Neurotensin Stimulates Protein Kinase C-dependent Mitogenic Signaling in Human Pancreatic Carcinoma Cell Line PANC-1[1]

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ABSTRACT

Neuropeptides and their corresponding G protein-coupled receptors are increasingly implicated in the autocrine/paracrine stimulation of growth of human cancers. Using *K-Ras* mutated human pancreatic ductal adenocarcinoma cell line PANC-1 as a model system, we demonstrate that neurotensin (NT) induced translocation of phosphorylated extracellular signal-regulated kinases (ERK-1 and ERK-2) to the nucleus, rapid dose-dependent activation of dual-specificity mitogen and ERK-1 and ERK-2 kinase-1/2 (MEK-1/2), and striking stimulation of c-Raf-1 but not pan-Ras. Furthermore, treatment of PANC-1 cells with protein kinase C (PKC) inhibitors, GF-1 and Ro 31–8220, completely abrogated NT-induced ERK-1 and ERK-2 activation, and significantly attenuated NT-induced c-Raf-1 stimulation. Interestingly, NT did not stimulate epidermal growth factor receptor transactivation, and epidermal growth factor receptor tyrosine kinase or Src inhibitors did not affect NT-induced ERK activation in PANC-1 cells. Our results indicate that NT potently stimulates c-Raf-1-MEK-ERK in PANC-1 cells through a PKC-dependent signaling pathway. Furthermore, we show that NT-induced DNA synthesis in PANC-1 cells is ERK-dependent. Finally, we demonstrate that NT stimulated clonal growth of PANC-1 cells in semisolid medium, which is abrogated by both GF-1 and the MEK-1/2 inhibitor, U0126. Collectively our results suggest that PKC-mediated stimulation of ERK-1 and ERK-2 play a pivotal role in NT-induced growth of PANC-1 cells harboring activating *K-Ras* mutation.

INTRODUCTION

Ductal adenocarcinoma of the pancreas, which comprises 90% of all human pancreatic cancers, is an extremely lethal disease, with an overall 5-year survival rate of only 3–5% and a median survival time after diagnosis of <6 months (1). In 2002, an estimated 30,300 new cases of pancreatic ductal adenocarcinoma will be diagnosed, and 29,700 people will die from the disease. Its intransigence to currently used therapeutics renders it nearly 100% lethal, making it now the fourth leading cause of cancer death in both men and women (1). Thus, novel therapeutic strategies are needed, and these could arise from defining the factors and signaling pathways that stimulate unrestrained proliferation of ductal pancreatic cancer cells (2).

A variety of neuropeptides, including bombesin or GRP,[4] gastrin,

CCK, bradykinin, vasopressin, and NT can 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 small cell lung cancer (4, 5). We have reported recently that multiple neuropeptides, including NT, bombesin/GRP, CCK, and angiotensin induce rapid Ca^{2+} mobilization from intracellular stores in human ductal pancreatic cancer cell lines (6). A salient feature of these results is that all of the pancreatic cancer cells examined express functional receptors for NT (7), including PANC-1 cells, an extensively studied model of human ductal pancreatic cancer (6–12). Interestingly, NT-binding sites have been detected by autoradiography of frozen sections in ~75% of human pancreatic cancer specimens (13). In contrast, no NT receptors were found in chronic pancreatitis or normal pancreatic tissues, including pancreatic acini, ducts, and islets (13). Furthermore, the expression of mRNA for the NTR-1 was shown markedly increased in ductal pancreatic cancer samples as compared with normal ductal epithelial controls (14–16). NT immunoreactivity has been identified in neuroendocrine N cells, in nerve fibers of the enteric nervous system, and in intrapancreatic ganglia in both normal and cancer tissues (17). Thus, NT can stimulate its cognate receptor, NTR-1, which is highly up-regulated on ductal epithelial cells of pancreatic cancer tissues in an autocrine and/or paracrine manner. Therefore, it is important to characterize the NT-mediated mitogenic signal transduction pathways in human pancreatic cancers, which may identify potential targets for novel therapeutic interventions.

Neuropeptides, including NT, bind to seven transmembrane domain GPCRs and induce phospholipase Cβ-mediated phosphoinositide hydrolysis to generate the two second messengers, inositol-1,4,5-triphosphate and DAG, which mobilize Ca^{2+} from intracellular stores and PKC, respectively (3, 4). Pancreatic cancer cells including PANC-1 express multiple DAG- and phorbol ester-sensitive (classic and novel), and DAG-insensitive (atypical) PKC isoforms, including α, β_1, ϵ, η, and ζ (7). Phorbol ester-mediated PKC activation has been reported to either promote or inhibit growth of human pancreatic cancer cells (18–21). In contrast, the effect of GPCR-mediated PKC activation on pancreatic cancer cell growth has not been examined.

The MAPKs constitute a family of highly conserved serine-threonine kinases that are activated in response to many extracellular signals (22). The best-characterized pathway leads to the activation of the ERKs. There are two ERK isoforms, ERK-1 and ERK-2 (also called p44mapk and p42mapk, respectively), which are directly activated by phosphorylation of a -Thr-Glu-Tyr- motif in the activation loop by the dual-specificity ERK kinase (or MEK; Ref. 23). Several pathways leading to MEK activation have been identified. Tyrosine kinase receptors stimulate ERK activation via SOS-Grb2-mediated accumulation of Ras-GTP, which then recruits c-Raf-1 to the plasma membrane and activates a kinase cascade comprising c-Raf-1, MEK, and ERK (23). GPCRs are known to induce ERK activation through multiple signal transduction mechanisms (4, 5) including PKC-dependent and PKC-independent pathways (24). The precise mechanism(s) by which PKC activates the ERKs remains incompletely understood (25). In some cases, PKC appears to directly phosphorylate and activate c-Raf-1 (26), whereas in others, PKC induces ERK activation via SOS-Grb2-mediated accumulation of Ras-GTP (27).

Neuropeptide agonists also stimulate PKC-independent ERK activation via rapid tyrosine phosphorylation of Shc, which recruits the SOS-Grb2 complex leading to Ras activation. Several neuropeptide-stimulated tyrosine kinases have been implicated as upstream regulators of this pathway, including Src, FAK (28, 29), and EGFR (30, 31). Activated ERKs directly phosphorylate and activate various enzymes, including p90rsk (32) and ELK-1 (33), which have been strongly implicated in the control of cell proliferation (34, 35). Although the mechanisms by which mitogenic GPCR agonists activate ERK have been extensively investigated, little is known about the pathways activated by these receptors in pancreatic cancer cells harboring activating *K-Ras* mutations. We showed recently that NT signaling through NTR-1 stimulated ERK-1 and ERK-2 activation in PANC-1, cells and subsequently promoted DNA synthesis (6). However, nothing is known about the signaling pathway by which NTR-1 induces ERK activation in *K-Ras* mutated PANC-1 cells.

In the present study, using PANC-1 cells as our model system, we report that NT stimulates translocation of phosphorylated ERKs to the nucleus, rapid and striking activation of MEK-1/2 and c-Raf-1, but not pan-Ras activation or EGFR transactivation. Furthermore, PKC inhibitors in PANC-1 cells significantly abrogate NT-induced c-Raf-1 and subsequent ERK activation. We also show that activated ERKs mediate NTR-1-initiated DNA synthesis in these cells. Finally, NT promoted clonal growth of PANC-1 cells through a PKC-ERK dependent pathway.

