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PKD, PKD2, and p38 MAPK Mediate Hsp27 Serine-82 Phosphorylation Induced by Neurotensin in Pancreatic Cancer PANC-1 Cells

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Abstract It is widely recognized that Hsp27 is a downstream substrate of the p38 MAPK cascade whereas the role of PKD family members in mediating receptor-stimulated Hsp27 Ser-82 phosphorylation has not been evaluated. Here, we show that neurotensin induced a rapid and striking increase in Hsp27 Ser-82 phosphorylation in PANC-1 cells, which was closely correlated with stimulation of activation loop phosphorylation of PKDs and p38 MAPK Thr180/Tyr182 phosphorylation. Treatment of PANC-1 cells with either the selective PKC inhibitor GF-I or the p38 MAPK inhibitor SB202190 partially reduced neurotensin-induced Hsp27 Ser-82 phosphorylation. However, treatment of the cells with a combination of GF-I and SB202190 virtually abolished neurotensin-induced Hsp27 Ser-82 phosphorylation. Overexpression of PKD in stably transfected PANC-1 cells increased the magnitude and prolonged the duration of Hsp27 Ser-82 phosphorylation in response to neurotensin. Either PKD or PKD2 gene silencing utilizing siRNAs targeting distinct PKD or PKD2 sequences reduced neurotensin-stimulated Hsp27 Ser-82 phosphorylation, but cotransfection of siRNAs targeting both, PKD and PKD2, markedly decreased neurotensin-induced Hsp27 Ser-82 phosphorylation. Knockdown of PKD and PKD2 abolished Hsp27 phosphorylation in cells treated with SB202190. Thus, neurotensin induces Hsp27 Ser-82 phosphorylation through p38 MAPK- and PKC/PKD-dependent pathways in PANC-1 cells. Our results demonstrate, for the first time, that neurotensin induces a striking increase in Hsp27 phosphorylation on Ser-82 in PANC-1 cells through convergent p38 MAPK, PKD, and PKD2 signaling. J. Cell. Biochem. 103: 648–662, 2008.

Key words: G protein-coupled receptors; protein kinase C; phorbol esters

The small heat shock proteins (Hsps), including human Hsp27 and mouse Hsp25, are widely expressed proteins that play an important role in the regulation of many cellular functions in response to stress, cytokines, growth factors, and G protein-coupled receptor (GPCR) agonists. These include refolding of denatured proteins by acting as a molecular chaperone [Georgopoulos and Welch, 1993], regulation of actin and intermediate filament cytoskeleton [Guay et al., 1997; Schafer et al., 1998; Perng et al., 1999; Geum

Abbreviations used: Hsp, heat shock protein; PKD, protein kinase D; PKC, protein kinase C; p38 MAPK, p38 MAP kinase; MK2/MAPKAPK2, MAP kinase-activated protein kinase-2; GPCR, G protein-coupled receptor; G proteins, guanine nucleotide-binding regulatory proteins; PLC, phospholipase C; PDBu, phorbol 12,13-dibutyrate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; siRNA, small interfering RNA.

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et al., 2002; Evgrafov et al., 2004], epithelial cell–cell adhesion [Berkowitz et al., 2006], cell cycleprogression [Parcellier et al., 2006], cell migration [Rousseau et al., 1997; Tangkijvanich et al., 2002; McMullen et al., 2005] and pro-inflammatory gene expression [Alford et al., 2007]. The level of Hsp27 is markedly increased in many cancer cells and its expression contributes to the malignant properties of these cells, including increased tumorigenicity and treatment resistance [Rocchi et al., 2004; Chen et al., 2004a; Shin et al., 2005; McCollum et al., 2006; Xu and Bergan, 2006] and inhibition of apoptosis [Bruey et al., 2000; Benn et al., 2002; Paul et al., 2002]. Since many of the functions attributed to Hsp27 require its phosphorylation, especially at Ser-82 [Guay et al., 1997; Geum et al., 2002; Kubisch et al., 2004; Berkowitz et al., 2006; Zheng et al., 2006], the identification of the protein kinases that mediate Hsp27 phosphorylation is critical for understanding the regulation of its multiple cellular functions and for the identification of potential targets for therapeutic intervention.

It is recognized that Hsp27 is a downstream substrate of the p38 MAPK cascade [Widmann et al., 1999; Chang and Karin, 2001] and activation of p38 MAPK leads to Hsp27 phosphorylation that changes its intracellular distribution [Geum et al., 2002], quaternary structure [Kato et al., 1994; Lambert et al., 1999; Vertii et al., 2006], and interaction with other proteins [Park et al., 2003; Zheng et al., 2006]. In response to stressors, GPCR agonists or cytokines, p38 MAP kinase is activated by dual phosphorylation on a Thr-Gly-Tyr motif by MKK3 and MKK6 [Schaeffer and Weber, 1999; Widmann et al., 1999; Ono and Han, 2000]. Activated p38α, the most studied member of the p38 MAPKs, phosphorylates MAP kinase-activated protein kinase-2 (MAPKAPK2 or MK2), which in turn phosphorylates Hsp27 [Stokoe et al., 1992; Rouse et al., 1994]. In addition to the well established p38/MK2/Hsp27 pathway, other studies demonstrated that tumor promoting phorbol esters also stimulate the phosphorylation of Hsp27 [Welch, 1985; Faucher et al., 1993; Kato et al., 1994; Minowada and Welch, 1995; Maizels et al., 1998; Doppler et al., 2005; Shin et al., 2005] through a pathway apparently independent of p38/MK2/Hsp27 [Maizels et al., 1998]. It remains unclear whether phorbol esters induce Hsp27 phosphorylation via direct PKC-mediated phosphorylation or through a protein kinase(s) that lies downstream of PKCs.

PKD (also known initially as PKCμ) is a serine/threonine protein kinase with structural, enzymological and regulatory properties different from the PKC family members [Johannes et al., 1994; Valverde et al., 1994]. PKD can be activated in intact cells by multiple stimuli, including tumor promoting phorbol esters, GPCR agonists, growth factors, T and B antigen-receptor engagement and oxidative stress [reviewed in Reference Rozengurt et al., 2005]. In many cases, rapid PKD activation is mediated by PKC-dependent phosphorylation of Ser-744 and Ser-748 within the activation loop of the PKD catalytic domain [Iglesias et al., 1998; Waldron et al., 1999, 2001; Waldron and Rozengurt, 2003; Yuan et al., 2003; Rey et al., 2004]. These findings indicate that PKD lies downstream of PKCs in a signal transduction pathway activated by multiple extracellular signals [Rozengurt et al., 2005]. PKD are increasingly implicated in the regulation of multiple cellular functions and several substrates, including Kidins220 [Iglesias et al., 2000], RIN1 [Wang et al., 2002] and class II histone deacetylases [Vega et al., 2004; Matthews et al., 2006], have been identified. Recently, PKD has been implicated in the phosphorylation of Hsp27 on Ser-82 in HeLa cells exposed to oxidative stress [Doppler et al., 2005], a condition shown to activate PKD [Waldron and Rozengurt, 2000; Storz et al., 2004; Waldron et al., 2004]. Although an extensive literature illustrates that oxidative stress also potently activates the p38 MAPK/MK2 pathway in a variety of systems [Huot et al., 1997; Clerk et al., 1998; Dasari et al., 2006; Ito et al., 2006; Kefaloyianni et al., 2006], the relative contribution of each of these pathways (i.e. p38/MK2 and PKD) to Hsp27 Ser-82 phosphorylation induced by oxidative stress in HeLa cells was not evaluated [Doppler et al., 2005].

