Molecular mechanisms underlying Ca\(^{2+}\)-mediated motility of human pancreatic duct cells

Hui Dong,1* Ki-Nam Shim,5 Jenny M. J. Li,1 Christine Estrema,1 Tiffany A. Ornelas,1 Flang Nguyen,1 Shanglei Liu,1 Sonia L. Ramamoorthy,6 Samuel Ho,3 John M. Carethers,1,2,3,4* and Jimmy Y. C. Chow1

1Division of Gastroenterology, Department of Medicine, University of California, San Diego, 2Rebecca and John Moores Comprehensive Cancer Center, and 3Department of Veterans Affairs San Diego Healthcare System, San Diego, California; 4Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan; 5Department of Internal Medicine, School of Medicine, Ewha Womans University, Seoul, Korea; and 6Department of Surgery, University of California, San Diego, California

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Dong H, Shim KN, Li JM, Estrema C, Ornelas TA, Nguyen F, Liu S, Ramamoorthy SL, Ho S, Carethers JM, Chow JY. Molecular mechanisms underlying Ca\(^{2+}\)-mediated motility of human pancreatic duct cells. Am J Physiol Cell Physiol 299: C1493–C1503, 2010. First published September 29, 2010; doi:10.1152/ajpcell.00242.2010.—We recently reported that transforming growth factor-β (TGF-β) induces an increase in cytosolic Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) in pancreatic cancer cells, but the mechanisms by which TGF-β mediates [Ca\(^{2+}\)]\(_{\text{cyt}}\) homeostasis in these cells are currently unknown. Transient receptor potential (TRP) channels and Na\(^+\)/Ca\(^{2+}\) exchangers (NCX) are plasma membrane proteins that play prominent roles in controlling [Ca\(^{2+}\)]\(_{\text{cyt}}\) homeostasis in normal mammalian cells, but little is known regarding their roles in the regulation of [Ca\(^{2+}\)]\(_{\text{cyt}}\) in pancreatic cancer cells and pancreatic cancer development. Expression and function of NCX1 and TRPC1 proteins were characterized in BxPc3 pancreatic cancer cells. TGF-β induced both intracellular Ca\(^{2+}\) release and extracellular Ca\(^{2+}\) entry in these cells; however, 2-aminoethoxydiphenyl borate [2-APB; a blocker for both inositol 1,4,5-trisphosphate (IP\(_3\)) receptor and TRPC], LaCl\(_3\) (a selective TRPC blocker), or KB-R7943 (a selective inhibitor for the Ca\(^{2+}\) entry mode of NCX) markedly inhibited the TGF-β-induced increase in [Ca\(^{2+}\)]\(_{\text{cyt}}\). 2-APB or KB-R7943 treatment was able to dose-dependently reverse membrane translocation of PKCα induced by TGF-β. Transfection with small interfering RNA (siRNA) against NCX1 almost completely abolished NCX1 expression in BxPc3 cells and also inhibited PKCα serine phosphorylation induced by TGF-β. Knockdown of NCX1 or TRPC1 by specific siRNA transfection reversed TGF-β-induced pancreatic cancer cell motility. Therefore, TGF-β induces Ca\(^{2+}\) entry likely via TRPC1 and NCX1 and raises [Ca\(^{2+}\)]\(_{\text{cyt}}\) in pancreatic cancer cells, which is essential for PKCα activation and subsequent tumor cell invasion. Our data suggest that TRPC1 and NCX1 may be among the potential therapeutic targets for pancreatic cancer.

transient receptor potential canonical; sodium/calcium exchanger; transforming growth factor-beta; pancreatic cancer

cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) is a ubiquitous cellular messenger, and receptor-mediated Ca\(^{2+}\) entry is a critical component of the cellular Ca\(^{2+}\) signaling in normal cells. Growing evidence suggests that some Ca\(^{2+}\)-mediated signaling pathways are implicated in tumorigenesis and tumor progression, such as metastasis, invasion, and angiogenesis (40). A remodeling of [Ca\(^{2+}\)]\(_{\text{cyt}}\) homeostasis is increasingly considered important in the process of malignant transformation, and it is expected that alteration of the expression and/or function of Ca\(^{2+}\) regulators, such as plasma membrane Ca\(^{2+}\) channels and exchangers, likely has a role in the process of tumorigenesis (8, 35).

[Ca\(^{2+}\)]\(_{\text{cyt}}\) homeostasis in normal cells is controlled by multiple proteins, which include the plasma membrane transient receptor potential receptor potential (TRP) channels and a Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) (6, 55). The TRP family is composed of seven subfamilies (TRPC, TRPV, TRPM, TRPA, TRPML, TRPP, and TRPN), and these TRP proteins display diverse properties, mode of regulation, and physiological functions (60). There are seven isoforms (TRPC1 to 7) in the transient receptor potential canonical (TRPC) subfamily that are expressed in mammalian cells (1, 3). NCX is a family of membrane transporters that can exchange Na\(^+\) and Ca\(^{2+}\) (or K\(^+\)) in either direction depending on transmembrane electrochemical gradients (6, 55). Two subfamilies have been described in mammalian cells; one in which Ca\(^{2+}\) movement is dependent only on Na\(^+\) (NCX1–3) and the other in which Ca\(^{2+}\) transport is also dependent on K\(^+\) (NCX1–6). TRPC and NCX have been described in mammalian tissues for decades, but very little is known about the expression and function of these proteins in human pancreatic cancer cells (4, 22, 32). Although the role of TRP channels has been reported in colorectal, colon, thyroid, breast, ovarian, and prostate cancer (7, 64), the involvement of TRP channels and NCX in pancreatic cancer development is currently unexplored.

