

Interlude of cGMP and cGMP/Protein Kinase G Type 1 in Pancreatic Adenocarcinoma Cells

Svetlana Karakhanova, PhD,* Marina Golovastova, MS,† Pavel P. Philippov, PhD,† Jens Werner, MD,* and Alexandr V. Bazhin, PhD*

Objective: cAMP and cGMP signaling is important both for normal and cancer cells. This signaling is controlled by adenylyl and guanylyl cyclases and cyclic nucleotide phosphodiesterases. One of the direct targets for cGMP is protein kinase G (PKG). The main aim of this work was to investigate cGMP and PKG signaling in pancreatic adenocarcinoma (PDAC) cells.

Methods: The PKG activity, cGMP, and calcium level were measured with the CycLex Cyclic GMP dependent protein kinase (cGK) Assay Kit, the DetectX Cyclic GMP Colorimetric EIA Kit, and the Fluo-4 NW Calcium Assay Kit, respectively. The Proteome Profiler Array was done using Human Phospho-Kinase Array and Human Phospho-MAPK Array Kits.

Results: This study shows for the first time that functional PKG1 is expressed in PDAC cells. It demonstrates that the specific PKG1 inhibitor, DT3, induces cytotoxicity through necrosis and reduces proliferation and migration of PDAC cells. Moreover, ERK1/2 and p38 can be considered as potential targets for PKG1 in PDAC cells. In addition, the study shows that phosphodiesterases and nitric oxide-guanylyl cyclases regulate the cGMP level in PDAC cells, affecting the proliferation of the cells.

Conclusions: The cGMP and PKG signaling may be a target for developing new therapeutic approaches for PDAC.

Key Words: pancreatic adenocarcinoma, protein kinase G, guanylyl cyclases, cyclic nucleotide phosphodiesterases, cGMP

(*Pancreas* 2014;43: 784–794)

Intracellular signaling, as a complicated regulatory network, plays a major role during normal and pathologic cell responses. The cAMP and cGMP were the first molecules to be described as second messengers.¹ Their levels are controlled during their synthesis by adenylyl and guanylyl cyclases (GC) and through the activation of cyclic nucleotide phosphodiesterases (PDEs). The cAMP and cGMP regulate multiple intracellular targets (eg, protein kinases A and G), cyclic nucleotide-gated channels, and PDEs themselves.

One of the direct targets of cGMP is protein kinase G (PKG).² The 2 PKG (sub)families (PKG1 and PKG2) are derived from separate genes. The PKG1 family consists of 2 isoforms PKG1 α and PKG1 β . The PKG1 isoforms are widely distributed,

whereas the expression of PKG2 is generally restricted to the brain, intestine, and kidneys. The PKG1 is more commonly activated when cGMP signaling is mediated by nitric oxide (NO).³ The NO at nanomolar concentration binds to haem, a prosthetic group of soluble GC, and causes a 100- to 200-fold activation of the enzyme.⁴ Activation of NO-GC increases conversion of GTP to cGMP, resulting in the elevation of cGMP, which initiates the cGMP signaling pathway and subsequent physiologic changes.⁵ These physiologic changes, such as the relaxation of the vascular and gastrointestinal smooth muscles, the inhibition of platelet aggregation, and other processes, are mediated through the phosphorylation of specific targets of PKG³ (Fig. S1, <http://links.lww.com/MPA/A301>). Furthermore, PKG1 signaling could be involved in cytoprotection through different mechanisms. Such cytoprotective effects have been observed in heart cells, neurons and glia, and epithelial cells, which are probably mediated through an increase in the opening of mitochondrial K⁺/ATP channels in heart cells, activating extracellular-signal regulated kinase (ERK) signaling, and Bcl-2-associated death promoter (BAD) protein phosphorylation.³

The elevation of cGMP and activation of PKG1 promote apoptosis in colon cancer.⁶ The investigators claim that PKG1 activation in colon cancer cells results in phosphorylation and activation of the mitogen-activated protein kinase kinase (MEKK1), activation of the stress-activated protein/ERK kinase 1 (SEK1), and activation of the c-Jun NH₂-terminal kinase 1 (JNK1) pathway. Recently, it has been demonstrated that the basal activity of PKG is essential for preventing spontaneous apoptosis.⁷ These data are in contrast with the proposed antiproliferative effect of PKG mentioned previously. Fraser et al speculated that, in colon cancer cell lines, the growth-inhibitory responses were initiated by either overexpression of PKG (especially the PKG1 β isoform) or the elevation of PKG1 β activity (but not the basal PKG activity). Thus, there is growing evidence that the different isoforms of PKG1 may regulate cell proliferation in different ways: PKG1 α activity (especially at the basal level) promotes cell proliferation, whereas PKG1 β (overexpressed or hyperactivated) inhibits cell proliferation.

Taken together, there is generally only poor and contradicting information on the phenotypical effects of activation/inhibition of PKG and their underlying molecular mechanisms in cancer cells. In addition, there are no data on the expression, function, and effect of inhibition/activation of PKG in pancreatic carcinoma cells.

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest cancers in the world. The PDAC mortality increases modestly in countries of the southern and central/eastern Europe, as well as in women.⁸ Patients with PDAC have a very poor prognosis with a 5-year survival rate of less than 1% and a median survival of 6 months.⁹ Even after surgical intervention, the 5-year survival rate is reached at best 15% without or 25% with adjuvant chemotherapy in specialized centers.¹⁰ The reasons for this poor prognosis are its early dissemination, lack of early specific symptoms, and late diagnosis.¹¹ In addition,

From the *Department of General Surgery, University Hospital Heidelberg, Germany; and †Department of Cell Signalling, Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia. Received for publication May 7, 2013; accepted January 14, 2014.

Reprints: Alexandr V. Bazhin, PhD, Department of General Surgery, University Hospital Heidelberg, Im Neuenheimer Feld 430, 69120 Heidelberg, Germany (e-mail: alexandr.bazhin@med.uni-heidelberg.de).

This study was supported by a grant from Wilhelm Sander-Stiftung (2011.036.1) to A.V.B. and J.W. and by the grant from the Russian Foundation for Basic Research (#12-04-00922) to P.P.P.

The authors declare no conflict of interest.

Supplemental digital contents are available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.pancreasjournal.com).

Copyright © 2014 by Lippincott Williams & Wilkins

TABLE 1. Expression of *PKG* and Their Activity in Pancreatic Malignant and Benign Cell Lines

Cell Lines	mRNA		Protein	Activity, u/mg	IC50 for DT3, μ M
	<i>PKG1</i>	<i>PKG2</i>	<i>PKG1</i>		
BXPC3	+	—	+	0.91 \pm 0.15	n.d.
Capan-1	+	—	+	1 \pm 0.3	0.5625
Dan-G	+	—	+	0.5 \pm 0.1	n.d.
MiaPaCa	+	—	+	0.8 \pm 0.05	1.782
Panc1	+	—	—	0.007 \pm 0.001	n.d.
ACBRI515	+	—	—	u.d.l.	n.d.
HPNPCC	+	—	—	u.d.l.	n.d.

n.d. indicates no data; u.d.l., under detection limit.

PDAC is highly resistant to chemotherapy and targeted therapy. It is for this reason that new therapeutic strategies are urgently needed. In this work, the roles of cGMP and *PKG1* in PDAC cells are investigated.