Ductal adenocarcinoma of the pancreas is an extremely lethal disease, with an overall 5-year survival rate of only 3–5%. Its refractoriness to currently used therapeutics renders it nearly 100% lethal, making it the fourth leading cause of cancer fatalities in both men and women [Jemal et al., 2002]. Novel therapeutic and diagnostic strategies for this devastating disease are urgently needed. Recent studies demonstrated that the GPCR agonist

neurotensin induces PKC/PKD activation [Guha et al., 2002; Rey et al., 2003b], rapid and transient ERK pathway activation [Ryder et al., 2001; Guha et al., 2003], DNA synthesis [Ryder et al., 2001; Guha et al., 2003] and anchorage-independent growth [Guha et al., 2003; Kisfalvi et al., 2005] in PANC-1 cells, an extensively studied model of ductal pancreatic adenocarcinoma cells. Interestingly, neurotensin-binding sites have been detected by autoradiography of frozen sections in ~75% of human pancreatic cancer specimens [Reubi et al., 1998] and the expression of mRNA for the neurotensin receptor is markedly increased in ductal pancreatic cancer samples [Ehlers et al., 2000; Elek et al., 2000; Wang et al., 2000]. It is therefore important to characterize neurotensin-mediated signal transduction pathways in human pancreatic cancer cells.

In the present study, we demonstrate that neurotensin induces a striking increase in Hsp27 phosphorylation on Ser-82 through convergent p38 MAPK, PKD and PKD2 signaling in pancreatic cancer PANC-1 cells.

EXPERIMENTAL PROCEDURES

Cell Culture

PANC-1, obtained from the American Type Culture Collection, is a less well-differentiated line established from human ductal pancreatic adenocarcinoma. PANC-1 cells were grown in DMEM (Life Technologies, Inc.) with 4 mM of glutamine, 1 mM of Na-pyruvate, and 10% fetal bovine serum (FBS) at 37°C with a humidified atmosphere containing 10% CO2. For experimental purposes, cells were plated in media containing 10% FBS and were allowed to grow to confluency (5–7 days) and then washed and changed to serum-free media for 4–6 h prior to the experiments.

Generation of Clonal PANC-1 Cells That Inducibly Express PKD

Generation of the inducible expression plasmid, pINDsp1-PKD, has been described previously [Hurd and Rozengurt, 2001]. PANC-1 cells were cultured to 40–60% confluency on 100 mm plates and then dually transfected with 6 μg pVgRxR and pINDsp1-PKD using lipofectin reagent according to instructions from Gibco-BRL. Cells were cultured 48 h, trypsinized, and plated at a density of 3×10^4 cells/100 mm plate in dually selective media containing 400 μg/ml of both zeocin and hygromycin-B. Subsequently, cells were continuously maintained in the presence of these antibiotics. Resistant clones were selected after approximately 14 days and sub-cultured in 35 mm plates. At approximately 50% confluency, they were trypsinized and transferred to 100 mm plates. To screen for inducible PKD expression, clonal cells were passaged, cultured to 60% confluency in 60 mm plates, and then treated with 1 μM ponasterone-A or ethanol carrier for 24 h. Cells were lysed in 500 μl SDS sample buffer and analyzed by Western blot for induced expression of PKD with PKD C-20 antibody. Clonal cells were cultured in low salt (without sodium pyruvate) DMEM supplemented with 400 μg/ml zeocin and hygromycin-B supplemented with 10% FBS. For experimental purposes, cells were plated in this media in 12-well dishes and allowed to grow to confluency (5–7 days). To induce PKD expression during experiments, 2 μl of 1 mM ponasterone-A or ethanol vehicle was added directly to cells cultured in 1 ml media (0.2% ethanol, 2 μM ponasterone-A, final concentration) 22–24 h prior to treatment.

Western Blot Analysis

Serum-starved cultures of PANC-1 cells grown on 35-mm or 12-well dishes were treated as described in individual experiments. The cells were then lysed in 50 mM Tris/HCl pH 7.6, 2 mM EGTA, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM 4-(2-aminoethyl)-benzenesulfonyl
fluoride hydrochloride and 1% Triton X-100 (lysis buffer A). Cell lysates were clarified by centrifugation at 15,000*g* for 10 min at 4°C. The supernatant of the cell lysate samples was solubilized by boiling in 2× SDS–PAGE sample buffer (200 mM Tris/HCl, pH 6.8, 0.1 mM sodium orthovanadate, 1 mM EGTA, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol). Following SDS–PAGE on 10% gel, proteins were transferred to Immobilon-P membranes (Millipore) and blocked by 3–6 h incubation with 5% non-fat dried milk in PBS, pH 7.2. Membranes were incubated overnight at 4°C with the respective primary antibody, at a dilution of 1:500 to 1:1,000 in PBS containing 5% nonfat dried milk and 0.1% Tween 20, including the antibody that specifically recognizes the different PKD isoforms (PKD C-20

and PKD2 antibody), the anti-phospho-antibody that specifically recognizes the phosphorylated state of activation loop serines of the three PKD isoforms (PKD: Ser-744/Ser748; PKD2: Ser-706/Ser-710; PKD3: Ser-731/Ser-735), the antibody that recognizes the phosphorylated state of Ser-82 of Hsp27, or the monoclonal antibody that specifically recognizes the phosphorylated state of Thr180/tyr182 p38 MAPK. Immunoreactive bands were visualized using horseradish peroxidase-conjugated anti-rabbit IgG and subsequent enhanced chemiluminescence detection reagents (Amersham). Autoradiograms were scanned using a Laser Densitometer and the labeled bands were quantified using ImageQuant 5.2 software program (Molecular Dynamics).

Knockdown of PKD and PKD2 Via siRNA

To inhibit PKD and PKD2 protein expression, subconfluent cultures (~ 40–60% confluence) of PANC-1 cells were transfected with small interfering RNA (siRNA). PKD and PKD2 siRNAs from Dharmacon (Chicago, IL) were used. The sequence for human PKD siRNAs (sense strands) are as follows: GAAGAGAUGUAGCUAUUAAUU; GAAAGAGUGUUUGUUGUUAUU; GAAUGCAGCUUUCAUGUAUUU; and GGAAGGAAAUAUCUCAUGAUU. The sequence for human PKD2 siRNA oligonucleotides (sense strands) are as follows: UGAGACACCUUCACUUCAUU, CAAGAACAUUGUCCACUGUUU, and GGAAGAUGGGAGAGCGAUAUU. si*CONTROL* Non-Targeting siRNA Pool (pool of 4 duplexes) was purchased from Dharmacon and used as a control. Transfection of duplex siRNAs was performed using Trans IT-TKO Reagent (Mirus, Madison, WI) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were used for experiments and subsequent Western blot analysis.

