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CCK causes PKD1 activation in pancreatic acini by signaling through PKC- δ and PKC-independent pathways

Marc J. Berna^a, K. Martin Hoffmann^a, Jose A. Tapia^b, Michelle Thill^c, Andrea Pace^d, Samuel A. Mantey, and Robert T. Jensen^a

a Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (NIH), Bethesda, Maryland, 20892-1804, USA

b Departamento de Fisiología, Universidad de Extremadura, Cáceres, 10071, Spain

c National Eye Institute, NIH, Bethesda, Maryland, 20892-1804, USA

d Medizinische Klinik I, Universitätsklinikum Hamburg-Eppendorf, 20246 Hamburg, Germany

Summary

Protein kinase D1 (PKD1) is involved in cellular processes including protein secretion, proliferation and apoptosis. Studies suggest PKD1 is activated by various stimulants including gastrointestinal (GI) hormones/neurotransmitters and growth factors in a protein kinase C (PKC)-dependent pathway. However, little is known about the mechanisms of PKD1 activation in physiologic GI tissues. We explored PKD1 activation by GI hormones/neurotransmitters and growth factors and the mediators involved in rat pancreatic acini. Only hormones/neurotransmitters activating phospholipase C caused PKD1 phosphorylation (S916, S744/748). CCK activated PKD1 and caused a time- and dose-dependant increase in serine phosphorylation by activation of high- and low-affinity CCK_A receptor states. Inhibition of CCK-stimulated increases in phospholipase C, PKC activity or intracellular calcium decreased PKD1 S916 phosphorylation by 56%, 62% and 96%, respectively. PKC inhibitors GF109203X/Go6976/Go6983/PKC- ζ pseudosubstrate caused a 62/43/49/0% inhibition of PKD1 S916 phosphorylation and an 87/13/82/0% inhibition of PKD1 S744/748 phosphorylation. Expression of dominant negative PKC- δ , but not PKC- ϵ , or treatment with PKC- δ translocation inhibitor caused marked inhibition of PKD phosphorylation. Inhibition of Src/PI3K/MAPK/tyrosine phosphorylation had no effect. In unstimulated cells, PKD1 was mostly located in the cytoplasm. CCK stimulated translocation of total and phosphorylated PKD1 to the membrane. These results demonstrate that CCK_A receptor activation leads to PKD activation by signaling through PKC-dependent and PKC-independent pathways.

Keywords

PKD1 activation; Pancreas; Cholecystokinin; Gastrointestinal hormones; Growth factors; PKC

Correspondence to: Dr. Robert T. Jensen, Digestive Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bldg. 10, Room 9C-103, 10 CENTER DR MSC 1804, BETHESDA MD 20892-1804, Tel: 301/496-4201; Fax: 301/402-0600 E-mail: robertj@bdg10.niddk.nih.gov

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1. Introduction

Protein kinase D1 (PKD1), PKD2 and PKD3 constitute the recently identified PKD family, a subclass of the AGC family of serine/threonine kinases, with structural and enzymological properties different from those of members of the PKC family [1,2]. PKD1 is composed of different domains: a N-terminal region, two cysteine-rich zinc-finger regions, a region rich in negatively charged amino acids, a pleckstrin-homology domain and a Ser/Thr kinase catalytic domain (for review, see [3]). PKD1 can be activated by growth factors, oxidative stress, thrombin, bioactive lipids, cross-linking of B- and T-cell receptors and some G-protein coupled receptors (GPCR) [4–7]. For most of these stimuli, direct phosphorylation by a member of the PKC family is reported to be the major mechanism of PKD1 activation *in vivo* [8]. This transphosphorylation occurs at the serine residues 744/748 in the activation loop of the kinase domain [8]. Mutational studies have shown that phosphorylation of these residues is required and sufficient for PKC-mediated PKD1 activation by a number of stimulants [9]. A second important phosphorylation event in PKD1 activation concerns serine 916 in the C-terminal region. This residue is autophosphorylated by activated PKD1, leading to a change in conformation that stabilizes PKD1 in an active state and influences duration of kinase activation [10]. Phosphorylation of S916 has been shown to tightly correlate with PKD1 activation status [11]. Whereas in resting cells, PKD1 is located mainly in the cytoplasm, upon stimulation, PKD1 migrates to the membrane where it is activated [12–14]. Activation of PKD1 leads to regulation of multiple cellular functions including Golgi organization and protein secretion [15], cell proliferation [16], invasion [17] and regulation of apoptosis [18].

Although PKD1 has been extensively studied in multiple cellular systems, several aspects of GPCR-mediated PKD1 activation remain controversial or not determined, including the role of changes in intracellular calcium levels in mediating PKD1 activation, the existence and relevance of PKC-independent pathways of PKD1 activation by GPCRs, the PKC isoforms or intracellular mediators other than PKC involved in PKD1 activation by CCK and the changes in the intracellular distribution of PKD1 upon activation. Furthermore, comparative data on kinetic and stoichiometric variables and intracellular distribution of the two phosphorylated PKD1 forms (S916 and S744/748) are lacking and only very limited information is available on PKD1 activation in native GI cells or tissues and the intracellular signaling cascades involved.

Intracellular signaling mechanisms for gastrointestinal (GI) growth factors as well as GI hormones/neurotransmitters have been extensively studied in dispersed pancreatic acini because these cells are highly responsive to various stimuli [19]. A number of GI hormones/neurotransmitters and growth factors that are known to alter pancreatic acinar cell function have been reported to activate PKD1 in other cells [20–22]. As PKD1 may play a central role in the regulation of protein trafficking, cell proliferation and induction of apoptosis in other cell systems [15–18], an understanding of the involvement of PKD1 in the signaling cascades of these GI hormones/neurotransmitters or growth factors in pancreatic tissue is important, because many of these stimuli play an important role in pancreatic disease. Therefore, to address the issues on PKD1 activation detailed above, we have analyzed the role of GI hormones/neurotransmitters and growth factors in PKD1 activation in pancreatic acinar cells and the intracellular signaling cascades involved.