MATERIALS AND METHODS

Cell Culture. PANC-1, obtained from American Type Culture Collection, is a less well-differentiated line established from human ductal pancreatic adenocarcinoma. PANC-1 cells were grown in DMEM (Life Technologies, Inc.) with 4 mM glutamine, 1 mM Na-pyruvate, and 10% FBS at 37°C with a humidified atmosphere containing 10% CO_2. IEC-18 stock cultures, purchased from American Type Culture Collection, are routinely maintained in our laboratory in DMEM with 5% 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) and lysed in 50 mM Tris-HCl (pH 7.4), 1% NP40, 0.25% Na-deoxycholate, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 1 mM Na_3VO_4, and 1 mM NaF (Lysis buffer-A). The cell lysates were clarified by centrifugation at $15,000 \times g$ for 10 min at 4°C. EGFR was then IP at 4°C for 2–4 h with the anti-EGFR polyclonal antibody. The immune complexes were recovered using protein A coupled to agarose.

Western Blot Analysis. Confluent cultures of PANC-1 cells grown on 6-cm dishes were washed twice with PBS, then replaced with serum-free DMEM for 6 h and treated as described in individual experiments. To detect the activation of MEK-1/2, ERK-1, and ERK-2, or the presence of Pyk2, the cells were directly lysed in 2× SDS-PAGE sample buffer [200 mM Tris-HCl (pH 6.8), 2 mM EDTA, 0.1 M Na_3VO_4, 6% SDS, 10% glycerol, and 4% 2-mercaptoethanol], followed by SDS-PAGE on 10% gels and transfer to Immobilon-P membranes (Millipore). For Western blot analysis of EGFR IP, the immunocomplexes were washed thrice in Lysis buffer-A, extracted for 10 min at 95°C in 2× SDS-PAGE sample buffer, and resolved by 8% SDS-PAGE. Western blots were then performed on membranes incubated overnight with antibodies that recognize dual phosphorylation sites of MEK-1/2, rabbit phospho-MEK-1/2 (Ser217/Ser221) polyclonal antibody, ERK-1 and -2, and mouse phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody at a dilution of 1:1000 in PBS containing 5% nonfat dried milk and 0.1% Tween 20, or with antiphosphotyrosine antibody (4G10) at a dilution of 1:500 in PBS containing 5% nonfat dried milk and 0.1% Tween 20, and anti-Pyk2 rabbit polyclonal antibody at a dilution of 1:1000 in PBS containing 5% nonfat dried milk and 0.1% Tween 20. The immunoreactive bands were detected with enhanced chemiluminescence reagents (Amersham). The same membranes were subsequently stripped and probed in a similar fashion with anti-MEK-1/2 and anti-ERK-2 polyclonal antibodies at a dilution of 1:3000 or anti-EGFR

polyclonal antibody at a dilution of 1:5000 in PBS containing 5% nonfat dried milk and 0.1% Tween 20.

Immunofluoresence. NT-stimulated and unstimulated cells were fixed in 4% paraformaldehyde in TBS for 30 min at 25°C. Cells were subsequently permeabilized with 0.1% Triton X-100 in TBS for 5 min at 25°C. Cells were then incubated with 0.2% glycine for 5 min at 25°C to quench the fixative. After quenching, the cells were washed three times with TBS and incubated for 1 h at 25°C in blocking buffer (TBS containing 2% FBS and 1% BSA). The fixed and permeabilized cells were incubated overnight at 4°C with dual-specific phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody at 1:200 in blocking buffer containing 0.1% Tween 20. The cells were then washed five times in TBS with 0.1% Tween 20 and stained at 25°C for 2 h with fluorescein-conjugated goat-antimouse antibody diluted at 1:500 in blocking buffer containing 0.1% Tween 20. After washing with TBS containing 0.1% Tween 20 and distilled water, the stained cells were examined with a Zeiss Axioskope 2 inverted microscope using ×63 objective lens. Images were obtained using a Spot digital camera and associated software (Spot; Image Diagnostics), and additionally processed using Adobe PhotoShop 6.0.

RAS Activation Assay. Confluent cultures of PANC-1 cells grown on 6-cm dishes were washed twice with PBS, then replaced with serum-free DMEM for 6 h and treated as described in individual experiments. The cells were lysed in ice-cold MLB [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, and 10% glycerol]. The lysate was precleared with glutathione agarose, and activated RAS was IP with c-Raf-1 RBD agarose beads. Subsequently, the proteins were extracted in 2× SDS-PAGE sample buffer, followed by SDS-PAGE on 12.5% gels and transfer to Immobilon-P membranes (Millipore). Western blots were then performed on membranes incubated overnight with 1 μg/ml of anti-RAS polyclonal antibody, clone RAS10 (Upstate Biotechnology) diluted in PBS containing 5% nonfat dried milk, and 0.1% Tween 20. The immunoreactive bands were detected with enhanced chemiluminescence reagents (Amersham).

c-Raf-1 Immunoprecipitation Kinase Cascade Assay. Confluent cultures of PANC-1 cells grown on 6-cm dishes were washed twice with PBS, then replaced with serum-free DMEM for 6 h and treated as described in individual experiments. The cells were lysed in ice-cold Buffer-B [50 mM Tris (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50 mM NaF, 5 mM Na PP$_i$, 10 mM sodium glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μM Microcystin]. Activated c-Raf-1 was IP with anti-c-Raf-1 antibody (Upstate Biotechnology) bound to protein G agarose. The coupled kinase assay was performed by mixing of c-Raf-1 immunocomplexes with the ADBI [20 mM 4-morpholinepropanesulfonic acid (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4 and 1 mM DTT], magnesium-ATP mixture [500 μM ATP and 75 mM $MgCl_2$ in ADBI], inactive MEK1 (recombinant enzyme expressed in *Escherichia coli*), and inactive ERK2 (recombinant enzyme expressed in *E. coli*) in a shaking incubator for 30 min at 30°C. An aliquot of supernatant containing activated ERK2 was then added to a phosphorylation mixture containing ADBI, MBP substrate, and diluted [γ-^{32}P]ATP and incubated for 10 min at 30°C. An aliquot of the reaction mixture was transferred to P81 phosphocellulose squares, and the amount of radiolabelled MBP was then measured in a scintillation counter.

[^{3}H]Thymidine Incorporation. PANC-1 cells (5×10^4) were plated and grown in 3.5-cm tissue culture plates for 5 days in DMEM (Life Technologies, Inc.) with 4 mM glutamine, 1 mM Na-pyruvate, and 10% FBS. The cells were washed twice and incubated in serum-free medium for 6 h. To start the experiment, fresh medium containing the specified concentration of agonist/antagonist or solvent was added after washing twice with PBS (four cultures for each condition), the cells were incubated for 16 h, and then pulsed for 6 h with [^{3}H]thymidine (0.25 μCi/ml). The cells were fixed with 5% trichloroacetic acid and washed twice with ethanol. Finally, cell residues were dissolved in 0.1 N NaOH with 1% SDS and counted in a liquid scintillation counter.

Clonogenic Assay. PANC-1 cells, 3–5 days after passage, were washed and resuspended in DMEM. Cells were then disaggregated by two passes through a 19-guage needle into an essentially single-cell suspension as judged by microscopy. Cell number was determined using a Coulter counter, and 5×10^4 cells were mixed with DMEM containing 1% FBS, 7.5% NaHCO$_3$, 4 mM glutamine, 1 mM Na-pyruvate, 100 nM NT, and 0.3% agarose in the presence or absence of 5 μM U 0126 or 10 μM GF-1, and layered over a solid base of 0.5% agarose in the same medium containing 100 nM NT in the

presence or absence of 5 μM U 0126 or 10 μM GF-1 in 33-mm dishes. The cultures were incubated in humidified 10% CO_2:90% air at 37°C for 21 days and then stained with the vital stain 1% methylene blue. Colonies of >120 μm in diameter (16 cells) were counted using a microscope.