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States, and nearly all patients diagnosed with pancreatic cancer die from a cancer-related death (24, 26, 52), with a 3% 5-year survival rate following diagnosis (43). Metastases are common in the process, particularly to the lymph nodes and liver (9, 19, 61). Histopathological analysis shows a ductal histology that makes up >90% of the cases of pancreatic adenocarcinoma (50). Since pancreatic cancer is characterized by poor survival rates and resistance to radiochemotherapy, novel therapeutic strategies are required to treat this devastating disease. The overall pathogenesis of pancreatic cancer is unclear; however, it is generally accepted that alterations to transforming growth factor-β (TGF-β) signaling play an important role in pancreatic cancer development (59). TGF-β suppresses cell growth via the TGF-β-SMAD pathway, but the ligand also promotes growth in pancreatic cancer cells, especially with disrupted SMAD signaling, with the latter corresponding to an invasive phenotype (20, 21, 53). The majority of pancreatic cancers are known to have SMAD4

*H. Dong (h2dong@ucsd.edu) and J. M. Carethers (jcarethe@umich.edu) are equal senior authors for this work.

Address for reprint requests and other correspondence: J. Y. C. Chow, Univ. of California, San Diego, University Center 303, MC 0063, 9500 Gilman Dr., La Jolla, CA 92093-0063 (e-mail: ycchow@ucsd.edu).

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deleted (17). The TGF-β-SMAD4 signaling pathway is pivotal to growth suppression in various epithelial cells (20, 21).

In a previous study (11), we found that TGF-β was able to mediate cell motility through [Ca\(^{2+}\)]\(_{cyt}\) immobilization and PKC\(\alpha\) activation in BxPc3 human pancreatic cancer cells; however, the underlying mechanisms by which TGF-β mediates [Ca\(^{2+}\)]\(_{cyt}\) homeostasis in human pancreatic cancer cells and the mechanisms by which TGF-β regulates cell motility via Ca\(^{2+}\) signaling pathway in human pancreatic cancer cells required further study. In the present study, we sought to further investigate the regulatory mechanisms of [Ca\(^{2+}\)]\(_{cyt}\) homeostasis in human pancreatic cancer cells and the underlying mechanisms by which TGF-β induces cell motility. Here, we demonstrate that TGF-β induces an increase in [Ca\(^{2+}\)]\(_{cyt}\) through TRPC1 channels and NCX1 followed by PKC\(\alpha\) activation, which mediates TGF-β-induced cell motility in SMAD4-null pancreatic cancer cells.

MATERIALS AND METHODS

Materials and reagents. G6-6976, a PKC inhibitor, was purchased from Pharmingen (San Diego, CA). A solution of G6-6976 in DMSO (Sigma, St. Louis, MO) was prepared. The solution was used after dilution in cell culture medium for each assay. All other reagents were purchased from Sigma.

Cell cultures. BxPc3 and CAPAN-1 cells were obtained from American Type Culture Collection and were maintained respectively in RPMI and Iscove’s DMEM (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS, GIBCO-BRL) in an incubator at 37°C and 5% CO\(_2\). They are both derived from human ductal pancreatic adenocarcinoma (15, 36), and both of them are SMAD4 null (54). These pancreatic cancer cell lines were grown to 70–80% confluence in medium containing 10% FCS. During the experiments, cells were washed twice in PBS, incubated for 30 min in serum-free medium, and treated for 24 and 48 h with 10 ng/ml TGF-β1 or medium alone without serum throughout the experiment.

Subcellular fractionation. Cells were lysed and separated into various compartments so as to determine the translocation of PKC isoforms when these enzymes were activated. This was carried out with a Cell Compartment Kit (FITEvenf Biochemicals, San Diego, CA), and experimental procedures were based on the manufacturer’s instructions. After treatment, cells were lysed in Cytoplasmic Fractionation Reagent A on ice for 10 min. This step disrupted the plasma membrane without solubilizing the cells. The lysates were then centrifuged at 1,000 g for 10 min at 4°C. The supernatants were removed, and this fraction contained cytosolic proteins. The pellet was resuspended in Membrane Fractionation Reagent B, which solubilized the plasma membrane, as well as all organelle membranes but not the nuclear membrane, by pipetting up and down with a 1-ml pipette tip. The lysates were then incubated at 4°C for 30 min on a shaker. Lysates were then centrifuged at 18,000 g for 20 min at 4°C. The supernatants, which contained the membrane proteins, were again transferred to new Eppendorf tubes and used for experiments.

Small interfering RNA transfection. We used validated small interfering RNA (siRNA) with inhibitory activities to NCX1 and TRPC1, respectively, as well as all organelle membranes but not the nuclear membrane, by pipetting up and down with a 1-ml pipette tip. The lysates were then incubated at 4°C for 30 min on a shaker. Lysates were then centrifuged at 18,000 g for 20 min at 4°C. The supernatants, which contained the membrane proteins, were transferred to new Eppendorf tubes and used for experiments.