2. Materials and Methods

2.1. Materials

Male Sprague-Dawley rats (150–250 g) were obtained from the Small Animals Section, Veterinary Resources Branch, National Institutes of Health (NIH), Bethesda, MD. Rabbit anti-phospho-PKD1-pS916, rabbit anti-phospho-PKD1-pS744/748, rabbit anti-PKD antibodies,

rabbit anti-phospho-Src family (Y416), mouse anti-phospho p44/42 MAPK (T202/Y204) and mouse anti-phospho Akt (S473) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Rabbit anti-PKD (C20), rabbit anti-PKD1 (N20), bovine anti-goat horseradish peroxidase (HRP)-conjugate and anti-rabbit-HRP-conjugate antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-E-Cadherin, anti PKC-delta and anti PKC-epsilon were purchased from BD Biosciences (San Jose, CA). Anti-Calpain was from BioSource International, Inc. (Camarillo, CA). Rabbit anti-PKD2 was from Bethyl Laboratories (Montgomery, TX). Tris/HCl pH 8.0 and 7.5 were from Mediatech, Inc. (Herndon, VA). 2-mercaptoethanol, protein assay solution and SDS were from Bio-Rad Laboratories (Hercules, CA). CaCl₂ and MgCl₂ were from Quality Biological, Inc. (Gaithersburg, MD). Dulbecco's phosphate buffered saline (DPBS), glutamine (200 mM), Tris/glycine/SDS buffer (10x), and Tris/glycine buffer (10x) were from Biosource. Minimal essential media (MEM) vitamin solution, basal medium Eagle (BME) amino acids 100x and Tris-Glycine gels were from Invitrogen (Carlsbad, CA). COOH-terminal octapeptide of cholecystokinin (CCK-8), hepatocyte growth factor (HGF), bombesin, insulin-like growth factor 1 (IGF-I), basic fibroblast growth factor (bFGF), vasoactive intestinal peptide (VIP), endothelin and secretin were from Bachem Bioscience Inc. (King of Prussia, PA). EGF, thapsigargin, PDGF, GF109203X, A23187, LY294002, Go6976, Go6983, PKC ζ pseudosubstrate inhibitor, wortmannin, PD98059, U0126, AG527 (B44), PP2, PP3, syntide-2, U-74321, U-74343 and deoxycholic acid were from Calbiochem (La Jolla, CA). Carbachol, insulin, TGF- β 1, dimethyl sulfoxide (DMSO), 12-O-tetradecanoylphorbol-13-acetate (TPA), L-glutamic acid, fumaric acid, pyruvic acid, trypsin inhibitor, HEPES, Triton-X, TWEEN[®], phenylmethanesulfonylfluoride (PMSF), EGTA, sucrose, sodium-orthovanadate and sodium azide were from Sigma-Aldrich, Inc. (St. Louis, MO). Albumin standard, Protein G beads, Super Signal West (Pico, Dura) chemiluminescent substrate and stripping buffer were from Pierce (Rockford, IL). Protease inhibitor tablets were from Roche (Basel, Switzerland). Purified collagenase (type CLSPA) was from Worthington Biochemicals (Freehold, NJ). ATP and γ ³²P-ATP were from Amersham (Piscataway, NJ). P81 phosphocellulose paper was from Upstate (Charlottesville, VA). Nitrocellulose membranes were from Schleicher and Schuell BioScience, Inc. (Keene, NH). Alexa 594-conjugated anti-rabbit secondary antibody was from Molecular Probes (Eugene, OR). Poly-L-lysine coated slides and sample chambers were from Wescor (Logan, UT). Ad5-CMV-PKC-delta-DN and Ad5-CMV-PKC-epsilon-DN were from Seven Hill Bioreagents (Cincinnati, OH), Ad5-CMV-GFP and Ad5-CMV were from QBiogene (Montreal, Canada).

2.2. Methods

Tissue Preparation—Pancreatic acini were obtained by collagenase digestion as previously described [23]. Standard incubation solution contained 25.5 mM HEPES (pH 7.45), 98 mM NaCl, 6 mM KCl, 2.5 mM NaH₂PO₄, 5 mM sodium pyruvate, 5 mM sodium glutamate, 5 mM sodium fumarate, 11.5 mM glucose, 0.5 mM CaCl₂, 1 mM MgCl₂, 1 mM glutamine, 1% (w/v) albumin, 0.01% (w/v) trypsin inhibitor, 1% (v/v) vitamin mixture and 1% (v/v) amino acid mixture.

Acini Stimulation—After collagenase digestion, dispersed acini were pre-incubated in standard incubation solution for 2 hrs at 37 °C with or without inhibitors as described previously [23]. After pre-incubation 1 ml aliquots of dispersed acini were incubated at 37 °C with or without stimulants. Cells were lysed in lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml). After sonication, lysates were centrifuged at 10,000x g for 15 min at 4 °C and protein concentration was measured using the Bio-Rad protein assay reagent. Equal amounts of samples were analyzed by SDS-PAGE and Western blotting.

Subcellular Fractionation—After stimulation acini were resuspended in membrane lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1% sodium azide, 1 mM EGTA, 0.4 mM EDTA, 0.2 mM sodium orthovanadate, 1 mM PMSF, and one protease inhibitor tablet per 10 ml). After homogenization for 30 seconds (Polytron homogenizer, Brinkmann Instruments) lysates were centrifuged at 500x g for 15 min to separate nuclei, debris and fat. The supernatant (cytosol and membrane fraction) was centrifuged for 30 min at 4°C and 60,000x g to separate cytosol and membrane fraction. The pellet containing the membrane fraction was washed twice in membrane lysis buffer, resuspended in lysis buffer (with detergents as described above), sonicated for 3 seconds and then centrifuged at 10,000x g for 15 min at 4°C. Protein concentration was measured using Bio-Rad protein assay reagents and equal amounts of protein were subjected to SDS-PAGE and analyzed by Western blotting.

Western Blotting—Whole cell lysates, immunoprecipitates or lysates of subcellular fractions were subjected to SDS-PAGE using 10% and 4–20% Tris-glycine gels. After electrophoresis, protein was transferred to nitrocellulose membranes. Membranes were blocked in blocking buffer (50 mM Tris/HCl pH 8.0, 2 mM CaCl₂, 80 mM NaCl, 0.05% Tween® 20, 5% nonfat dry milk) at 4°C overnight or at room temperature for one hour. Membranes were then incubated with primary antibody. Membranes were incubated with HRP-conjugated secondary antibody (anti-mouse, anti-rabbit, anti-goat) for 45 minutes. Membranes were then washed twice in blocking buffer and twice in washing buffer (50 mM Tris/HCl pH 8.0, 2 mM CaCl₂, 80 mM NaCl, 0.05% Tween® 20), incubated with chemiluminescence detection reagents and finally exposed to Kodak Biomax film (XAR, MR). The intensity of the protein bands was measured using Kodak ID Image Analysis. For re-probing, membranes were incubated in Stripping buffer for 30 minutes at room temperature, washed in washing buffer, blocked for 1 hour in blocking buffer and re-probed as described above.