Materials. [γ-^{32}P]ATP (370 MBq/ml) was obtained from Amersham International. Bisindolylmaleimide I (GF-1), Bisindolylmaleimide V (GF-V), Ro 31–8220, wortmannin, rapamycin, AG 1478, PD 158780, AG 825, GM 6001, PP-2, U 0124, U 0126, and genistein were purchased from Calbiochem. NT and PDB were obtained from Sigma. Protein A and protein G agarose were purchased from Boehringer Mannheim-Roche. Antiphosphotyrosine antibody (4G10), anti-Pyk2 polyclonal rabbit antibody, anti-EGFR polyclonal antibody, RAS activation assay kit, c-Raf-1 immunoprecipitation kinase cascade assay kit, and myelin basic protein were purchased from Upstate Biotechnology. Anti-ERK-2 polyclonal antibody was obtained from Santa Cruz Biotechnologies. Anti-MEK-1/2 polyclonal antibody, the phospho-MEK-1/2 (Ser217/Ser221) polyclonal antibody, and the phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody were obtained from Cell Signaling Technology. Fluorescein-conjugated goat-antimouse antibody was obtained from Sigma. All of the other reagents were of the purest grade available.

RESULTS

NT Stimulates ERK-1 and ERK-2 Nuclear Translocation and MEK-1/2 Activation 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 (6–12). We showed recently that addition of NT to serum-starved PANC-1 cells induced rapid and dose-dependent ERK-1 and ERK-2 phosphorylation, which is completely abrogated by specific MEK-1/2 inhibitors, PD 098059 and U 0126 (6). ERK activation requires translocation from the cytosol to the nucleus to target transcription factors that are strongly implicated in the control of cellular proliferation (36, 37). To determine whether the increase in ERK-1 and ERK-2 phosphorylation induced by NT in PANC-1 cells is also associated with enhanced nuclear translocation of ERKs and thereby activation of ERKs, confluent cultures of serum-starved PANC-1 cells were fixed, permeabilized, and stained with phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody. As illustrated by immunofluorescent microscopy in Fig. 1A, phosphorylated ERKs are undetectable in the nuclei of unstimulated serum-starved cells. Stimulation with NT for 2 h caused a striking increase in nuclear translocation of ERKs (Fig. 1A). Having established that phosphorylated ERKs translocate to the nucleus in response to NT in PANC-1 cells, we analyzed the signaling pathways initiated by NTR-1 that lead to ERK-1 and ERK-2 activation.

Initially we determined the effect of NT on activation of MEK-1/2, a dual-specific ERK kinase (35). Confluent cultures of PANC-1 cells were washed, transferred to serum-free medium, and stimulated with 50 nM NT. After various times of incubation, cells were lysed, and the active forms of MEK-1/2 were detected by Western blotting using phospho-MEK-1/2 (Ser 217/221)-specific polyclonal antibody. As shown in Fig. 1, B and C, minimal MEK-1/2 phosphorylation was detected in unstimulated PANC-1 cells, indicating that the mutated K-Ras present in these cells (38) is not sufficient to promote constitutive activation of the ERK pathway, in agreement with other reports (12, 21, 39).

Stimulation of PANC-1 cells with NT induced a striking increase in phosphorylation (activation) of MEK-1/2, in a time-dependent manner (Fig. 1B, top panel). Maximal effect was achieved after 1 min of exposure to NT. Western blotting with anti-MEK-1/2 antibody confirmed that similar amounts of MEK protein were loaded into the gel after treatment with NT for different time-periods (Fig. 1B, bottom panel). In view of a previous report indicating that NT-mediated mitogenic signaling in pancreatic cancer cell lines is sharply concentration-dependent with inhibition at high NT concentrations (40), we
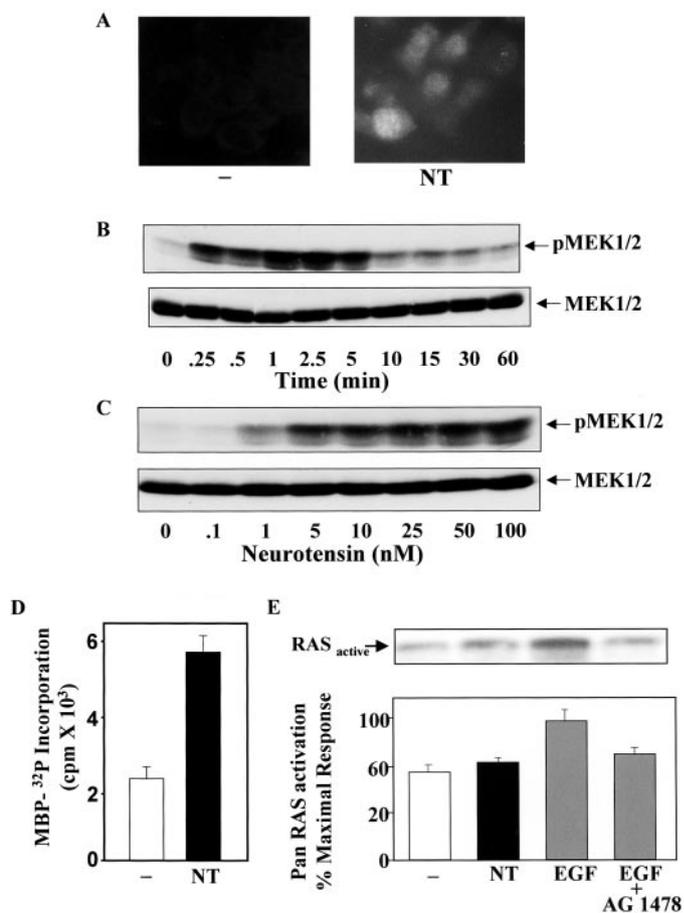

Fig. 1. NT stimulates ERK-1 and ERK-2 nuclear translocation, and activation of MEK-1/2 and c-Raf-1 but not pan-Ras in PANC-1 cells. Confluent cells were washed twice with Dulbecco's PBS and incubated in serum-free DMEM for 6 h. *A,* phospho-ERK-1/2 nuclear translocation. The cells were either stimulated with 50 nM NT or an equivalent amount of solvent (−) for 2 h, and were subsequently fixed, permeabilized, and quenched as described in "Materials and Methods." The fixed and permeabilized cells were incubated (see "Materials and Methods" for details) with dual-specific phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody followed by staining with fluorescein-conjugated goat-antimouse antibody. As described in "Materials and Methods" the samples were examined with an inverted Zeiss Axioskope microscope using the X100 aqueous objective lens. *B,* NT activates MEK-1/2 in PANC-1 cells, time course. Serum-starved cells were stimulated with 50 nM NT for the indicated time-periods at 37°C. The cells were then washed in cold PBS, lysed in 2× SDS-PAGE sample buffer, and transferred to Immobilon membranes (as indicated in "Materials and Methods"). Samples were analyzed for MEK-1/2 activation using the phospho-MEK-1/2 (Ser217/221) specific polyclonal antibody (*B, top*). The same membrane was stripped and probed with MEK-1/2 polyclonal antibody (*B, bottom*). *Arrows* indicate the positions of pMEK-1/2 and total MEK-1/2. Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. *C,* NT activates MEK-1/2 in PANC-1 cells, dose-response. Serum-starved PANC-1 cells were stimulated for 2.5 min at 37°C with NT at the indicated concentrations. As above, the cells were analyzed for MEK-1/2 activation using the phospho-MEK-1/2 (Ser217/221)-specific polyclonal antibody (*C, top*). The same membrane was stripped and probed with MEK-1/2 polyclonal antibody (*C, bottom*). *Arrows* indicate the positions of phospho-MEK-1/2 and total MEK-1/2. Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. *D,* NT stimulates c-Raf-1 activation. The cultures were stimulated with 50 nM NT or an equivalent amount of solvent (−) for 10 min at 37°C. The cells were subsequently washed twice with cold PBS and lysed in ice-cold Buffer-B as described in "Materials and Methods." c-Raf-1 was IP by anti-Raf-1 antibody bound to protein G agarose, and subsequently coupled kinase assay was performed using MBP as a substrate with the protein G-Raf-1 immunocomplex as described in "Materials and Methods." *E,* EGF, not NT, stimulates pan-RAS activation. Serum-starved cells were incubated for 1 h with 250 nM AG 1478 and subsequently challenged with 10 ng/ml EGF. Parallel sets of cultures were stimulated with either 50 nM NT or 10 ng/ml EGF for 10 min at 37°C. Control cells (−) received equivalent amounts of solvent. Activated pan-RAS was IP by Raf-1 RBD and transferred to Immobilon membranes as described in "Materials and Methods." Immunoblot analysis was performed with pan-Ras monoclonal antibody (*E, top*). *Arrow* indicates the position of Ras. Graphed data (*E, bottom*) represents average values (*n* = 3) of the level of pan-Ras activation obtained from scanning densitometry and are expressed as percentages of the maximal increase in activation obtained with 10 ng/ml EGF for 10 min at 37°C; *bars,* ±SE.