Total RNA extraction and semiquantitative reverse transcription-polymerase chain reaction. Total RNA was extracted from control or TGF-β-treated cells with TRIzol reagent (Invitrogen). Cells were grown on six-well plates and lysed. Lysates were combined with TRIzol reagent and precipitated with isopropanol and 75% ethanol and then air dried. Two micrograms of total RNA was reverse transcribed into cDNA and amplified by PCR for NCX1 and TRPC1—7 expression (SuperScript II, Invitrogen). Brieﬂy, after inactivation at 65°C for 10 min, 1 µl of the reaction mixture was incubated in buffer containing dATP, dCTP, dGTP, and dTTP at 0.2 mM each, 0.2 µM concentrations each of oligonucleotide primers, 3 mM MgCl\(_2\), and a 10X buffer consisting of 200 mM Tris-HCl (pH 8.0), 500 mM KCl, and 1 U of Taq DNA polymerase isolated from Thermus aquaticus YT1 (Invitrogen). PCR primers of the NCX1 genes were used based on the literature (62), and primers of TRPC channels are listed in Table 1. The conditions for PCR reactions for these genes were similar to those previously described (11). Primers used for TRPC2 were designed from mouse DNA because human beings do not express TRPC2.

Western blotting. BxPc3 cells were washed three times with ice-cold PBS. Cells were then lysed with total lysis buffer [in mM: 150 NaCl, 10 Tris-HCl (pH 7.8), 1 EDTA, and 1 sodium orthovanadate, with 0.5% Triton X-100] containing protease inhibitors (1 µg/ml leupeptin and 100 µg/ml PMSF). Cells were then incubated at 4°C for 30 min with constant shaking. Cells were then scraped into microcentrifuge tubes, and the samples were centrifuged at 12,000 g for 15 min to remove insoluble material. The protein content in each sample was determined and adjusted. For immunoprecipitation studies, lysates were incubated with the immunoprecipitating antibody for 1 h at 4°C, followed by another 1-h incubation with protein A-agarose at 4°C. Pellets were then resuspended in 2× loading buffer [50 mM Tris (pH 6.8), 2% SDS, 100 mM dithiothreitol, 0.2% bromphenol blue, and 20% glycerol] and boiled for 5 min. Loading buffer supernatants from immunoprecipitation studies or cell lysates were then resuspended in 2× gel loading buffer, boiled for 5 min, and then separated by SDS-polyacrylamide gel electrophoresis (9% polyacrylamide). Resolved proteins were transferred overnight at 4°C onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Membranes were then blocked with a 5% solution of skim milk for 30 min at room temperature, followed by further incubation with specific monoclonal antibodies (PKC\(\alpha\), 1:1,000 (BD Transduction Laboratories, San Diego, CA), TRPC1, 4, and 6, 1:500 (Alomone Labs), phospho-PKC\(\alpha\) 1:1,000 (Upstate Biotechnology), and GAPDH 1:1,000 (Millipore, Billerica, MA). Membranes were then probed with primary antibodies overnight. Membranes were then washed three times for 10 min each in TBS-T (2×) and incubated with secondary antibodies. Membranes were then washed again, and bands were visualized using enhanced chemiluminescence (ECL) Western blotting reagents (GE Healthcare, Pittsburgh, PA) followed by autoradiography.

Table 1. Primer sequences of genes used for RT-PCR to determine gene expression

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<th>Target Gene</th>
<th>Accession No.</th>
<th>Primer Sequence</th>
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| NCX1 | NM_021097 | Sense 5'-TCTGAGCTTCGACAGTCTGCA-3'  
Antisense 5'-TCTCAGGACTGCTCAGAGTGT-3' |
| TRPC1 | NM_003304 | Sense 5'-TCTCTGCAAACACTGCTTTG-3'  
Antisense 5'-AACCTTTTGCTTCACTGGAC-3' |
| mTRPC2 | AF111107 | Sense 5'-ACCAGAGAAGAATCACTAAG-3'  
Antisense 5'-ACCAGAGAAGAATCACTAAG-3' |
| TRPC3 | NM_003305 | Sense 5'-CTTCTGCTTGCCAGAAAAAG-3'  
Antisense 5'-GGTACTGGAATGACAGAGAAG-3' |
| TRPC4 | NM_016179 | Sense 5'-GCTGAGGAAAGACAGACTGG-3'  
Antisense 5'-GCTGAGGAAAGACAGACTGG-3' |
| TRPC5 | NM_012471 | Sense 5'-ATCTGGACTGCGAGAGAC-3'  
Antisense 5'-ATGATCACTGCGAGAGAC-3' |
| TRPC6 | NM_004621 | Sense 5'-GCCCTGAGATGACTTGTCAATA-3'  
Antisense 5'-GCCCTGAGATGACTTGTCAATA-3' |
| TRPC7 | NM_020389 | Sense 5'-GTAAAACGCTGCCAGAAACT-3'  
Antisense 5'-GTAAAACGCTGCCAGAAACT-3' |
| GAPDH | NM_002046 | Sense 5'-GACAGAGTCTCAGTTGACAC-3'  
Antisense 5'-GACAGAGTCTCAGTTGACAC-3' |
1:5,000 (Ambion). After being washed with PBS with 1% Tween (PBST), the secondary antibody was applied to the membrane. After being washed with PBST, the membrane was treated with a chemiluminescent solution (FIVephoto Biochemicals) according to manufacturer's instructions and exposed to X-ray film. Densitometric analysis of the blots was performed with the use of an AlphaImager digital imaging system (Alpha Innotech, San Leandro, CA).

