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Possible Role of Duration of PKC-Induced ERK Activation in the Effects of Agonists and Phorbol Esters on DNA Synthesis in Panc-1 Cells

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Abstract Protein kinase C (PKC) and extracellular signal-regulated kinase (ERK) have been implicated in the effects of regulatory peptides on proliferation. We studied how ERK was activated by PKC following regulatory peptide or phorbol ester stimulation and we also investigated the effect of ERK activation on proliferation in Panc-1 cells. Panc-1 cells transfected with CCK_1 receptors were treated with cholecystokinin (CCK), neurotensin (NT), or phorbol 12-myristate 13-acetate (PMA). DNA synthesis was studied by measuring tritiated thymidine incorporation. PKC isoforms were selectively inhibited with Gö6983 and 200 nM Ro-32-0432, their translocation was detected by confocal microscopy and by subcellular fractionation followed by immunoblotting. ERK cascade activation was detected with phosphoERK immunoblotting and inhibited with 20 μM PD98059. PMA and CCK inhibited, NT stimulated DNA synthesis. These effects were inhibited by Ro-32-0432 but not by Gö6983 suggesting the involvement of PKCε in proliferation control. Confocal microscopy and subcellular fractionation demonstrated that PMA, CCK, and NT caused cytosol to membrane translocation of PKCε and ERK activation that was inhibited by Ro-32-0432 but not by Gö6983. ERK activation was prolonged following PMA and CCK, but transient after NT treatment. PMA, CCK, and NT all activated cyclinD1, while p21CIP1 expression was increased by only PMA and CCK, but not by NT; each of these effects is inhibited by PD98059. In conclusion, our results provide evidence for PKCε-mediated differential ERK activation and growth regulation in Panc-1C cells. Identification of the mechanisms by which these key signaling pathways are modulated could provide a basis for the development of novel therapeutic interventions to treat pancreatic cancer. J. Cell. Biochem. 98: 1667–1680, 2006.

Key words: cholecystokinin; neurotensin; protein kinase C; Panc-1; pancreas; extracellular signal-regulated kinase; proliferation; gene expression

Protein kinase C (PKC) is a multi-gene family with at least 10 members that differ in their tissue expression, subcellular localization, activator and cofactor requirements, and substrate specificity [Dekker and Parker, 1994]. Conventional isoforms (cPKCα, -βI, -βII, and -γ) are activated by calcium, diacylglycerol (DAG), and phospholipids; novel isoforms (nPKCδ, -ε, -η ,and -θ) are regulated by DAG and phospholipids but not calcium; and atypical isoforms (aPKCζ and -λ) require neither DAG nor calcium for activation [Dekker and Parker, 1994]. Isoforms of PKC can be activated indirectly by regulatory peptides acting on G protein-coupled receptors (GPCRs). Phospholipase Cβ is activated downstream of GPCRs and hydrolyses phosphoinositides to generate DAG which activates cPKCs and nPKCs, and inositol-1,4,5-triphosphate (IP_3) that releases calcium from intracellular stores [Rozengurt, 1998]. Pharmacologically, cPKCs and nPKCs can be directly activated by phorbol esters which are analogs of DAG [Nishizuka, 1988].

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Members of the PKC family are involved in diverse cellular functions including cell growth and differentiation. In the pancreas, direct PKC activation by phorbol esters has been reported either to inhibit [Detjen et al., 2000] or stimulate [Ishino et al., 2002] the growth of human pancreatic cancer cells, whereas indirect PKC activation by neurotensin (NT) acting on its cognate receptor natively expressed on Panc-1 pancreatic cancer cells stimulates proliferation [Guha et al., 2003].

Mitogen-activated protein kinases (MAPK) are serine-threonine protein kinases that are activated by a range of extracellular signals via protein phosphorylation cascades [Widmann et al., 1999]. Extracellular signal-regulated kinase (ERK1) and ERK2 are the best-characterized family members. The ERK cascade can be activated through several pathways, including stimulation of receptor tyrosine kinases such as the EGF or PDGF receptor [Widmann et al., 1999]. GPCRs are also capable of activating ERKs through multiple mechanisms [Rozengurt, 1998], including PKC-independent and PKC-dependent pathways [Gutkind, 1998]. PKC can activate the ERK cascade either directly, by phosphorylating Raf-1 [Cai et al., 1997], or in a Ras-dependent manner [Marais et al., 1998]. By directly phosphorylating and thereby activating several enzymes and transcription factors such as p90RSK, Elk-1, and c-Myc [Davis, 1993], ERKs play important roles in the control of key cellular processes such as differentiation, apoptosis, and proliferation [Seufferlein, 2002]. In the Panc-1 human pancreatic adenocarcinoma cell line, the proliferation in response to PKC activation by NT has been shown to depend on ERK [Guha et al., 2003]. Furthermore, in MiaPaCa-2 human pancreatic cancer cells, direct PKC activation by phorbol esters has been shown to promote anchorage-independent growth in an ERK-dependent manner [Ishino et al., 2002]. However, the role of the ERK cascade in the inhibition of anchorage-dependent growth by phorbol esters in MiaPaCa-2 [Ishino et al., 2002] or in DanG [Detjen et al., 2000] human pancreatic cancer cells has not been addressed.

It is now clear that the magnitude and temporal organization of ERK activity can determine specific biological responses [Marshall, 1995; Roovers and Assoian, 2000; Murphy et al., 2002, 2004]. Specifically, transient ERK activation induced by epidermal growth factor (EGF) leads to proliferation, while prolonged ERK activity induced by neuronal growth factor (NGF) results in differentiation in PC12 cells [Traverse et al., 1992]. Furthermore, in intestinal epithelial cells, ERK activity is transient in response to growth factors and results in cell growth, but is strong and sustained after PKC activation when it leads to cell cycle arrest [Clark et al., 2004].

The role of the kinetics of ERK activation in the proliferation of human pancreatic cancer cells has been less investigated. Kisfalvi et al. [2005] have recently shown that simultaneous stimulation of Panc-1 and MiaPaCa-2 cells with NT and EGF results in a synergistic increase in DNA synthesis due to an increased duration of ERK activation which lasts for up to 5 h in MiaPaCa-2 and for up to 3 h in Panc-1 cells. However, given that the effects on DNA synthesis and growth are sustained for days, it is also relevant to consider ERK activation over longer periods.

The current study was designed (i) to examine the role of different PKC isoforms in growth regulation and ERK cascade activation and (ii) to study the involvement of ERK activation kinetics in the regulation of proliferation in Panc-1 human pancreatic cancer cells. Our results show that PKC activation, either directly by a phorbol ester or indirectly by the activation of native or transfected neuropeptide receptors, regulates growth in a PKCε- and ERK-dependent manner. Furthermore, the stimulatory or inhibitory effects of different PKC activators appear to be determined by the duration of ERK activation in this system.

MATERIALS AND METHODS

Cell Culture

Panc-1 cells transfected with the human cholecystokinin (CCK-1) receptor (Panc-1C) [Detjen et al., 1997] were kindly provided by Dr. Craig D. Logsdon (University of Michigan, Ann Arbor, MI). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma) with 10% fetal bovine serum (FBS) in the presence of 200 μg/ml G418 at 37°C in a humidified atmosphere containing 5% CO_2.