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also examined MEK-1/2 activation in response to increasing concentrations of NT. As illustrated in Fig. 1*C, top panel,* maximal MEK-1/2 activation was induced by addition of NT at 5 nM, and it remained at a maximum when the concentration of this peptide agonist was increased from 5 to 100 nM. Again, Western blotting with anti-MEK-1/2 antibody confirmed that similar amounts of MEK protein were loaded into the gel after treatment with increasing concentrations of NT (Fig. 1*C, bottom panel*). These results demonstrate that NT potently stimulated MEK-1/2 activation in *K-Ras*-mutated PANC-1 cells with minimal basal MEK-1/2 activity.

NT Stimulates c-Raf-1 Activation in PANC-1 Cells. We next analyzed NT-induced c-Raf-1 activation in PANC-1 cells. The Raf protein serine-threonine kinases play central roles in the response of cells to extracellular growth factors (23). Activation of Raf downstream of protein tyrosine-kinase receptors is mediated by the Ras-GTP binding proteins, which are required for stimulation of Raf kinase activity. Raf then phosphorylates and activates MEK, initiating a protein kinase cascade that leads to ERK-1 and ERK-2 activation, and culminates in proliferation or differentiation of a variety of cell types (23). As shown in Fig. 1*D,* NT induced striking activation of c-Raf-1 over basal level as determined by the sequential immune complex kinase assay using MBP as a substrate of activated ERKs. B-Raf somatic missense mutations were reported recently in majority of malignant melanoma patients independent of Ras function (41). Using a similar assay, we did not detect a significant increase in NT-induced B-Raf activation in PANC-1 cells, although the basal level of B-Raf activation was significantly higher as compared with c-Raf-1 (data not shown). Thus, our results suggest that NT potently stimulates c-Raf-1-MEK-ERK signaling pathway in *K-Ras*-mutated PANC-1 cells.

Raf serine-threonine kinase activity is stimulated by the Ras-GTP binding proteins functioning downstream of the growth-factor tyrosine-kinase receptors in variety of cell types, including pancreatic cancer cells (10, 12). However, GPCR-mediated pan-Ras activation in the pancreatic cancer cells harboring mutant *K-Ras* has not been investigated. So, to determine pan-Ras activation by NT in PANC-1 cells, we used a fusion protein coupled to agarose that includes the RBD of c-Raf-1, which specifically interacts with the GTP-bound form of all of the Ras isoforms with high affinity (12). Interestingly, as shown in Fig. 1*E,* NT did not significantly increase pan-Ras activation in PANC-1 cells as detected by pan-Ras-specific monoclonal antibody. In contrast, EGF significantly increased pan-Ras activation over basal level, which is attenuated by EGFR tyrosine kinase inhibitor AG 1478. As expected, we observed a high basal level of pan-Ras activation in PANC-1 cells that is potentially contributed by the activating *K-Ras* mutation in these cells (Fig. 1*E*). Unlike EGF, NT did not increase Ha-Ras activation in these cells as detected by Ha-Ras-specific monoclonal antibody (data not shown). Collectively, the results presented in Fig. 1 demonstrate that NT-induced ERK-1 and ERK-2 activation is significantly dependent on c-Raf-1-MEK-1/2-mediated signaling pathway.

NT Stimulates c-Raf-1 and ERK Activation in PANC-1 Cells through a PKC-dependent Signaling Pathway. Having established that NT strikingly stimulates c-Raf-1 but not pan-Ras in PANC-1 cells, we next examined the role of PKCs in mediating ERK-1 and ERK-2 activation in these cells. We showed previously that NT significantly stimulated PKC and PKD, a novel family of serine-threonine kinase that functions downstream of PKC in a unique signal transduction pathway (7). To determine whether NT can stimulate PKC-dependent ERK-1 and ERK-2 activation in PANC-1 cells, confluent cultures of PANC-1 cells were treated with various concentrations of either Ro 31–8220 or GF-1, selective inhibitors of phorbol ester-sensitive isoforms of PKC (7, 42). Treatment with either Ro

31–8220 (Fig. 2*A*) or GF-1 (Fig. 2*B*) potently blocked ERK-1 and ERK-2 activation induced by subsequent addition of NT in a concentration-dependent manner. Half-maximal inhibitory concentrations of Ro 31–8220 and GF-1 were 0.5 μM and 1 μM, respectively. Western blotting with anti-ERK2 antibody confirmed that similar amounts of ERK-1 and ERK-2 protein were loaded into the gel after treatment with increasing concentrations of either Ro 31–8220 (Fig. 2*A, inset, bottom panel*) or GF-1 (Fig. 2*B, inset, bottom panel*). This result implies that Ro 31–8220 interferes with NT-mediated ERK-1 and ERK-2 activation in PANC-1 cells by blocking PKC.
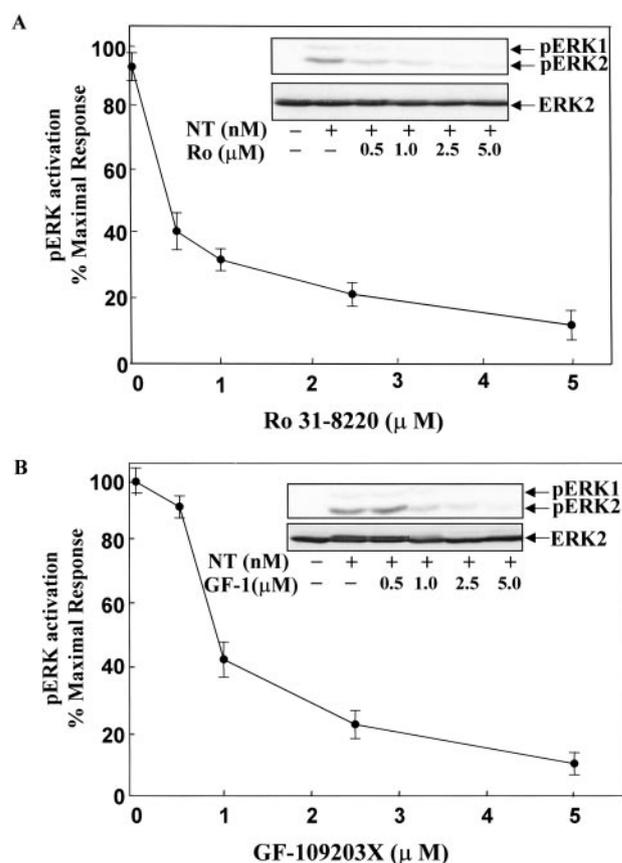