Materials

The ecdysone inducible expression kit, pINDsp1 plasmid, ponasterone-A, hygromycin-B, and zeocin were purchased from Invitrogen. Bisindolylmaleimide I (also known as GF-1 or GF 109203X) and SB202190 were purchased from Calbiochem. Neurotensin, PDBu, and anisomycin were obtained from Sigma Chemical Co. The PKD2 polyclonal antibody used for Western blotting was obtained from Upstate (Lake Placid, NY). Antibody for PKD C-20 was obtained from Santa Cruz Biotechnology (Palo Alto, CA). Phosphoserine 744/748 PKD antibody, anti-phospho-p38 MAPK (Thr180/Tyr182) mAb, and anti-Hsp27 (G31) mAb were obtained from Cell Signaling Technology (Beverly, MA). Anti pS82 Hsp27 antibody was obtained from ALEXIS Biochemicals (San Diego, CA). Other items were from standard suppliers or as indicated in text.

RESULTS

Neurotensin Induces Phosphorylation of Hsp27 and Activation of PKD and p38 MAPK in a Time- and Concentration-Dependent Manner

To determine whether neurotensin stimulation induces Hsp27 phosphorylation at Ser-82 in PANC-1 cells, serum-depleted cultures of these cells were stimulated with this GPCR agonist for various times (1–120 min), lysed and the extracts were analyzed by Western blotting using a site-specific antibody that detects the phosphorylated state of Hsp27 at Ser-82. As shown in Figure 1, Hsp27-Ser(P)-82 immunoreactivity was virtually absent before neurotensin stimulation, indicating that Hsp27 phosphorylation on Ser-82 was very low in cultures of PANC-1 cells. Upon neurotensin stimulation, Hsp27-Ser(P)-82 immunoreactivity of a single protein band migrating in SDS–PAGE at the expected apparent molecular mass for Hsp27 (27 kDa) increased dramatically in a time-dependent manner. An increase in Hsp27 phosphorylation at Ser-82 was detected as early as 1 min after the addition of the agonist, reached a maximum within 5-10 min, and declined toward base-line levels within 120 min. The maximal increase of Hsp27 phosphorylation at Ser-82 induced by neurotensin was ~10-fold, as compared with the unstimulated level. Immunoblotting with anti-Hsp27 antibody verified that similar loading of the gels after different times of neurotensin treatment (Fig. 1A).

Interestingly, neurotensin also stimulated a rapid activation of PKD and p38 MAPK family members, as shown by Western blotting with antibodies that detected the phosphorylated state of the activation loop serines of PKD family members (Ser-744 and Ser-748 in PKD) and of the threonine and tyrosine residues (Thr-Gly-Tyr) of p38 family members. Thus, neurotensin-stimulated striking Hsp27

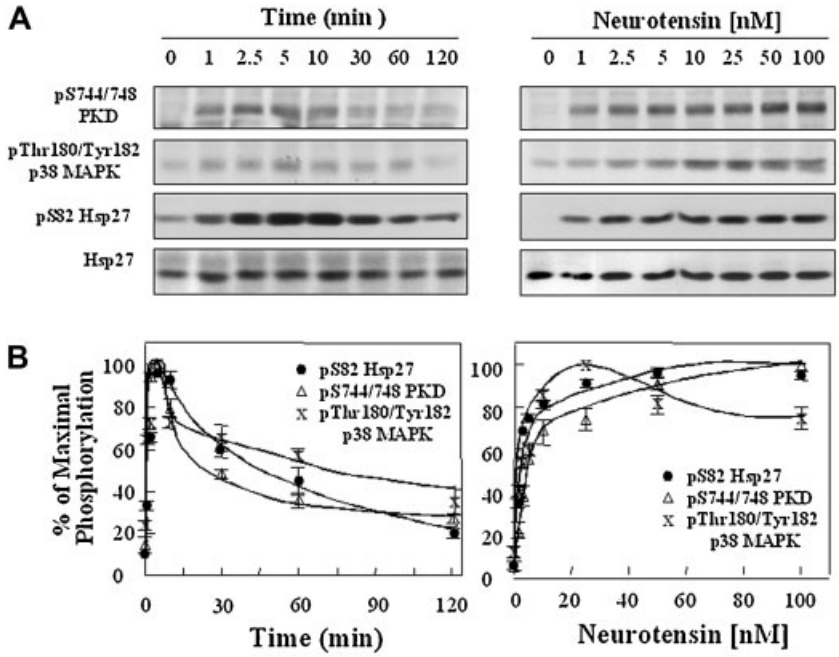

Fig. 1. Neurotensin induces Hsp 27 Ser-82 phosphorylation, PKD activation loop phosphorylation and p38 MAPK Thr180/Tyr182 phosphorylation in PANC-1 cells. Confluent PANC-1 cells were washed and incubated in serum-free medium (DMEM) for 4–6 h. **A**, time course and dose response of neurotensin (NT)-induced PKD activation loop phosphorylation, p38 MAPK Thr180/Tyr182 phosphorylation, and Hsp27 Ser-82 phosphorylation. Serum-starved cells were stimulated with 50 nM neurotensin for the indicated time periods or with increasing concentrations of neurotensin for 10 min at 37°C and lysed. Cell extracts were fractionated by SDS–PAGE followed by Western blot analysis as described in ''Experimental Procedures Section,'' using PKD pS744/748 antibody (which detects the phosphorylated state of the activation loop of all members of the PKD family), pThr 180/Tyr 182 p38 MAPK antibody, or pS82 Hsp27 antibody. The Western blots shown are representatives of at least three independent experiments. **B**: The graphs show the means (n = 3) of the level of Hsp27 Ser 82 phosphorylation (●), PKD Ser 744/748 phosphorylation (Δ), and p38 MAPK Thr180/Tyr182 phosphorylation (X) obtained from scanning densitometry of the Western blot results and are expressed as percentages of the maximal increase in phosphorylation obtained by stimulation with 50 nM NT for 5–10 min at 37°C.

phosphorylation that coincided with robust increases in the phosphorylation of both PKD and p38 MAPK pathways in PANC-1 cells.

Neurotensin-stimulated Hsp27 Ser-82 phosphorylation and activation loop phosphorylation of PKD and p38 in a concentration-dependent manner (Fig. 1). Half-maximal stimulatory effects of Hsp 27 phosphorylation, PKD and p38 activation were achieved at ~3 nM neurotensin. As a first step to identify the pathways that mediate Hsp 27 Ser-82 phosphorylation in response to neurotensin, we next examined whether stimulation of PKC/PKD or p38 MAPK cascades induces Hsp 27 Ser-82 phosphorylation in PANC-1 cells.