MATERIALS AND METHODS

Materials

The membrane-permeant peptide-based inhibitor of *PKG1* α —DT3—was purchased from BIOLOG Life Science Institute (Germany). The soluble GCs inhibitor 1H-[1,2,4]oxodiazolo[4,3-a]quinoxalin-1-one (ODQ) and the NO donor 4-ethyl-2E-(hydroxyimino)-5-nitro-3E-hexenamide (FK-409) were obtained from Cayman Chemical. The endogenous peptide activator of intestinal GC—guanylin—was purchased from TOCRIS Bioscience (United Kingdom). Carbachol (carbamoylcholine chloride) was purchased from Sigma-Aldrich (Germany). A goat polyclonal (monospecific) antibody against *PKG1* α/β was purchased from Santa Cruz Biotechnology, Inc. Antibodies against phosphorylated and unphosphorylated forms of CREB, ERK1/2, GSK-3, JNK, and P38 were purchased from Cell Signaling Technology, Inc. Human Total RNA Panel II, Human Small Intestine Total RNA, and Human Small Intestine Protein Medley were obtained from Clontech Laboratories, Inc.

Cell Cultures

Pancreatic carcinoma cell lines (Table 1) were purchased from DSMZ (Germany) or from ATCC. Cells were cultivated in a RPMI-1640 medium with 10% of fetal calf serum, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin (whole medium), obtained from PAA Laboratories (Germany). The human pancreatic epithelial cell line ACBRI515 was purchased from Applied Cell Biology Research Institute (ACBRI), and the cells were cultivated in calf serum free complete medium provided by the manufacturer. The human pancreatic normal primary cell culture

(HPNPCC) was obtained from Celprogen, and the cells were cultivated in the HPNPCC complete growth media with serum provided by the manufacturer. Cell lines were cultivated at 37°C and 5% of carbon dioxide. Cells were routinely checked for mycoplasma contamination and commercially authenticated by Multiplexon GmbH (Germany).

RNA Isolation and Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA from cell lines was isolated using an RNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. The RNA concentrations were determined using a NanoPhotometer Pearl (SERVA Electrophoresis, Germany). The reverse transcription polymerase chain reaction (RT-PCR) analysis of *PKG1* and *PKG2* was performed as previously described by Sengenès et al.¹² Briefly, 1 μ g of the total RNA from cell lines or tissues was reverse-transcribed by using the first strand cDNA synthesis kit (Roche Diagnostics, Germany) at 42°C for 50 minutes, as described by the manufacturer. The PCR amplification was performed using 1 μ L from the RT-reaction mixture in 25 μ L of the PCR mixture containing 50 pmol of sense and antisense primers. After the initial incubation at 94°C for 2 minutes, 33 cycles of amplification were carried out for *PKG1* and *PKG2* as well as special isoforms of GC and PDE (Table 2) and 21 cycles for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). At least 3 independent PCR experiments were running. Primer sequences annealing temperatures and sizes of PCR products for the *PKG1* and *PKG2* were published by Sengenès et al.¹² The same parameters for GAPDH were published previously by us.¹³ The RT-PCR primers for GC and PDE were purchased from Qiagen (Germany).

Western Blot Analysis

Western blot analysis was performed as described elsewhere.¹³ Briefly, 10 μ g of total protein per lane were separated in 12.5% of polyacrylamide gel. After sodium dodecyl sulfate polyacrylamide gel electrophoresis, separated proteins were electrotransferred from the gel to nitrocellulose membranes (Hybond-C; Amersham Bioscience) in a Tris-glycine-methanol buffer (pH 8.3). After blocking with 10% (w/v) of delipidated dry milk in phosphate buffered saline, containing TWEEN-20, the membranes were incubated overnight with an antibody against protein of the interest or for 1.5 hours with GAPDH (as a loading control). All antibodies were diluted as suggested by the manufacturer. Then, membranes were incubated for 1.5 hours with an appropriate peroxidase conjugated secondary antibody: anti-goat Immunglobuline G (IgG) (1:30000), anti-rabbit IgG 1:10000, or anti-mouse IgG for β -actin 1:5000. All secondary antibodies were obtained from Santa Cruz Biotechnology. Immunoreactive bands were visualized by an

TABLE 2. PDE and GC mRNA Expression in PDAC Cell Lines

Cell Lines	PDEs						GCs				
	1A1	2A1	3A1	5A1	9A1	11A1	1A2	1A3	1B2	1B3	GC-C
BXPC3	pos	pos	pos	pos	pos	pos	pos	pos	neg	pos	pos
Capan-1	pos	pos	pos	pos	pos	pos	neg	neg	pos	neg	pos
Dan-G	pos	pos	pos	pos	pos	pos	neg	pos	pos	pos	neg
MiaPaCa	pos	pos	pos	neg	pos	pos	neg	neg	pos	neg	pos
Panc1	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg

enhanced chemiluminescence system (Amersham Bioscience) according to the method described by the manufacturer.

Measurement of PKG Activity

Measurement of the PKG activity in lysates of PDAC cells was done using the CycLex Cyclic GMP dependent protein kinase (cGK) Assay Kit (CycLex Co, Ltd, Japan), as described by the manufacturer. Briefly, the cell pellet ($5\text{--}10 \times 10^6$ cells) was resuspended in phosphate buffered saline with a proteinase inhibitor cocktail. The cells were lysed by 3 freeze-thaw cycles followed by centrifugation at 400 g for 10 minutes at 4°C. Then, 100 μL of supernatant (10 mg/mL of total proteins) were incubated for 10 minutes with or without DT-3 at concentrations mentioned in the figure legends. Then, 10 μL of supernatant were added into the wells precoated with a substrate corresponding to the recombinant PKG substrate. Afterward, 90 μL of the cGMP plus Kinase Reaction Buffer from the kit were added, and the wells were incubated for 30 minutes at 30°C. After washing the wells, 100 μL of the horseradish peroxidase conjugated Detection Antibody 10H11 were added into each well, and the plate was incubated at room temperature for 60 minutes. After a washing step, 100 μL of the substrate reagent were pipetted and incubated at room temperature for 15 minutes. Finally, 100 μL of the stop solution was added into each wells, and the absorbance was measured using a plate reader at 450 nm. The cGK positive control-1 (catalytic domain) obtained from CycLex Co, Ltd (Japan), was used as a positive control. As a negative control, a probe without adding of the protein supernatant was used. The PKG activity expressed in optical density (OD) was measured in duplicate, and the final activity was calculated from 3 independent experiments. Being lower than 0.05, the OD of a negative control was not subtracted from the experimental values.

cGMP Measurement

The cGMP was measured with the DetectX Cyclic GMP Colorimetric EIA Kit from ArborAssays in a regular format as described by the manufacturer. Briefly, harvested cells (approximately 10^7 cells) were lysed with the provided sample of the diluents; 50 μL of the lysates or standards (provided with the kit) were added into the IgG precoated wells. After incubation with different reagents from the kit at room temperature, the plates were read on a plate reader. Standard concentrations of cGMP (from 0.5 pmol/mL to 32 pmol/mL) were simultaneously proceeded to estimate cGMP level in the probes of interest. Each cell line was measured in duplicate, and the final concentration of cGMP normalized on cell numbers was determined from at least 3 independent experiments.

Calcium Measurement

The free intracellular calcium level was measured with a Fluo-4 NW Calcium Assay Kit (Molecular Probes) as described by the manufacturer. Briefly, 4×10^4 cells were grown overnight in a 96-well microplate (Greiner Bio-One, Germany). Then, the growth medium was removed, and 100 μL of loading solution from the kit was added. The plate was incubated during different times at 37°C in the dark. Cell stimulation with 100 nM of carbachol was used as a positive technical control. Measurement of fluorescence at 480 nm of excitation and 520 nm of emission was performed with TECAN SPECTRAFluor Plus. The relative fluorescence was used for estimation of changes in the intracellular calcium level calculated from at least 3 independent experiments.

Cell Viability Assays

Cell viability after drug treatment was measured both with an EZ4U Kit (Biomedica, Austria) and with an ApoTox-Glo Triplex Assay Kit (Promega) in the whole medium as described by the manufacturers. Briefly, for the EZ4U assay, 20,000 cells

per well were seeded in 96-well plates. After overnight growing (about 90% of cells attached), a drug of interest was added without the medium exchange. After a 24- or 48-hour incubation period, a substrate compound from the kit was added, and the cells were incubated for 5 hours at 37°C to convert the yellow-colored tetrazolium to its red formazan derivative by living cells. Finally, the absorbance was measured at 450 nm.

