D Activation and DNA Synthesis in Human Pancreatic Carcinoma Cell Line PANC-1

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ABSTRACT

Signal transduction pathways through protein kinase C (PKC) may play a significant role in DNA synthesis and proliferation of human pancreatic cancers. Treatment of human pancreatic ductal adenocarcinoma cell line PANC-1 with biologically active phorbol-12,13-dibutyrate led to striking activation of protein kinase D (PKD), a member of a novel family of serine/threonine kinases distinct from PKC isoforms. Using PANC-1 as a model system, we demonstrate that neurotensin (NT) induced a rapid and striking activation of PKD as determined by in vitro kinase assay and by in vivo phosphorylation of serines 744, 748, and 916. PKD activation induced by NT was abrogated by treatment of PANC-1 cells with PKC inhibitors GF-1 and Ro 31–8220. NT induced a rapid and transient translocation of PKD from the cytosol to the plasma membrane. Inhibiting PKC activity blocked the reverse translocation of PKD from the plasma membrane to the cytosol. Finally, we show that NT-induced DNA synthesis in PANC-1 cells is PKC-dependent. Collectively our results demonstrate, for the first time, the existence of a functional PKC/PKD signaling pathway in human ductal pancreatic carcinoma cells and suggest that PKCs mediate the mitogenic signaling process initiated by NT.

INTRODUCTION

Pancreatic cancer is a devastating disease, with an overall 5-year survival rate of only 3–5% (1). Ductal adenocarcinoma of the pancreas comprises 90% of the human exocrine pancreatic cancers and is the fourth leading cause of cancer death in men and the fifth leading cause in women in most Western countries. Surgery and current conventional chemotherapy offer very limited survival benefits, and novel therapeutic strategies are urgently required to treat this aggressive disease (2). These could arise from a detailed understanding of the factors and signaling pathways that stimulate the unrestrained proliferation of ductal pancreatic cancer cells.

Neuropeptides including NT, gastrin, cholecystokinin, and mammalian bombesin-like peptides act as potent cellular mitogens for a variety of cell types (3–5). These peptides have been implicated as autocrine/paracrine growth factors for some human cancer cells, notably SCLC (5, 6). We have reported recently that multiple neuropeptides, including NT, induce rapid Ca2+ mobilization from intracellular stores in human ductal pancreatic cancer cell lines (7). In particular, NT stimulated activation of ERK1 and ERK2, and DNA synthesis in PANC-1 cells, an extensively studied model of human ductal pancreatic cancer (7–13). Interestingly, NT-binding sites have been detected by autoradiography of frozen sections in ~75% of human pancreatic cancer specimens (14). More recently, the expression of mRNA for the NTR-1 has been shown to be markedly increased in pancreatic cancer samples as compared with normal controls (15, 16). It is therefore important to characterize the NT-mediated signal transduction pathways in human pancreatic cancers, which may identify potential targets for therapeutic interventions.

Neuropeptides, including NT, bind to heptahelical GPCRs and trigger the activation of multiple signal transduction pathways including the phospholipase C-mediated generation of the second messengers, inositol (1, 4, 5) trisphosphate, which induces the rapid release of Ca2+ from internal stores and DAG, which activates PKC. PKC plays a central role in signal transduction pathways that mediate action of growth factors, neuropeptides, tumor promoters, and cellular oncogenes (17, 18). Molecular cloning has demonstrated the existence of multiple related PKC isoforms, which are subdivided in three groups based on their structural homology and mode of activation (reviewed in Ref. (19). The conventional isoenzymes (α, β1, β2, and γ) require DAG and calcium as coactivators in addition to phosphtidylinositol-4,5-bisphosphate and calcium-dependent. Both conventional and novel PKC isoforms function as major intracellular receptors for phorbol esters. In contrast, the atypical PKCs (ξ, ζ, and η) are DAG- and calcium-dependent. Phorbol ester-mediated PKC activation has been reported to either promote or inhibit growth of human pancreatic cancer cells (20–22). In contrast, the effect of GPCR-mediated PKC activation on pancreatic cancer cell growth has not been examined. Furthermore, the immediate downstream signaling events stimulated by PKCs in human pancreatic cancer cells as well as in other cancer cells remain poorly characterized.

PKD, also known as PKCθ, is a serine/threonine protein kinase with distinct structural features and unique enzymological properties (23, 24). In particular, the catalytic domain of the PKD shows very low homology to the conserved kinase subdomain of the PKCs and displays distinct substrate specificity (25, 26). In contrast to all of the known PKCs, the NH2-terminal region of PKD contains a pleckstrin homology domain that regulates enzyme activity (27) and lacks a sequence with homology to the typical PKC autoinhibitory pseudosubstrate motif (28). However, the proximal NH2-terminal region of PKD contains a tandem repeat of cysteine-rich, zinc finger-like motifs that binds phorbol esters with high affinity (28, 29) and mediates PKD translocation to the cell membrane (29). PKD/PKCθ can be activated in vitro by DAG/phorbol esters in the presence of phosphatidylinositol-4,5-bisphosphate (30, 31), indicating that PKD/PKCθ is a phorbol ester/DAG-stimulated kinase (32). Two other members have been included recently in this family of serine/threonine kinases, PKCγ (33) and PKD2 (34), lending additional support to the notion that the PKDs comprise a novel family of protein kinases.