[^{3}H]-Thymidine Incorporation

Panc-1C cells (2×10^4) were plated in 24-well plates and grown overnight. On the second day,

medium was changed and cells were incubated in serum-free medium for 24 h. Cells were then incubated in fresh medium with or without the specified concentrations of agonists and/or inhibitors for 24 h. Subsequently, cells were pulse labeled with 1 μCi/ml [^{3}H]-thymidine for 2 h. Cells were then washed twice with ice-cold phosphate buffered saline (PBS) and fixed with trichloroacetic acid. Precipitates were then dissolved in 0.1 M NaOH and the incorporated radioactivity was determined by liquid scintillation counting.

Immunoblotting

Panc-1C cells were plated in 6-cm dishes at equal cell numbers (4×10^5 cell/dish), grown overnight and made quiescent by serum deprivation for 24 h. Subconfluent, quiescent cells were treated with agonists and/or inhibitors as described in individual experiments. Cells were then rinsed with ice-cold PBS and directly lysed in 1 × SDS–PAGE sample buffer (100 mM TrisHCl pH6.8, 1 mM EDTA, 3% SDS, 5% glycerol, and 2% 2-mercaptoethanol) followed by SDS–PAGE on 6% (all PKC isoforms, Raf-1), 10% (ERK2, phospho-ERK, cyclin D1), or 12% (p21Cip1) gel and transfer to polyvinyl difluoride membranes (Fluka). Blots were then blocked, probed with primary antibodies appropriately diluted (1:5,000 for Anti-ACTIVE MAPK and 1:1,000 for the rest of the antibodies) in Tris-buffered saline (TBS) containing 0.1% Tween-20 and 2% ECL Advance Blocking Reagent (Amersham, Budapest, Hungary), washed, and incubated with secondary antibody. After additional washing, immunoreactive bands were visualized with the Amersham ECL Advance chemiluminescent detection system.

Subcellular Fractionation

For subcellular fractionation, Panc-1C cells were cultured in 10-cm dishes and experiments were carried out as described above for immunoblotting. To obtain subcellular fractions, cells were scraped in 300 μl of buffer A containing 50 mM HEPES pH 7.5, 250 mM sucrose, 10 mM EDTA, 1 mg/ml aprotinin, 0.5 mg/ml leupeptin, and 20 mM phenylmethylsulphonyl fluoride (PMSF). Plasma membranes were disrupted by repeated passing through a 25-gauge needle (35 times). Nuclei were then collected by centrifugation at 800*g* for 10 min, resuspended in 300 μl buffer A and purified by centrifugation through 1 M sucrose in buffer A. The resulting pellet was finally dissolved in 300 μl buffer B (50 mM HEPES pH 7.5, 100 mM NaF, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 2 mM EGTA, 2 mM Na-orthovanadate, 10 mM Na-pyrophosphate, 1 mg/ml aprotinin, 0.5 mg/ml leupeptin, and 20 mM PMSF) and designated as the nuclear fraction. The post-nuclear supernatant was spun at 21,000*g* for 45 min and the resulting supernatant was designated as the cytosolic fraction. The remaining pellet was washed in buffer A and then resuspended in 300 μl buffer B to yield the crude membrane fraction. Subcellular fractions were mixed with equal amounts of 2 × SDS–PAGE sample buffer and analyzed by Western blotting.

Confocal Microscopy

Panc-1C cells were cultured on glass coverslips and treated with agonists or inhibitors as described above. Cells were then rinsed with ice-cold PBS and fixed with methanol at −20°C for 20 min. Non-specific binding sites were blocked by incubation with 4% bovine serum albumine (BSA) in PBS containing 0.1% Na-azide (blocking solution) for 2 h. Coverslips were then incubated overnight with primary antibodies diluted at 1:1,000 in blocking solution, washed, and incubated with appropriate Alexa Fluor 488 Secondary Antibodies (Molecular Probes) diluted at 1:500 in blocking solution. Nuclei were stained with propidium iodide (0.01 mg/ml). After additional washing and mounting with Mowiol mounting medium (Calbiochem), cells were examined using an Olympus FLUOROVIEW FV/300 (Olympus, Hamburg) confocal microscope.

Materials and Antibodies

Dulbecco's Modified Eagle Medium (DMEM), G418, propidium iodide, cholecystokinin octapeptide, NT, phorbol 12-myristate 13-acetate (PMA), PD98059, Gö6976, and Gö6983 were all obtained from Sigma-Aldrich, Budapest, Hungary. Ro-32-0432 was from Biomol. Rabbit polyclonal Anti-ACTIVE MAPK antibodies were from Promega, whereas rabbit polyclonal primary antibodies against PKCα, -δ and -ε, ERK2, Raf-1, cyclin D1 and p21Cip1, and the HRP-conjugated goat anti-rabbit IgG, were all from Santa Cruz Biotechnology. All other reagents were from Sigma and of the highest grade available.

RESULTS

PKCε Can Mediate Proliferation or Growth Arrest in Panc-1 Human Pancreatic Cancer Cells

We first asked what PKC isoforms were responsible for the proliferation induced by NT, and the growth inhibition caused by CCK-1 receptor activation or direct PKC activation by phorbol esters, in Panc-1 human pancreatic cancer cells stably transfected with CCK-1 receptors (Panc-1C). The dose for each treatment was chosen based on previous studies, where 100 nM PMA [Detjen et al., 2000], 10 nM CCK [Detjen et al., 1997], and 50 nM NT [Ryder et al., 2001; Guha et al., 2002] had been shown to exert maximal effects on cell growth.

As expected, NT stimulated, whereas CCK moderately and PMA dramatically inhibited DNA synthesis (Fig. 1). This is in accordance with the earlier results of Guha et al. [2003] and Detjen et al. [1997, 2000], respectively. To study the involvement of different PKC isoforms, we pretreated the cells before experiments for 30 min with isoform-selective PKC inhibitors. Gö6983 is a bisindolylmaleimide with the following IC_{50} values for specific PKC isozymes: α and β: 7 nM, γ: 6 nM, δ: 10 nM, ζ: 60 nM, and μ: 20 μM [Gschwendt et al., 1996]. We therefore used 20 nM Gö6983, which is expected to inhibit PKCα, -β, -γ, and -δ but has negligible effect on ζ and μ in both in purified preparations [Gschwendt et al., 1996] and in intact cells [Peterman et al., 2004; Schreckenberg et al., 2004]. Ro 32-0432 displays an IC50 of 9 nM for PKCα, 28 nM for βI, and 108 nM for ε [Wilkinson et al., 1993]. We used 200 nM Ro 32-0432 to inhibit PKCα, -βI, and -ε. Ro 32-0432 Gö6976 is an indolocarbazole that inhibits PKCα with an IC50 of 2.3 nM, PKCβI with an IC50 of 6.2 Nm, and PKCμ with an IC50 of 20 nM, but fails to inhibit PKC δ and -ε [Martiny-Baron et al., 1993]; we used 40 nM Gö6976 to inhibit PKCα, -βI, and -μ. While Ro 32-0432 prevented the effects of PMA, CCK, and NT on the proliferation of Panc-1C cells, Gö6983 was without effect (Fig. 1). Like Gö6983, Gö6976 also did not prevent the effects of PMA, CCK, and NT (data not shown). Taken together, these results suggest that the same isoform, namely PKCε, is responsible for the growth regulatory effect of PKC activation in Panc-1 pancreatic cancer cells, whether PKC is activated directly by PMA or indirectly through native NT- or transfected CCK-receptors.