Immunoprecipitation and PKD1 kinase assay—After treatment with or without 10 nM CCK for 5 minutes as indicated, pancreatic acini were lysed (50 mM Tris-HCl pH 7.5, 1% Triton-X, 2 mM EGTA, 2 mM EDTA, 2 mM DTT), lysates were sonicated, cleared by centrifugation at 10,000x g for 15 min at 4 °C and protein concentration was measured using the Bio-Rad protein assay reagent. 1800 µg of protein were incubated with a PKD antibody for 2 hours (C-20 from Santa-Cruz, diluted 1:100), conjugates were immunoprecipitated using protein G coupled to agarose beads and immunoprecipitates were washed twice in lysis buffer and twice in kinase buffer (30 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 2 mM DTT). For assessment of PKD1 autophosphorylation, 10 µl of kinase buffer containing 2.5 µCi ³²P-ATP/sample (diluted with cold ATP to give a final concentration of 100 µM) was added to 20 µl of beads. Samples were incubated at 30°C for 10 minutes and the reaction was stopped by addition of 1 ml of ice-cold kinase buffer. Immunoprecipitates were recovered and an equal amount of 2x running buffer was added. Samples were submitted to SDS-PAGE and gels were dried prior to autoradiography. To determine phosphorylation of the synthetic substrate syntide-2, immunoprecipitates (20 µl) were incubated with 30 µl of kinase buffer containing 2 µCi/sample ³²P-ATP (diluted with cold ATP to give a final concentration of 100 µM) and 5 µg/µl syntide-2 for 10 minutes at 30°C. Reactions were terminated by addition of 100 µl phosphoric acid (0.75%) and 75 µl of the supernatant was spotted onto P81 phosphocellulose squares. These were washed thoroughly 5 times 5 minutes in 0.75% phosphoric acid and then 5 minutes in acetone. Squares were allowed to dry and incorporation of ³²P into syntide-2 was assessed by Cerenkov counting.

Immunocytochemistry and immunofluorescence imaging—After treating pancreatic acini with or without stimulants as indicated, cells were fixed, transferred to glass slides and blocked as previously reported [24]. Slides were incubated with an anti-PKD

antibody (C-20, Santa Cruz) at a dilution of 1:500 and an anti-E-cadherin antibody (1:100) overnight at 4° C. Reactivity was demonstrated by incubation with Alexa Fluor555-conjugated goat anti-rabbit and Alexa Fluor468-conjugated goat anti mouse secondary antibodies at a dilution of 1:500 for 2 hours at room temperature. Negative controls consisted of replacement of primary antibody with an isotype-matched control. Slides were analyzed as previously reported [24].

Pancreatic acinar isolation, infection and culture—Pancreatic acini were isolated as described above, infected with adenovirus at various titers and cultured on dishes coated with rat tail collagen in Waymouth's medium containing 0.5% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.2 mg/ml soybean trypsin inhibitor as described previously [25]. After 24 hours, stimulants were added and cells lysed as described above.

Statistical Analysis—All experiments were performed at least 4 times. Data are presented as mean ± SEM and were analyzed using the Student's t-test for paired data using the software StatView (SAS Institute, Cary, NC). P values <0.05 were considered significant.

3. Results

3.1. PKD1 is expressed and phosphorylated by CCK in rat pancreatic acini

We first determined whether PKD1 was present in pancreatic acinar cells and whether it was phosphorylated in response to stimulation by CCK, a major physiological stimulant of these cells [26]. Using a specific PKD1 antibody for Western blotting on whole cell lysates from rat pancreatic acini treated with 10 nM CCK for 5 min, a single 115 kD protein was detected (Fig. 1, lane 1), whereas a single 105 kD protein was present when a specific PKD2 antibody was used (Fig. 1, lane 4), demonstrating the presence of both PKD1 and PKD2. When these lysates were submitted to Western blotting with specific antibodies directed against PKD1 phosphorylated at S916 and S744/748, proteins co-migrating with PKD1, but not PKD2, were visualized (Fig. 1, lanes 2 and 3), showing the presence of both forms of phosphorylated PKD1 in pancreatic acini treated with CCK. These findings demonstrate that PKD1 and PKD2 are present in rat pancreatic acini and that PKD1 is phosphorylated at S916 and S744/748 in response to CCK.

3.2. PKD1 is phosphorylated on S916 and S744/748 by CCK, carbachol and bombesin, but not by growth factors in rat pancreatic acini

Numerous GI growth factors as well as GI hormones/neuropeptides activate pancreatic acinar cells [19]. To assess whether stimulation by GI hormones or growth factors can cause phosphorylation of PKD1, we analyzed the alterations in the phosphorylation of S916 (a PKD1 autophosphorylation site reflecting activation) and S744/748 (a site for transphosphorylation by PKC isoforms) in whole cell lysates from rat pancreatic acini treated with various stimuli (Fig. 2). Phosphorylation of PKD1 at both S916 and S744/748 was markedly increased after treatment with GI hormones causing mobilization of intracellular calcium and PKC activation (i.e. CCK, carbachol, bombesin) (Fig. 2, top panel, lanes 2–4) [19], but not after treatment with GI hormones that increase cellular cyclic AMP and cause PKA activation (i.e. secretin, VIP) (Fig. 2, top panel, lanes 5–8) [19] or treatment with various pancreatic growth factors (Fig. 2, bottom panel). To confirm that each stimulus tested can alter acinar cell function, we showed that each GI hormone stimulated an increase in phosphorylation of MAPK or Akt (Fig. 2) and that each growth factor stimulated an increase in Akt phosphorylation (Fig. 2), indicating that each stimulus had an effect on acinar cells in our assay.

3.3. PKD1 is activated by CCK in rat pancreatic acini

We next wanted to determine if CCK not only causes PKD1 phosphorylation, but also activation. In-vitro kinase assays monitoring PKD autophosphorylation and phosphorylation of the synthetic substrate syntide-2 by PKD have been used in other experimental models to determine PKD activation by a given stimulus [5,27]. Incubation with 10 nM CCK for 5 minutes caused an 8.6 ± 0.5 -fold increase in PKD1 autophosphorylation (Fig. 3, upper panel) and a highly significant ($p < 0.001$) increase in phosphorylation of the synthetic substrate syntide-2 (Fig. 3, lower panel), demonstrating activation of PKD kinase activity by CCK.