Recently, a second mechanism of PKD activation has been identified that involves PKD phosphorylation (31, 35–37). Specifically, treatment of intact fibroblasts with biologically active phorbol esters (31), bryostatin (38), growth factors, and GPCR agonists (39–41) induces PKD activation that persists during cell disruption and immunoprecipitation. Treatment with PKC-selective inhibitors prevents...
PKD activation by all of these factors (31, 32, 38, 41). Furthermore, cotransfection of PKD with constitutively active mutants of PKC\(\varepsilon\) and PKC\(\eta\) dramatically increases the catalytic activity of PKD (31, 42) and leads to complex formation between PKD and PKC\(\varepsilon\) (37). It has been proposed previously that phosphorylation of Ser744 and Ser748 within the activation loop of PKD plays a critical role in mediating PKC-dependent PKD activation (35, 37). It has been demonstrated that PKD Ser744/Ser748 phosphorylation is rapidly induced by stimulation with PDB or via receptor-mediated pathways in a variety of model systems (43). Also, Ser744/Ser748 phosphorylation requires PKC but not PKD activity, indicative of transphosphorylation (43). In addition, PKCs play an important role in regulating the rapid release of activated PKD from the cell membrane (44). These findings reveal a link between PKCs and PKD, and implied that PKD lies downstream of PKCs in a novel signal transduction pathway (32, 37). The existence of a functional receptor-mediated PKC/PKD pathway in human cancers was first noted in SCLC cells (45). However, nothing is known about the PKC/PKD signaling pathway in human pancreatic cancer cells.

In the present study, we demonstrate that multiple PKC isoforms are expressed in PANC-1 and other human ducal pancreatic cancer cell lines along with PKD/PKC\(\mu\). We then identify that PKD is rapidly activated in these cells in response to biologically active PDB. Using PANC-1 cell as a model system, we additionally demonstrate that NT induced rapid and striking activation, multisite phosphorylation, and membrane translocation of PKD in these cells. Our results also show that NT induced DNA synthesis in PANC-1 cells through a PKC-dependent pathway. Thus, PKCs play a critical role in mediating PKD activation and mitogenic signaling from the NT receptor in PANC-1 cells.

MATERIALS AND METHODS

Cell Culture. PANC-1, obtained from American Type Culture Collection, is a less well-differentiated line established from human ducal pancreatic adenocarcinoma. PANC-1 cells were grown in DMEM (Life Technologies, Inc.) with 4 mM of glutamine, 1 mM of Na-pyruvate, and 10% FBS at 37°C with a humidified atmosphere containing 10% CO\(_2\).

Immunoprecipitation. PANC-1 cells cultured for 4–6 days were washed twice in ice-cold PBS (Sigma Chemical Co.) and lysed in 50 mM of Tris-HCl (pH 7.6), 2 mM of EDTA, 2 mM of EDTA, 1 mM of DTT, 10 \(\mu\)g/ml leupeptin, 10 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml leupeptin, 1 mM of 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 1% Triton X-100 (lysis buffer). The immunoprecipitation was carried out with the PA-1 antipeptide antiserum (1:100) in the absence or the presence of the immunizing peptide (10 \(\mu\)g/ml) as described before (26, 31).

Kinase Assay of PKD. PKD autophosphorylation was determined in an IGV assay by mixing 20 \(\mu\)l of PKD immunocomplexes with 10 \(\mu\)l of a phosphorylation mixture containing (final concentration) 100 mM [\(\gamma\)-\(\text{32P}\)]ATP (specific activity 400–600 CPM/\(\mu\)mol), 30 mM Tris-HCl (pH 7.4), 10 mM MgCl\(_2\), 1 mM DTT, and followed by SDS-PAGE analysis (26, 31). The gels were dried, and the 110-kDa radioactive band corresponding to autophosphorylated PKD was visualized by autoradiography. Autoradiographs were scanned in a GS-710 Calibrated Imaging Densitometer (Bio-Rad), and the labeled band was quantified with the Quantity One software program.