Stimulation of Hsp27 Phosphorylation by Phorbol Esters and Anisomycin in PANC-1 Cells

In order to determine whether stimulation of PKCs leads to an increase in Hsp27 Ser-82 phosphorylation in PANC-1 cells, cultures of these cells were treated with the tumor promoting agent phorbol-12, 13-dibutyrate (PDBu), a direct activator of PKC, for various times. As shown in Figure 2A, treatment with PDBu caused a striking, time-dependent, increase in Hsp27 phosphorylation at Ser-82. The time-course of Hsp27 phosphorylation was similar to the increase in the activation loop phosphorylation of PKD family members, evaluated with the pS744/748 antibody (Fig. 2A). Furthermore, PDBu stimulated Hsp27 Ser-82 phosphorylation and PKD family activation loop phosphorylation at similar concentrations (Fig. 2A).

In response to stressors, p38 MAP kinase is activated by dual phosphorylation on a Thr-Gly-Tyr motif by MKK3 and MKK6 [Widmann et al., 1999; Ono and Han, 2000]. Activated p38 phosphorylates and activates MK2, which in turn phosphorylates Hsp27 at Ser-82 [Stokoe et al., 1992; Rouse et al., 1994]. Accordingly, exposure to the protein synthesis inhibitor anisomycin strikingly stimulated Hsp27 Ser-82 phosphorylation in

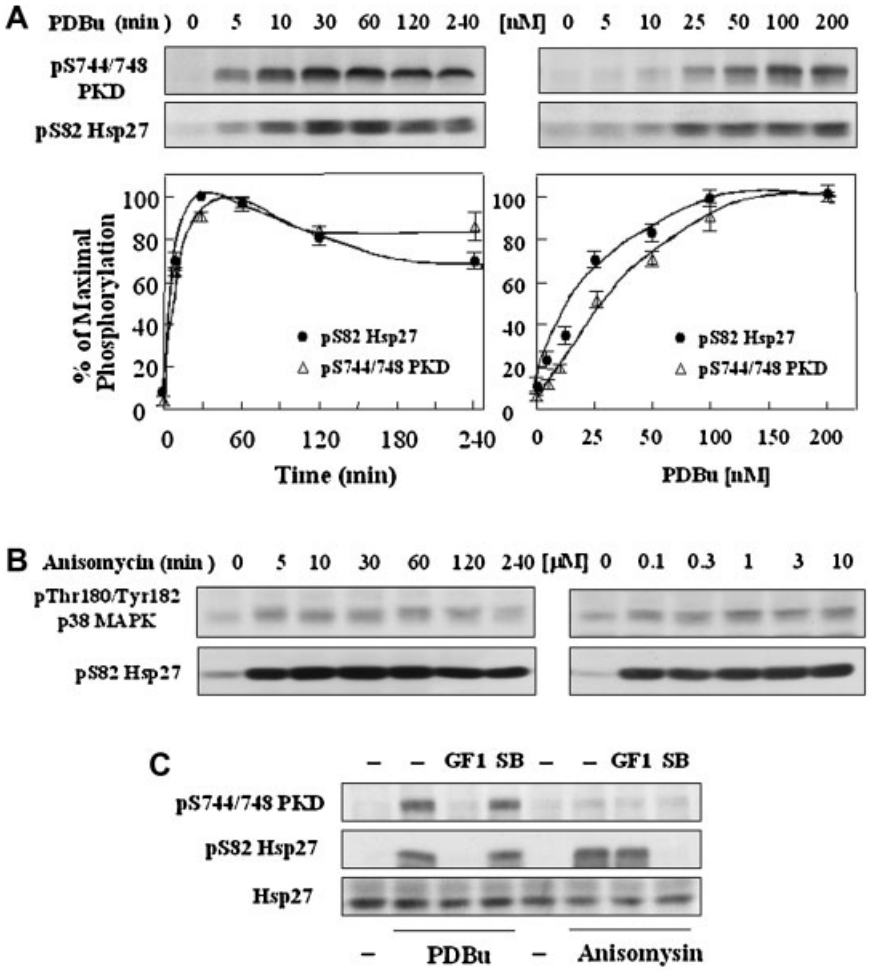

Fig. 2. Treatment with PDBu or anisomycin stimulates Hsp27 Ser-82 phosphorylation in PANC-1 cells. Confluent PANC-1 cells were washed and incubated in serum-free medium (DMEM) for 4–6 h. **A**: time-course (**left**) and dose response (**right**) of PDBu-induced PKD family activation loop phosphorylation and Hsp27 Ser-82 phosphorylation. Serum-starved cells were stimulated with 200 nM PDBu for the indicated time periods or with increasing concentrations of PDBu for 10 min at 37°C and then lysed. The cell extracts were analyzed by Western blotting using PKD pS744/748 antibody and pS82 Hsp27 antibody. The Western blots shown are representatives of at least three independent experiments. The curve graphs in A shows the means (n = 3) of the level of PKD Ser 744/748 phosphorylation (Δ) and Hsp27 Ser-82 phosphorylation (●), obtained from scanning densitometry and are expressed as percentages of the maximal increase in phosphorylation obtained with 200 nM PDBu for 10 min at 37°C. **B**: time-course and dose response of anisomycin-induced p38 MAPK phosphorylation and Hsp27 Ser-82 phosphorylation. Serum-starved cells were exposed to 3 μM anisomysin for the indicated time periods or increased concentrations at 37°C. Cell lysates were analyzed by Western blotting using pThr180/Tyr182 p38 MAPK antibody or pS82 Hsp27 antibody. **C**: Effect of GFI or SB202190 on HSP 27 Ser-82 phosphorylation induced by either PDBu or anisomycin. PANC-1 cells were treated for 1 h with 3 μM GF-1 or 2 μM SB202190. The cultures were subsequently stimulated with either 200 nM PDBu or 3 μM anisomycin for 10 min at 37°C and lysed. The phosphorylation of PKD or Hsp27 was determined by Western blot analysis using the pS744/748 PKD and pS82 Hsp27 antibodies, respectively. The Western blots shown are representative of at least two independent experiments.

a time- and concentration-dependent manner in PANC-1 cells (Fig. 2B).

The results presented in Figure 2 suggest that Hsp27 phosphorylation on Ser-82 can be induced through PKC- and p38 MAPK-dependent pathways in PANC-1 cells. In order to corroborate this interpretation, PANC-1 cells were treated with the selective PKC inhibitor GF I (also known as GF 109203X or bisindolylmaleimide I) or the selective p38 inhibitor SB202190 prior to exposure to PDBu or anisomycin. Treatment of the cells with GF I prevented PKD activation loop phosphorylation and potently blocked Hsp27 Ser-82 phosphorylation induced by PDBu but it did not interfere with the increase in Hsp27 phosphorylation produced by anisomycin (Fig. 2C). Reciprocally, treatment with SB202190 completely blocked

the increase in Hsp27 Ser-82 phosphorylation induced by anisomycin but did not affect Hsp27 Ser-82 phosphorylation or PKD loop phosphorylation produced by PDBu. These results indicate that Hsp27 phosphorylation at Ser-82 can be induced via separate PKC-dependent and p38 MAP kinase-dependent pathways in PANC-1 cells.