Fig. 2. The PKC inhibitors, GF-1 and Ro 31–8220, prevent ERK-1 and ERK-2 activation by NT in PANC-1 cells. Confluent cells were washed twice with PBS and incubated in serum-free DMEM for 6 h. *A,* Ro 31–8220 inhibits ERK-1 and ERK-2 activation. Serum-starved PANC-1 cells were incubated for 1 h with different concentrations of the PKC inhibitor Ro 31–8220. Control cells (−) received equivalent amounts of solvent. The cultures were subsequently stimulated with 50 nM NT for 10 min at 37°C. The cells were then washed in cold PBS, lysed in 2× SDS-PAGE sample buffer, and transferred to Immobilon membranes (as indicated in "Materials and Methods"). Samples were analyzed for ERK-1 and ERK-2 activation using the dual-specific phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) antibody (*A, inset, top*). The same membrane was stripped and probed with ERK-2 polyclonal antibody (*A, inset, bottom*). *Arrows* indicate the positions of pERK-1, pERK-2, and total ERK-2. Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. Graphed data represents average values (*n* = 3) of the level of ERK-1 and ERK-2 activation obtained from scanning densitometry, and are expressed as percentages of the maximal increase in phosphorylation obtained with 50 nM NT for 10 min at 37°C; *bars,* ±SE. *B,* GF-1 inhibits ERK-1 and ERK-2 activation. Serum-starved PANC-1 cells were incubated for 1 h with different concentrations of a structurally unrelated PKC inhibitor GF-1. Control cells (−) received equivalent amounts of solvent. The cultures were subsequently stimulated with 50 nM NT for 10 min at 37°C. The cells were then washed in cold PBS, lysed in 2× SDS-PAGE sample buffer, and transferred to Immobilon membranes (as indicated in "Materials and Methods"). Samples were analyzed for ERK-1 and ERK-2 activation using the dual-specific phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody (*B, inset, top*). The same membrane was stripped and probed with ERK-2 polyclonal antibody (*B, inset, bottom*). *Arrows* indicate the positions of pERK-1, pERK-2, and total ERK-2. Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. Graphed data represents average values (*n* = 3) of the level of ERK-1 and ERK-2 activation obtained from scanning densitometry, and are expressed as percentages of the maximal increase in phosphorylation obtained with 50 nM NT for 10 min at 37°C; *bars,* ±SE.
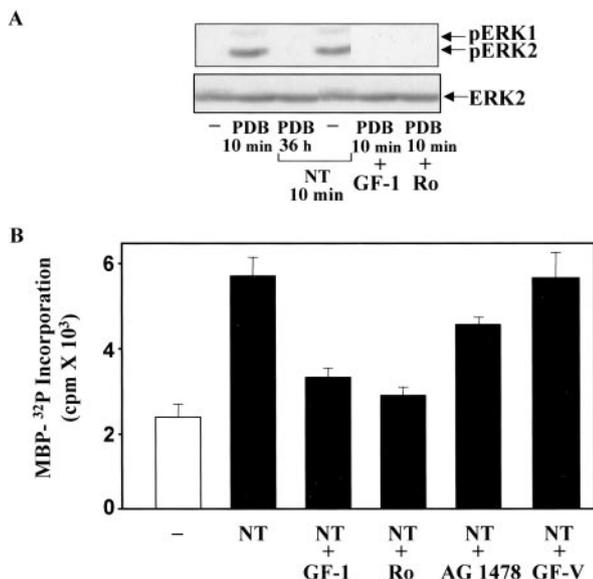
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Fig. 3. NT stimulates ERK-1 and ERK-2 activation in PANC-1 cells through a PKC-dependent pathway. *A,* prolonged PDB treatment prevents NT-induced ERK activation. Confluent cultures of PANC-1 cells were incubated for 36 h with 200 nM PDB. The cultures were washed with serum-free DMEM and incubated for 2 h at 37°C in serum-free DMEM. The cells were subsequently 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. Another set of cultures of were treated with 5 μM GF-1 and 5 μM Ro 31–8220 for 1 h, and then subsequently challenged with 200 nM PDB for 10 min at 37°C. The cells were then washed in cold PBS, lysed in 2× SDS-PAGE sample buffer, and transferred to Immobilon membranes (as indicated in "Materials and Methods"). Samples were analyzed for ERK-1 and ERK-2 activation using the dual-specific phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody (*A, top*). The same membrane was stripped and probed with ERK-2 polyclonal antibody (*A, bottom*). *Arrows* indicate the positions of pERK-1, pERK-2, and total ERK-2. Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. *B,* PKC significantly mediates NT-induced c-Raf-1 activation in PANC-1 cells. Confluent cells were washed twice with PBS and incubated in serum-free DMEM for 6 h. Serum-starved PANC-1 cells were incubated for 1 h with 5 μM GF-1, 5 μM Ro 31–8220, 500 nM AG 1478, and 5 μM GF-V. Control cells (−) received equivalent amounts of solvent. The cultures were subsequently stimulated with 50 nM NT for 10 min at 37°C. The cells were washed twice with cold PBS and lysed in ice-cold buffer A as described in "Materials and Methods." c-Raf-1 was IP by anti-Raf-1 antibody bound to protein G agarose, and subsequently coupled kinase assay was performed using MBP as a substrate with the protein G-Raf-1 immunocomplex as described in "Materials and Methods." *Columns,* mean ^{32}P incorporation of MBP from three separate dishes; *bars,* ±SE. Shown here is a representative plot from one experiment. Similar results were obtained in three independent experiments.

To confirm the requirement of PKC in mediating NT-induced ERK-1 and ERK-2 activation, PANC-1 cells were treated with PDB for 36 h to produce extensive depletion of classic and novel PKCs (7, 43), and then challenged with NT for 10 min. In a parallel set of experiments, cells were either treated with GF-1 and Ro 31–8220 for 1 h and stimulated subsequently with PDB for 10 min, or were treated without (−) and with PDB alone for 10 min. As shown in Fig. 3*A, top panel,* the increase in ERK-1 and ERK-2 activation induced by acute exposure to NT was completely abrogated by previous chronic treatment with PDB for 36 h. Stimulation with PDB for 10 min predictably increased ERK-1 and ERK-2 activation, which was completely abrogated by pretreatment with the PKC inhibitors. Western blotting with anti-ERK2 confirmed that similar amounts of ERK-1 and ERK-2 protein were loaded into the gel (Fig. 3*A, bottom panel*). This result provides additional evidence indicating the requirement of PKC(s) for ERK-1 and ERK-2 activation.

Having documented that NT-induced ERK-1 and ERK-2 activation in PANC-1 cells is PKC-dependent, we next investigated whether NT stimulates PKC-dependent c-Raf-1 activation in these cells. As shown in Fig. 3*B,* NT alone or in the presence of GF-V, an inactive structural analogue of GF-1, induced striking activation of c-Raf-1 over basal

level as determined by a sequential immune complex kinase assay using MBP as a substrate of activated ERK-1 and ERK-2. However, treatment of the cells with the PKC inhibitors GF-1 and Ro 31–8220 significantly attenuated NT-induced c-Raf-1 activation in PANC-1 cells. Interestingly, EGFR tyrosine kinase inhibitor AG 1478 minimally decreased NT-induced c-Raf-1 activation in these cells.