3.4. Time-dependence and dose-dependence of PKD1 phosphorylation by CCK

Phosphorylation of both S916 and S744/748 of PKD1 rapidly increased after CCK stimulation with a detectable effect after 30 s and a maximal effect after 10–15 min of stimulation, lasting over 60 min (Fig. 4). The increase in S916 phosphorylation occurred significantly faster ($p < 0.05$) than the increase in S744/748 phosphorylation (Table 1). The dose-response of PKD1 S916 and S744/748 phosphorylation by CCK was comparable for both phosphorylation sites (Fig. 5). Increase in phosphorylation was detectable at concentrations greater than 0.01 nM CCK and reached a maximum at 10 nM CCK (Fig. 5). Further increasing the CCK concentration to 100 or 1000 nM CCK did not increase the level of PKD1 S916 or S744/748 phosphorylation (Fig. 5). With maximal CCK stimulation, the fold increase over basal phosphorylation levels was significantly greater ($p = 0.013$) for S916 than for S744/748PKD1 (Table 1).

3.5. CCK-induced PKD1 serine phosphorylation is mediated both by the high- and the low-affinity CCK_A receptor

The CCK_A receptor on pancreatic acini can exist in both a high and low affinity receptor state [19,26]. To analyze the possible role of each receptor state in causing CCK mediated PKD1 phosphorylation, we analyzed the effect of CCK-JMV, a CCK synthetic analog which in rat pancreatic acini functions as an agonist of the high-affinity CCK_A state and as an antagonist at the low-affinity CCK_A receptor state [28,29], on PKD1 serine phosphorylation. CCK-JMV caused an increase in PKD S916 and S744/748 phosphorylation detectable above 10 nM and reaching a maximum at 100 nM (Fig. 5). For both sites, the maximal stimulation by CCK-JMV was one-fifth of the maximal stimulation by CCK (Fig. 5, Table 1). Co-stimulation with CCK (1 nM) and CCK-JMV at variable concentrations led to a dose-dependent inhibition of CCK-mediated PKD1 S916 and S744/748 phosphorylation by CCK-JMV. After co-stimulation with CCK (1 nM) and maximal CCK-JMV concentrations (10 μ M), phosphorylation of both S916 and S744/748PKD1 was comparable to phosphorylation stimulated by 10 μ M CCK-JMV alone (Fig. 5). Together, these data demonstrate that 80% of the effect of CCK on PKD1 phosphorylation is mediated by activation of the low-affinity CCK_A receptor and that 20% is mediated by activation of the high-affinity CCK_A receptor state.

3.6. CCK-induced PKD1 phosphorylation is partly dependant on PKC and strongly inhibited by depletion of intracellular calcium

Binding of CCK to its receptor causes activation of PLC- β , leading to an increase of intracellular calcium levels and activation of PKC through diacylglycerol [26,30]. To assess the respective roles of PKC and intracellular calcium signaling on CCK-mediated PKD1 phosphorylation, we analyzed the effect of GF109203X, an inhibitor of classical and novel PKC isoforms [31], and thapsigargin, an agent that specifically inhibits the endoplasmic reticulum ATPase, which depletes Ca⁺⁺ from intracellular compartments in a calcium-free medium after 1 h of incubation [23]. Furthermore, we analyzed the effect of TPA, an activator of classical and novel PKC isoforms, on PKD1 phosphorylation. TPA (1 μ M) stimulated PKD1 S916 and S744/748 phosphorylation to a comparable degree to stimulation by 10 nM CCK

(Fig. 6, top panel). TPA-induced PKD1 phosphorylation was completely inhibited by pre-incubation of pancreatic acini with GF109203X (Fig. 6, top panel), demonstrating effective inhibition of classical/novel PKC isoforms by GF109203X under our experimental conditions. In contrast, CCK-induced PKD1 phosphorylation was only partly inhibited by pre-incubation with GF109203X (Fig. 6, top panel). Specifically, CCK-stimulated pS916 PKD1 phosphorylation was inhibited by $62 \pm 7\%$ by GF109203X, which was significantly ($p < 0.026$) less than the $87 \pm 2\%$ inhibition of pS744/748 PKD1 phosphorylation. Depletion of intracellular calcium by thapsigargin caused a $96 \pm 2\%$ and $87 \pm 3\%$ inhibition of CCK-induced PKD1 S916 and S744/748 phosphorylation, respectively (Fig. 6, top panel), but did not have a significant effect on TPA-mediated PKD1 phosphorylation (Fig. 6, top panel). Both CCK- and TPA-mediated PKD1 phosphorylation were completely inhibited after pre-incubation of pancreatic acini with GF109203X and thapsigargin in combination (Fig. 6, top panel). An acute increase in intracellular calcium by short-term incubation with thapsigargin in calcium-containing media did not cause PKD1 phosphorylation but potentiated TPA-mediated PKD1 phosphorylation (Fig. 6, bottom panel) as did the calcium ionophore A23187 (data not shown). These results demonstrate that CCK-stimulated PKD1 phosphorylation is mediated by activation of both arms of the phospholipase C-mediated cascade with participation of both PKC-dependent and -independent pathways, however, the contribution of these different pathways varies for the two serine phosphorylation sites. Furthermore, these data suggest an important role for intracellular calcium in CCK-mediated PKD1 activation.

3.7. PKD1 activation by CCK is partly dependent on PLC-mediated PKC activation

CCK-induced PKC activation is mediated by PLC beta in pancreatic acini [30]. To analyze the role of PLC in PKD1 activation by CCK, we studied the influence of the PLC inhibitor U-73122 on CCK-mediated PKD1 phosphorylation. U-73122 caused an almost complete inhibition ($88.3 \pm 3.9\%$) of CCK-mediated PKD1 S744/748 and a partial inhibition ($55.6 \pm 5.5\%$) of S916 phosphorylation (Fig. 7, top panel).