PKC-Dependent and p38 MAPK-Dependent Pathways in the Stimulation of Hsp27 Phosphorylation Induced by Neurotensin in PANC-1 Cells

The results presented in Figures 1 and 2, prompted us to determine the role of PKC- and p38 MAP kinase-dependent pathways in mediating Hsp27 Ser-82 phosphorylation induced by neurotensin in PANC-1 cells. Cultures of these cells were treated without or with 3μM GFI, 2μM SB202190 or with a combination of both inhibitors and then challenged with neurotensin for various times (5–240 min). Treatment with GFI, at a concentration that completely prevented PKD family activation, as shown by the total inhibition of PKD loop phosphorylation, markedly decreased Hsp27 Ser-82 phosphorylation at earlier times of neurotensin stimulation (~70% inhibition at 30 min) and virtually abolished Hsp27 phosphorylation at later times (120 and 240 min). Treatment of parallel cultures with SB202190, at a concentration that did not affect PKD family activation, also attenuated Hsp27 Ser-82 phosphorylation in response to neurotensin (Fig. 3). Interestingly, treatment with both GFI and SB202190

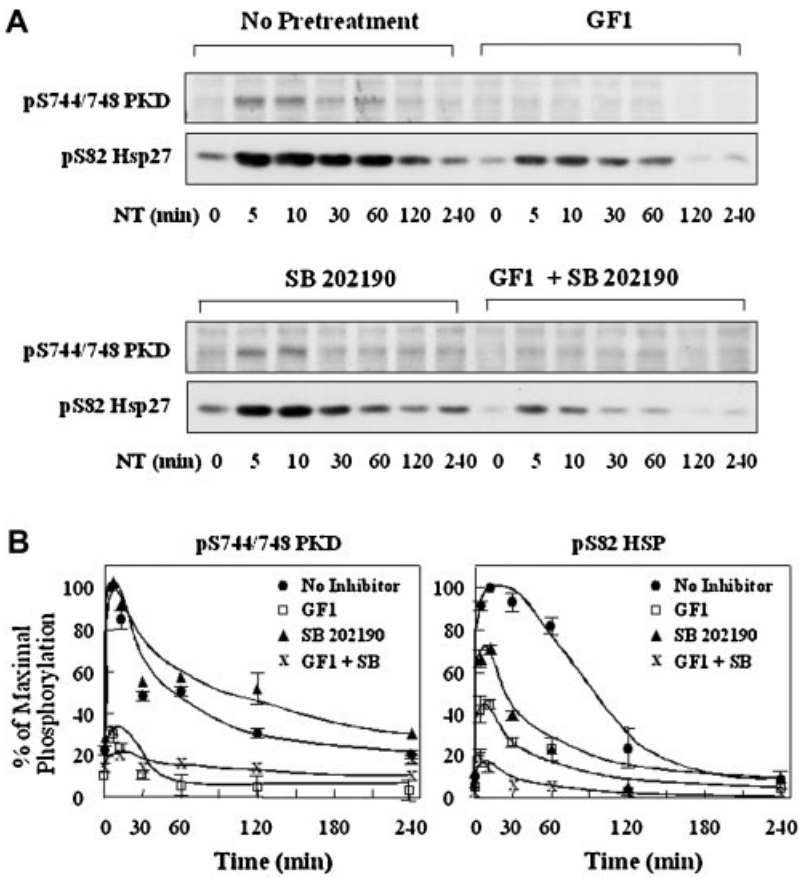

Fig. 3. Neurotensin stimulates Hsp27 Ser-82 phosphorylation through both p38 MAPK- and PKC/PKD-dependent pathways in PANC-1 cells. **A**: Confluent PANC-1 cells were washed and incubated in serum-free medium (DMEM) for 4-6 h. The serum-starved PANC-1 cells were incubated for 1 h with the PKC inhibitor GF-1 (3 μM), the p38 MAPK inhibitor SB202190 (2 μM), or the combination of GF-1 (3 μM) and SB202190 (2 μM). Control cells received equivalent amounts of solvent. The cultures were subsequently stimulated with 50 nM neurotensin (NT) for the indicated time periods at 37°C and lysed. Cell extracts were analyzed by Western blotting using pS744/748 PKD antibody and pS82 Hsp27 antibody. **B**: The values in the plots represent the means (n = 3) of the level of PKD pS744/748 phosphorylation (**left panel**) and Hsp27 pS82 phosphorylation (**right panel**) obtained from scanning densitometry and are expressed as percentages of the maximal increase in phosphorylation obtained by cell stimulation with 50 nM neurotensin for 10 min at 37°C.

strikingly reduced neurotensin-induced Hsp27 phosphorylation, virtually abolishing it after 30 min of agonist stimulation (Fig. 3).

The results shown in Figure 3 suggest that Hsp27 Ser-82 phosphorylation induced by neurotensin is mediated by both, PKC-dependent and p38 MAP kinase-dependent pathways in PANC-1 cells. While the role of p38 MAP kinase/MK-2 pathway in promoting Hsp27 phosphorylation is well established, the precise element(s) implicated in the PKC-dependent pathway, the predominant pathway in neurotensin-stimulated PANC-1 cells, remains incompletely defined. We next examined whether PKDs play a role in mediating PKC-dependent Hsp27 Ser-82 phosphorylation in these cells.

Overexpression of PKD Potentiates Hsp27 Phosphorylation Induced by PDB or Neurotensin

We have developed a model system in which PKD can be inducibly expressed under the control of a modified ecdysone receptor enhancer/promoter in stably transfected PANC-1 cells [No et al., 1996; Hurd and Rozengurt, 2001; Hurd et al., 2002; Hurd and Rozengurt, 2003]. In order to determine whether overexpression of PKD enhances the ability of PDBu to promote Hsp27 Ser-82 phosphorylation, PANC-1 cells were treated with the ecdysone analog ponasterone-A for 22 h to induce PKD and then with increasing concentrations of PDBu (5–200 nM) for 10 min. Whole cell lysates were prepared and analyzed by Western blotting for Hsp27 Ser-82 phosphorylation and induced expression of PKD phosphorylated at the activation loop. As shown in Figure 4A, PDBu-induced Hsp27 Ser-82 phosphorylation was markedly enhanced in cells treated with ponasterone-A to induce PKD overexpression. These results imply that PKD mediates PDBu-induced Hsp27 phosphorylation at Ser-82.

Next, we determined whether PKD overexpression enhances Hsp27 Ser-82 phosphorylation stimulated by the GPCR agonists neurotensin. PANC-1 cells that inducibly express PKD were treated with increasing concentrations of ponasterone-A for 22 h and then with 50 nM neurotensin for 10 min. As shown in Figure 4B, induction of PKD expression by increasing concentrations of ponasterone caused a further enhancement of neurotensin-induced Hsp27 phosphorylation at Ser-82. These results suggest that PKD expression facilitates neurotensin-induced Hsp 27 phosphorylation.