NT Does Not Stimulate EGFR Transactivation, or Src or Pyk2 Activation in PANC-1 Cells. We showed in Fig. 1*E* that NT, unlike EGF, did not significantly induce pan-Ras activation in PANC-1 cells. To additionally substantiate this result, we examined GPCR agonists-mediated transactivation of EGFR in these cells. EGFR tyrosine autophosphorylation by the transactivation process can lead to ERK-1 and ERK-2 activation by GPCRs, at least in some cell types (30, 44). In pancreatic cancer cells, which harbor activating *K-Ras* mutations and produce EGFR agonists (*e.g.,* TGF-α), this process of GPCR cross-talk with EGFR has not been investigated previously. As shown in Fig. 4*A,* EGF significantly stimulated tyrosine phosphorylation of EGFR above a high basal level, which is expected in *K-Ras* mutated PANC-1 cells. Interestingly, NT did not mediate an increase in EGFR tyrosine phosphorylation, which effectively correlates with the result
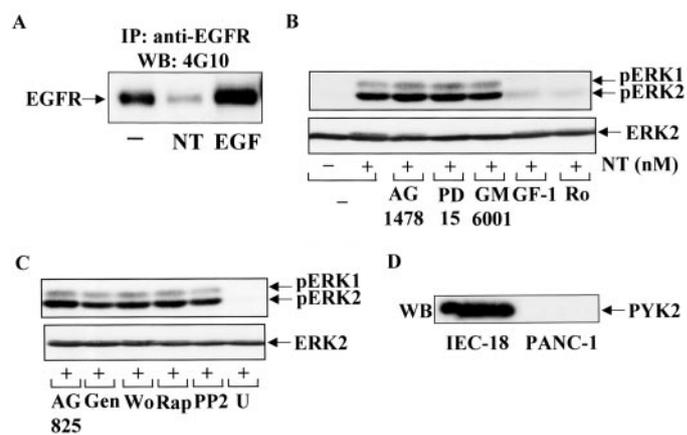

Fig. 4. NT-induced ERK-1 and ERK-2 activation in PANC-1 cells is independent of EGFR transactivation, Src, and Pyk2 stimulation. Confluent PANC-1 cells were washed twice with PBS and incubated in serum-free DMEM for 6 h. *A,* NT does not stimulate EGFR transactivation. Cells were stimulated with 50 nM NT, 10 ng/ml EGF, or an equivalent amount of solvent (−) for 10 min at 37°C. EGFR was IP, and Western blot was then performed with antiphosphotyrosine antibody (4G10) as described in "Materials and Methods." Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. *B,* EGFR tyrosine kinase and broad-spectrum metalloprotease inhibitors do not abrogate NT-induced ERK-1 and ERK-2 activation. Serum-starved cultures were incubated for 1 h with 500 nM AG 1478, 500 nM PD 158780 (PD 15), 10 μM GM 6001, 5 μM GF-1, 5 μM Ro 31–8220 (Ro), or an equivalent amount of solvent (−). Cells were subsequently challenged for 10 min with 50 nM NT at 37°C and lysed with 2× SDS-PAGE sample buffer. Western blot was performed as indicated in "Materials and Methods." Samples were analyzed for ERK-1 and ERK-2 activation using the dual-specific phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody (*B, top*). The same membrane was stripped and probed with ERK-2 polyclonal antibody (*B, bottom*). *Arrows* indicate the positions of pERK-1, pERK-2, and total ERK-2. Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. *C,* HER-2 tyrosine kinase, broad-spectrum tyrosine kinase, phosphatidylinositol 3′-kinase, p70^{s6k}, and Src inhibitors do not abrogate NT-induced ERK activation. Confluent PANC-1 cells were washed twice with PBS and incubated in serum-free DMEM for 6 h. Serum-starved cultures were incubated for 1 h with 50 μM AG 825, 50 μM Genistein (*Gen*), 100 nM Wortmannin (*Wo*), 20 nM Rapamycin (*Rap*), 10 μM PP-2, 5 μM U 0126 (*U*), or an equivalent amount of solvent (−). Cells were subsequently challenged for 10 min with 50 nM NT at 37°C and lysed with 2× SDS-PAGE sample buffer. Western blot was performed as indicated in "Materials and Methods." Samples were analyzed for ERK-1 and ERK-2 activation using the dual-specific phospho-p44^{mapk}/p42^{mapk} (Thr202/Tyr204) monoclonal antibody (*C, top*). The same membrane was stripped and probed with ERK-2 polyclonal antibody (*C, bottom*). *Arrows* indicate the positions of pERK-1, pERK-2, and total ERK-2. Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments. *D,* absence of Pyk2 expression. Confluent PANC-1 and IEC-18 cells were washed twice with PBS, and Western blot was performed with anti-Pyk2 polyclonal antibody as described in "Materials and Methods." Shown here is a representative autoluminogram from one experiment. Similar results were obtained in three independent experiments.

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of non-Ras stimulation observed in Fig. 1*E*. In particular, NT consistently reduced EGFR tyrosine phosphorylation in PANC-1 cells. Next we examined NT-mediated ERK activation using EGFR tyrosine kinase inhibitors AG 1478 and PD 158780 (45, 46), the broad-spectrum metalloprotease inhibitor GM 6001 (47) that blocks the generation of EGFR ligands, and the broad-spectrum protein tyrosine kinase inhibitor genistein (48) in PANC-1 cells. Fig. 4*B, top panel,* demonstrates that NT-induced ERK-1 and ERK-2 activation in these cells is completely abrogated by the PKC inhibitors GF-1 and Ro 31–8220 but not by the inhibitors of the EGFR transactivation pathway. Western blotting with anti-ERK2 confirmed that similar amounts of ERK-1 and ERK-2 protein were loaded into the gel (Fig. 4*B, bottom panel*).

Src activation has been postulated to act both upstream as well as downstream of EGFR (49). Having established that NT does not mediate EGFR transactivation, we next investigated the role of Src kinase family members in NT-induced ERK-1 and ERK-2 activation in PANC-1 cells. As shown in Fig. 4*C, top panel,* treatment with Src tyrosine kinase inhibitor PP-2 (50) did not inhibit NT-induced ERK-1 and ERK-2 activation in these cells. 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 other signaling pathways and examined ERK-1 and ERK-2 activation after subsequent challenge with NT. Treatment with phosphatidyl 3′-kinase inhibitor wortmannin (51), the p70 ribosomal S6 kinase ($p70^{s6k}$) inhibitor rapamycin (52) and HER-2 tyrosine kinase inhibitor AG 825 (53) did not affect ERK-1 and ERK-2 activation in response to NT in PANC-1 cells (Fig. 4*C, top panel*). Again, Western blotting with anti-ERK2 confirmed that similar amounts of ERK-1 and ERK-2 protein were loaded into the gel (Fig. 4*C, bottom panel*).

Pyk2 is a member of the nonreceptor tyrosine kinase family that is stimulated by multiple GPCR agonists in certain cell types (54) and links Src to downstream signaling pathways including the activation of MAPK (54). So, we next determined whether Pyk2 is expressed in PANC-1 cells. Fig. 4*D* demonstrates that Pyk2 was not detected by Western blot analysis in PANC-1 cells as compared with a control lysate from IEC-18 cells known to express Pyk2 (55). So, these results illustrate not only the specificity of the PKC inhibitors but also indicate that EGFR transactivation or other kinases, including Src and Pyk2, are not upstream regulators of NT-induced ERK-1 and ERK-2 activation in PANC-1 cells. Together, our results reinforce the notion that PKC-dependent signal transduction pathway predominantly mediates NT-induced ERK-1 and ERK-2 activation in PANC-1 cells.

Effect of MEK-1/2 Inhibitor U 0126 on NT-mediated DNA Synthesis in PANC-1 Cells. The effect of GPCR-mediated ERK 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 (6). Subsequently, we showed that NTR-1-mediated DNA synthesis is dependent on PKC activity in these cells (7). Now, we examined whether NT-induced DNA synthesis in PANC-1 cells is ERK-dependent.