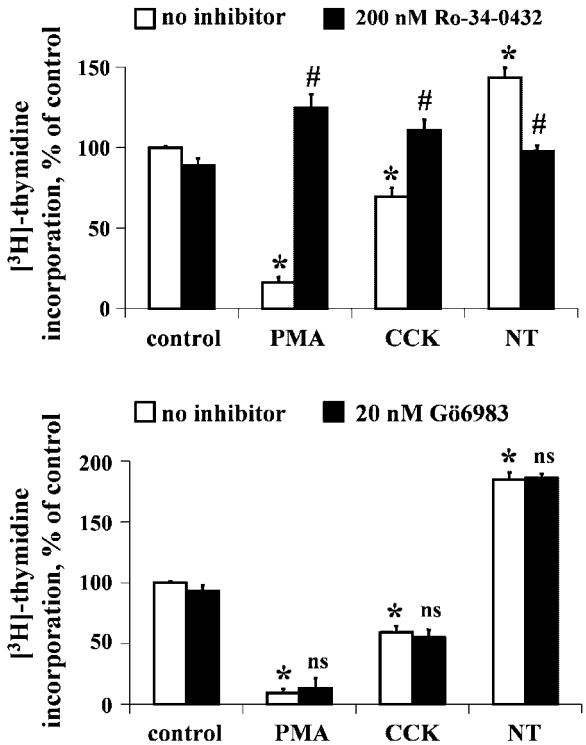

Fig. 1. Phorbol-12-myristate-13-acetate (PMA), cholecystokinin (CCK), and neurotensin (NT) regulate DNA synthesis in a PKCε-dependent manner in Panc-1 cells stably transfected with CCK-1 receptors (Panc-1C). Quiescent, subconfluent Panc-1C cells were preincubated for 30 min with 200 nM Ro-32-0432 or solvent (**upper panel**) or with 20 nM Gö6983 or solvent (**lower panel**), and then treated with or without 100 nM PMA or 10 nM CCK or 50 nM NT for 24 h. Cells were then pulse-labeled with 1 μCi/ml [^{3}H]-thymidine for 2 h and the rate of DNA synthesis was determined by measuring the radioactivity incorporated into acid precipitable material. Ro-32-0432 (200 nM), which inhibits cPKCα, -βI, and nPKCε (upper panel) but not 20 nM Gö6983, which inhibits cPKCα, -β, -γ, and nPKCδ (lower panel), prevented the effects of PMA, CCK, and NT on the DNA synthesis of Panc-1C cells. Data were analyzed by ANOVA followed by Bonferroni's post-test. $*P < 0.05$ compared to control. $\#P < 0.05$ compared to treatments in the absence of inhibitors. ns, not significant. The results shown are representative of three independent experiments.

Translocation of Several PKC Isoforms in Response to PMA, CCK, and NT in Panc-1C Cells

Several PKC isoforms (α, δ, and ε) have previously been implicated in the growth regulation of human pancreatic cancer cells [Detjen et al., 2000; Ishino et al., 2002]. Activation of PKC isoenzymes is usually associated with their translocation from the cytoplasm to the membrane or another intracellular compartment [Clemens et al., 1992]. We therefore studied the activation of PKCα, -δ, and -ε in

response to PMA, CCK, and NT in Panc-1C cells by examining their translocation from the cytosol to the membrane and the nucleus. Quiescent Panc-1C cells were treated with PMA or CCK or NT for 0, 2, 6, and 30 min. Cytosolic, nuclear, and membrane fractions were prepared and analyzed by immunoblotting with antibodies to PKCα, -δ, and -ε (Fig. 2A). PKCα became more abundant in the nuclear fraction in response to PMA whereas it practically disappeared from the nucleus by 30 min after treatment with CCK or NT (Fig. 2A, PKCα immunoblot middle panel, see also quantification). PMA increased the amount of PKCα in the membrane fraction. In contrast, CCK and NT, after a slight increase at 2 min, dramatically decreased the amount of PKCα in the membrane fraction (Fig. 2A, PKCα immunoblot right panel, see also quantification). PKCδ gradually became less abundant in the nuclear fraction in response to PMA, CCK, and NT, while the amount in the membrane fraction was strikingly increased by PMA, moderately increased by NT, and not substantially changed by CCK (Fig. 2A, PKCδ immunoblot). PKCε became less abundant in the nuclear fraction in response to PMA and NT, while its level increased in the membrane fraction in response to PMA, CCK, and NT (Fig. 2A, PKCε immunoblot, see also quantification). PKC translocation was also assessed by confocal microscopy. Quiescent Panc-1C cells on glass coverslips were treated for 0 or 2 min as described above, fixed, and processsed for confocal microscopy. Nuclei were stained with propidium iodide, while PKC isoforms were immunolabeled with the same

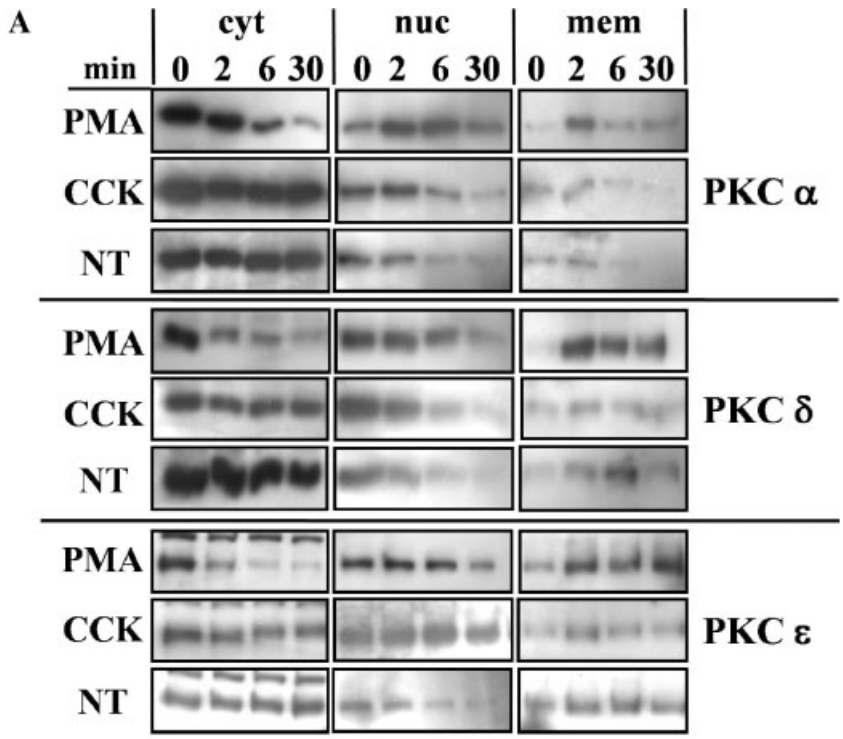

Fig. 2. Translocation of the PKC isoforms α, δ, and ε in response to PMA, CCK, and NT in Panc-1C cells. **A**: Quiescent Panc-1C cells were treated with 100 nM PMA, 10 nM CCK, or 50 nM NT for 0, 2, 6, and 30 min. Cytosolic, nuclear, and membrane fractions were prepared and analyzed by immunoblotting with antibodies to PKCα, -δ, and -ε. Results were quantified by densitometry and expressed as percent of the untreated control of each fraction. Results are representative of three independent experiments. **B**: Panc-1C cells were seeded on glass coverslips and serum-deprived for 24 h. Quiescent cells were treated with 100 nM PMA, 10 nM CCK, or 50 nM NT for 0 or 2 min, fixed, and processed for confocal microscopy. Nuclei were stained with propidium iodide; primary antibodies to PKCα, -δ, and -ε were used at a dilution of 1:1,000; while Alexa Fluor 488-labeled secondary antibodies were used at a dilution of 1:500. Results were quantified by drawing a line between a point (represented by a white arrow) outside the cell and the nucleus, and plotting the green pixel fluorescent density profile along the line. Density profiles are shown under each micrograph. Sections of the density plots corresponding to the membrane, cytosol, and nucleus are labeled with m, c, and n, respectively. Results are representative of two to three independent experiments. Bars represent 10 μm. **C**: Control untreated Panc-1C cells were processed as described in B, except that the primary antibody was omitted. Bar represents 10 μm.