In order to substantiate that PKD overexpression enhances Hsp27 phosphorylation in response to neurotensin, PANC-1 cells treated without or with ponasterone-A for 22 h to induce PKD, were subsequently stimulated with 50 nM neurotensin for various times (Fig. 5A) or with increasing concentrations of neurotensin for 10 min (Fig. 5B). As shown in Figure 5, PKD overexpression markedly augmented neurotensin-induced Hsp27 phosphorylation (~2-fold at 30 min) and strikingly prolonged the duration of Hsp27 Ser-82 phosphorylation in response to neurotensin stimulation in PANC-1 cells. Similarly, PKD overexpression enhanced Hsp27 phosphorylation at Ser-82 in cells stimulated with various concentrations of neurotensin.

Knockdown of PKD and PKD2 Abrogates Neurotensin-Induced Hsp27 Ser-82 Phosphorylation in PANC-1 Cells Treated With SB202190

PKD is the founding member of a new family of serine/threonine protein kinases, including PKD2 and PKD3 [reviewed in Reference Rozengurt et al., 2005]. These kinases, which share similarities in overall structure, primary sequence, and enzymological properties [Hayashi et al., 1999; Sturany et al., 2001; Rey et al., 2003b], are also activated through PKC [Sturany et al., 2002; Rey et al., 2003a,b; Yuan et al., 2005; Chiu et al., 2007]. In order to determine whether endogenous PKD family members play a role in mediating neurotensin-induced Hsp27 phosphorylation at Ser-82 in PANC-1 cells, cultures of these cells were trasnfected with either non-targeting siRNA or with siRNAs targeting PKD or PKD2. Parallel cultures were transfected with a combination of siRNAs targeting PKD and PKD2. Each set of cultures was treated in the absence or in the presence of 2 μM SB202190 for 1 h and then stimulated with 50 nM neurotensin, as indicated in Figure 6. The cultures were then lysed and the extracts were analyzed by Western blotting with antibodies directed against PKD, PKD2, Hsp27 pSer-82 and Hsp27.

As illustrated in Figure 6, PKD and PKD2 protein expression was markedly decreased by transfection of siRNAs targeting distinct PKD and PKD2 sequences, as compared with the

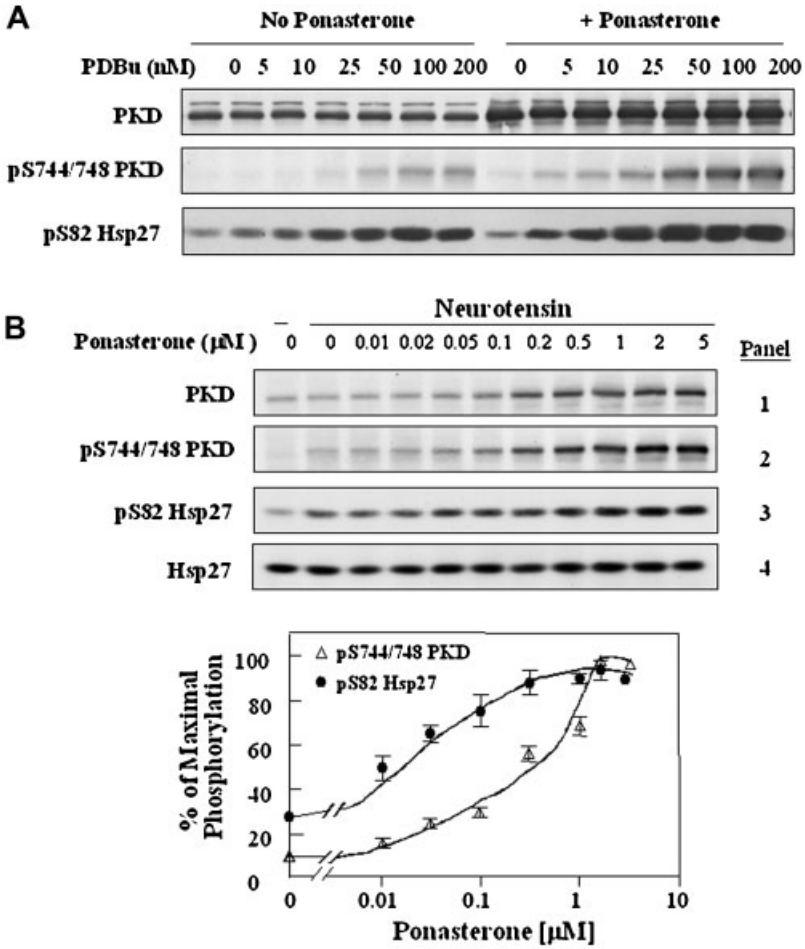

Fig. 4. PKD overexpression enhances Hsp27 Ser-82 phosphorylation induced by PDBu or neurotensin in PANC-1. **A**: Confluent PANC-1 cells stably transfected with PKD were treated without or with 2 μM ponasterone-A (an analog of the insect hormone ecdysone) for 22 h and then washed and incubated in serum-free medium (DMEM) for 2–4 h. The cells were stimulated without or with increasing concentrations of PDBu for 10 min and lysed. PKD expression level and the phosphorylation of either PKD at the activation loop or Hsp27 at Ser-82 were determined by Western blot analysis using PKD C-20 antibody (**upper panel**), pS744/748 PKD antibody (**middle panel**), and pS82 Hsp27 antibody (**bottom panel**), respectively. **B**: Confluent PANC-1 cells stably transfected with PKD were treated without (0) or with different concentrations of ponasterone-A for 22 h. The cells were washed and incubated in serum-free medium (DMEM) for 2–4 h. and then stimulated without (–) or with 50 nM neurotensin (NT) for 10 min at 37°C and lysed. Western blot analyses were performed to determine the inducibly expressed PKD level (**panel 1**), PKD pS744/748 phosphorylation (**panel 2**), and Ser-82 phosphorylation of Hsp27 (**panel 3**), using anti-PKD C-20 antibody, PKD pS744/748 antibody, and Hsp27 pS82 antibody respectively. The same blot was stripped and reblotted with monoclonal antibody against Hsp27 (G31) to assess the expression level of Hsp27 (**panel 4**). The curve graph shows the means (n = 3) of the level of Hsp27 Ser-82 phosphorylation (●) and PKD Ser-744/748 phosphorylation (Δ), obtained from scanning densitometry and are expressed as percentages of the maximum increase in phosphorylation obtained from the cells treated with 2 μM ponasterone-A.

expression levels of the proteins in PANC-1 cells transfected with non-targeting siRNA. Gene silencing of either PKD or PKD2 utilizing siRNAs attenuated neurotensin-stimulated Hsp27 Ser-82 phosphorylation in cells treated with SB202190. Cotransfection of siRNAs targeting both, PKD and PKD2, markedly diminished Hsp27 Ser-82 phosphorylation in PANC-1 cells stimulated with 50 nM neurotensin (by ~50%) and virtually abrogated agonist-induced HSP27 phosphorylation on Ser-82 in cells pretreated with SB202190. Collectively, these results indicate that neurotensin-induced Hsp27 phosphorylation on Ser-82 is mediated through p38 MAPK, PKD, and PKD2 signaling in PANC-1 cells.