For the ApoTox-Glo test, 20,000 cells per well were seeded in 96-well plates. After overnight growing and subsequent drug treatment, a glycyl-phenylalanyl-amino-fluorocoumerin substrate compound from the kit was added, and the cells were incubated for 30 minutes at 37°C to allow for substrate cleaving by live-cell protease. Then, the fluorescence was measured at 360 nm of excitation and 480 nm of emission with TECAN SPECTRAFluor Plus; 30 $\mu\text{g/mL}$ of digitonin was used as a positive control for viability decrease. Absorbance or fluorescence intensities were proportional to the amount of living cells. The viability tests were performed for at least 3 independent experiments for each assay.

Apoptosis and Necrosis Assays

Apoptosis after DT3 treatment was measured using the ApoTox-Glo Triplex Assay Kit (Promega) as described by the manufacturer. Briefly, after the viability measurement (see above), the Caspase-Glo 3/7 reagent was added to the wells, and the plates were incubated for 30 minutes at room temperature. Afterward, the luminescence that is proportional to the amount of caspase activity was measured using TECAN SPECTRAFluor Plus.

For the simultaneous analysis of apoptosis and necrosis, Annexin V and ethidium homodimer III staining were performed using the Apoptotic/Necrotic Cells Detection Kit (PromoKine, Germany) as described by the manufacturer. Briefly, FITC-Annexin V and ethidium homodimer III solutions were added to 200,000 cells. After 15 minutes of incubation in the dark, the binding buffer from the kit was added, and the cells were analyzed with a BD FACSCanto II flow cytometer. Then, 10 μM of staurosporin or 100 μM of ionomycin were used as a positive control for apoptosis or necrosis, respectively. The apoptosis and necrosis tests were performed for at least 3 independent experiments.

Cell Proliferation Assay

Proliferation of cell lines after drug treatment was analyzed with a Bromodeoxyuridine (BrdU) Cell Proliferation Assay kit (Millipore) as described by the manufacturer. Then, 20,000 cells were seeded in 96-well plates. After overnight growing and 24-hour drug incubation, the BrdU reagent was added, and the cells were incubated for 12 hours at 37°C to allow the BrdU incorporation into proliferating cells. After incubation, the cells were fixed and washed, then a detector antibody was added, and the plates were incubated for 1 hour at room temperature. After washing, a goat anti-mouse IgG peroxidase conjugate from the kit was added, and the plates were incubated for 30 minutes at room temperature. After further washing, the cells were incubated for 30 minutes at room temperature in the dark with the 3,3',5,5'-tetramethylbenzidine peroxidase substrate. The reaction was stopped by adding the acid stop solution from the kit. Finally, the absorbance was measured at 450 nm. Cell proliferation assays were performed for 3 independent experiments.

Cell Migration and Invasion Assays

Cell migration and invasion assays were performed with CytoSelect Cell Migration and Invasion Assay kit (Cell Biolabs, Inc) as described by the manufacturer. For the cell migration assay, the polycarbonate inserts (8- μm pore size) from the kit were used. For the cell invasion assay, the polycarbonate inserts

(8- μ m pore size) coated with a uniform layer of dried basement membrane matrix solution from the kit were used. For both assays, 300,000 cells in a serum-free medium were present per well. Plates were incubated for 24 hours at 37°C, and the migrated or invaded cells were stained and washed. After that, the

extraction solution from the kit was added, and the intensity of color was measured at 560 nm using a TECAN SPECTRAFluor Plus. Absorbance intensity was proportional to the amount of migrated or invaded cells. Cell migration and invasion assays were performed for 3 independent experiments.

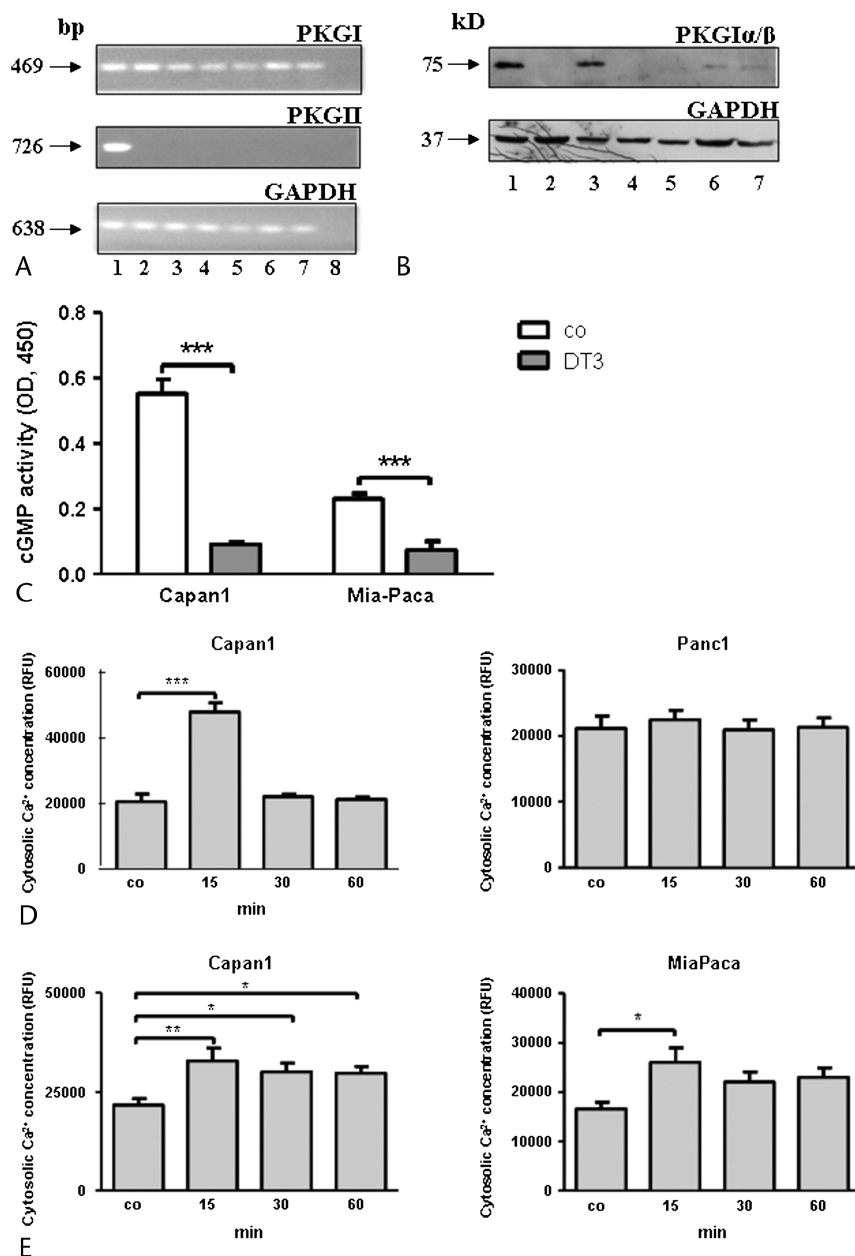


FIGURE 1. Functional *PKG1* but not *PKG2* is expressed in PDAC cell lines. A and B, Analysis of *PKG* expression at mRNA and protein levels. A, A representative picture of an agarose gel electrophoresis (1.5%) after conventional RT-PCR analysis of PDAC cell lines from Table 1 with specific *PKG1*, *PKG2*, and GAPDH (as a control) primer pairs: 1, a positive control (sample of human small intestine); 2, Panc1; 3, Capan-1; 4, ACBRI; 5, HPNPPC; 6, MiaPaCa; 7, Dan-G; 8, water (a negative control). B, A representative picture of a Western blotting membrane stained with a *PKG1*α/β or a GAPDH antibody: 1 to 7, the same as 1 to 7 in A. C, Measurement of *PKG* activity (high kinase activity corresponds to high OD measured photometrically) in lysates of PDAC cell lines without (co) and with (DT3) 1- μ M DT3. D and E, Ca²⁺ measurement (corresponds to relative fluorescence units) in PDAC cell lines measured fluorometrically before (co) and after 15-, 30-, and 60-minute incubation with 1- μ M DT3 (E) or with 100-nM carbachol as a positive control (D, left) or the *PKG1*-negative PDAC cell line Panc1 after incubation with 1- μ M DT3 as a negative control (D, right). For C, D, and E, the data of 3 independent experiments are presented with SE and analyzed with unpaired 2-tailed *t* test (C) and 1-way ANOVA with the Dunnett post hoc test (D). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Proteome Profiler Array