Chronic treatment with phorbol ester for 24 hours has been shown to cause down-regulation of novel/classical PKC isoforms, but not PKD [32]. In pancreatic acini, TPA ($1 \mu\text{M}/24\text{h}$) caused complete down-regulation of PKC- δ and PKC- ϵ and completely inhibited TPA and CCK-stimulated PKD1 S744/748 phosphorylation (Fig. 7, bottom panel). However, after 24h TPA treatment, CCK could still trigger an increase in PKD1 S916 phosphorylation, which corresponds to 19% of the maximal increase without TPA pre-treatment. Together, these findings suggest that approximately 80% of PKD1 activation is mediated by a PLC-PKC dependent pathway but that 20% is mediated by a PKC-independent pathway.

3.8. Differential effect of inhibitors of classical, novel and atypical PKC isoforms on TPA- and CCK-induced PKD1 phosphorylation

Four PKC isoforms ($\alpha, \delta, \epsilon, \zeta$) have been reported to occur in pancreatic acini [33–36]. To elucidate which PKC isoforms are involved in CCK-mediated PKD1 phosphorylation, we used Go6976, a selective inhibitor of classical PKC isoforms and PKD1 [37], Go6983, a general PKC inhibitor [37] and PKC- ζ pseudosubstrate, a specific cell-permeable inhibitor of PKC- ζ [38]. TPA-mediated phosphorylation of the PKD1 auto-phosphorylation site S916 was significantly inhibited by classical PKC/PKD inhibitor Go6976 (Fig. 8, lane 8), demonstrating that this inhibitor was effective in our experimental system. However, TPA-mediated phosphorylation of the PKD1 trans-phosphorylation site S744/748, which does not require PKD1 catalytic activity, was not significantly altered by Go6976 (Fig. 8, lane 8), demonstrating that classical PKC isoforms are not involved in TPA-mediated PKD1 phosphorylation. Similarly, Go6976 had no significant effect on CCK-mediated PKD1 S744/748 phosphorylation, demonstrating classical PKC isoforms are not involved in CCK-mediated S744/748 phosphorylation. The general PKC inhibitor Go6983 completely inhibited TPA-

mediated phosphorylation of both phospho-specific PKD1 sites studied (Fig. 8, lane 9). In contrast, Go6983 only partly inhibited CCK-induced PKD1 S744/748 phosphorylation and had a weak effect on CCK-mediated S916 phosphorylation (Fig. 8, right panel and lane 9). These results remained unchanged when different agonist concentrations and incubation times were used (i.e. CCK 100 nM for 5 min and TPA 1 μ M for 10 min) (data not shown), suggesting that these results are not explained simply by different concentrations or incubation times of the agonists used. PKC- ζ pseudosubstrate, which has been shown to inhibit PKC- ζ kinase activity in rat pancreatic acini under experimental conditions identical to those used in the present study [33], had no significant effect on TPA- or CCK-induced PKD1 S916 or S744/748 phosphorylation (Fig. 8). These data support the conclusion that CCK-mediated PKD1 phosphorylation is partly dependant on novel PKC isoforms.

3.9. Effect of isoform-specific PKC translocation inhibitors and dominant-negative PKC isoforms on CCK-mediated PKD1 phosphorylation

To determine which novel PKC isoform(s) mediate(s) PKD1 phosphorylation, we used two independent approaches. First, we analyzed the effect of isoform-specific PKC translocation inhibitors [33] on CCK-mediated PKD1 phosphorylation of the S744/748 transphosphorylation site. While the PKC- δ translocation inhibitor δ V1-1 caused a strong inhibition of PKD1 phosphorylation, the PKC- ϵ translocation inhibitor ϵ V1-2 had no significant effect (Fig. 9). Second, using an adenoviral vector, we expressed dominant negative PKC- δ and PKC- ϵ isoforms as described previously [25]. These dominant negative (DN) mutants encode the full-length protein, but contain a single amino acid substitution in a critical ATP binding residue, rendering the kinase inactive [39]. As reported previously [25], infection with 10^9 pfu/mg of acinar protein leads to expression of the protein of interest in 100% of cells (Fig. 9, panel D). Infection with an empty adenoviral vector or with Ad-5-DN PKC- ϵ had no effect on PKD1 phosphorylation, but infection with Ad-5-DN PKC- δ strongly inhibited CCK-mediated PKD1 phosphorylation (Fig. 9). These data further demonstrate that PKC- δ is the main PKC isoform involved in PKD1 activation.

3.10. CCK-stimulated PKD1 phosphorylation is not dependant on Src, PI3K, MAPK or tyrosine phosphorylation

To further characterize possible signaling cascades contributing to PKD1 phosphorylation in rat pancreatic acini, we analyzed the effect on CCK-stimulated PKD1 phosphorylation of the inhibition of several pathways reported to be important in mediating PKD1 activation in other cellular systems with other stimulants [4,5,40]. Inhibition of Src by PP2, PI3K by wortmannin or LY294002, MAPK by PD98059 or U0126 and inhibition of tyrosine phosphorylation by B44, a wide-spectrum tyrosine kinase inhibitor [41] had no effect on CCK-mediated S916 or S744/748 phosphorylation (Fig.10). These inhibitors have all been reported to inhibit CCK-mediated signaling cascades in pancreatic acini [42–45]. To ensure that this lack of effect was not due to a general non-responsiveness of our pancreatic acinar preparation to these inhibitors, controls to verify specific inhibition of a typical phosphorylation event were included for each inhibitor (Fig. 10).

3.11. PKD1 translocates to the membrane after CCK stimulation in rat pancreatic acini

PKD membrane translocation is reported to be a crucial step in PKD activation in response to different stimuli in numerous cellular models [5,12,13,46]. To analyze intracellular distribution of total PKD in quiescent and stimulated intact pancreatic acini, cells were submitted to immunocytochemistry-immunofluorescence. In non-stimulated acini, PKD1 was distributed diffusely in the cytoplasm, with only minimal staining at the membrane (Fig. 11, panels B1-B2). After stimulation with CCK (Fig. 11, panels C1-2, D1-2) or TPA (Fig. 11, panels E1-2, F1-2), there was a rapid translocation to the membrane, obvious at 5 min (Fig. 12, panels C1-2,