DISCUSSION

Hsps, including Hsp27, are highly expressed in many cancer cells and tissues and the

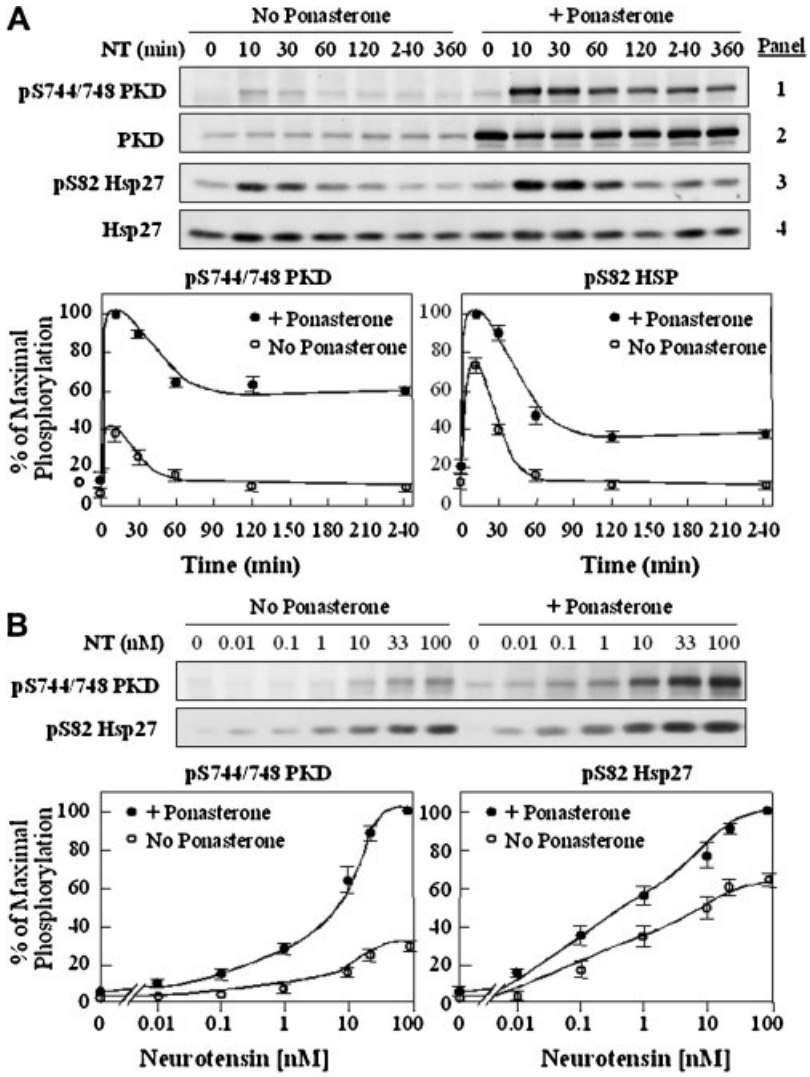

Fig. 5. Overexpression of PKD prolongs the duration of NT-induced HSP27 pS82 phosphorylation and enhances the dose response of HSP27 pS82 phosphorylation to neurotensin. Confluent PANC-1 cells stably transfected with PKD were treated without or with 2 μM ponasterone-A for 22 h. The cells were washed and incubated in serum-free medium (DMEM) for 2–4 h. **A**: cells pretreated with or without ponasterone-A were stimulated without (0) or with 50 nM neurotensin (NT) for the indicated time periods at 37°C. Cells were lysed with lysis buffer and Western blot analyses with specific antibodies were performed to determine the inducibly expressed PKD level (with PKD C-20 antibody, **panel 2**), PKD pS744/748 phosphorylation (with PKD pS744/748 antibody**, panel 1**), pS82 phosphorylation of Hsp27 (with pS82 Hsp27 antibody, **panel 3**), and Hsp27 expression level (with Hsp27 G31 antibody, **panel 4**). **B**: ponasterone-A pretreated cells and non-ponasterone-A pretreated cells were stimulated without or with increased concentrations of neurotensin (NT) for 10 min. Cells were lysed with lysis buffer and the phosphorylation of PKD or Hsp27 was determined by Western blot analysis using pS744/748 PKD antibody and pS82 HSP27 antibody. The curve graphs in A,B, show the means (n = 3) of the levels of PKD Ser 744/748 phosphorylation (**left panels**) and HSP27 Ser 82 phosphorylation (**right panels**) in cells treated with (●) and without (○) ponasterone-A, obtained from scanning densitometry and are expressed as percentages of the maximum increase in phosphorylation obtained from the ponasterone-A treated cells stimulated with 50 nM NT for 10 min at 37°C.

expression of these proteins contributes to the malignant properties of cancer cells, including increased tumorigenicity and treatment resistance [Rocchi et al., 2004; Chen et al., 2004a; Shin et al., 2005; McCollum et al., 2006; Xu and Bergan, 2006] and inhibition of apoptosis [Bruey et al., 2000; Benn et al., 2002; Paul et al., 2002]. Consequently, targeting these proteins and/or their upstream kinases may provide novel approaches for therapeutic intervention [Schmitt et al., 2007]. Although it is widely recognized that Hsp27 is a substrate of the p38 MAPK/MK2 cascade [Widmann et al., 1999; Chang and Karin, 2001], other studies