Western Blot Analysis. Serum-starved cultures of PANC-1 cells grown on 6-cm dishes were washed twice with PBS then replaced with DMEM and treated as described in individual experiments. To detect different PKC isoenzymes and PKD, the cells were directly lysed in 2 \(\times\) SDS-PAGE sample buffer, followed by SDS-PAGE on 8% gels and transfer to Immobilon-P membranes (Millipore). For Western blot analysis of PKD IP, the samples were washed twice in lysis buffer followed by twice in kinase buffer. Western blots were then performed on membranes incubated overnight with antibodies that recognize the different PKC isoforms and PKD (C-20) at a dilution of 1 \(\mu\)g/ml, or an antiserum that specifically recognizes the dual-phosphorylated state of Ser744/Ser748 in PKD (pS744/S748) at a concentration of 1 \(\mu\)g/ml, or for 3 h with an antiserum that specifically recognizes the phosphorylated state of serine 916 position in PKD (pS916) at a dilution of 1:500 in PBS containing 5% nonfat dried milk and 0.1% Tween 20. The immunoreactive bands were detected with enhanced chemiluminescence reagents (Amersham).

Immunocytochemistry. PANC-1 cells plated in Lab-Tek (Nalge Nunc International, Naperville, IL) double-chambers per each slide were fixed for 15 min at 25°C in 10% buffered formalin phosphate and permeabilized with 0.2% Triton X-100 in PBS for 5 min at 25°C. The fixed cells were incubated for 18 h at 25°C in BB (PBS-1% gelatin-0.05% Tween 20) and then stained at 37°C for 60 min with rabbit polyclonal PKD antibody (C-20) diluted in BB. Subsequently, the cells were washed with PBS containing 0.05% Tween 20 at 25°C and stained at 37°C for 60 min with fluorescein-conjugated goat-anti rabbit antibody diluted in BB. After washing with PBS containing 0.05% Tween 20 and PBS, the samples were mounted with a gelvatol-glycerol solution containing 2.5% 1,4-diazobicyclo-[2.2.2]octane with a humidified atmosphere containing 10% CO\(_2\). It has been proposed previously that phosphorylation of Ser744 and Ser748 within the activation loop of PKD plays a critical role in mediating kinase activation in PANC-1 cells.

RESULTS

Expression of Multiple PKC Isoforms, PKD, and PDB-Mediated Activation of PKD in PANC-1 Cells. PANC-1 cells have been used extensively as a model system to study the effects of growth factors on the biological behavior of human pancreatic cancer cells (7–13). To identify PKC isoforms expressed in PANC-1 cells, lysates from these cells were subjected to Western blot analysis using PKC isoform-specific antibodies. As shown in Fig. 1A, a major immunoreactive band corresponding to the predicted molecular masses of PKC\(\alpha\), PKCa\(_{\beta}\), PKC\(\varepsilon\), and PKC\(\eta\) were detected in PANC-1 cells. Isoform-specific matched controls were used to detect the presence of a band corresponding to different PKCs (results not shown). PKC\(\delta\), which inhibits growth and induces apoptosis in certain cancer cells (46, 47), is notably not detected by Western blot analysis in these cells as compared with a control lysate from IEC-6 cells, known to express PKC\(\delta\) (48). A similar pattern of expression of PKC isoforms was noted in other human pancreatic ductal adenocarcinoma cell lines.
HPAF-II and MIAPaCa-2 (results not shown). Our results differ from those previously reported with antisera in the absence of the immunizing peptide (100 μM PDB for 10 min at 37°C). The cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum (as indicated in “Materials and Methods”). The lysates were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blots were performed with the use of isotype-specific polyclonal antisera against different PKCs, as described in “Materials and Methods.” Isomorph-specific matched controls were used to detect the presence or absence of a band corresponding to different PKCs (data not shown). Equivalent amount of protein (measured by Bradford assay) was loaded in each lane except for PKC α, which was one-third of the rest. B, Western blot analysis of the cell lysates, PKD IP and PKD activation. **Left panel,** confluent cultures of PANC-1 cells were washed twice with Dulbecco’s PBS. The cells were then solubilized in 2× SDS-PAGE sample buffer (as indicated in “Materials and Methods”). The lysates were analyzed by SDS-PAGE and transferred to Immobilon membranes. Western blot was performed using the polyclonal antibody against the COOH-terminal region of mouse PKD (C-20), Equivalent amount of protein (measured by Bradford assay) was loaded. **Middle panel,** confluent cultures PANC-1 cells were lysed with lysis buffer (as indicated in “Materials and Methods”). The lysates were immunoprecipitated with the PA-1 antiserum in the absence or presence (++) of the immunizing peptide (10 μg/ml) that corresponds to the COOH-terminal region of the predicted amino acid sequence of PKD. The IPs were analyzed by Western blotting using the polyclonal PKD antiserum (C-20) as described in “Materials and Methods.” **Right panel,** PKD activation in PANC-1 cells. Confluent cultures of PANC-1 cells, washed with PBS and serum-free medium (DMEM), were incubated in the absence (−) or in the presence (+) of 200 nM PDB for 10 min at 37°C. The cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum in the absence (−) or presence (++) of the immunizing peptide (10 μg/ml). The PKD activity in the immunocomplexes was determined by IVK assay as described in “Materials and Methods,” followed by SDS-PAGE and autoradiography.