E1-2) and lasting at least 30 min (Fig. 11, panels D1-2, F1-2). The membrane translocation of PKD1 after stimulation is confirmed by a co-localization with the membrane marker E-cadherin (Fig. 11, panels C2-F2). To quantitate the PKD1 translocation response, subcellular fractions of rat pancreatic acini were analyzed by Western blotting (Fig. 12). In non-stimulated acini, $80 \pm 7\%$ of total PKD was found in the cytoplasmic fraction (Fig. 12, lanes 1, 6), confirming results of the immunocytochemistry. After stimulation with TPA, there was a marked increase of total PKD in the membrane fraction detectable at 1 min (data not shown), obvious at 5 min (Fig. 12, lane 7) and lasting at least 30 min (Fig. 12, lane 9) whereas PKD completely disappeared from the cytoplasmic fraction (Fig. 12, lanes 2, 4), indicating a pronounced and durable translocation of PKD to the membrane. Similarly, after CCK stimulation, total PKD increased by $209 \pm 57\%$ at 1 min (data not shown), $357 \pm 105\%$ at 5 min and $305 \pm 75\%$ at 30 min in the membrane fraction (Fig. 12, lanes 8, 10). A marked increase in PKD1 phosphorylated at S916 and S744/748 was detectable in the membrane fraction after 1 (data not shown), 5 and 30 min CCK and TPA stimulation (Fig. 12, lanes 7–10) and in the cytoplasmic fraction after CCK stimulation only (Fig. 12, lanes 2–5). To verify separation of cytosolic and membrane fractions and equal loading of protein, lysates from subcellular fractions were analyzed for E-Cadherin (located in cell membranes) and Calpain (located in the cytosol). This analysis confirmed the purity of the subcellular fractions and showed equal loading.

4. Discussion

Our results demonstrate that PKD1 is present in rat pancreatic acini and is phosphorylated in response to GI hormones/neurotransmitters that activate phospholipase C, but not by GI hormones/neurotransmitters stimulating other transduction cascades or by pancreatic growth factors. We showed that CCK induced a 8.6-fold increase in PKD1 autophosphorylation and a 2.2-fold increase in phosphorylation of an exogenous substrate by PKD1, demonstrating PKD1 activation by CCK. These increases in PKD activity are comparable to the 4- to 8- fold increase in autophosphorylation and the 2.6–4 fold increase in syntide-2 phosphorylation reported in the literature [7,20,47]. The lower increase in activity of these kinase assays when compared to phosphorylation assays probably reflects different sensitivities of these assays. As rat pancreatic acini express exclusively CCK_A and not CCK_B receptors, our results for the first time show that CCK_A receptors can lead to PKD1 activation. Moreover, because the extent of PKD1 S916 phosphorylation has been shown to closely reflect the activation status of PKD1 [11,32,48], the presence of phosphorylated S916 PKD1 after stimulation with CCK, carbachol or bombesin supports the conclusion that PKD1 is activated by these stimuli in pancreatic acini. Our results, showing lack of effect of endothelin or growth factors in pancreatic acini, differ from studies in other, mostly transformed and/or transfected cells which report activation of PKD1 can also be mediated by endothelin [20] or by various growth factors including PDGF [27] or by VEGF [4,49]. These data suggest that not only may the cellular basis of PKD1 activation by a given growth factor or GI hormone/neurotransmitter vary from one tissue to another, it may also vary from normal cells to transfected and/or neoplastic cells.

There are no studies that have compared in detail the kinetics and stoichiometry of PKD1 S916 (autophosphorylation site) and S744/748 (PKC transphosphorylation site) by a given stimulus. In general, for the individual serine phosphorylation sites, our kinetic and stoichiometric results agree with previous studies [21,50], which show a rapid stimulation of PKD1 activation with a sustained effect lasting over 60 min and an EC₅₀ of most stimulants in the nanomolar range. However, we found that CCK stimulation of the serine phosphorylation sites S744/748 and S916 differed in kinetics and efficacy. Specifically, CCK caused a significantly faster onset of S916 phosphorylation by CCK ($t_{1/2}=28.9$ vs. 103 s, $p=0.0031$, Table 1) with a comparable t_{max} . This result is somewhat unexpected because studies with PKD1 mutants have shown that S744/748 phosphorylation is required for PKC-mediated PKD1 activation [51] and in a recent

model of PKD1 activation by GPCRs, S744/748 phosphorylation was proposed to be an essential phosphorylation step leading to PKD1 activation and autophosphorylation of the S916 site of PKD1 [52]. However, evidence exists from a study of BMP-2, which binds to a cell surface serine-threonine kinase receptor, leading to PKD1 activation by stimulating S916 serine phosphorylation without affecting S744/748 phosphorylation [53]. While multiple explanations for the more rapid serine phosphorylation of S744/748 by CCK could be proposed, including different levels of amplification, an important possibility is that PKC-dependent and -independent pathways of PKD1 phosphorylation with different kinetics could exist in rat pancreatic acini.

In pancreatic acini the CCK_A-receptor has been shown to exist in both a low-affinity and a high-affinity receptor state, each of which can activate multiple, distinct downstream signaling cascades [19,54–56]. Our data support the conclusion that CCK-induced PKD1 activation and serine phosphorylation at both S744/748 and S916 is dependent on activation of both the low (80%) and the high affinity state (20%) of the CCK_A-receptor. This is relevant because activation of the high-affinity CCK_A receptor state by JMV does not lead to PLC or PKC activation in pancreatic acini [57], suggesting the participation of PLC/PKC-independent pathways of PKD1 activation in these cells. Nevertheless, 80% of PKD1 activation in pancreatic acini is mediated by the low affinity CCK_A receptor, which is coupled to PLC activation, and the important role of PLC is illustrated by the pronounced inhibition of PKD1 S744/748 phosphorylation by the PLC inhibitor U-73122.