Measurement of \([Ca^{2+}]_{cyt}\) by digital Ca²⁺ imaging. \([Ca^{2+}]_{cyt}\) in human BxPc3 cells was measured by fura-2 fluorescence ratio digital imaging. Briefly, BxPc3 cells grown on coverslips were loaded with 5 µM fura-2 AM dissolved in 0.001% Pluronic F-127 plus 0.1% DMSO in physiological salt solution (PSS, described below) at room temperature (22°C) for 50 min and then washed in PSS for 30 min. Thereafter, the coverslips with BxPc3 cells were mounted in a perfusion chamber on a Nikon microscope stage. The ratio of fura-2 fluorescence with excitation at 340 or 380 nm (F340/F380) was followed over time and captured with an intensified charge-coupled device camera (ICCD200) and the MetaFluor Imaging System (Universal Imaging, Downingtown, PA). The PSS solution used in digital \(Ca^{2+}\) measurement contained the following (in mmol/l): 140 Na⁺, 5 K⁺, 2 Ca²⁺, 147 Cl⁻, 10 HEPES, and 10 glucose. For the \(Ca^{2+}\)-free solution, Ca²⁺ was omitted and 0.5 mM EGTA was added to prevent possible Ca²⁺ contamination. BAPTA-AM, a chelator of calcium, was used in some experiments at concentrations of 2 and 20 µM before TGF-β treatment. The osmolalities for all solutions were ~284 mosmol/kgH₂O.

Transwell migration assay. After coating the Corning Costar insert chambers as well as Transwell 24-well plates (8-µm pores; Corning, Corning, NY) with 0.1% fibronectin (Sigma) and blocking with 1% bovine serum albumin in 1× PBS for 1 h at 37°C, we seeded BxPc3 cells in triplicate at 50,000 cells per well in serum-free medium containing 1% bovine serum albumin with or without TGF-β (10 ng/ml) and/or siRNA treatment. Cells were then allowed to migrate for 3 h. After removal of medium from both the chamber and the Transwell followed by three washes with 1× PBS, the chamber was gently wiped with a cotton swab. Migrated cells were fixed in 100% methanol for 1 h and then allowed to air dry overnight. Cell staining was performed with a modified Giemsa stain (Sigma) at 1:10 for 1 h. After carefully rinsing the Transwell and the chamber with water, we captured the images using an Axiovert 2000 microscope with an AxioCAM HRC Camera (both Zeiss Microimaging, Thornwood, NY). Images were taken from six microscopic fields at the center of each well, and the stained cells on the bottom of the chamber were counted.

Statistical analysis. All data are expressed as means ± SE for a series of n experiments. Data were analyzed by one-way ANOVA followed by the Student-Newman-Keuls post hoc test or by Student's t-tests for unpaired samples with GraphPad Prism 3.0 (San Diego, CA). P < 0.05 was considered statistically significant.

RESULTS

Expression of NCX and TRPC channel in pancreatic cancer cells. Although NCX expression and function have been demonstrated in intestinal epithelial cells (12) and in pancreatic β-islet cells (12), little is known about NCX in human pancreatic adenocarcinoma cells (22). Here, we demonstrated that both the mRNA transcript and protein of NCX1 are expressed in pancreatic cancer cells (Fig. 1). NCX1 mRNA expression in BxPc3 cells was lower compared with that in the brain tissue (Fig. 1A, left) that served as a positive control. However, expression levels of NCX1 proteins were comparable in BxPc3 cells and the brain tissue (Fig. 1A, right). Expression of NCX2 and NCX3 was not detected in BxPc3 cells (data not shown). On the other hand, the mRNA and protein of TRPC1, 4, and 6 were expressed in BxPc3 cells at relatively high levels, but TRPC2, 3, 5, and 7 mRNAs were not detected by RT-PCR (Fig. 1, B and C). Thus our data indicate that both NCX1 and specific TRPC channels are expressed in human pancreatic cancer cells (22).

Functional activity of NCX and TRPC channels in pancreatic cancer cells. Although expression of NCX1 and TRPC channels was identified, we sought to characterize their function in pancreatic cancer cells. As mentioned previously (6), NCX exchanges Na⁺ and Ca²⁺ in either direction depending on transmembrane electrochemical gradients. To test whether NCX1 functions in the Ca²⁺ entry mode to transport external Ca²⁺ into the cells, BxPc3 cells were superfused with solutions without extracellular Na⁺ or Ca²⁺. As shown in Fig. 2A, removal of extracellular Na⁺ only (0 Na⁺), by replacing Na⁺ in the solution with equimolar Li⁺ to increase a driving force for
operation of the Ca\(^{2+}\) entry mode of NCX1) caused a rapid increase in \([Ca^{2+}]_{\text{cyt}}\). The 0 Na\(^{-}\)-induced Ca\(^{2+}\) signaling is likely due to the result of activating the Ca\(^{2+}\) entry mode of NCX1 because 1) the Ca\(^{2+}\) signal was dependent on loss of extracellular Na\(^{+}\), 2) the 0 Na\(^{-}\)-induced Ca\(^{2+}\) signaling absolutely depended on extracellular Ca\(^{2+}\) (data not shown), and 3) extracellular application of KB-R7943 (10 \(\mu\)M), a selective inhibitor of NCX (especially in its Ca\(^{2+}\) entry mode), significantly attenuated the 0 Na\(^{-}\)-induced increase in \([Ca^{2+}]_{\text{cyt}}\) (Fig. 2B). Figure 2C summarizes the effects of KB-R7943 (10 \(\mu\)M) on 0 Na\(^{-}\)-induced rise in \([Ca^{2+}]_{\text{cyt}}\) in pancreatic cancer cells.