NT Induces PKD Activation in PANC-1 Cells. We reported recently that NT stimulated rapid Ca2+ fluxes, MAP kinase, and DNA synthesis in PANC-1 cells through NTR-1 (7). To additionally elucidate the signaling events downstream of NTR-1 in PANC-1 cells, we examined whether NT can stimulate PKD activation in intact PANC-1 cells. We treated serum-starved cultures of PANC-1 cells with NT for increasing times, lysed the cells, immunoprecipitated PKD with the PA-1 antiserum, and performed IVK assay. As shown in Fig. 2A, top panel, PKD isolated from unstimulated PANC-1 cells had low basal catalytic activity. Treatment of PANC-1 cells with NT induced a rapid and striking increase in PKD kinase activity that was maintained during cell lysis and immunoprecipitation. PKD activation was detectable within 1 min and reached a maximum (~9-fold) after 15 min of NT stimulation. These results demonstrate that PKD activation is one of the early events induced by NT in pancreatic cancer cells.

Recently, an antiserum specifically recognizing the phosphorylated form of a PKD COOH-terminal residue, serine 916, was developed and used to detect in vivo autophosphorylation at this site by active PKD (36). Thus, pS916 antiserum provides a novel approach for detecting conversion of PKD to an active form within cells. Here, lysates from PANC-1 cells stimulated with NT for various times were analyzed by SDS-PAGE followed by Western blot analysis with the pS916 antiserum. NT stimulation induced a dramatic increase in the immunoreactivity of the PKD band indicative of phosphorylation at serine 916 (Fig. 2A, middle panel). An increase in PKD autophosphorylation was detectable within 2.5 min and reached a maximum (~10-fold) after 10–15 min of NT stimulation. NT-induced PKD activation within cells declined gradually toward baseline levels. In contrast, PKD activation induced by PDB persisted for at least 180 min of treatment (results not shown). Thus, NT-induced PKD activation is transient when compared with that induced by PDB. Western blotting with PKD specific antiserum (C-20) confirmed that similar amounts of PKD protein were loaded into the gel after treatment with NT for different times (Fig. 2A, bottom panel).

Stimulation of intact PANC-1 cells with increasing concentrations of NT for 10 min induced a striking dose-dependent increase in PKD activation, as judged by assays of IVK activity after immunoprecipitation or by Western blotting with pS916 antibody to detect autophosphorylation in intact cells (Fig. 2B). Again, Western blot analysis with a PKD-specific antibody (C-20) confirmed that equal amounts of PKD protein were loaded into the gel after treatment with increasing concentrations of NT for 10 min. Half-maximal PKD activation by NT was achieved at 10 nM (Fig. 2B).

NT Increases Transient and Rapid Translocation of PKD from the Cytosol to the Plasma Membrane in PANC-1 Cells. In unstimulated cells, PKD has been localized to the cytosol and to several intracellular compartments including Golgi and mitochondria (29, 50–55). It has been demonstrated recently that treatment of Swiss 3T3 fibroblasts with the GPCR agonist bombesin induces a striking and transient translocation of PKD from the cytosol to the plasma membrane (29, 44, 55). Here, we analyzed the effect of NT stimulation on...
Fig. 2. NT activates PKD in PANC-1 cells in a time- and dose-dependent manner. Confluent PANC-1 cells were washed with PBS and incubated in serum-free medium (DMEM) for 6 h, A, time course. Serum-starved cells were stimulated with 50 nM NT for the indicated time periods at 37°C. Top, cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. The PKD activity in the immunocomplexes was determined by IVK assay as described in “Materials and Methods,” followed by SDS-PAGE and autoradiography. The autoradiogram shown is representative of three independent experiments. Middle, an identical experiment was carried out in parallel where Western blot analysis using pS916 antiserum was performed after lysis of the treated cells with 2 × SDS-PAGE sample buffer as described in “Materials and Methods.” The same membrane was stripped and probed with polyclonal PKD antibody (C-20) in a similar fashion. The Western blot shown is representative of at least three independent experiments. Bottom, scanning densitometry. The results shown are means (n = 3) of the level of PKD activation by IVK obtained from scanning densitometry and are expressed as percentages of the maximum increase in phosphorylation obtained from cells incubated with 50 nM NT for 15 min at 37°C; bars, ±SE. B, dose response. Serum-starved cells were stimulated with various concentrations of NT for 10 min at 37°C. Top, cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. The PKD activity in the immunocomplexes was determined by IVK assay as described in “Materials and Methods,” followed by SDS-PAGE and autoradiography. The autoradiogram shown is representative of three independent experiments. Middle, an identical experiment was carried out in parallel where Western blot analysis using pS916 antiserum was performed after lysis of the treated cells with 2 × SDS-PAGE sample buffer as described in “Materials and Methods.” The same membrane was stripped and probed with polyclonal PKD antibody (C-20) in a similar fashion. The Western blot shown is representative of at least three independent experiments. Bottom, scanning densitometry. The results shown are means (n = 3) of the level of PKD activation by IVK obtained from scanning densitometry and are expressed as percentages of the maximum increase in phosphorylation obtained with 100 nM NT at 37°C; bars, ±SE.