While the importance of PKC stimulation in mediating PKD1 activation has received considerable attention [52], the role of changes in intracellular calcium in mediating PKD1 activation is unclear because the few studies investigating the role of calcium in PKD1 activation and serine phosphorylation gave contradictory results. In pancreatic acini, activation of the CCK_A-receptor leads to PLC activation, which in turn results in the formation of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP₃) [26,57,58]. DAG binds and activates pPKCs, while IP₃ causes an increase in intracellular calcium levels [19,59]. Several lines of evidence in our study support the conclusion that CCK stimulation of both arms of the PLC-pathway, i.e. PKC activation and changes in cytosolic calcium, mediate PKD1 serine phosphorylation and that both PKC-dependent and PKC-independent signaling mechanisms are important. First, we found a >80% suppression of CCK-induced PKD1 serine phosphorylation at both the S744/748 and the 916 site after inhibiting CCK-mediated changes in cytosolic calcium using thapsigargin in a calcium-free medium. This result was not due to a toxic effect of thapsigargin on pancreatic acini because TPA-mediated PKD1 serine phosphorylation at neither S744/748 nor the S916 site was altered. Second, the general (classical, novel) PKC inhibitor GF109203X [31,38] caused only a partial inhibition of CCK-induced PKD1 S916 phosphorylation (Fig. 6). This lack of complete inhibition was not due to incomplete PKC inhibition because GF109203X was used at concentrations that completely inhibited S916 phosphorylation induced by the classical/novel PKC activator TPA. Third, CCK caused PKD1 activation through stimulation of both the high (20% of maximum) and low affinity (80% of maximum) CCK_A receptor states. However, only activation of the low affinity CCK_A receptor state is coupled to cascades activating PKC in pancreatic acini [56–58], supporting the conclusion that 20% of maximal PKD1 activation is mediated by a PKC-independent mechanism. Fourth, after 24h of TPA incubation, there was a complete down-regulation of novel PKC isoforms and, consequently, of PKD1 S744/748 transphosphorylation. However, CCK could still cause an increase in PKD1 S916 phosphorylation (20% of maximal increase), suggesting again that 20% of PKC activation is mediated independently of PKC. Our results, showing that both PKC-dependent and PKC-independent pathways are important in mediating serine phosphorylation of PKD1 at S916 and S744/748 sites, are in contrast to those of others showing complete or almost complete inhibition of PKD1 activation by different stimuli after pre-incubation with GF109203X in Swiss 3T3 cells or rat-1 cells transfected with

the rat CCK_B receptor [21,22] and in a number other cells [20,60–62], including rat parotid acinar cells stimulated with carbachol [63]. Our results therefore contrast with the conclusion of a number of other studies in other tissues reporting that stimulation of PKD1 by GPCR activation is exclusively PKC-mediated [21,22,60,63]. However, our finding of PKD1 activation mediated partly through PKC-independent signaling cascades in rat pancreatic acini is consistent with other studies of PKD1 activation in response to the growth hormone BMP-2 in osteoblasts and mesenchymal stem cells [53,64], the cytostatic agent Ara-C in myeloid leukemia cells [65] or oxidative stress in HeLa cells [40]. Different mechanisms of PKC-independent PKD activation have been described including direct interaction with G_{βγ} [66], cleavage by caspase-3 [65] or tyrosine phosphorylation of PKD [40]. The present study does not provide further insight into the exact mechanism(s) involved, but our results for the first time provide strong support for the conclusion that PKC-independent pathways can be important in GPCR agonist-mediated activation of PKD1. The marked differences in PKD1 activation pathways found in different cells in response to various stimuli suggest that not only the cell, but also the stimulus used can have an effect on the possible PKD1 activation pathways stimulated.

Previous studies have shown the presence of one classical PKC isoform (PKC- α), two novel PKCs (PKC- δ , ϵ) and one atypical PKC (PKC- ζ) in rat pancreatic acini [33–36]. A number of our results support the conclusion that PKC- δ is the important PKC isoform in mediating CCK-dependent PKD1 activation. First, GF109203X, which has been shown to be an inhibitor of classical and novel PKC isoforms in pancreatic acini and other cells [31,33,38] or the classical/novel PKC inhibitor Go6983 [37] significantly inhibited CCK stimulated PKD1 S744/748 phosphorylation, suggesting the involvement of either PKC- α , δ or ϵ in PKD1 phosphorylation in pancreatic acini. Second, Go6976, an inhibitor of classical PKC isoforms and PKD1 activity [37] had no significant effect on PKD1 S744/748 phosphorylation by CCK. This result supports the conclusion that the conventional isoform, PKC- α , is not involved in CCK-mediated PKD1 activation in pancreatic acini. This conclusion is consistent with most [33,35,36], but not all [34] recent studies which report CCK_A receptor activation does not activate PKC- α in pancreatic acini. Third, PKC- ζ pseudosubstrate, a specific PKC- ζ inhibitor shown to inhibit PKC- ζ kinase activity in rat pancreatic acini under the same experimental conditions used in this study [33], had no effect on CCK mediated PKD1 S744/748 phosphorylation, illustrating that PKC- ζ is not involved in CCK-mediated PKD1 activation. Together, these data suggest that only the novel PKC isoforms δ and/or ϵ can mediate PKD activation. Our studies with isoform-specific PKC translocation inhibitors and dominant negative PKC isoforms clearly show that PKC- δ mediates CCK-induced PKD1 phosphorylation. This proposal is consistent with previous studies that demonstrate CCK is a potent activator of PKC- δ in pancreatic acini stimulating its tyrosine phosphorylation, translocation to plasma and nuclear membranes and its kinase activity [43,67]. This result is similar to recent studies showing PKD1 activation in rat aortic smooth muscle pcells, HeLa cells, BON neuroendocrine cells and intestinal epithelial cells is mediated by PKC- δ [48,68,69], but differs from other reports which show PKD1 activation can be mediated through PKC- α [49], PKC- ϵ [14,70], PKC- η [70,71] or PKC- θ [72] in other cells with other stimulants. These results suggest that PKD1 activation can not only vary in different cells with different stimulants in terms of the presence or absence of PKC-independent activation, but also in the PKC isoform mediating activation.

Various agents that activate PKD1 are reported to stimulate its translocation to membranes in transfected and tumor cells [5,12,13,46]. However, there are no studies in normal cells and there is no data on the distribution of the serine-phosphorylated PKD1 forms. In normal, unstimulated pancreatic acinar cells, we found that PKD1 was almost entirely located in the cytoplasm, which is similar to results reported in other cells. Furthermore, we found that both TPA and CCK stimulated total PKD translocation to membranes, however, there were several differences. With TPA, all detectable PKD translocates rapidly and durably to the membrane.