Human Phospho-Kinase Array and Human Phospho-MAPK Array Kits were purchased from R&D, and the assays were performed according to the manufacturer's instructions. Briefly, 1.15×10^6 to 2.5×10^6 cells were solubilized in the lysis buffer from the kit and rocked gently at 4°C for 30 minutes. After centrifugation at 14,000 g for 5 minutes, the whole protein concentration in the supernatants was quantified, and samples were diluted to 200 µg/mL. Afterward, the cell lysates were mixed with a cocktail of biotinylated detection antibodies and incubated overnight with membranes captured with "capture" and control antibodies. After that, the membranes were washed, and streptavidin-horseradish peroxidase and chemiluminescent detection reagents were applied. A signal was produced at each capture spot corresponding to the amount of a phosphorylated protein bound. For the detailed list of kinases, refer to http://www.rndsystems.com/product_detail_objectname_MAPKArray.aspx for MAPK and to http://www.rndsystems.com/product_detail_objectname_kinaseantibodyarray.aspx for phosphokinases.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 5.01. Distributions of continuous variables were described by their means and SE and were presented as column bar graphs. The null hypothesis (mean values were equal) versus the alternative hypothesis (mean values were not equal) was tested by unpaired 2-tailed *t* test (for 2 groups) and by 1-way analysis of variance (ANOVA) with the Dunnett post hoc test (for more than 2 groups). A *P* < 0.05 was considered significant.

RESULTS

Functional *PKG1* but not *PKG2* Is Expressed in PDAC Cell Lines

Conventional RT-PCR analysis revealed the *PKG1* but not the *PKG2* expression at the RNA level in the PDAC and normal pancreatic cell lines tested (Table 1, Fig. 1A). The *PKG1* was also expressed at the protein level in all cell lines tested except in Panc1 and cells of a normal pancreas (Table 1, Fig. 1B). All PDAC cell lines positive for *PKG1* showed a kinase activity, whereas the *PKG1*-negative cell line did not (Table 1). Moreover, the *PKG1*-specific inhibitor DT3 at 1 µM significantly reduced the *PKG* activity in the PDAC cell lines tested (Fig. 1C). Thus, functional *PKG1* is actually expressed in many PDAC cell lines. One of the consequences of the functional *PKG* inhibition can be an increase of the Ca^{2+} amount¹⁴ (Fig. S1, <http://links.lww.com/MPA/A301>). Indeed, incubation of PDAC cell lines in the presence of 1-µM DT3 led to an increase in the Ca^{2+} level with the maximum reached after 15-minute incubation (Fig. 1E). It should be stressed that the Ca^{2+} level in the *PKG*-negative cell line Panc1 was not affected by DT3 (Fig. 1D).

DT3 Induces Cytotoxicity Through Necrosis and Reduces Proliferation and Migration, but not Invasion of PDAC Cells

Next, the viability, proliferation, migration, and invasion of PDAC cells after *PKG1* inhibition with DT3 were investigated. This drug at 1 µM induced cytotoxicity of PDAC cells measured both with the ApoTox-Glo Triplex Assay Kit (Fig. 2A) and with a conventional EZ4U test (data not shown). The IC₅₀ for the DT3 cytotoxicity was measured for 2 PDAC cell lines, and the data are summarized in Table 1. The DT3-induced cell death mechanism was not apoptosis (measured with the ApoTox-Glo Triplex Assay Kit; Fig. 2D) but necrosis (measured with the Apoptotic/Necrotic Cells Detection Kit; Fig. 2C, data not shown).

In addition, the flow cytometry analysis confirmed cell death after treatment with DT3 at IC₅₀ concentrations (Fig. 2C, data not shown). In these experiments, DT3 reduced the proliferation (Fig. 3A) and migration (Fig. 3B) but did not affect the invasion of the PDAC cells tested (Fig. 3C).

ERK1/2 and JNK as Potential Targets of *PKG1*

To identify potential targets for *PKG1* in PDAC cells, a proteome profiler assay for human kinases and specific MAP kinases was performed. With this method, 6 proteins (CREB, ERK1/2, GSK-3, JNK1, JNK2/3, and P38) were identified whose phosphorylated forms had a tendency to be down-regulated in Capan1 and MiaPaCa cell lines after 48-hour treatment with the IC₅₀ concentration of DT3 (Fig. 4, data not shown). Down-regulation of ERK1/2, JNK1, and JNK2/3 was confirmed by Western blot analysis (Fig. 4, data not shown). Thus, ERK1/2, JNK1, and JNK2/3 could be potential targets for *PKG1* in PDAC cells.

PDEs Regulate the cGMP Level in PDAC Cells but Have no Influence on the Viability or Proliferation of the Cells

The PDEs regulate the cGMP level in the cell and thus can potentially be involved in the regulation of *PKG* activity in PDAC cells. First, the mRNA expression of all cGMP-PDEs (Table 2) was analyzed, and it was found that *PDE1A1*, *PDE2A1*, *PDE3A1*, *PDE9A*, and *PDE11A1* were expressed at the mRNA level in all cell lines tested and *PDE5A1* in 4 from 5 of the cell lines analyzed. In vitro 24-hour incubation of the cell lines with the unspecific inhibitor of PDE—3-isobutyl-1-methylxanthine (IBMX)—at 10 µM led to an increase in cGMP level in the PDAC cells (Fig. 5A). These data demonstrate that PDEs are responsible for maintaining a certain cGMP level in PDAC cells. Simultaneously, a 48-hour incubation of MiaPaCa and Capan1 cells at different concentrations of IBMX did not have any effects on the proliferation (Fig. 5B) and viability (data not shown) of the cells. Because it was found that PDEs are important for maintaining the cGMP level in the PDAC cells, IBMX was further used to inhibit the undesirable PDE effect.

GC-C Is Involved in the Regulation of cGMP Level in PDAC Cells but Has no Influence on the Viability or Proliferation of the Cells

Next, the mRNA expression of different GC in the PDAC cell lines listed in Table 2 was analyzed. A heterogenic expression of NO-GC isoforms (*GC1A3*, *GC1B2*, and *GC1B3*) was found in the cell lines tested (Table 1). It is interesting to note that the expression of intestinal GC-C was detected in 3 cell lines from the 5 tested. To discover whether GC-C could be involved in maintaining the cGMP homeostasis of PDAC cells, the Capan1 and MiaPaCa cell lines were treated with the endogenous peptide activator of GC-C: guanylin. The guanylin treatment increased cGMP level in the PDAC cell lines tested (Fig. 5C). Thus, GC-C is involved in the regulation of the cGMP level in the PDAC cells. However, the treatment of Panc1 and MiaPaCa cell lines with different (35 nM–35 µM) concentrations of guanylin with or without 10-µM IBMX did not reveal any effect on the viability and proliferation of the cells (Fig. 5D, data not shown).