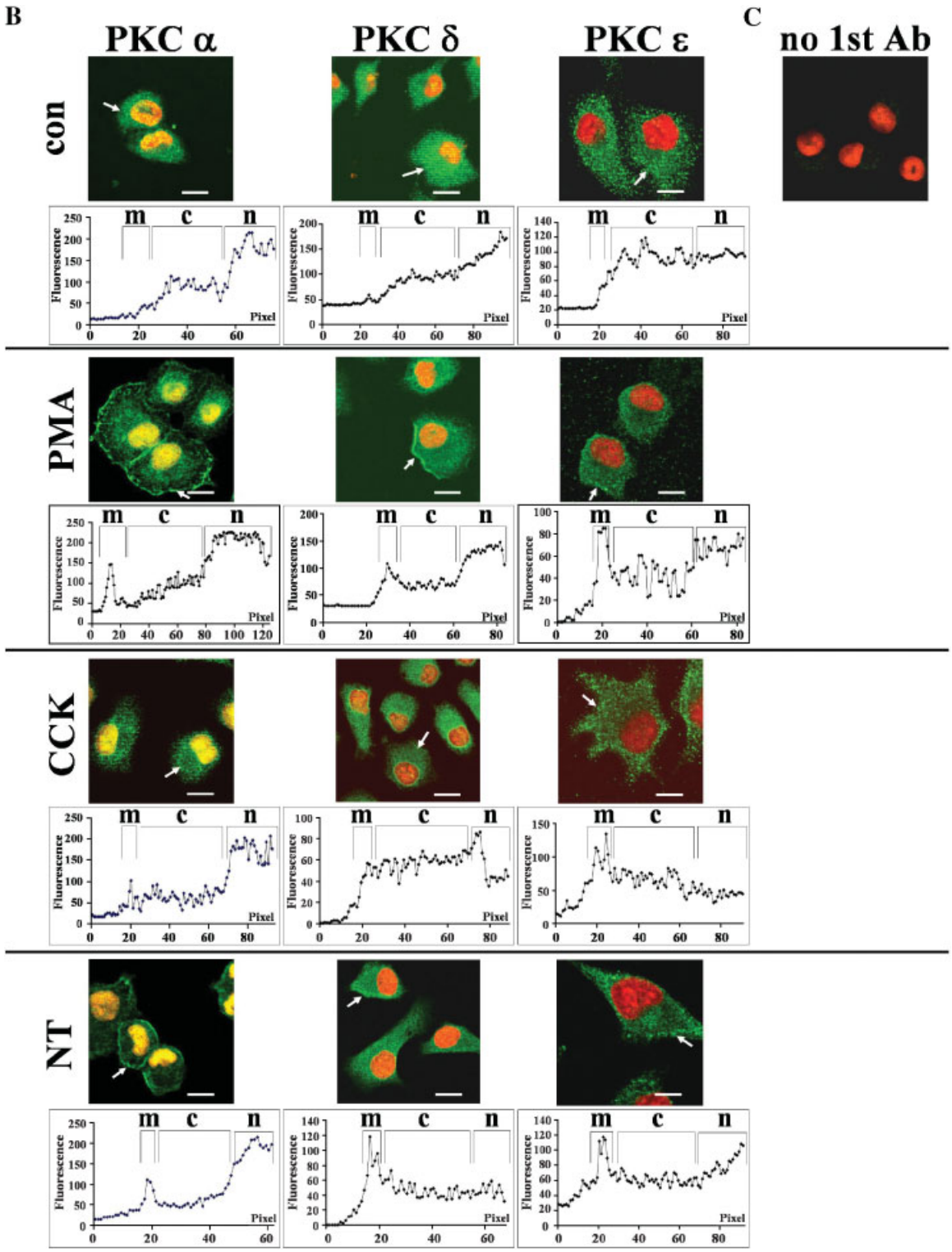

Fig. 2. (*Continued*)

antibodies as for immunoblotting. The results shown on Figure 2B essentially confirm the results obtained by immunoblots on the subcellular fractions: after 2 min treatments PKCα and -ε became more abundant in the membrane in response to PMA, CCK, and NT, and PKCδ translocated to the membrane in response to PMA and NT but not to CCK.

PMA, CCK, and NT Stimulate the ERK Cascade and Regulate Growth in an ERK-Dependent Manner in Panc-1C Cells

The ERK cascade is of paramount importance in the regulation of key cellular processes such as proliferation or differentiation [Davis, 1993] and is thought to be involved in the regulation of apoptosis, proliferation, and metastasis in pancreatic cancer cells [Seufferlein, 2002]. We therefore studied whether direct or indirect stimulation of PKC activated ERK in Panc-1C cells. Cells were made quiescent by serum deprivation for 24 h, treated with 100 nM PMA, 10 nM CCK, or 50 nM NT over a time course ranging from 2 min to 6 h and then processed for immunoblotting. Blots were then probed with antibodies recognizing either pan ERK (anti-ERK2) or phosphorylated, that is, activated, ERK (Anti-ACTIVE MAPK). The results in Figure 3A show that the ERK cascade was activated with different kinetics: an increase in the level of phospho-ERK was induced for at least 6 h by PMA, for up to 30 min by CCK and for as little as 2 min by NT. The level of pan ERK, on the other hand, remained constant (Fig. 3A). Our findings suggest that the kinetics of ERK activation correlates with the effects on DNA synthesis: PMA induces prolonged ERK activation and dramatically reduces proliferation, NT causes very transient ERK activation followed by growth stimulation, whereas the intermediate duration ERK activation induced by CCK is associated with a moderate inhibitory effect on cell growth.

To determine whether PKC activation regulates DNA synthesis in an ERK-dependent manner in Panc-1C cells, we used 20 μM PD98059 (an inhibitor of MEK that prevents ERK phosphorylation). Again, NT stimulated, whereas CCK moderately and PMA dramatically inhibited DNA synthesis (Fig. 3B, empty bars). Pretreatment of cells with 20 μM PD98059 in itself reduced DNA synthesis by more than 50% (Fig. 3B, control, closed bar), whereas it prevented the stimulatory effect of NT restoring [^{3}H]-thymidine incorporation to levels close to what was obtained by incubation with PD98059 alone. Thymidine incorporation in the presence of PD98059 and either PMA or CCK was similar to the value obtained with PD98059 alone. This is consistent with the inhibitory effects of both agents on DNA synthesis being wholly (CCK) or largely (PMA) mediated through ERKs.