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. Now, to determine the role of ERKs in NT-induced DNA synthesis, PANC-1 cells were treated with the selective MEK-1/2 inhibitor U 0126 and U 0124, an inactive analogue of U 0126, before addition of NT. DNA synthesis, as measured by [³H]thymidine incorporation, was induced by NT and was not affected by addition of U 0124 (Fig. 5*A*). NT alone stimulated DNA synthesis to the same
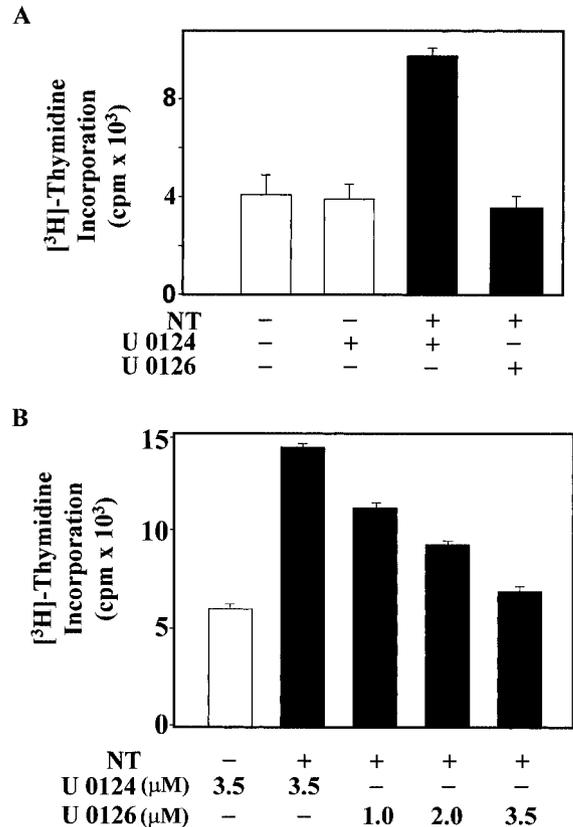

Fig. 5. NT-induced DNA synthesis in PANC-1 cells is ERK-dependent. Cells incubated in serum-free medium were pulse-labeled with [³H]thymidine (0.25 μCi/ml), and the radioactivity incorporated into acid-insoluble pools was counted in a scintillation counter as described in "Materials and Methods." *A,* effect of MEK-1/2 inhibitor, U 0126, on DNA synthesis in cells stimulated with NT. Cells were incubated with 5 μM U 0124 (+) or 5 μM U 0126 (+) 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. *B,* dose-response for increasing concentration of U 0126 on NT-stimulated DNA synthesis. Cells were incubated with 3.5 μM U 0124 or indicated concentrations of U 0126 for 1 h at 37°C. DNA synthesis was measured in cells additionally stimulated without (−) and with (+) 50 nM NT at 37°C. Shown here is a representative plot from one experiment for each panel. Similar results were obtained in three independent experiments; *bars,* ±SE.

level as depicted in Fig. 5*A* (result not shown). In striking contrast, U 0126 blocked NT-induced DNA synthesis in PANC-1 cells (Fig. 5*A*). The addition of U 0126 prevented the NT-induced increase in the incorporation of [³H]thymidine in a dose-dependent fashion (Fig. 5*B*). Increasing concentrations of U 0126 attenuated NT-induced DNA synthesis as compared with cells treated with highest concentration of U 0124 (Fig. 5*B*). The results demonstrate that NTR-1-mediated activation of DNA synthesis is ERK-dependent in PANC-1 cells.

Effect of U 0126 and GF-1 on NT-induced Colony Formation in PANC-1 Cells. Several reports indicate that activation of MAPK cascade plays a central role in mediating mitogenesis in various cell lines including ductal pancreatic cancer cells (34, 35). Growth factors including EGF and TGF-α are known to stimulate colony formation of ductal pancreatic cancer cells in semisolid medium (10, 12, 21). However, a GPCR agonist including NT-mediated colony formation of these cells has not been examined previously. Consequently, we determined whether NT stimulated colony formation of PANC-1 cells in semisolid medium. As shown in Fig. 6*A,* NT significantly increased colony formation of these cells, and the MEK-1/2 inhibitor U 0126 notably attenuates this effect. This MEK-1/2 inhibitor also caused a profound decrease in basal colony formation of PANC-1 cells. Because inhibition of PKC activity appreciably diminished DNA synthesis in PANC-1 cells (7), it could be expected that inhibition of these
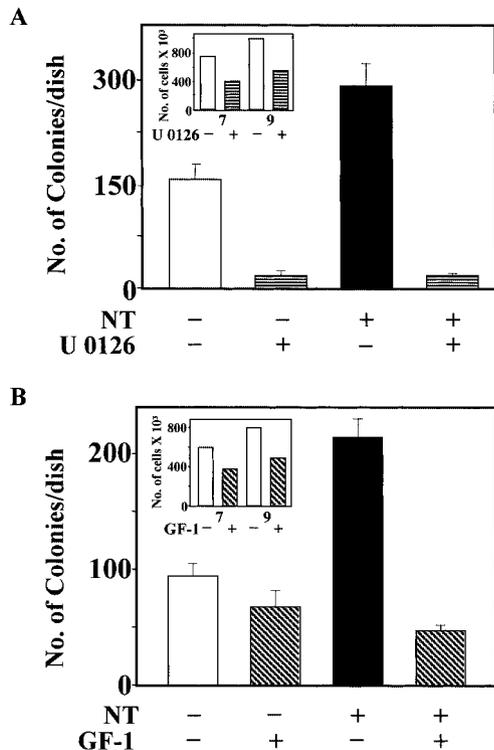
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A

B

Fig. 6. Effect of U 0126 and GF-1 on colony formation in response to NT in PANC-1 cells. *A*, a single-cell suspension of PANC-1 cells was plated in agarose medium containing 1% FBS at a density of 5×10^4 cells/dish in the absence ($-$) or presence ($+$) of 100 nM NT, and without ($-$) or with ($+$) 5 μM U 0126 and incubated for 3 weeks as described in "Materials and Methods." *Inset*, PANC-1 cells were plated in DMEM with 0.5% FBS in the presence ($+$) or absence ($-$) of 5 μM U 0126. Cell number was determined from a minimum of 3 plates per condition when cells were growing exponentially at days 7 and 9 using a Coulter counter. *B*, a single-cell suspension of PANC-1 cells was plated in agarose medium containing 0.5% FBS at a density of 1×10^4 cells/dish in the absence ($-$) or presence ($+$) of 100 nM NT, and without ($-$) or with ($+$) 10 μM GF-1 and incubated for 3 weeks as described in "Materials and Methods." *Inset*, PANC-1 cells were plated in DMEM with 0.5% FBS in the presence ($+$) or absence ($-$) of 10 μM GF-1. Cell number was determined from a minimum of 3 plates per condition when cells were growing exponentially at days 7 and 9 using a Coulter counter. *Columns*, mean number of colonies formed on four separate dishes; *bars*, $\pm$SE. Shown here is a representative plot from one experiment for each panel. Similar results were obtained in three independent experiments.

enzymes should also prevent colony formation. Fig. 6*B* shows that GF-1 markedly inhibited colony growth in response to NT in PANC-1 cells. We also verified that U 0126 and GF-1 reduced the proliferation of PANC-1 cells in liquid culture by 40 and 50%, respectively (Fig. 6, *A* and *B, insets*). Thus, our results demonstrate that PKC-mediated signaling pathway stimulates NT-induced colony growth in PANC-1 cells.

DISCUSSION

Neuropeptides, a structurally diverse set of signaling molecules, including bombesin/GRP, bradykinin, CCK, gastrin, and NT, have been identified as potent cellular growth factors, and are increasingly implicated as autocrine and/or paracrine growth factors for a variety of clinically aggressive tumors (3–5). We have reported recently that multiple neuropeptides, including NT, bombesin/GRP, CCK, and angiotensin induce rapid Ca^{2+} mobilization from intracellular stores in human ductal pancreatic cancer cell lines (6). In particular, NT stimulated ERK-1 and ERK-2 activation and DNA synthesis in PANC-1 cells (6), an extensively studied model of human ductal pancreatic cancer (6–12). Subsequently, we have shown that multiple PKC isoforms are expressed in PANC-1 cells, and NTR-1-mediated activation of DNA synthesis in these cells is PKC-dependent (7). This

prompted us to examine the downstream signaling pathway from NTR-1 leading to ERK-1 and ERK-2 activation, and to determine whether PKC mediates this process and promotes mitogenesis in PANC-1 cells.

Activation of ERK-1 and ERK-2, as one major signaling event responsible for cell cycle entry initiated by mitogenic stimuli, is closely connected with the translocation of phosphorylated ERKs to the nucleus. So, we first examined the subcellular localization of phosphorylated ERKs in PANC-1 cells after NT stimulation. Our results show that NT significantly induced translocation of phosphorylated ERKs to the nucleus, and thereby resulted in ERK-1 and ERK-2 activation in PANC-1 cells.