the intracellular distribution of endogenous PKD in PANC-1 cells. As illustrated by the confocal image presented in Fig. 3A, endogenous PKD expressed in unstimulated PANC-1 cells was distributed throughout the cytosol and mostly excluded from the nucleus with basal fluorescent signal localized to the plasma membrane. The basal level of fluorescence seen in unstimulated cells (Fig. 3A) represents plasma membrane localized serum-activated PKD. Stimulation with NT for 5 min induced rapid translocation of PKD to the plasma membrane, causing a striking increase in localized fluorescence in the plasma membrane at the cell periphery (Fig. 3B). The association of endogenous PKD with the plasma membrane was transient. The reverse translocation from the plasma membrane to the cytosol was nearly complete within 15 min of NT stimulation (Fig. 3C). The results presented in Fig. 3 demonstrate that NT induces a prominent and transient translocation of endogenous PKD to the plasma membrane in PANC-1 cells.

NT Induces PKD Activation in PANC-1 Cells through a PKC-dependent Pathway. Having established that NT induces striking PKD activation in PANC-1 cells, our next step was to determine the role of PKCs in mediating PKD activation in this system. Cultures of PANC-1 cells were treated with various concentrations of either GF-1 or Ro 31–8220, selective inhibitors of phosphor ester-sensitive isoforms of PKC (56–58) but not PKD (31, 38, 41). As shown in Fig. 4A, treatment with GF-1 potently blocked PKD activation induced by subsequent addition of NT in a concentration-dependent manner. In contrast, GF-1 added directly to the IVK assay, even at concentrations (0.1–1 μM) that abrogated NT-induced PKD activation in intact PANC-1 cells did not inhibit PKD activity. Fig. 4B depicts similar results obtained with a structurally distinct PKC inhibitor Ro 31–8220 instead of GF-1. Ro 31–8220 blocked NT-induced PKD activation at a lower concentration than GF-1 (half-maximal inhibition obtained with Ro was at 0.25 μM as compared with 0.5 μM of GF-1). These results imply that GF-1 and Ro 31–8220 do not inhibit PKD activity directly but interfere with NT-mediated PKD activation in intact PANC-1 cells by blocking PKC.

To confirm the requirement of PKC in mediating NT-induced PKD activation, we treated PANC-1 cells with PDB for 36 h to produce extensive depletion of classic and novel PKCs but not of PKD (48) and then challenged with NT for 10 min. In a parallel set of experiments, cells were stimulated with either NT or PDB for 10 min. As shown in Fig. 5A, the increase in PKD activity induced by acute exposure to PDB or NT was completely abrogated by previous chronic treatment with PDB for 36 h. This result provides additional evidence indicating the requirement of PKC(s) for PKD activation.

To additionally substantiate the specificity of the results obtained with PKC inhibitors GF-1 and Ro 31–8220, we treated PANC-1 cells with inhibitors of multiple pathways and examined PKD activation after the subsequent challenge with NT. Treatment with the phosphatidy1 3'-kinase inhibitor wortmannin (59, 60), the p70 ribosomal protein/ERK kinase inhibitor genistein (64), and the mitogen-activated protein/ERK kinase inhibitor PD 98059 (65), which prevents activation of ERKs, did not affect PKD activation in response to NT in PANC-1 cells (Fig. 5B). In contrast, GF-1, a selective PKC inhibitor, abrogated NT-induced PKD activation as measured both by the IVK assay and pS916 immunoreactivity in Western blot (Fig. 5B). These results demonstrate the specificity of the PKC inhibitors and indicate that these kinases are not upstream regulators of PKD. So, activation of PKD by NT in PANC-1 cells is dependent on phorbol ester-regulated PKC(s).