Passive depletion of Ca\(^{2+}\) stores from the endoplasmic reticulum (ER) with a combination of Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase [sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)] inhibitors [such as cyclopiazonic acid (CPA)] and ryanodine receptor activators (such as caffeine) caused Ca\(^{2+}\) influx through store-operated channels (SOC), usually referred to as capacitative Ca\(^{2+}\) entry (CCE) (42). Although the molecular identity of SOC has hitherto not been clear, TRPC channels are believed to participate in the formation of functional SOC in epithelial cells (1, 3). Therefore, we sought to use store depletion-mediated Ca\(^{2+}\) influx to identify the function of TRPC-encoded SOC in the pancreatic cancer cells since this has been widely accepted as a standard way for the functional identification of TRPC-encoded SOC in many types of cells (42, 48). In the absence of extracellular Ca\(^{2+}\), application of CPA (10 \(\mu\)M) plus caffeine (10 mM) induced an increase in \([Ca^{2+}]_{\text{cyt}}\) due to Ca\(^{2+}\) leakage from the ER to the cytosol. When the store was depleted, restoration of extracellular \([Ca^{2+}]\) (to 2 mM) induced another increase in \([Ca^{2+}]_{\text{cyt}}\) due to Ca\(^{2+}\) entry (Fig. 3A).

Extracellular application of 2-aminoethoxydiphenyl borate (2-APB, 100 \(\mu\)M) or LaCl\(_3\) (La\(^{3+}\), 10 \(\mu\)M), both selective blockers of SOC (29, 37, 44), significantly attenuated CCE without altering intracellular Ca\(^{2+}\) release from the ER (Fig. 3, C).
inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 in pancreatic cancer cells.

TGF-β induced intracellular Ca\(^{2+}\) release from ER and TGF-β mediated Ca\(^{2+}\) signaling via PKCα activation in pancreatic cancer cells. Activation of G protein-coupled receptors (GPCRs) or tyrosine kinase receptors leads to activation of phospholipase C (PLC-β and PLC-γ) and to synthesis of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 induces Ca\(^{2+}\) mobilization from the ER by activating IP3 receptors, while diacylglycerol activates PKC and mediates Ca\(^{2+}\) signaling (18). Since TGF-β induces Ca\(^{2+}\) signaling and activates PKC (11, 18), we first tested whether TGF-β-induced Ca\(^{2+}\) signaling was prevented by pretreatment of the cells with Gö-6976 (10 µM), a selective PKCα inhibitor, indicating the involvement of PKCα in TGF-β-mediated Ca\(^{2+}\) signaling in the pancreatic cancer cells.

TGF-β-induced Ca\(^{2+}\) entry is likely through TRPC-encoded SOC and NCX1 in pancreatic cancer cells. It is known that the store depletion-mediated opening of TRPC-encoded SOC would promote not only Ca\(^{2+}\) influx but also Na\(^{+}\) influx, because SOC are permeable to both Ca\(^{2+}\) and Na\(^{+}\) (42). Since Ca\(^{2+}\) entry via NCX depends greatly on cytosolic Na\(^{+}\) concentration ([Na\(^{+}\)]\(_{cyt}\)), the store depletion-mediated Na\(^{+}\) influx through SOC would increase [Na\(^{+}\)]\(_{cyt}\), ultimately activate the Ca\(^{2+}\) entry mode of NCX, and enhance Ca\(^{2+}\) entry (6). We designed experiments to test whether or not TGF-β induces extracellular Ca\(^{2+}\) entry through the plasma membrane TRPC-encoded SOC and NCX1. As shown in Fig. 5A, TGF-β (10 ng/ml) induced the first phase of [Ca\(^{2+}\)]\(_{cyt}\) in the Ca\(^{2+}\)-free solution. Restoration of the external Ca\(^{2+}\) (2 mM CaCl\(_2\)) ~5 min after the first phase of [Ca\(^{2+}\)]\(_{cyt}\) declined (i.e., when the store was depleted) induced the second phase of [Ca\(^{2+}\)]\(_{cyt}\) that was due to the external Ca\(^{2+}\) entry (Fig. 5A). In a control