We further investigated the role of the ERK cascade in the growth regulatory effect of PKC activation by assessing the expression of two key cell cycle genes, cyclin D1 and the p21Cip1 tumor suppressor. Panc-1C cells were made quiescent by serum deprivation for 24 h, incubated with 20 μM PD98059 or solvent for 30 min and treated with 100 nM PMA, 10 nM CCK, or 50 nM NT for 0, 5, 16, and 24 h. Cells were then lysed and the expression of cyclin D1 and p21Cip1 was studied by immunoblotting. Figure 3C shows that cyclin D1 expression was upregulated by PMA, CCK, and NT, whereas p21Cip1 expression was increased by PMA and CCK but not by NT (Fig. 3D). Furthermore, pretreatment with PD98059 reduced the above effects of PMA, CCK, and NT on the expression of the cell cycle genes (Fig. 3C, D). These results further confirm that direct or indirect PKC activation regulates the growth of Panc-1 pancreatic cancer cells via the ERK cascade.

Both Transient and Sustained ERK Activation Is Accompanied by Sustained Nuclear Translocation of ERK in Panc-1C Cells

ERKs can translocate to the nucleus upon activation [Chen et al., 1992]. In PC12 rat phaeochromocytoma cells, sustained ERK activation results in differentiation and is associated with translocation to the nucleus, whereas transient ERK activation, which favors proliferation, does not lead to nuclear translocation [Marshall, 1995]. To determine whether transient and sustained ERK activation is associated with such differences in the translocation of ERK in Panc-1C cells, we treated quiescent Panc-1C cells with PMA, CCK or NT for 0, 2, 15, and 60 min and studied ERK translocation from the cytosol to the nucleus by confocal microscopy and by subcellular fractionation followed by immunoblotting. Figure 4 shows that each treatment results in ERK2 nuclear translocation by 2 min, which persists for at least 60 min. These results suggest that both transient and sustained PKC-dependent ERK activation leads to nuclear translocation, and differential localization is therefore unlikely to underlie the opposite effects of PMA and NT on the growth of Panc-1C pancreatic cancer cells.

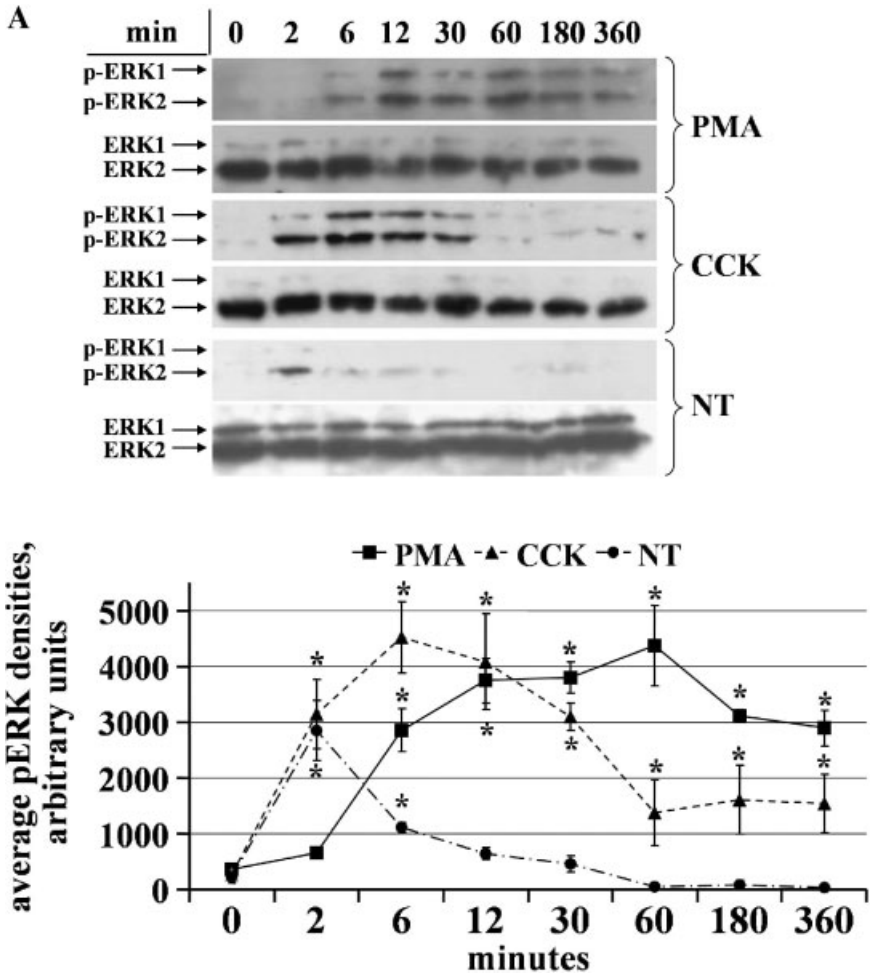

Fig. 3. PMA, CCK, and NT activate the ERK cascade with different kinetics and regulate cell growth in an ERK-dependent manner in Panc-1C cells. **A**: Quiescent Panc-1C cells were treated with 100 nM PMA, 10 nM CCK, or 50 nM NT over a time course ranging from 2 min to 6 h. Cells were then directly lysed in 1 × SDS–PAGE sample buffer, the samples were subjected to electrophoresis, transferred to PVDF membranes, and immunoblotted with antibodies to phospho-ERK1/2 and ERK2. **Upper panel**: arrows indicate the positions of phospho-ERK1/2, and total ERK1/2. Shown are results representative of three independent experiments. **Lower panel**: shown are results obtained by quantifying three independent phospho-ERK1/2 immunoblots. Data were analyzed by ANOVA followed by Dunnett post-test. $*P < 0.05$ compared to control. **B**: Quiescent, subconfluent Panc-1C cells were preincubated for 30 min with 20 μM PD98059 (closed bars) or solvent (open bars), and then treated with or without 100 nM PMA, 10 nM CCK, or 50 nM NT for 24 h. Cells were then pulse-labeled with 1 μCi/ml [^{3}H]-thymidine for 2 h and the rate of DNA synthesis was determined by measuring the radioactivity incorporated into acid precipitable material. Data were analyzed by ANOVA followed by Bonferroni's post-test. $*P < 0.05$ compared to control. $\#P < 0.05$ compared to treatments in the absence of PD98059. ns, not significant. Results are representative of three independent experiments. **C**, **D**: Quiescent, subconfluent Panc-1C cells were preincubated for 30 min with 20 μM PD98059 or solvent, and then treated with 100 nM PMA or 10 nM CCK or 50 nM NT for 0, 5, 16, and 24 h. Cells were then directly lysed in 1 × SDS–PAGE sample buffer, the samples were subjected to electrophoresis, transferred to PVDF membranes and immunoblotted with antibodies to cyclin D1 (C) or p21Cip1 (D). Results are representative of two to three independent experiments. WB, Western blotting. PD, 20 μM PD98059; tr, treatment; h, hours.