NO-GC Regulates cGMP Level and Proliferation of PDAC Cells

We suggested that NO-GC could be important for the regulation of *PKG1* activity in PDAC cells as it was found to be so for other cancer cells. Indeed, treatment of Capan1 and

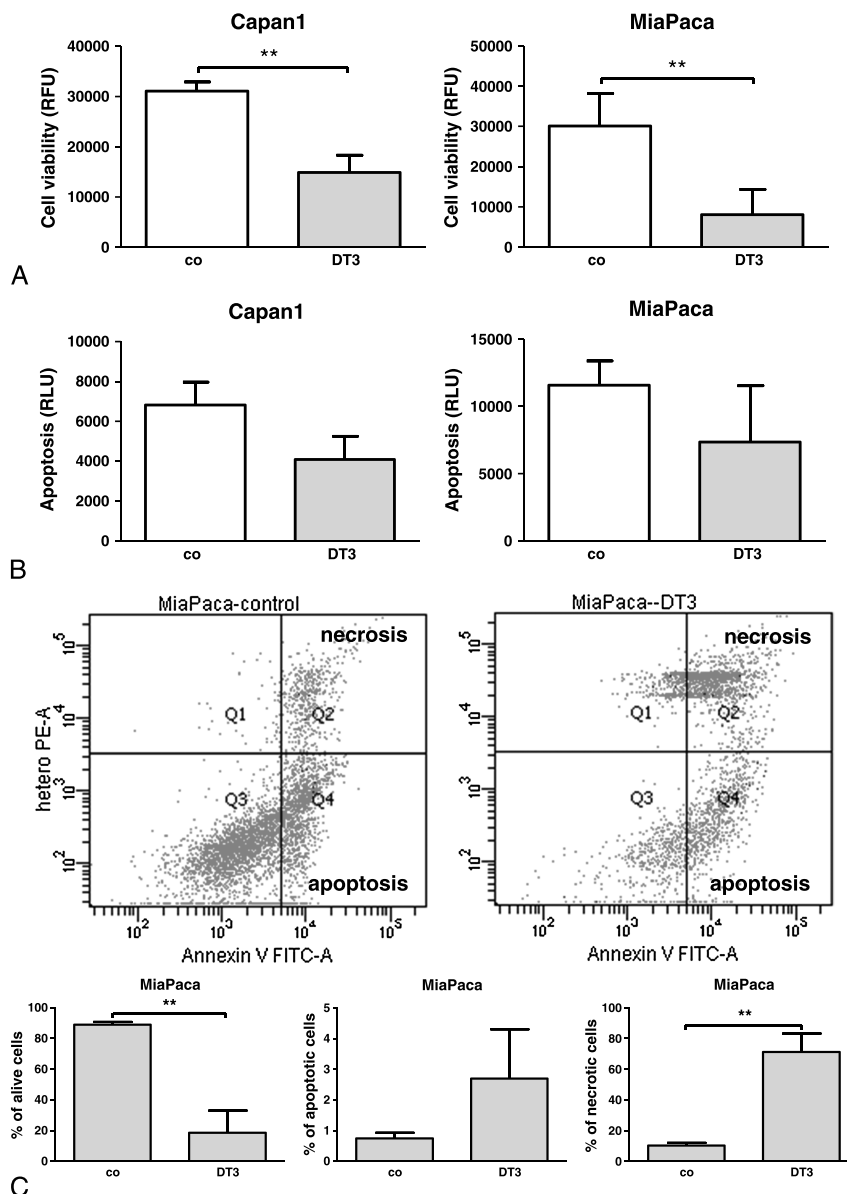


FIGURE 2. DT3 induces cytotoxicity through necrosis of PDAC cell lines. A, Analysis of cell viability (high cell viability corresponds to high relative fluorescence unit measured fluorometrically) after 24-hour incubation with 1- μ M DT3. B, Analysis of apoptosis (high apoptosis rate corresponds to high relative luminescence units measured luminometrically) after 24-hour incubation with DT3 at IC50 concentrations from Table 1 (1.8 μ M for MiaPaCa and 0.5 μ M for Capan-1). C, Analysis of apoptosis and necrosis measured with flow cytometry: top, a result of 1 representative experiment; bottom, summary of all experiments done; co, untreated control. The data of 4 independent experiments are presented with SE and analyzed with unpaired 2-tailed *t* test (C). **P* < 0.05 and ***P* < 0.01.

MiaPaCa with the NO donor FK409 at 450 nM led to an increase in cGMP levels in the cells as compared with the effect of IBMX alone (Fig. 6A), which should lead to the *PKG1* activation. Anyway, the treatment of the PDAC cells with different (45 nM–4.6 μ M) concentrations of FK409 did not reveal any changes in either the proliferation (Fig. 6B) or the viability (data not shown) of the cells. In vitro incubation of Capan1 and MiaPaCa cells with the NO-GC inhibitor ODQ had a tendency to reduce the intracellular cGMP level when compared with the treatment with IBMX (Fig. 6C), indicating the high impact of NO-GC on the cGMP homeostasis. At the same time, a 48-hour treatment with different (100 nM–10 μ M) concentrations of ODQ in the combination with 10- μ M IBMX slightly but

significantly reduced the proliferation of Capan1 and MiaPaCa cells (Fig. 6D) but did not affect the viability (data not shown) of the cells. The ODQ as well as IBMX alone had no effect on the proliferation of the cells (data not shown, Fig. 5A).

DISCUSSION

The *PKG* is the central effector for cGMP signaling in a multitude of processes in healthy cells as well as in cancer cells. However, cGMP/*PKG* signaling was not so far investigated for PDAC. Thus, the main aim of the present work was to investigate such a signaling in this type of cancer.

The expression of *PKG1* has been documented for colon cancer cells,¹⁵ ovarian cancer cells,^{7,16} and recently, nonsmall

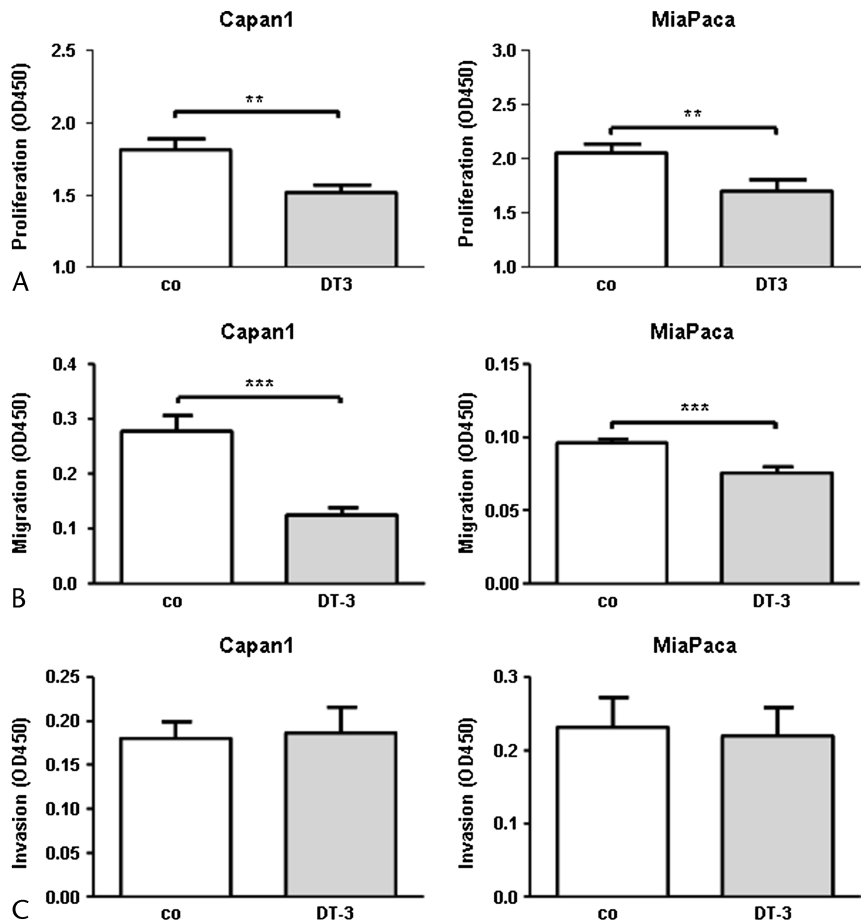


FIGURE 3. DT3 reduces proliferation and migration but not invasion of PDAC cell lines. Analysis of cell proliferation (A), migration (B), and invasion (C) (high level of the tested parameters corresponds to high OD measured photometrically) before and after 48-hour treatment with DT3 by IC50 concentrations from Table 1 (1.8 μ M for MiaPaCa and 0.5 μ M for Capan-1); co, untreated control. The data of 4 (A) or 3 (B and C) independent experiments are presented with SE and analyzed with unpaired 2-tailed *t* test (C). ***P* < 0.01 and ****P* < 0.001.