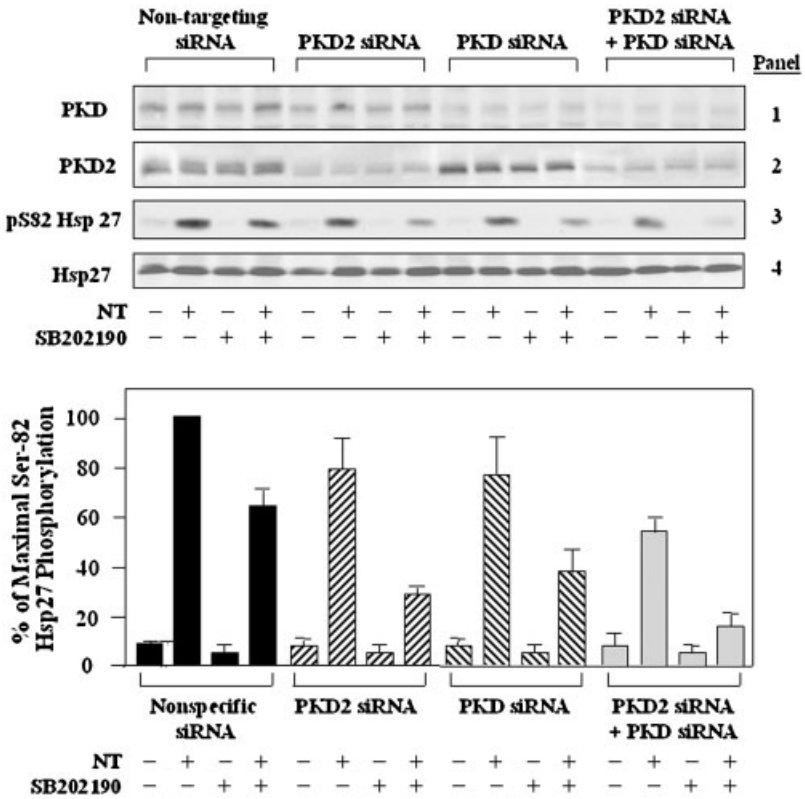

Fig. 6. Knockdown of PKD and PKD2 abrogates neurotensin-induced Hsp 27 Ser-82 phosphorylation in PANC-1 cells treated with SB202190. PANC-1 cells were transiently transfected with PKD siRNA or PKD2 siRNA, or cotransfected with both PKD siRNA and PKD2 siRNA. The control cells were transfected with non-targeted negative control duplex, as indicated in the figure. Two days after transfection, the cultures were incubated with (+) or without (−) the p38 MAPK inhibitor SB202190 (+SB, 2.5 μM) for 1 h and were subsequently unstimulated (−) or stimulated (+) with 50 nM neurotensin (NT) for 10 min and lysed. PKD and PKD2 protein levels were assessed by Western blot analysis using PKD C-20 antibody (**panel 1**) and PKD2 antibody (**panel 2**). Western blot analysis for Ser-82 phosphorylation of Hsp27 was also performed (**panel 3**). The same membranes were stripped and reblotted with Hsp27 antibody to indicate the equal loading of the samples (**panel 4**). The bar graph shows the level of Ser-82 phosphorylation of Hsp27 obtained from scanning densitometry expressed as percentage of the maximal phosphorylation. The results shown are representatives of three independent experiments.

demonstrated that phorbol esters also stimulate the phosphorylation of Hsp27 in a variety of cell types [Welch, 1985; Faucher et al., 1993; Kato et al., 1994; Minowada and Welch, 1995; Maizels et al., 1998; Doppler et al., 2005; Shin et al., 2005] via a PKC-dependent but p38/MK2-independent pathway [Maizels et al., 1998]. However, the possibility that these pathways act simultaneously in promoting Hsp27 phosphorylation in response to physiological stimuli has received little attention. Furthermore, the precise role of PKCs in the direct phosphorylation of Hsp27 remains unclear, raising the possibility that phorbol ester-induced Hsp27 phosphorylation is mediated by a protein kinase(s) that lies downstream of PKCs in intact cells.

PKD is the founding member of a novel serine/threonine protein kinase family with structural, enzymological and regulatory properties different from the PKC family [Rozengurt et al., 2005]. PKD can be activated in intact cells via PKC-dependent phosphorylation of Ser-744/748 within the PKD activation loop [reviewed in Reference Rozengurt et al., 2005]. Recently, PKD has been implicated in the phosphorylation of Hsp27 on Ser-82 in HeLa cells exposed to oxidative stress [Doppler et al., 2005], a condition previously shown to activate PKD [Waldron and Rozengurt, 2000; Storz et al., 2004; Waldron et al., 2004]. However, the role of PKD in mediating Hsp27 phosphorylation in response to physiological stimuli rather than stress has not been evaluated in any system.

The present study was designed to elucidate the relative contribution of the p38 MAPK and PKC/PKD pathways to the phosphorylation

of Hsp27 on Ser-82 induced by the mitogenic GPCR agonist neurotensin in PANC-1 cells [Guha et al., 2002, 2003; Kisfalvi et al., 2005], a cellular model of pancreatic adenocarcinoma. Initially, we demonstrated that neurotensin induces a rapid and striking increase in Hsp27 Ser-82 phosphorylation in PANC-1 cells, which closely correlated with activation loop phosphorylation of PKDs and p38 MAPK. Treatment of PANC-1 cells with the selective PKC inhibitor GF-I or the p38 MAPK inhibitor SB202190 partially reduced neurotensin-induced Hsp27 Ser-82 phosphorylation. These results suggest that neurotensin induces Hsp27 phosphorylation via PKC-dependent and p38 MAPK-dependent pathways. In line with this possibility, treatment of the cells with a combination of GF-I and SB202190 virtually suppressed neurotensin-induced Hsp27 Ser-82 phosphorylation.

These results prompted us to elucidate whether PKD family members play a role in mediating PKC-dependent Hsp27 phosphorylation induced by neurotensin in PANC-1 cells. We produced several lines of evidence supporting this hypothesis. Using clones of stably transfected PANC-1 cells that inducibly express PKD, we showed that overexpression of PKD increased the magnitude and prolonged the duration of Hsp27 Ser-82 phosphorylation in response to neurotensin. The enhanced Hsp27 Ser-82 phosphorylation was closely correlated to the level of expressed PKD protein. These results suggest that PKD plays a role in mediating Hsp27 Ser-82 phosphorylation in response to the GPCR agonist neurotensin.

Previously, we demonstrated that PANC-1 cells endogenously express PKD and PKD2 [Rey et al., 2003a,b]. Here, we found that gene silencing of PKD and PKD2 utilizing siRNAs targeting distinct PKD or PKD2 sequences markedly reduced neurotensin-stimulated Hsp27 Ser-82 phosphorylation in PANC-1 cells. In particular, knockdown of both PKD and PKD2, virtually abolished neurotensin-induced Hsp27 Ser-82 phosphorylation in PANC-1 cells treated with SB202190, to eliminate the p38 MAPK/MK-2 pathway. These results demonstrate that neurotensin induces Hsp27 phosphorylation on Ser-82 via simultaneous operation of at least two separate pathways in PANC-1 cells and that members of the PKD family, PKD, and PKD2, play a critical role in mediating one of the pathways.

A recent report from another laboratory using HeLa cells subjected to oxidative stress [Doppler et al., 2005], proposed that PKD is the relevant upstream kinase that phosphorylates Hsp27 on Ser-82 but the contribution of the p38 MAPK/MK2 pathway was not evaluated. Our results using PANC-1 cells stimulated with neurotensin support a different model, namely that neurotensin induces Hsp27 phosphorylation on Ser-82 through p38 MAPK, PKD, and PKD2 pathways. In addition to neurotensin, many Gq-coupled receptor agonists that stimulate Gαq-mediated activation of β isoforms of phospholipase C to produce $Ins(1,4,5)P_3$ that mobilizes Ca^{2+} from internal stores and diacylglycerol that activates PKC/PKD [Exton, 1996; Rhee, 2001], also promote Hsp27 phosphorylation in a variety of cell types [Larsen et al., 1997; Schèafer et al., 1998; Williams, 2001; Shigeru Akamatsu et al., 2004; Chen et al., 2004b]. GPCR agonists also activate the p38 MAP kinase cascade and this pathway has been implicated in GPCR-induced Hsp27 phosphorylation in intact cells [Larsen et al., 1997; Schèafer et al., 1998; Akamatsu et al., 2004; Chen et al., 2004b]. However, the contribution of the PLC/PKC/PKD pathway to the phosphorylation of Hsp27 in response to GPCR agonists was not examined in any of these previous studies. We propose that Hsp 27 phosphorylation on Ser-82 in response to GPCR stimulation is mediated by the simultaneous operation of p38/MAPKAPK2 and PKC/PKD pathways, a proposition that warrants further experimental work.

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