PMA, CCK, and NT Stimulates the ERK Cascade in Panc-1C Cells in a PKC-Dependent Manner

We next wanted to confirm that PMA, CCK, and NT stimulate ERK in a PKC-dependent manner. We therefore treated quiescent Panc-1C cells with PMA, CCK, and NT with or without prior 30-min pretreatment with PKC isoform-selective inhibitors. The duration of the treatment was chosen to be 6 min for PMA and 2 min for CCK and NT since these durations were sufficient for strong ERK activation to occur (Fig. 3A). As shown in Figure 5, pretreatment with 200 nM Ro-32-0432, but not with

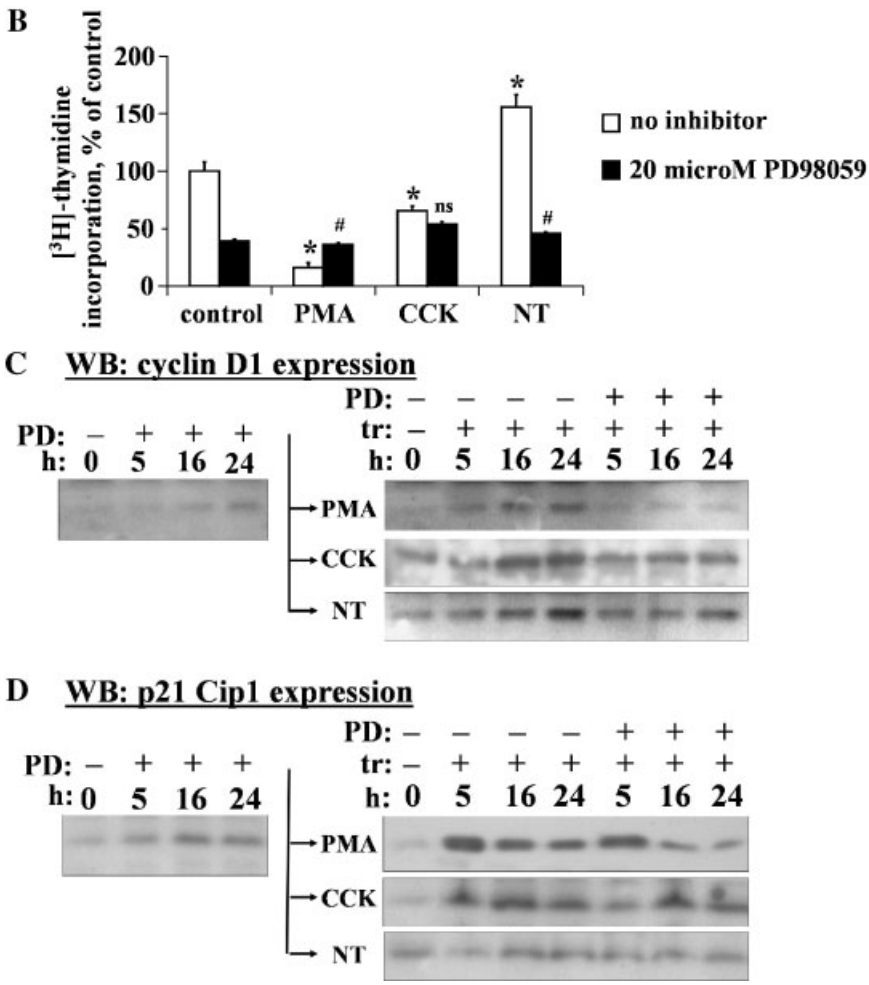

Fig. 3. (*Continued*)

20 nM Gö6983 or 40 nM Gö6976, reduced the effect of PMA, CCK, and NT on ERK activation. This suggests that the novel isoform PKCε may be reponsible for the activation of ERK cascade.

DISCUSSION

Protein kinase C participates in growth regulation in a range of cell types [Livneh and Fishman, 1997; Rozengurt, 1998, 2002]. Phorbol ester-mediated and GPCR-mediated activation of PKC have been implicated in both positive and negative growth regulation of human pancreatic cancer cells. Specifically, 12-O-tetradecanoylphorbol-13-acetate (TPA) inhibits anchorage-dependent growth of the DanG, AsPc-1, Capan-2, Panc-1, and MiaPaCa-2 human pancreatic cancer cell lines [Detjen et al., 2000], and it stimulates anchorage-independent growth in MiaPaCa-2 [Ishino et al., 2002]. Furthermore, indirect PKC activation by NT acting on its cognate GPCR has been shown to stimulate anchorage-dependent growth in Panc-1 human pancreatic cancer cells [Guha et al., 2002, 2003]. Several mechanisms may underlie the heterogeneity of the results outlined above. The cellular context may be important, since different sets of PKC isoforms may be expressed and activated in response to TPA, or different additional pathways may be activated in different cell lines. Alternatively, experimental conditions may also vary among the above studies. Therefore, our purpose was first to investigate the effects of different ways of PKC activation on DNA synthesis in a single cell line under the same experimental conditions. Panc-1 cells have been shown to possess native NTR-1 NT receptors [Ryder et al., 2001], to be devoid of native CCK receptors [Detjen et al.,