cell lung cancer cells.¹⁷ The *PKG2* expression has been found in gastric cancer cells.^{18–20} Expressions of both isoforms have been reported for breast cancer cells by Fallahian et al²¹ and recently by Karami-Tehrani et al.²² In this work, it has been shown for the first time that functional *PKG1* but not *PKG2* is also expressed in another cancer type, namely in PDAC.

The most interesting result obtained is that the specific *PKG1* inhibitor DT3 induced cytotoxicity of the PDAC cells. DT3 inhibits *PKG1* at a different site from the catalytic domain, making DT3 a competitive/noncompetitive inhibitor at nmol concentration.²³ Also, DT3 reduced the proliferation and migration of PDAC cells. Our results are partially in agreement

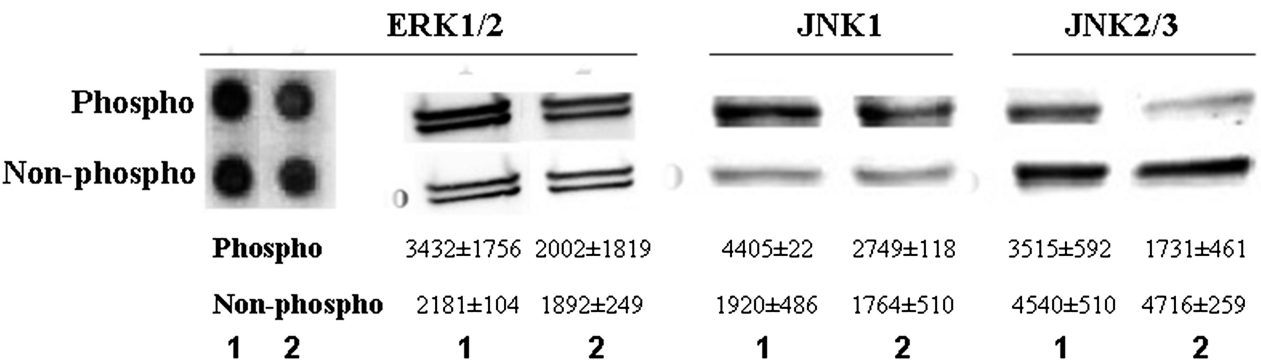


FIGURE 4. DT3 inhibits phosphorylation of ERK1/2, JNK1, JNK2/3, and p38. Representative pictures of a Proteome Profiler Array membrane (for ERK1/2) and Western blotting membrane (for ERK1/2, JNK1, JNK2/3, and p38) stained with antibodies specific for phosphorylated (Phospho) or unphosphorylated (Non-phospho) forms of the proteins tested in MiaPaCa: 1, untreated sample; 2, DT3-treated sample. Bottom presents the summarized data of 2 independent experiments in mean and SD.

with the data of Fiscus' group on the cytotoxic effects of *PKG1* inhibition in ovarian cancer cells.^{7,16} The molecular mechanism of such effects described by the authors was apoptosis⁷ and the inhibition of DNA synthesis/cell proliferation.¹⁶ We also showed a reduced proliferation of PDAC cells after the DT3 treatment. In any case, in our model, the cytotoxic effect of DT3 was mainly due to necrosis. We do not know what is the basis of the abovementioned difference in molecular mechanisms of the cell death due to *PKG1* inhibition, but it cannot be excluded that

this effect could be cancer-type specific. Our data are in contrast with the earlier work of Deguchi et al, which showed the inhibition of growth of colon cancer cells after *PKG* activation. Here, we share the opinion of Fiscus' group¹⁶ who speculated that, in colon cancer cell lines, the growth-inhibitory responses were initiated either by overexpression of *PKG* (especially the *PKG1β* isoform) or by the elevation of *PKG1β* activity (but not basal *PKG* activity). In addition, it can be assumed that the difference in the effects of activated/inhibited *PKG* can be