**Fig. 4.** Transforming growth factor-β (TGF-β)-induced intracellular Ca\(^{2+}\) release from the endoplasmic reticulum (ER) and the role of PKCα in the regulation of TGF-β-induced Ca\(^{2+}\) signaling in BxPc3 pancreatic cancer cells. A: cells were superfused with normal physiological salt solutions, and then addition of TGF-β (10 ng/ml) induced significant Ca\(^{2+}\) signaling in the absence of external Ca\(^{2+}\) (0 Ca\(^{2+}\)). B: pretreatment with 2-APB (100 µM) prevented the TGF-β-induced Ca\(^{2+}\) signaling in the absence of external Ca\(^{2+}\) (0 Ca\(^{2+}\)). C: cells were superfused with normal physiological salt solutions, and then addition of TGF-β (10 ng/ml) induced significant Ca\(^{2+}\) signaling. D: pretreatment with Gö-6976 (10 µM) prevented the TGF-β-induced Ca\(^{2+}\) signaling in normal physiological salt solutions. E: summarized data showing the amplitudes of TGF-β-induced Ca\(^{2+}\) rise in the presence or the absence of Gö-6976. Data are shown as means ± SE of 20–40 cells for each group. **P < 0.01 vs. the control in the absence of inhibitor.
experiment, switching of superfusion solutions with or without external Ca\(^{2+}\) in the absence of TGF-β could not alter the baseline (data not shown). Induction of intracellular Ca\(^{2+}\) release followed by external Ca\(^{2+}\) entry (CCE) after activation of the plasma membrane receptors is a well-documented response in mammalian cells (46, 51, 58). Since most nonexcitable cells (such as pancreatic cancer cells) do not express voltage-gated Ca\(^{2+}\) channels, CCE is considered as an important mechanism to control [Ca\(^{2+}\)]\(_{cyt}\) homeostasis and regulation of Ca\(^{2+}\)-dependent biological processes in epithelial cells (48).

The second set of experiments was designed to further test whether TGF-β-induced extracellular Ca\(^{2+}\) entry is due to activation of the plasma membrane TRPC-encoded SOC and NCX1. As shown in Fig. 5B, pretreatment of cells with La\(^{3+}\) (30 μM), a selective blocker for SOC, partially inhibited intracellular Ca\(^{2+}\) release but totally abolished TGF-β-induced CCE. Figure 5, C and D, summarize the effects of La\(^{3+}\) (30 μM), 2-APB (100 μM), also a SOC blocker, and KB-R7943 (10 μM), a selective inhibitor of NCX, on the rise in [Ca\(^{2+}\)]\(_{cyt}\) in the pancreatic cancer cells. All tested pharmacological inhibitors partially inhibited the TGF-β-induced rise in [Ca\(^{2+}\)]\(_{cyt}\) via release but totally abolished TGF-β-induced rise in [Ca\(^{2+}\)]\(_{cyt}\) via influx. Taken together, these results suggest that TRPC-encoded SOC and NCX1 may have a functional coupling and play an important role in TGF-β-induced [Ca\(^{2+}\)]\(_{cyt}\) in pancreatic cancer cells.

Inhibition of TRPC channel or NCX1 prevents TGF-β-induced PKCα activation. Because both TRPC and NCX are involved in regulation of [Ca\(^{2+}\)]\(_{cyt}\) homeostasis in pancreatic cancer cells, we examined the role of the TRPC channels on Ca\(^{2+}\)-dependent PKCα activation induced by TGF-β. Cells were pretreated with 2-APB (10 and 100 μM), followed by TGF-β treatment for 30 min. As previously reported by us (11), TGF-β induced PKCα activity in the pancreatic cancer cells (Fig. 6A). While 2-APB treatment alone at either concentration did not affect PKCα translocation from cytosol to the plasma membrane, 2-APB pretreatment at 10 and 100 μM dose-dependently and significantly reversed TGF-β-induced PKCα translocation (Fig. 6A). Similarly, inhibition of NCX1 with KB-R7943 (10 and 30 μM) alone did not affect PKCα translocation but significantly reversed PKCα translocation induced by TGF-β (Fig. 6B). In another experiment, phosphorylated PKCα antibody was used to further study the role of NCX1 in TGF-β-induced PKCα activity both in BxPc3 and CAPAN-1 cells (Fig. 6, C and D). Similar to the data shown in Fig. 6B, inhibition of NCX by KB-R7943 was able to suppress TGF-β-induced PKCα serine phosphorylation. These data clearly indicate that both the TRPC channels and NCX1 play important roles in regulating PKCα activity induced by TGF-β.

To further confirm the important role of NCX1 protein in this process, siRNA to specifically inhibit NCX1 expression was used to test its effect on PKCα activation. Cells were transfected with either a scramble siRNA or siRNA against NCX1 for 24 h. While NCX1 protein expression was not altered in pancreatic cancer cells transfected with the scramble siRNA, NCX1 protein expression was significantly suppressed in the cells that were transfected with the siRNA against NCX1 (Fig. 7A). The cells were then treated with either TGF-β or PKC activator phorbol 12-myristate 13-acetate (PMA, 1 μM) as a positive control. PMA significantly induced PKCα phosphorylation as predicted (Fig. 7B). Transfection of the scramble siRNA did not alter the effect of TGF-β on PKCα activity. However, transfection of the siRNA against NCX1 completely reversed TGF-β-induced PKCα phosphorylation (Fig. 7B). Therefore, our findings obtained from genetic knockdown experiments are consistent with those obtained from our pharmacological study.