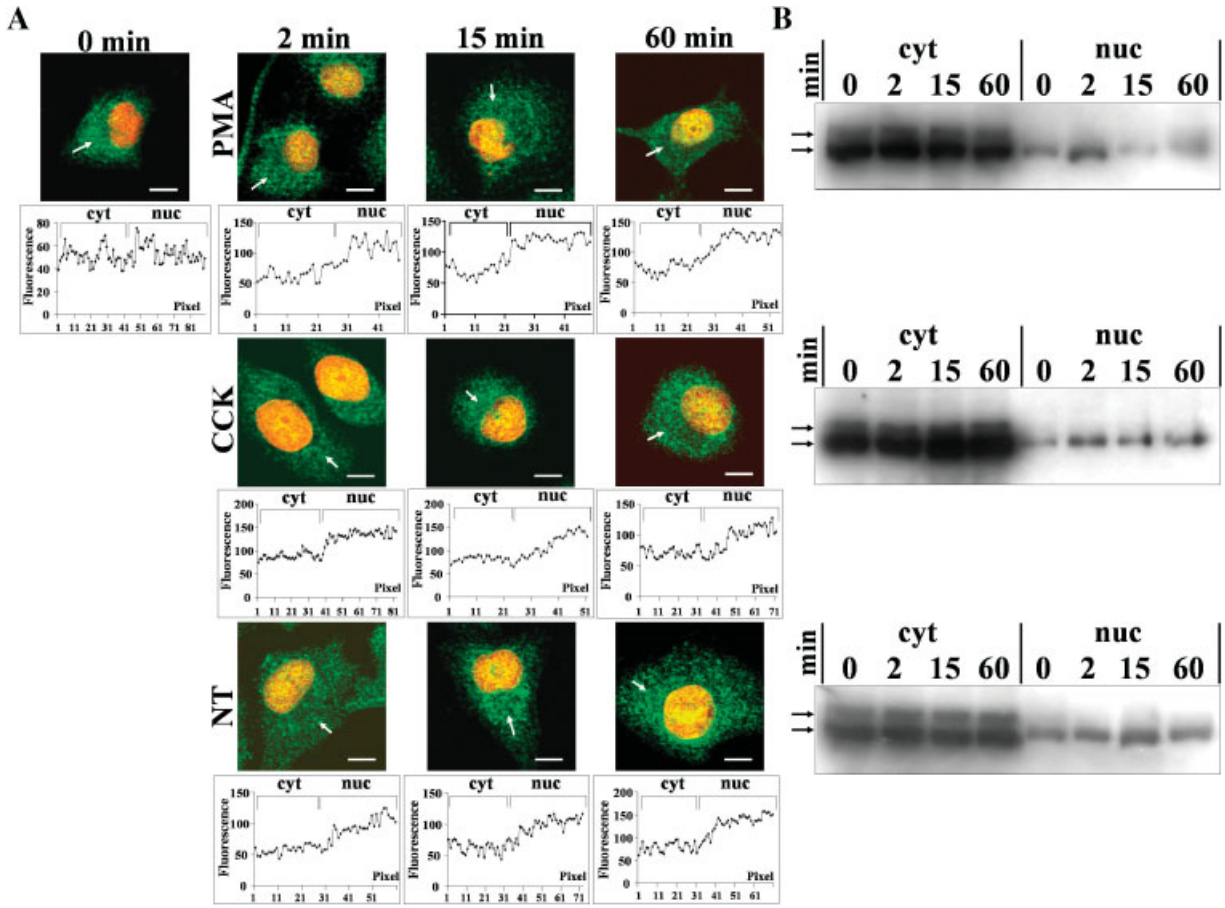

Fig. 4. PMA, CCK, and NT induce ERK nuclear translocation in Panc-1C cells. Panc-1C cells were seeded on glass coverslips (**A**) or in culture dishes (**B**), serum-deprived for 24 h, and then treated with 100 nM PMA (**top**), 10 nM CCK, (**middle**) or 50 nM NT (**bottom**) for 0, 2, 6, and 30 min. A: For confocal microscopy, cells were fixed, nuclei were stained with propidium iodide, and cells were stained with primary antibodies to ERK2 at a dilution of 1:1,000, followed by labeling with Alexa Fluor 488-labeled secondary antibodies diluted to 1:500. Results were quantified as described for Figure 2B, using green pixel density profiles plotted along lines between cell nuclei and white arrows. Density profiles are shown under each micrograph. Bars represent 10 μm. B: cytosolic and nuclear fractions were submitted to SDS–PAGE, transferred to PVDF membranes, and immunoblotted with ERK2 antibodies. Of the double arrows, the upper represents ERK1 whereas the lower ERK2. Results are representative of three independent experiments.

1997] and to express multiple PKC isoforms [Guha et al., 2002]. In the present study, we used Panc-1 cells stably transfected with CCK-1 receptors (Panc-1C) to examine the effects of PKC activation by native G protein-coupled NTR-1 receptors, transfected CCK-1 receptors, or phorbol esters, on the DNA synthesis of adherent cells. Our results demonstrate that in quiescent, adherent Panc-1C cells, different forms of PKC activation have opposite effects on cell growth. In accordance with previous observations [Detjen et al., 1997, 2000; Guha et al., 2003], phorbol ester-mediated direct activation of PKC leads to a striking decrease in DNA synthesis, while activation of transfected CCK-1 receptors moderately inhibits, and stimulation of natively expressed NT receptors stimulates, cell growth.

To examine whether different PKC isoforms are responsible for the opposite effects of PMA, CCK, and NT on cell growth, we used isoform-selective PKC inhibitors. Using a similar strategy to that used previously by others in intact cells [Peterman et al., 2004; Schreckenberg et al., 2004], we showed that inhibition of classical PKC isoforms (cPKCs) and nPKCε, but not cPKCs and nPKCδ, prevents the effects of PMA, CCK, and NT on the DNA synthesis of Panc-1C cells. Our results therefore suggest that the effects on proliferation are mediated by PKCε. However, a more specific approach, such as overexpression of a dominant negative

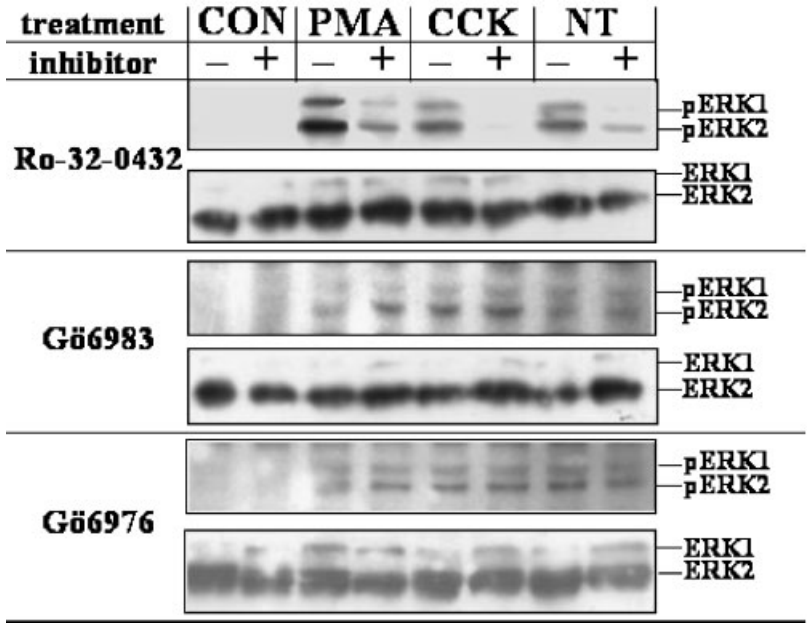

Fig. 5. PMA, CCK, and NT regulate the activation of the ERK cascade in a PKCε-dependent manner in Panc-1C cells. Quiescent Panc-1C cells were preincubated for 30 min with 200 nM Ro-32-0432 or solvent (**upper panel**), or with 20 nM Gö6983 or solvent (**middle panel**), or with 40 nM Gö6976 (**lower panel**), then treated with 100 nM PMA (6 min), 10 nM CCK or 50 nM NT (both 2 min). Cells were then directly lysed in 1 × SDS–PAGE sample buffer, samples were subjected to electrophoresis, transferred to PVDF membranes and immunoblotted with antibodies to phospho-ERK1/2 and ERK2. Arrows indicate the positions of phospho-ERK1/2, and total ERK1/2. Shown are results representative of three independent experiments.

protein or small interfering RNA-mediated downregulation, will be needed to confirm the role of PKCε unequivocally.

Several PKC isoforms have been reported to be involved in the growth regulation of human pancreatic cancer cells. The growth inhibitory effect of phorbol esters has been attributed to PKCα in DanG human pancreatic cancer cells [Detjen et al., 2000], while PKCδ, along with PKCα and ε, promoted anchorage-independent growth in MiaPaCa-2 human pancreatic cancer cells [Ishino et al., 2002]. Thus, we still cannot exclude the possibility of differential activation of PKC isoforms in addition to ε. Alternatively, since activation of PKC isoenzymes is usually associated with their translocation from the cytoplasm to the membrane or another intracellular compartment [Clemens et al., 1992], opposite growth regulatory effects could conceivably arise even from the differential localization of the same PKC isoform, PKCε. Following different stimuli to test these possibilities, we compared the subcellular redistribution pattern of PKCα, -δ, and -ε in Panc-1C cells in response to treatment with PMA, CCK or NT. Our results show that, in response to all treatments, PKCε followed an essentially similar pattern of redistribution, that is, cytosol to membrane translocation. On the other hand, PKCα and -δ differentially redistributed in response to PMA, CCK, and NT. However, the significance of these differences remains to be established since, as discussed above, differential growth regulation in Panc-1C cells apparently depends on PKCε, rather than on PKCα or -δ. Taken together, the results presented here suggest that it is not differences in the pattern of activated isoforms, or differential localization of isoforms, that underlies the variable growth effects of different ways of activating PKC.