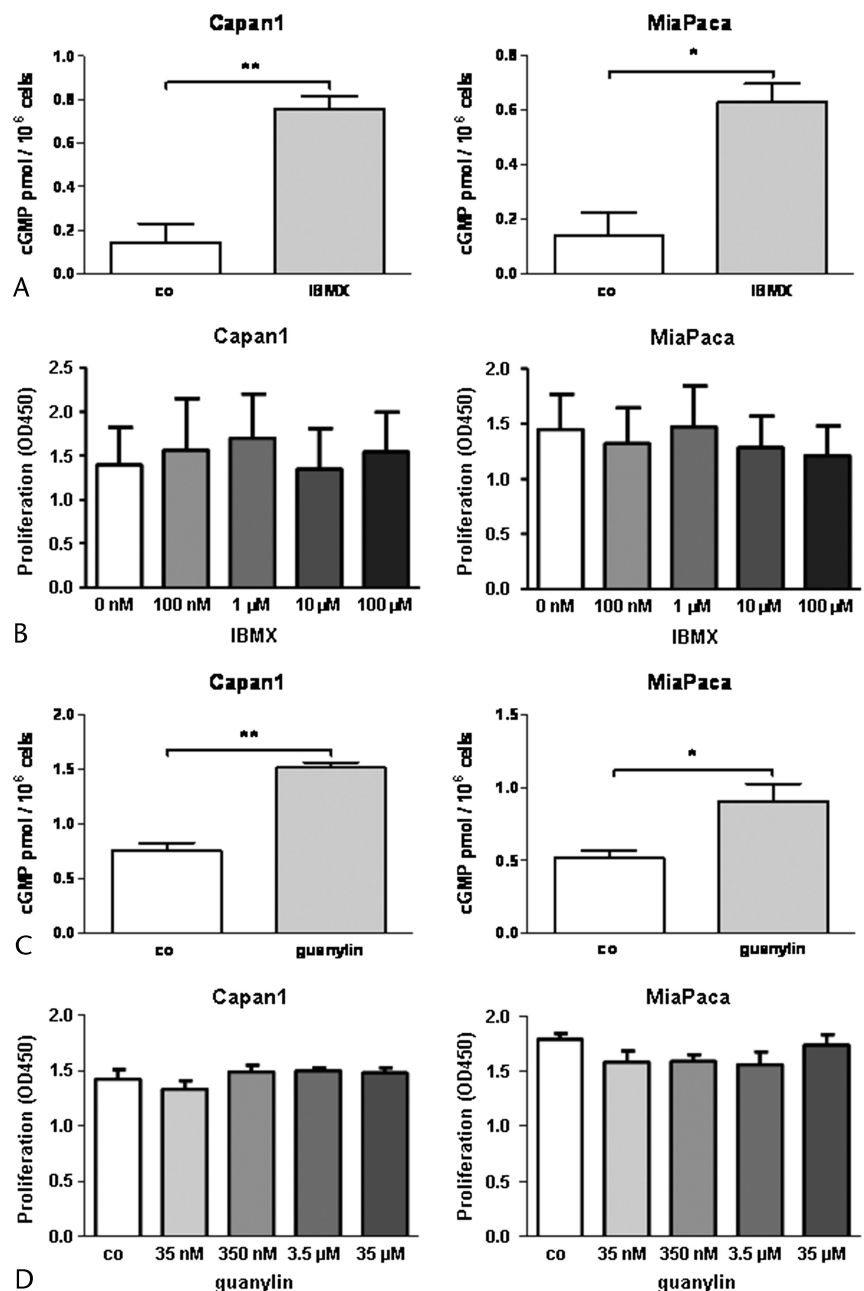


FIGURE 5. PDEs regulate cGMP level and *PKG* activity in PDAC cells but have no influence on viability or proliferation of the cells. A and C, In vitro cGMP measurement in the PDAC cell lines after 24-hour treatment with 10-μM IBMX (A) or with a combination of 3.5-μM guanylin and 10-μM IBMX; co, only 10-μM IBMX (C). B and D, Proliferation analysis (high level of proliferation corresponds to high OD measured photometrically) before and after 48-hour treatment with different concentrations of IBMX (C) or guanylin with 10-μM IBMX (D). The data of 3 independent experiments are presented with SE and analyzed with unpaired 2-tailed *t* test (A and C) and with 1-way ANOVA with the Dunnett post test (B and D). **P* < 0.05 and ***P* < 0.01.

dependent on 1 and 2 isoforms of *PKG*, because Chen et al¹⁸ showed that the activation of *PKG2* inhibits proliferation of gastric cancer cells. Thus, growing evidence shows that the different isoforms of *PKG* may regulate cell proliferation in different ways: *PKG1* α activity promotes cell proliferation, whereas *PKG1* β and *PKG2* inhibit cell proliferation.

It has been recently shown for teratocarcinoma^{24,25} and melanoma¹⁴ cells that the inhibition of *PKG* leads to an increase of calcium concentration in the cells. In addition, for the

teratocarcinoma cells, such calcium elevation increases the nuclear factor of activated T-cells-sensitive transcription.²⁴ This cascade of transcriptional regulation is ruled by the Wnt5a/ Frizzled-2 pathway.^{14,24,25} In the PDAC cells, we found calcium elevation after the DT3 treatment. Thus, this study suggests that this regulatory cascade could also take place in this type of cancer cells. Involvement of the noncanonical Wnt/Frizzled pathway in *PKG1* activation in PDAC cells should be investigated in more detail in the future. The molecular target(s) for

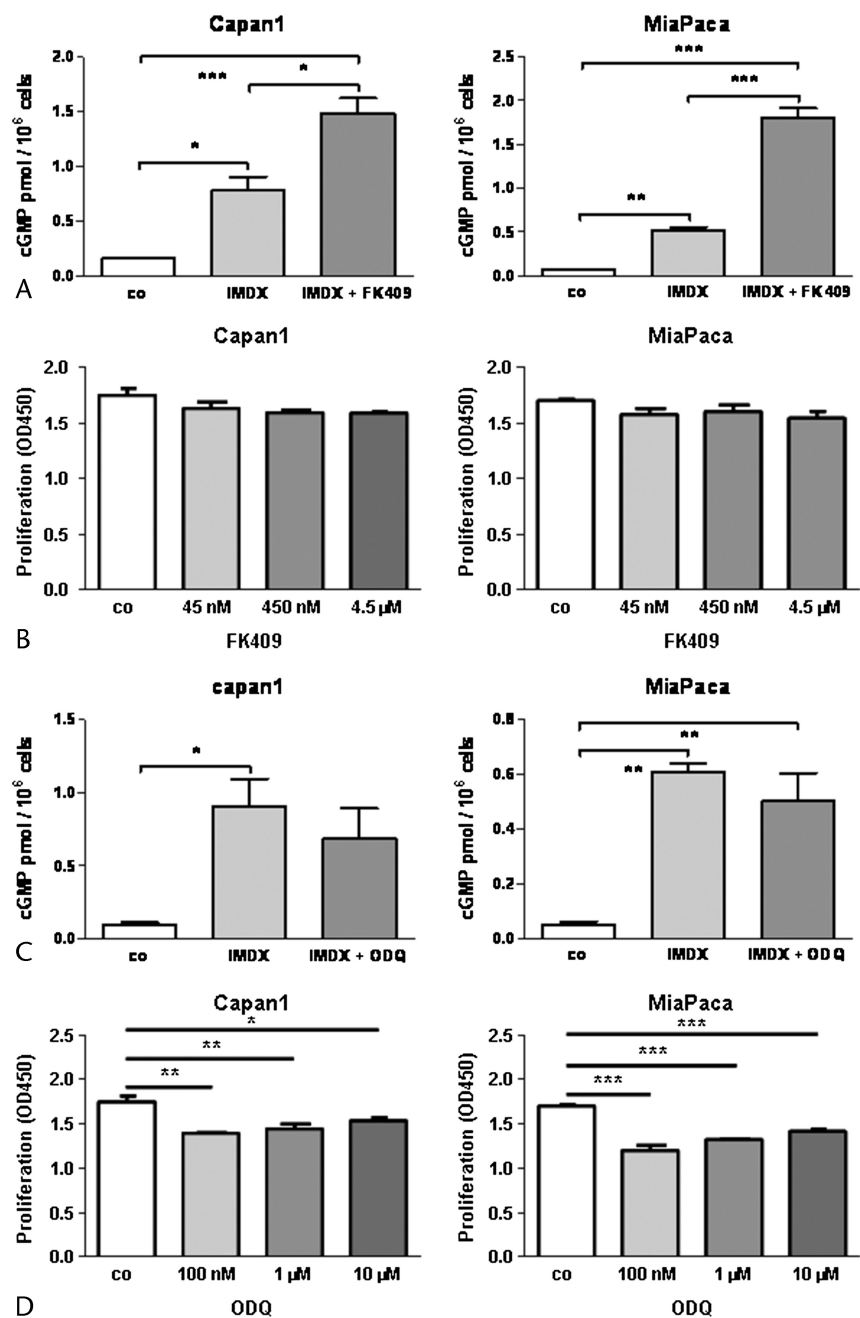


FIGURE 6. NO-GC is involved in the regulation of cGMP level and proliferation of PDAC cells. A and C, In vitro cGMP measurement in the PDAC cell lines after 24-hour treatment with 10-μM IBMX and 450-nM FK409 (A) or 10-μM IBMX and 1-μM ODQ (C). B and D, Proliferation analysis of the PDAC cell lines after 48-hour treatment with 10-μM IBMX and different concentrations of FK409 (B) or 10-μM IBMX and different concentrations of ODQ (D); co, untreated control. Data of 3 independent experiments are presented with SE and analyzed with 1-way ANOVA with the Dunnett post test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

PKG essential for the calcium-nuclear factor of activated T-cells signaling are still not known.

In this work, an attempt has also been made to identify potential molecular substrates of PKG1 in PDAC cells. At present, there is experimental evidence that the molecular partners of PKG1 are cSrc, a member of Src family kinases in ovarian cancer cells,¹⁷ and CREB in nonsmall cell lung cancer cells.¹⁶ The PKG2 employs some members of the MAP kinase family as molecular substrates.^{19,20} Using a proteome profiler array for human kinases and specific MAP kinases with subsequent validation with Western blot, we identified ERK1/2, JNK1, and JNK2/3 as potential targets for PKG1 in PDAC cells. In this work, it has not been asked whether these kinases are involved directly in the regulation of calcium homeostases, cytotoxicity, reduced proliferation, and migration of PDAC cells induced by DT3. These questions will be answered in our future work.