Inhibition of TRPC or NCX suppresses TGF-β-induced cell motility. As demonstrated previously (11), TGF-β was able to induce pancreatic cancer cell motility in Boyden chambers via the activation of PKCα. In the present study, we found that TGF-β activates the Ca\(^{2+}\)-dependent PKCα likely via activation of the plasma membrane TRPC and/or NCX1. To further test the effect of NCX1 on cell motility, pancreatic cancer cells were either
transfected with the siRNA against NCX1 for 24 h or pretreated with KB-R7943 (10 μM) or 2-APB (100 μM) for 4 h. They were then seeded onto the Boyden chambers for cell motility assay. As shown in Fig. 8, A and B, KB-R7943 or 2-APB alone did not alter cell motility but significantly reversed TGF-β-induced motility in BxPc3 and CAPAN-1 cells. Similarly, while treatment with scramble siRNA or specific siRNA against NCX1 alone did not alter cell motility, knockdown of NCX1 protein by specific siRNA (Fig. 7A) significantly reversed TGF-β-induced pancreatic cancer cell motility (Fig. 8, C and D). Because we observed three TRPC isoforms expressed in BxPc3 cells (Fig. 1, B and C), we sought to examine the role of each of these isoforms on cell motility and therefore transfected the cells with specific siRNAs, which were able to effectively knock down these three TRPC isoforms (Fig. 9A). However, only transfection with specific siRNA against TRPC1 isoform could reverse TGF-β-induced cell motility but not siRNAs against TRPC4 and 6 isoforms (Fig. 9B). The data suggested that only the TRPC1 isoform plays a role in mediating TGF-β-induced cell motility.

DISCUSSION

TGF-β typically mediates its tumor suppressive effects through complex formation of SMAD2/3 and SMAD4. These complexes enter the nucleus to carry out TGF-β-responsive gene transcription (5). When the TGF-β-SMAD pathway is disrupted, the tumor suppressive phenotype is switched to tumor promotion as demonstrated in various cancers, including pancreatic cancer (21, 28). However, others have revealed a TGF-β-induced SMAD-independent ERK pathway that activates plasma membrane TRPC1 and NCX1 proteins that are responsible for mediating Ca²⁺ mobilization and led to induction of motility in SMAD4-null cells; however, the mechanisms underlying TGF-β-induced increase in [Ca²⁺]_{cyt} are not fully understood. In this study, we sought to examine the sources of Ca²⁺ that mobilizes pancreatic cancer cells and the precise mechanisms involved in these processes. We have demonstrated that TGF-β activates plasma membrane TRPC1 and NCX1 proteins that are responsible for mediating Ca²⁺-dependent PKCα activation followed by an increase in cell motility. Therefore, the TGF-β-induced Ca²⁺/PKCα signaling via activation of TRPC1 and NCX1 appears to be an important pathway that becomes dominant to enhance the metastatic tumor phenotype when the TGF-β-SMAD pathway is disrupted. The data suggested that only the TRPC1 isoform plays a role in mediating TGF-β-induced cell motility.
rupted in pancreatic cancer. A schematic of this activation is shown in Fig. 10.

We have shown that NCX1 protein is expressed in BxPc3 cells, which is consistent with a study by Hansen et al. (22) showing NCX1 expression in other human pancreatic cancer cell lines (CFPAC-1, PANC-1, and Capan-1). Although TRPC3 is suggested to play a role in the pathogenesis of acute pancreatitis (32), its role in the pathogenesis of pancreatic cancer is not explored. Here, we showed that human pancreatic cancer cells express TRPC1, 4, and 6 but not other isoforms. TRPC1 is believed to be a functional SOC in other cell types, including intestinal epithelial cells (38, 48), and TRPC6 to be a functional receptor-operated channel (ROC) (34, 45). Moreover, our data from the use of TRPC1 and NCX1 siRNAs strongly suggest coupling of TRPC1-encoded SOC and NCX1 in regulating human pancreatic cancer cell motility.

Our data show that TGF-β raises [Ca^{2+}]_{cyt} because of Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular space. The intracellular Ca^{2+} store is likely in the ER since 2-APB, a cell-permeant antagonist of IP3 receptor on ER, abolished TGF-β-induced Ca^{2+} signaling in the Ca^{2+}-containing solutions. TGF-β-induced Ca^{2+} entry likely comes from TRPC and NCX1 because Ca^{2+} signaling in the Ca^{2+}-containing solutions was abolished not only by 2-APB and La^{3+}, two widely used blockers for TRPC-encoded SOC (37, 44), but

**Fig. 7.** TGF-β-induced PKCα serine phosphorylation and prevention by specific knockdown of NCX1 with small interfering RNA (siRNA) in BxPc3 pancreatic cancer cells. A: cells were transfected with either scramble siRNA or siRNA against NCX1 for 24 h. The cells were lysed, resolved by 7.5% SDS-PAGE, and then transferred to PVDF membrane. The blots were then probed with anti-NCX1 antibody. With transfection of the siRNA against NCX1, NCX1 protein expression was significantly suppressed in cells. B: BxPc3 cells were treated with either TGF-β (10 ng/ml) or PKC activator phorbol 12-myristate 13-acetate (PMA; 1 μM), which was used as a positive control. Either TGF-β or PMA significantly induced PKCα phosphorylation as predicted. While transfection of the scramble siRNA did not affect TGF-β-induced PKCα activity, transfection with the siRNA against NCX1 completely reversed TGF-β-induced PKCα phosphorylation. Data were quantitated by image analysis and are means ± SE for 3 similar experiments. ***P < 0.001 vs. control; ++P < 0.001 vs. TGF-β treatment alone by 1-way ANOVA with the Student-Newman-Keuls post hoc test.