It has been reported recently that Ser744 and Ser748 located in the activation loop of PKD are transphosphorylated in vivo resulting in PKD activation (43). To determine whether a similar mechanism operates in human pancreatic cancer cells, we examined whether NT stimulated Ser744/Ser748 phosphorylation in PANC-1 cells. As shown in Fig. 5C, the selective PKC inhibitor, abrogated NT-induced PKD phosphorylation as measured both by the IVK assay and pS916 immunoreactivity in Western blot. These results demonstrate the specificity of the PKC inhibitors and indicate that these kinases are not upstream regulators of PKD. So, activation of PKD by NT in PANC-1 cells is dependent on phorbol ester-regulated PKC(s).
treatment with this agonist induced an electrophoretic mobility shift of PKD Ser744/Ser748, which is indicative of their phosphorylation and subsequent activation. Next, we assessed the role of PKC in the phosphorylation of Ser744/Ser748 of PKD after stimulation with NT and PDB in PANC-1 cells. NT-induced phosphorylation of Ser744/ Ser748 was potently blocked by the PKC inhibitors GF-1 and Ro 31–8220 in PANC-1 cells (Fig. 5C, bottom panel). A similar result was obtained when the cells were stimulated with PDB instead of NT (Fig. 5C, bottom panel). These results demonstrate that activation of PKD by NT is a rapid and transient event, and support a model of PKC-PKD phosphorylation cascade in PANC-1 cells.

**PKD Reverse Translocation from the Plasma Membrane Is PKC-dependent in PANC-1 Cells.** We have shown in Fig. 3 that NT induced a rapid translocation of PKD from the cytosol to the plasma membrane. As shown in Fig. 3C, the rapid translocation of PKD to the plasma membrane is transient. PKD returns to the cytosol after 15 min of exposure to NT. Next, we examined whether PKCs are required for these processes. PANC-1 cells grown in serum were treated with PKC inhibitors GF-1 (Fig. 6, A–C) and Ro 31–8220 (Fig. 6, D–F) and subsequently challenged with NT for 5 min (Fig. 6, B and E) and 15 min (Fig. 6, C and F). Cells treated with GF-1 (Fig. 6A) or Ro 31–8220 (Fig. 6D) alone demonstrated basal level of immunofluorescence indicative of serum-stimulated PKD localized to the plasma membrane. On stimulation with NT for 5 min, there was a significant increase in the level of localized fluorescence in the plasma membrane at the cell periphery (Fig. 6, B and E). These results indicate that PKC is not required for NT-induced translocation of PKD to the plasma membrane. Interestingly, the level of localized immunofluorescence noted on the plasma membrane in cells treated with PKC inhibitors did not appreciably decrease after stimulation with NT for 15 min (Fig. 6, C and F) in contrast to Fig. 3C. This demonstrates that PKC activity is required for the rapid reverse translocation of PKD from the plasma membrane to the cytosol, which is consistent with recent data shown in other nontransformed cells (44).

**Effect of the PKC Inhibitor GF-1 on NT-induced DNA Synthesis in PANC-1 Cells.** The effect of receptor-mediated PKC activation on the regulation of DNA synthesis has not been examined previously in human pancreatic cancer cells. We have reported earlier that NT promotes DNA synthesis in PANC-1 cells in a dose-dependent manner signaling via NTR-1 receptor subtype (7). Now, we examined whether NT receptor-mediated DNA synthesis is dependent on PKC activity in these cells.

Cultures of PANC-1 cells grown in medium containing 10% FBS were washed and transferred to serum-free medium for 6 h. To start the experiment, fresh serum-free medium containing NT, 10% FBS, or solvent were added to parallel cultures (n = 4 for each condition). After 16 h of incubation, the cultures were pulse labeled for 6 h with [3H]thymidine. As shown in Fig. 7A, NT induced a significant increase in the incorporation of [3H]thymidine into acid-insoluble pools. These results demonstrate that NT, at concentrations that induced PKC/PKD activation, also induced DNA synthesis in PANC-1 cells.

To determine the role of PKCs in NT-induced DNA synthesis, PANC-1 cells were treated with the selective PKC inhibitor, GF-1, and GF-V, an inactive analogue of GF-1, before addition of NT. DNA synthesis, as measured by [3H]thymidine incorporation, was again induced by NT and was not affected by addition of GF-V (Fig. 7B). NT alone stimulated DNA synthesis to the same level as depicted in Fig. 8A (result not shown). In striking contrast, GF-1 blocked NT-induced DNA synthesis in PANC-1 cells (Fig. 7B). The addition of GF-1 prevented the NT-induced increase in the incorporation of [3H]thymidine in a dose-dependent fashion (Fig. 7C). Increasing concentrations of GF-1 attenuated NT-induced DNA synthesis as compared with cells treated with highest concentration of GF-V (Fig. 7C). The results demonstrate that NT receptor-mediated activation of DNA synthesis is PKC-dependent in PANC-1 cells.

**DISCUSSION**

The PKC isoforms are implicated in the signal transduction of multiple cellular activities including differentiation, proliferation, cell cycle regulation, and apoptosis (18, 19, 66–69). PKCs also mediate multiple cellular functions induced by neuropeptides, which have been implicated as autocrine/paracrine growth factors for some human cancer cells, notably SCLC (5, 6). However, the downstream targets for PKCs that transmit signals to the cell interior and participate in these processes remain poorly characterized. We have reported earlier that functional NT receptors were present in multiple human pancreatic cancer cell lines (7). Now, we have used human pancreatic adenocarcinoma cell line PANC-1 as our model system to study the signaling pathways initiated by NT. Consequently, the present challenge is to identify downstream targets of PKCs induced by NT in these cells.