The PKG1 is the direct target for cGMP,² and this enzyme is more commonly activated by NO-GC, the effect of which is mediated by NO.³ However, the cellular cGMP level is generally controlled during cGMP synthesis, not only by NO-GC but also by other GC and, in addition, through the activation of PDE.²⁶ Are PDE and GC involved in regulation of the cGMP-PKG1 cascade in PDAC cells, and if yes, what types are they? We found that PDE (presumably PDE1, PDE2, PDE3, PDE9, and PDE11), NO-GC1, and GC-C are expressed in the cancer cells. The GC-C is the common GC of the gastrointestinal tissues.²⁷ Expression of GC-C has been shown in the exocrine cells of healthy pancreas.²⁸ Therefore, it is not surprising that we found the GC-C expression also in the PDAC cells. Using the endogenous activator of GC-C, guanylin,²⁹ the cGMP level could be elevated in the study's cellular model, which demonstrates the importance of GC-C for maintaining the cGMP homeostasis in cancer cells. Using pharmacologic inhibition or activation of NO-GC, we demonstrated that this soluble GC could regulate the cGMP level also in PDAC cells, influencing proliferation of the cells. Because PDEs, the catabolic arm of the cGMP, are also present in the PDAC cells, we investigated the impact of PDE on the activation of PKG1 using pharmacologic inhibition. As IBMX treatment led to an increase in cGMP concentration, we concluded that PDEs are important for the cGMP homeostasis in PDAC cells and virtually may regulate the PKG1 activity. At the same time, the IBMX treatment did not exert any influence on the cell proliferation. That is why we assume that, even maintaining of the cGMP homeostasis is obliged to NO-GC, GC-C, and PDE, the cytotoxic effect of PKG1 inhibition through cGMP anabolism is much higher than the PDE impact through cGMP catabolism.

In conclusion, based on the cytotoxic activity of the PKG1 inhibition, it is tempting to speculate that the cGMP/PKG signaling pathway might be a target for developing new therapeutic approaches for PDAC.

ACKNOWLEDGMENT

The authors thank Mr M. Herbst for his excellent technical assistance.

REFERENCES

- Sutherland EW, Rall TW. Fractionation and characterization of a cyclic adenosine ribonucleotide formed by tissue particles. *J Biol Chem*. 1958;232:1077–1091.
- Francis SH, Blount MA, Zoraghi R, et al. Molecular properties of mammalian proteins that interact with cGMP: protein kinases, cation channels, phosphodiesterases, and multi-drug anion transporters. *Front Biosci*. 2005;10:2097–2117.
- Francis SH, Busch JL, Corbin JD, et al. cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action. *Pharmacol Rev*. 2010;62:525–563.
- Cary SP, Winger JA, Derbyshire ER, et al. Nitric oxide signaling: no longer simply on or off. *Trends Biochem Sci*. 2006;31:231–239.
- Bryan NS, Bian K, Murad F. Discovery of the nitric oxide signaling pathway and targets for drug development. *Front Biosci*. 2009;14:1–18.
- Thompson WJ, Piazza GA, Li H, et al. Exisulind induction of apoptosis involves guanosine 3',5'-cyclic monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin. *Cancer Res*. 2000;60:3338–3342.
- Fraser M, Chan SL, Chan SS, et al. Regulation of p53 and suppression of apoptosis by the soluble guanylyl cyclase/cGMP pathway in human ovarian cancer cells. *Oncogene*. 2006;25:2203–2212.
- Bosetti C, Bertuccio P, Negri E, et al. Pancreatic cancer: overview of descriptive epidemiology. *Mol Carcinog*. 2012;51:3–13.
- Jemal A, Tiwari RC, Murray T, et al. Cancer statistics, 2004. *CA Cancer J Clin*. 2004;54:8–29.
- Warshaw AL, Fernandez-del Castillo C. Pancreatic carcinoma. *N Engl J Med*. 1992;326:455–465.
- Rosty C, Goggins M. Early detection of pancreatic carcinoma. *Hematol Oncol Clin North Am*. 2002;16:37–52.
- Sengenès C, Bouloumie A, Hauner H, et al. Involvement of a cGMP-dependent pathway in the natriuretic peptide-mediated hormone-sensitive lipase phosphorylation in human adipocytes. *J Biol Chem*. 2003;278:48617–48626.
- Bazhin AV, Schadendorf D, Owen RW, et al. Visible light modulates the expression of cancer-retina antigens. *Mol Cancer Res*. 2008;6:110–118.
- Bazhin AV, Tambor V, Dikov B, et al. cGMP-phosphodiesterase 6, transducin and Wnt5a/Frizzled-2-signaling control cGMP and Ca(2+) homeostasis in melanoma cells. *Cell Mol Life Sci*. 2010;67:817–828.
- Pitari GM, Li T, Baksh RI, et al. Exisulind and guanylyl cyclase C induce distinct antineoplastic signaling mechanisms in human colon cancer cells. *Mol Cancer Ther*. 2006;5:1190–1196.
- Leung EL, Wong JC, Jholls MG, et al. Protein kinase G type Ialpha activity in human ovarian cancer cells significantly contributes to enhanced Src activation and DNA synthesis/cell proliferation. *Mol Cancer Res*. 2010;8:578–591.
- Wong JC, Bathina M, Fiscus RR. Cyclic GMP/protein kinase G type-Ialpha (PKG-Ialpha) signaling pathway promotes CREB phosphorylation and maintains higher c-IAP1, livin, survivin, and Mcl-1 expression and the inhibition of PKG-Ialpha kinase activity synergizes with cisplatin in non-small cell lung cancer cells. *J Cell Biochem*. 2012;113:3587–3598.
- Chen YC, Ren F, Sang JR, et al. Type II cGMP-dependent protein kinase inhibits proliferation of the gastric cancer cell line BGC-823. *Mol Med Report*. 2010;3:361–366.
- Sang J, Chen Y, Jiang L, et al. Type II cGMP-dependent protein kinase inhibits ERK/JNK-mediated activation of transcription factors in gastric cancer cells. *Mol Med Report*. 2012;6:1190–1194.
- Wu Y, Chen Y, Qu R, et al. Type II cGMP-dependent protein kinase inhibits EGF-triggered signal transduction of the MAPK/ERK-mediated pathway in gastric cancer cells. *Oncol Rep*. 2012;27:553–558.
- Fallahian F, Karami-Tehrani F, Salami S, et al. Cyclic GMP induced apoptosis via protein kinase G in oestrogen receptor-positive and -negative breast cancer cell lines. *FEBS J*. 2011;278:3360–3369.

22. Karami-Tehrani F, Fallahian F, Atri M. Expression of cGMP-dependent protein kinase, PKGIalpha, PKGIbeta, and PKGII in malignant and benign breast tumors. *Tumour Biol.* 2012;33:1927–1932.
23. Dostmann WR, Taylor MS, Nickl CK, et al. Highly specific, membrane-permeant peptide blockers of cGMP-dependent protein kinase Ialpha inhibit NO-induced cerebral dilation. *Proc Natl Acad Sci U S A.* 2000;97:14772–14777.
24. Ma L, Wang HY. Suppression of cyclic GMP-dependent protein kinase is essential to the Wnt/cGMP/Ca²⁺ pathway. *J Biol Chem.* 2006;281:30990–31001.
25. Ma L, Wang HY. Mitogen-activated protein kinase p38 regulates the Wnt/cyclic GMP/Ca²⁺ non-canonical pathway. *J Biol Chem.* 2007;282:28980–28990.
26. Lucas KA, Pitari GM, Kazerounian S, et al. Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev.* 2000;52:375–414.
27. Basu N, Arshad N, Visweswariah SS. Receptor guanylyl cyclase C (GC-C): regulation and signal transduction. *Mol Cell Biochem.* 2010;334:67–80.
28. Kulaksiz H, Cetin Y. Uroguanylin and guanylate cyclase C in the human pancreas: expression and mutuality of ligand/receptor localization as indicators of intercellular paracrine signaling pathways. *J Endocrinol.* 2001;170:267–275.
29. Currie MG, Fok KF, Kato J, et al. Guanylin: an endogenous activator of intestinal guanylate cyclase. *Proc Natl Acad Sci U S A.* 1992;89:947–951.