The ERK cascade is of paramount importance in the regulation of key cellular processes such as proliferation or differentiation [Davis, 1993], and is involved in the regulation of apoptosis, proliferation, and metastasis in pancreatic cancer cells [Seufferlein, 2002]. NT-induced activation of PKC has been shown to stimulate cell growth in an ERK-dependent manner in Panc-1 pancreatic cancer cells [Guha et al., 2003], and PKC activation by phorbol esters stimulated anchorage-independent growth in MiaPaCa-2 pancreatic cancer cells via the ERK cascade [Ishino et al., 2002]. We therefore asked

whether the growth inhibition by PMA and CCK, and the proliferation induced by NT, was accompanied by ERK activation. Our results show that PMA, CCK and NT all stimulate the ERK cascade, but with strikingly different kinetics. The growth regulatory effects thus appear to correlate with the duration of ERK activation in Panc-1C cells: transient activation is associated with proliferation, whereas sustained ERK activation is associated with growth arrest.

These results are apparently at odds with those of Kisfalvi et al. [2005]. In Panc-1 cells, more sustained ERK activation in their hands caused stronger growth stimulation than did transient activation, whereas in our hands transient activation is associated with stimulation and prolonged activation with growth inhibition. However, we have found that, if we compare the effects of serum to those of NT, serum stimulation of Panc-1 cells leads to more prolonged ERK activation (>30 min vs. 2 min) and stronger growth stimulation (2–3 fold vs. 1.4–1.6 fold) (unpublished observations, data not shown). Thus, it is possible that there is an optimal duration of ERK activation that results in the highest increase in DNA synthesis, and that the differences between our study and that of Kisfalvi et al. reflect the slightly different choice of time points for measuring ERK activation.

To confirm that PMA, CCK, and NT regulate the growth of Panc-1C cells in an ERK-dependent manner, we used a specific inhibitor of the MEK1 MAP kinase kinase, PD98059. Pretreatment of the cells with this inhibitor before applying PMA, CCK, or NT restored DNA synthesis to levels close to what was obtained with PD98059 alone. These results indicate that ERK mediates the effects on proliferation. To further investigate the role of the ERK cascade in the growth regulatory effects of PKC activation, we studied the effects of PMA, CCK, and NT on the expression of two key cell cycle proteins in the presence or absence of PD98059. Our results show that PMA, CCK, and NT regulate the level of cyclin D1 and p21Cip1 in an ERK-dependent manner. These findings are in accordance with results obtained in colorectal cancer cells and fibroblasts [Roovers and Assoian, 2000; Park et al., 2003], where the duration of the ERK signal determines whether cyclin D1 alone is induced, leading to proliferation, or whether cylin D1 expression is accompanied by the induction of p21Cip1, which results in growth arrest.

Extracellular signal-regulated kinases can translocate to the nucleus upon activation [Chen et al., 1992]. In PC12 rat phaeochromocytoma cells, sustained ERK activation results in differentiation and is associated with translocation to the nucleus, whereas transient ERK activation, which favors proliferation, does not lead to nuclear translocation [Marshall, 1995]. In intestinal epithelial cells, direct PKC activation promotes cytoplasmic and nuclear accumulation of ERK activity, whereas growth factor-induced phospho-ERK is localized only in the cytoplasm [Clark et al., 2004]. We therefore asked whether the differential effects of PMA, CCK, and NT on cell growth were related to differential localization of ERK in response to the various stimuli. Our results demonstrate that this is probably not the case, since cytoplasmic to nuclear redistribution of ERK2 was observed in response to all treatments.

Taken together, the results presented here argue that ERK plays a key role in mediating both the positive and the negative that effects of PKC activation on the proliferation of Panc-1C human pancreatic cancer cells, and that it is the duration of ERK signal that determines the effect on DNA synthesis via differential induction of cell cycle genes. Such a role of the kinetics of ERK activation has been reported in several cell types. For example, sustained ERK activation is required for the proliferation of hamster fibroblasts [Meloche et al., 1992], whereas transient ERK activation induced by EGF leads to proliferation, and prolonged ERK activity induced by NGF results in differentiation, of PC12 cells [Traverse et al., 1992]. In intestinal epithelial cells, ERK activity is transient in response to growth factors and results in cell growth, but strong and sustained after PKC activation when it leads to cell cycle arrest [Clark et al., 2004].

How are the kinetics of ERK activation sensed by the cells? Recent studies have identified immediate early gene products as possible sensors of ERK signal duration in fibroblasts: sustained ERK signaling regulates the post-translational modifications of these sensors, regulating their stability during G1-S progression [Murphy et al., 2002, 2004]. However, the existence of such a sensory mechanism for ERK signal duration in human pancreatic cancer cells still remains to be established.

A further key question is how the dramatically different patterns of ERK activation are produced. There are potentially several different ways to generate transient versus sustained ERK signals. Small changes in ligand concentration, the rate of receptor internalization, receptor downregulation, or differences in receptor number may have such effects [Marshall, 1995]. This points to a potential serious limitation of studies using overexpression to study the function of a particular receptor. This is that the increased receptor number that results from overexpression, either by itself or by interfering with internalization and downregulation, can generate a signal with characteristics significantly different from that of native receptors; this can ultimately lead to very different decisions made by the cells in response to a particular stimulus. This may be true for the model system used in the present study, since in the cell line used CCK-1 receptors were stably transfected into Panc-1 human pancreatic cancer cells, which normally lack these receptors [Detjen et al., 1997]. In contrast to what is observed in rat pancreatic cells, where CCK is an important growth factor [Niederau et al., 1994; Williams, 2001], activation of the transfected receptor in this system resulted in growth inhibition. The opposite effects of CCK in human and rat pancreatic cells may arise from species-specific differences in the cellular context, but more probably reflect activation of the transfected receptors resulting in a signal with significantly altered characteristics. Indeed, our results show that natively expressed receptors for another regulatory peptide, NT, also generate an ERK signal but with different characteristics, and mediate proliferation.

Having established that growth regulation by the activation of CCK and NT receptors, or by the non-specific PKC activator PMA, depends on both PKCε and the ERK cascade, we wanted to confirm that ERK is activated in a PKCε-dependent manner. By detecting phosphorylated ERK in Panc-1C cells pretreated with isoform-selective inhibitors and subsequently treated with PMA, CCK, and NT, we have found that ERK phosphorylation depends on PKCε but not on classical PKC isoforms or PKCδ. We therefore conclude that the ERK cascade is activated by PKCε in Panc-1C human pancreatic cancer cells. These findings are in line with previous results of others [Cacace et al., 1996; Ueffing et al., 1997; Hamilton et al., 2001] who found that PKCε activated Raf-1 in mouse and rat fibroblasts. We propose the following model for the growth regulatory effect of PKC activation in Panc-1C human pancreatic cancer cells. Occupation of native NTR-1 or transfected CCK-1 receptors, as well as direct stimulation by phorbol esters results in the activation of several PKC isoforms, including PKCε, which is associated with Raf-1. PKCε then stimulates Raf-1, leading to the activation of the ERK cascade. The duration of the ERK signal is different in response to PMA, CCK and NT, which results in differential induction of cell cycle genes in order to either stimulate or inhibit proliferation. The mechanisms by which the ERK signal is modulated in response to different stimuli remain to be determined.

In conclusion, our results provide evidence for PKCε-mediated differential ERK activation and growth regulation in Panc-1C human pancreatic cancer cells. Identification of the mechanisms by which these key signaling pathways are modulated could provide a basis for the development of novel therapeutic interventions to treat pancreatic cancer.

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