PKD/PKC\(\mu\), a novel serine/threonine kinase, has been identified as a unique downstream effector of PKC-mediated signal transduction in variety of cell types. PKD is also expressed in PANC-1 cells. As a first step to elucidate the mechanism of activation of PKD in human pancreatic cancer cells, we examined the expression of various PKC isoforms in these cells. Our results demonstrate that multiple PKCs are expressed in PANC-1 ductal adenocarcinoma cells. This result
PKD in PANC-1 cells via receptor-mediated pathways. PANC-1 cells have been used extensively as a model of human pancreatic ductal adenocarcinoma in recent reports (7–13). We demonstrated that PKD activation occurs within minutes of NT stimulation in intact PANC-1 cells and, thus, is one of the early events induced by NT in these cells. This suggests that NT signaling through NTR-1 induced a rapid and physiological PKD activation. We found that treatment of these cells with the PKC inhibitors GF-1 and Ro 31–8220 before stimulation with NT strikingly prevents PKD activation. Importantly, these PKC inhibitors do not reduce PKD activity when added directly to the IVK assays, even at concentrations higher than those used in intact PANC-1 cells to block NT-induced PKD activation. PKD is not constitutively active in these cells but can be strikingly activated within these cells via PKC. These results suggest that NT promotes a rapid and concentration-dependent PKD activation in intact PANC-1 cells through a PKC-dependent pathway.

It is well established that long-term exposure to potent biologically active phorbol esters induces down-regulation of conventional and differs from the restrictive pattern of PKC isoforms expression in human pancreatic cancer cells proposed in earlier studies (49, 70).

Next, we examined the regulation of PKD in PANC-1 cells. Our results demonstrate that treatment of intact cells with the tumor-promoting phorbol ester PDB induces rapid PKD activation. To additionally investigate the physiological significance of PKD activation in pancreatic cancer cells, we then examined the regulation of

Fig. 4. The PKC inhibitors GF-1 and Ro 31–8220 prevent PKD activation by NT in PANC-1 cells. Confluent PANC-1 cells were washed with PBS and incubated in serum-free medium (DMEM) for 6 h. A, GF-1 inhibits PKD activity. Serum-starved PANC-1 cells were incubated for 1 h with different concentrations of the PKC inhibitor GF-1 (top blot and •). Control cells (−) received equivalent amounts of solvent. The cultures were subsequently stimulated with 50 nM NT for 10 min at 37°C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. The PKD activity in the immunocomplexes was determined by IVK assay as described in “Materials and Methods.” B, Western blot analysis using pS744/S748 dual-specific antiserum was performed after lysis. It is well established that long-term exposure to potent biologically active phorbol esters induces down-regulation of conventional and

Fig. 5. NT induces PKD activation in PANC-1 cells through a PKC-dependent pathway. A, NT induced PKD activity after prolonged PDB treatment. Confluent cultures of PANC-1 cells were incubated for 36 h either without (−) or with 200 nM PDB. The cultures were washed with DMEM and incubated for 2 h at 37°C in DMEM. The cells were then challenged for an additional 10 min with 50 nM NT at 37°C. Parallel set of cultures were incubated without (−) or with 200 nM PDB for 10 min at 37°C. Cells were lysed with lysis buffer and immunoprecipitated with PA-1 antiserum. The PKD activity in the immunocomplexes was determined by IVK assay as described in “Materials and Methods,” followed by SDS-PAGE and autoradiography. The autoradiogram shown is representative of two independent experiments.
novel PKC isoforms in mammalian cells (48). So, we examined whether chronic PDB stimulation abrogates the NT-induced PKD activation in PANC-1 cells. The result indicates that chronic PDB exposure leads to complete inhibition of NT-induced PKD activation in these cells. Inhibition of other signaling pathways, including the FRAP-p70 ribosomal S6 kinase, which is constitutively active in PANC-1 cells (71), or the p42\textsuperscript{mapk}/p44\textsuperscript{mapk} pathway, which is markedly stimulated by phorbol esters in HPAC cells via PKC (72), did not affect PKD activation by NT. Recently, new experimental evidence indicates that activation loop transphosphorylation at Ser744 and Ser748 occurs during PKD activation in vivo and requires PKC activity (43). We examined Ser744/Ser748 phosphorylation of PKD in NT-stimulated PANC-1 cells. Our results demonstrate that NT-induced Ser744/Ser748 phosphorylation of PKD is rapid and transient, indicating one of the early events induced by NT in PANC-1 cells. The PKC inhibitors GF-1 and Ro 31–8220 potently blocked NT-induced phosphorylation of Ser744/Ser748 in PKD. These findings strongly support the notion of a PKC-PKD phosphorylation cascade in signal transduction by NT in pancreatic cancer cells.