GPCRs are known to induce ERK activation through multiple signal transduction mechanisms (4, 24), but little is known about the pathways activated by these receptors in cells harboring *K-Ras* mutations (56). So, next we analyzed the signaling pathways initiated from NTR-1 in PANC-1 cells leading to ERK-1 and ERK-2 activation. Given that ∼90% of human ductal pancreatic carcinomas harbor activating *K-Ras* mutations, it seems plausible that the MEK-ERK signaling pathway will be constitutively active in these cells. Instead, we showed that MEK-1/2 are not dually phosphorylated in PANC-1 cells incubated in serum-free medium, in agreement with other reports (12, 21, 39). NT also induced a rapid, striking, and dose-dependent activation of MEK-1/2 in PANC-1 cells. Furthermore, we demonstrated a significant stimulation of c-Raf-1 by NT in these cells. Interestingly, NT did not induce pan-Ras activation in PANC-1 cells. Thus, our results suggest that NT potently stimulates c-Raf-1 and MEK-1/2 to activate ERK-1 and ERK-2 in PANC-1 cells.

We showed recently that NT stimulated rapid and transient activation of PKC and PKD, a novel serine-threonine kinase functioning downstream of PKC (7). In the current study, we demonstrated that NT strikingly stimulates c-Raf-1 activation but not pan-Ras in PANC-1 cells, suggesting involvement of a PKC-mediated signaling pathway leading to ERK-1 and ERK-2 activation. To determine the role of PKC, we treated PANC-1 cells with the PKC inhibitors GF-1 and Ro 31–8220 before stimulation with NT. Our results showed that the PKC inhibitors markedly prevent NT-induced ERK-1 and ERK-2 activation. These results suggest that NT promotes a rapid and striking ERK-1 and ERK-2 activation in 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 novel PKC isoforms in mammalian cells (7, 43). So, we examined whether chronic PDB stimulation abrogates the NT-induced ERK-1 and ERK-2 activation in PANC-1 cells. The result indicates that chronic PDB exposure leads to complete inhibition of NT-induced ERK-1 and ERK-2 activation in these cells suggesting a critical requirement of PKC.

In many cell types, GPCR agonists induce a rapid increase in EGFR tyrosine autophosphorylation (44, 57), a receptor cross-talk mediated, at least in part, by rapid proteolytic generation of EGFR ligands (*e.g.,* heparin-binding EGF) at the cell surface termed transactivation (44). Interestingly, our results suggest that NT did not transactivate EGFR in PANC-1 cells, which is in agreement with the finding that NT did not stimulate pan-Ras activation in these cells. In fact, NT decreased EGFR tyrosine phosphorylation in PANC-1 cells by a process termed transmodulation (58, 59). This process is partly mediated by PKC, at least in some cell types (58, 59). Given that NT strongly stimulates PKC in PANC-1 cells, it seems plausible that NT-induced EGFR transmodulation is mediated by PKC. We additionally validated our results with the use EGFR tyrosine kinase, broad-spectrum tyrosine kinase or broad-spectrum metalloprotease inhibitors, showing that NT-induced ERK-1 and ERK-2 activation is not mediated by EGFR transactivation in PANC-1 cells. Inhibition of other signaling path-

ways, including the p70 ribosomal S6 kinase (p70^{s6k}), which is constitutively active in PANC-1 cells (60), or Src family of tyrosine kinases, which is postulated to act both upstream as well as downstream of EGFR (49), did not affect ERK-1 and ERK-2 activation by NT. However, the PKC and MEK-1/2 inhibitors completely abrogated NTR-1-meadied ERK-1 and ERK-2 activation in PANC-1 cells. Pyk2, a nonreceptor tyrosine kinase, was shown to be involved in GPCR-mediated ERK activation via EGFR transactivation in certain cell types (54). Here, we did not detect Pyk2 expression in PANC-1 cells. Collectively, these findings strongly suggest a pivotal role mediated by PKC in signal transduction initiated by NT in pancreatic cancer cells.

Having demonstrated that NT did not transactivate EGFR but strongly induced c-Raf-1, we examined whether NT-induced c-Raf-1 activation in PANC-1 cells is PKC-dependent. Our results show that the PKC inhibitors significantly abrogated NT-induced c-Raf-1 activation in contrast with the EGFR tyrosine kinase inhibitor, which only had a partial effect. This strengthens the concept that a PKC-dependent pathway predominantly mediates the NT-induced c-Raf-1-MEK-ERK activation in PANC-1 cells.

We reported earlier that NT-induced DNA synthesis in PANC-1 cells is dependent on PKC (7). Here, we show that NT-mediated DNA synthesis is also ERK-dependent. Thus, our results suggest that a functional MAPK signaling cascade downstream of NTR-1 is necessary for DNA synthesis, which is an important surrogate marker for mitogenesis in the ductal pancreatic cancer cells. Next, we examined anchorage-independent growth of PANC-1 cells in semisolid medium, because it correlates well with cell tumorigenicity and appears to be a fundamental characteristic of cancer cells (61, 62). NT markedly stimulated the clonal growth of PANC-1 cells in semisolid medium. Our results show, for the first time, that a GPCR agonist markedly enhanced colony formation of human ductal pancreatic cancer cells. Phorbol ester has been shown recently to promote anchorage-independent growth of MIA PaCa-2 cells through a PKC-dependent pathway (21). However, the role of PKC in GPCR-mediated colony formation of pancreatic cancer cells has not been studied previously. Here, we demonstrate that both the MEK-1/2 inhibitor, U 0126, and the PKC inhibitor, GF-1, strikingly inhibited NT-induced colony formation in PANC-1 cells. In view of our results that NT-induced ERK activation, DNA synthesis, and colony formation in pancreatic cancer cells are PKC-dependent, it is conceivable that PKC predominantly mediates GPCR-initiated mitogenesis in these cells.

We propose a following model for the signal transduction pathways initiated by NTR-1 in PANC-1 cells harboring activating *K-Ras* mutations. NT, on binding NTR-1, stimulates PKC through a Gα_q-dependent pathway and subsequently activates downstream targets of PKC, including ERKs and PKD. Using a pharmacologic approach, our current results strongly suggest NT-induced PKC-dependent c-Raf-1 activation, which leads to MEK-ERK activation in PANC-1 cells. We did not demonstrate that transactivation of EGFR or activation of other signaling pathways including Src, phosphatidylinositol 3'-kinase, p70^{s6k}, and Pyk-2 mediate NT-induced ERK activation in these cells. Although we did not observe a significant pan-Ras stimulation by NT in PANC-1 cells, activating K-Ras mutation probably plays a permissive role in mediating c-Raf-1 activation in these cells. A plausible hypothesis to support this model is that activated Ras recruits c-Raf-1 to the plasma membrane but is not sufficient to induce downstream signaling events leading to ERK activation in PANC-1 cells. GPCR-activated PKCs then translocate to the plasma membrane and additionally bolster the effect of activated Ras on c-Raf-1, either directly or indirectly, ultimately leading to a strong activation of downstream signaling molecules including ERKs. Finally, the model depicts GPCR-activated, PKC-dependent increased DNA synthesis and colony formation in PANC-1 cells.

In conclusion, our results provide experimental evidence of NT-induced PKC-dependent c-Raf-1-MEK-ERK activation 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 pivotal role in mediating GPCR-initiated response. Our results, showing that NT-induced colony formation of PANC-1 cells in semisolid medium is PKC-dependent, raised the possibility that PKC mediates GPCR-initiated mitogenesis in PANC-1 cells. Collectively, it suggests that identification of specific targets of the PKC signaling pathway will lead to the development of novel chemotherapeutic interventions in human pancreatic cancer.

ACKNOWLEDGMENTS

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