**Fig. 8.** TGF-β-induced cell motility and prevention by either pharmacological inhibition or specific knockdown of NCX1 with siRNA in BxPc3 and CAPAN-1 pancreatic cancer cells. Cells were either pretreated with KB-R7943 (30 μM; A and B) or 2-APB (100 μM) or transfected with the siRNA against NCX1 (C and D) for 24 h. Cells were then seeded onto Boyden chambers and treated with TGF-β for 4 h. The reactions were then stopped by fixing the cells in methanol followed by staining in Giemsa. Once dried, cells on the bottom side of the membrane were counted with a microscope in 5 areas to assess the number of cells that crawl across the membrane through the pores. Either pharmacological inhibition of TRPC or NCX1 or specific knockdown of NCX1 with siRNA significantly reversed TGF-β-induced cell motility. Data were quantitated by image analysis and are means ± SE for 3 experiments. ***P < 0.001 vs. control; ++P < 0.001 vs. TGF-β treatment alone by 1-way ANOVA with the Student-Newman-Keuls post hoc test. HPF, high-power field.
also phosphorylate IP3 receptors (30, 39), TRPC1 (2), and the mode of NCX1 and observed a Na+/H+ exchange that does not affect intracellular Ca2+ concentrations that are sensitive to their modulators; and 2) in regard to nonspecific actions of these inhibitors, to test this possibility we removed external Na+ to directly activate the Ca2+ entry mode of NCX1 and observed a Na+-dependent Ca2+ signaling that is KB-R7943 sensitive (Fig. 2), indicating the real function of NCX1 in pancreatic cancer cells. We have also used store depletion-mediated Ca2+ entry as a standard way for the functional identification of TRPC1-encoded SOC in pancreatic cancer cells. In this study, selective SOC inhibitors significantly attenuated CCE without altering intracellular Ca2+ release from the ER (Fig. 3), indicating the function of TRPC-encoded SOC in these cells. Taken together, our results suggest that TGF-β raises [Ca2+]cyt in these cells via induction of ER Ca2+ release as well as Ca2+ entry through a functional coupling of TRPC1 and NCX1. It was reported recently that TGF-β1-mediated fibrogenesis is dependent on the Ca2+ entry mode operation of NCX1 (47), providing further support. Although the concentrations of the pharmacological inhibitors used in the present study were considered to be selective inhibition in the literatures, a specific knockdown of NCX1 or TRPC1, 4, 6 using the siRNA approaches has also been applied to verify our hypothesis. Consistent results obtained from these two strategies strongly support that TRPC1 and the Ca2+ entry mode of NCX1 play important roles in the development of TGF-β-mediated pancreatic cancer.

In summary, we found that 1) both TRPC1 and NCX1 are expressed and function in pancreatic cancer cells (Fig. 1), 2) TGF-β induces intracellular Ca2+ release from the ER (Fig. 4) and Ca2+ entry likely via TRPC1 (Figs. 3 and 5) and NCX1 (Figs. 2 and 5), 3) inhibition of TRPC and NCX1 reverses the TGF-β-induced Ca2+-dependent activation of PKCα (Fig. 6), 4) inhibition or knockdown of NCX1 reverses TGF-β-induced PKCα activation and then cell motility (Figs. 6–8), and, similarly, 5) knockdown of TRPC1 reverses TGF-β-dependent cell motility (Fig. 9). Our findings strongly support a novel SMAD-independent tumor-promoting model of TGF-β-induced Ca2+-dependent motility of pancreatic cancer cells via a TRPC1-NCX1-Ca2+-PKCα cascade (Fig. 10). If our hypothesis is verified, TRPC1 and NCX1 may be novel potential targets for human pancreatic cancer therapy.

![Fig. 9. TGF-β-induced cell motility is inhibited by knockdown of TRPC1 with siRNA in BxPc3 pancreatic cancer cells. A: cells were transfected with the siRNA against TRPC1, 4, or 6 for 24 h. Cells were then seeded onto Boyden chambers and treated with TGF-β for 4 h. The reactions were then stopped by fixing the cells in methanol, followed by staining in Giemsa. sc, scramble siRNA; si, siRNA against TRPC1, 4, or 6. B: Once dried, cells on the bottom side of the membrane were counted with a microscope in 5 areas to assess the number of cells that crawl across the membrane through the pores. Suppression of TRPC1 but not other TRPCs with siRNA significantly reversed TGF-β-induced cell motility. Data were quantitated by image analysis and are means ± SE for 3 experiments. *P < 0.05, **P < 0.01 vs. control by 1-way ANOVA with the Student-Newman-Keuls post hoc test.](http://ajpcell.physiology.org/)

![Fig. 10. Schematic diagram depicting the proposed mechanisms of TGF-β-mediated cell motility via Ca2+/PKCα signaling pathway in pancreatic cancer cells. Binding of TGF-β with its membrane receptor results in production of inositol 1,4,5-trisphosphate (IP3) that activates IP3 receptors (IP3R) on the ER membrane and induces Ca2+ release from the ER. Depletion of the ER Ca2+ then activates the TRPC-encoded SOC and causes both Na+ and Ca2+ entry. The accumulated intracellular Na+ activates the entry mode of NCX1 and induces the inward transportation of Ca2+ via NCX1. An increase in [Ca2+]cyt mediates cell motility directly or indirectly via activation of Ca2+-dependent PKCα.](http://ajpcell.physiology.org/)
REFERENCES