Activated PKD is transiently localized to the cell membranes but then returns to the cytosol where its activity is retained for extended periods (29, 44, 55). We investigated the plasma membrane translocation of endogenous PKD in PANC-1 cells. Our results demonstrate that NT-induced PKD translocation to the plasma membrane is a rapid and transient phenomena. The striking increase in plasma membrane fluorescence after NT stimulation declines quite rapidly in these cells. Treatment with the PKC inhibitors GF-1 and Ro 31–8220 did not prevent NT-induced rapid translocation of PKD to the plasma membrane but blocked the reverse translocation of PKD from the plasma membrane to the cytosol. PKC activity is required for reverse translocation of activated PKD from plasma membrane to the cytosol where activated PKD now can target multiple proteins to mediate biological functions. Thus, our result is consistent with recent evidence suggesting PKCs play an important role in regulating the rapid release of activated PKD from the cell membrane thereby mediating multiple cellular functions (44).

The effect of phorbol ester-induced PKC activation on the growth of human pancreatic cancer cells has been a subject of controversy. PKC\(\alpha\) was proposed as a potent inhibitor of the G\(1\)-S transition in phorbol ester-treated proliferating human pancreatic cancer cells (DanG; Ref. 22). In contrast, overexpression of PKC\(\alpha\) in HPAC cells was associated with increased tumorigenicity in an orthotopic model that was attenuated with antisense therapy toward PKC\(\alpha\) (21). We examined PKC-dependence on NT-induced DNA synthesis in serum-starved PANC-1 cells. We found that NT-induced DNA synthesis in PANC-1 cells is dependent on PKC. We conclude that receptor-mediated, unlike phorbol ester-induced stimulation of PKCs in human pancreatic cancer cells is growth-stimulatory. Recently, it has been demonstrated that overexpression of PKD in Swiss 3T3 fibroblasts selectively potentiates mitogenesis induced by GPCR agonists like bombesin and vasopressin (73). In view of our results that NT induced PKC/PKD activation and DNA synthesis in human pancreatic cancer cells, it is conceivable that PKD mediates some aspect(s) of PKC-dependent mitogenesis in these cells.

In conclusion, our results provide experimental evidence of NT-induced rapid and transient activation of PKD in PANC-1 cells, a model system for human pancreatic ductal adenocarcinoma. A salient feature of the results presented here is that PKC plays a critical role in mediating this response. Our results, showing that PKD can function downstream of PKC in these cells, raise the possibility that PKD mediates some of the biological responses elicited by PKC in the human pancreatic cancer cell line PANC-1. The results also demonstrate that NT promotes DNA synthesis in these cells, which is PKC dependent. Our findings indicate that receptor-mediated PKC stimulation promotes growth of human pancreatic cancer cell line PANC-1 and suggest that elucidation of the signal transduction pathways that mediate NT-induced mitogenesis may identify novel targets for therapeutic intervention.
50 nM NT, 10% FBS, and an equivalent amount of solvent (GF-1, on DNA synthesis in cells stimulated with NT. Cells were incubated with 1/2H9262 DNA synthesis was then measured in cells additionally stimulated without (A). A representative plot from one experiment for each panel. Similar results were obtained in three independent experiments; bars, ±SE.

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Fig. 7. NT-induced DNA synthesis in PANC-1 cells is PKC-dependent. Cells incubated in serum-free medium were pulse-labeled with [3H]thymidine (0.25 μCi/ml) and the radioactivity incorporated into acid-insoluble pools was counted in a scintillation counter as described in “Materials and Methods.” A, DNA synthesis in cells treated with 50 nM NT, 10% FBS, and an equivalent amount of solvent (−). B, effect of PKC inhibitor, GF-1, on DNA synthesis in cells stimulated with NT. Cells were incubated with 1 μM GF-V (−) or 1 μM GF-1 (+) and an equivalent amount of solvent (−) for 1 h at 37°C. DNA synthesis was then measured in cells additionally stimulated without (−) and with (+) 50 nM NT at 37°C. C, dose-response for increasing concentration of GF-1 on NT-stimulated DNA synthesis. Cells were incubated with 1 μM GF-V (−) or indicated concentrations of GF-1 for 1 h at 37°C. DNA synthesis was measured in cells additionally stimulated with 50 nM NT at 37°C. DNA synthesis was then measured in cells additionally stimulated without (−) and with (+) 50 nM NT at 37°C. The plot represents percentage maximal inhibition of DNA synthesis with GF-1 as compared with GF-V in NT-stimulated cells. Shown here is a representative plot from one experiment for each panel. Similar results were obtained in three independent experiments; bars, ±SE.


Neurotensin Induces Protein Kinase C-dependent Protein Kinase D Activation and DNA Synthesis in Human Pancreatic Carcinoma Cell Line PANC-1

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