This observation is in accordance with a previous study [12] showing a durable membrane translocation of a transfected GFP-PKD1 fusion protein after phorbol ester stimulation in Cos-7 cells. After TPA stimulation, PKD1 phosphorylated at S916 and S744/748 was detectable in the membrane fraction only. In contrast to our results with TPA, after CCK treatment, total PKD1 increased in membranes, however the total amount of PKD1 translocated was sufficiently small not to cause a change in the total PKD in the cytoplasm. PKD1 forms phosphorylated at S916 and S744/748 were absent in unstimulated cells and were found both in the membrane and in the cytoplasm after CCK stimulation. These results support the conclusion that after stimulation with TPA or CCK, PKD1 rapidly translocates to the plasma membrane, however, the magnitude and duration of translocation is much greater with TPA. While our results with CCK suggest that PKD1 serine phosphorylation could be occurring either in the cytoplasm or plasma membrane, our results with TPA suggest the phosphorylation is occurring in the membrane fraction, because no PKD1 was detected in the cytoplasm and the phosphorylated form of PKD1 was detected only in the membrane fraction. Similar to our study, transfected GFP-PKD1 translocated from the cytoplasm to the membrane after bombesin stimulation in fibroblasts and epithelial cells and then there was a rapid re-distribution from the membrane to the cytoplasm, but in contrast to our results, this re-distribution was complete within 10 min after stimulation [13]. In another study, transfected GFP-PKD1 translocated from the cytoplasm to the membrane in transfected ventricular fibroblasts and remained active in the membrane for over 90 min [46]. At present it is unknown whether the marked differences in the redistribution of PKD1 after stimulation with various agents in different cell systems are due to the fact that we are assessing a normal cell system, whereas others are analyzing transfected cells or whether there is a difference in the translocation of PKD1 with different stimulants in different cells.

With various stimulants in other cells, PKD1 activation is reported to be affected by Src kinases [14,68,73], PI3K [4] and tyrosine phosphorylation of various cellular proteins [5,6]. We found that CCK-mediated PKD1 activation is not dependent on Src, PI3K, MAPK, activation or stimulation of tyrosine phosphorylation. These results suggest that the importance of Src kinases, PI3K and tyrosine phosphorylation of various cellular proteins in PKD1 activation differs with different stimuli in various cells.

In conclusion, we report for the first time that PKD1 is expressed in rat pancreatic acini and activated by GI hormones/neurotransmitters leading to activation of phospholipase C, but not by GI hormones with other cellular mechanisms or by pancreatic growth factors. CCK-induced serine phosphorylation at both S744/748 and S916 sites was mediated by activation of both the high affinity (20%) and low affinity (80%) CCK_A receptor state. In contrast to PKD1 activation in most other cells studied, CCK-induced PKD1 activation was only partially mediated by PLC/PKC in rat pancreatic acini and the principal PKC isoform involved is PKC- δ . Our results show that CCK-mediated PKD1 activation is highly dependent on changes in intracellular calcium but is not mediated by Src, PI3K, MAPK or tyrosine phosphorylation as reported for a number of other stimulants in other cell systems. Upon stimulation by CCK, PKD1 translocates to the membrane and marked increases of phospho-S916 and phospho-S744/748 occurred both in the membrane and, to a lesser degree, in the cytoplasm. However with TPA, total translocation to the membrane occurs and phosphorylated PKD1 forms are only present in the membrane, suggesting phosphorylation is occurring at the membrane. In agreement with some studies using transfected cell systems, but not others, our results show that the majority of activated PKD1 persists at the membrane after activation, whereas a fraction is redistributed to the cytoplasm. Our results demonstrate a number of novel aspects of PKD1 activation in normal pancreatic acinar cells by GI hormones/neurotransmitters not generally reported in studies of tumor cells or transfected cells. Particularly important are the demonstration of the importance of PKC-independent stimulation, the demonstration of the importance of calcium-mediated cellular processes for activation of PKD1 and the lack of

involvement of a number of other cellular signaling cascades reported important with other stimuli. These results emphasize the importance of studying the effects of stimulants of PKD1 in normal cell systems.

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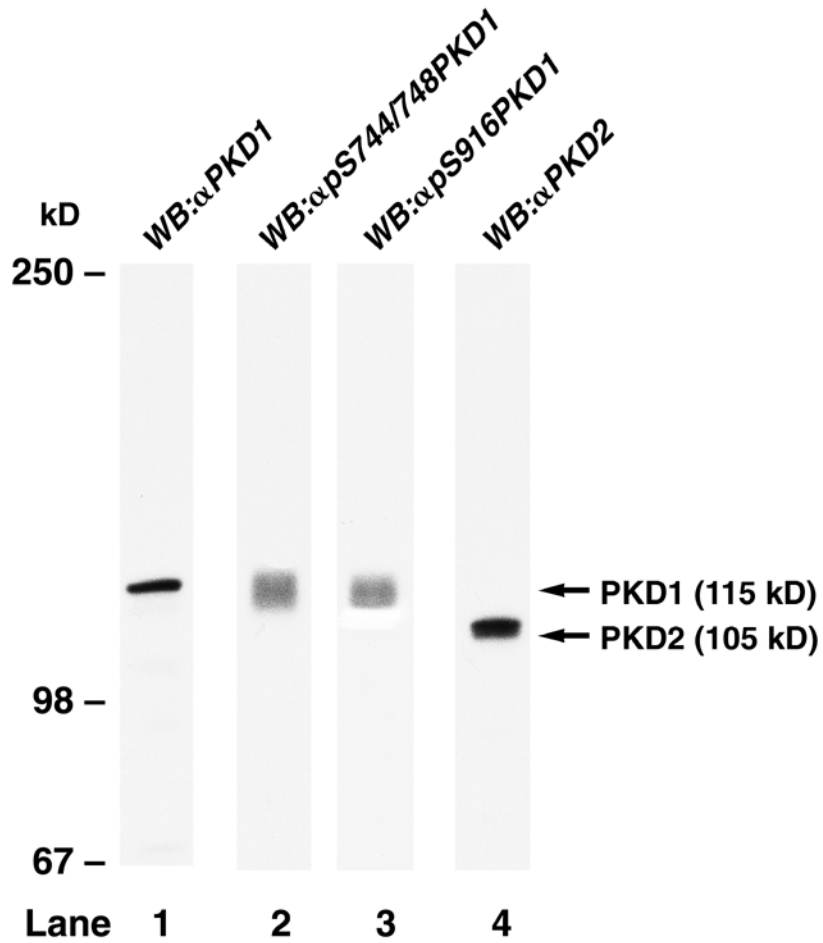


Fig 1. PKDs and their phosphorylated forms occur in rat pancreatic acini

Rat pancreatic acini were treated for 5 min with 10 nM CCK and then lysed. Western blots were analyzed using either anti-PKD1 antibody (Ab) (lane 1), anti-pS744/748 PKD1 Ab (lane 2), anti-pS916 PKD1 Ab (lane 3) or anti PKD2 Ab (lane 4). Bands were visualized using chemiluminescence. Positions of molecular mass markers are shown on the *left*. The position of PKD1 and PKD2 is indicated with *arrows*. Results are representative of 2 other experiments.

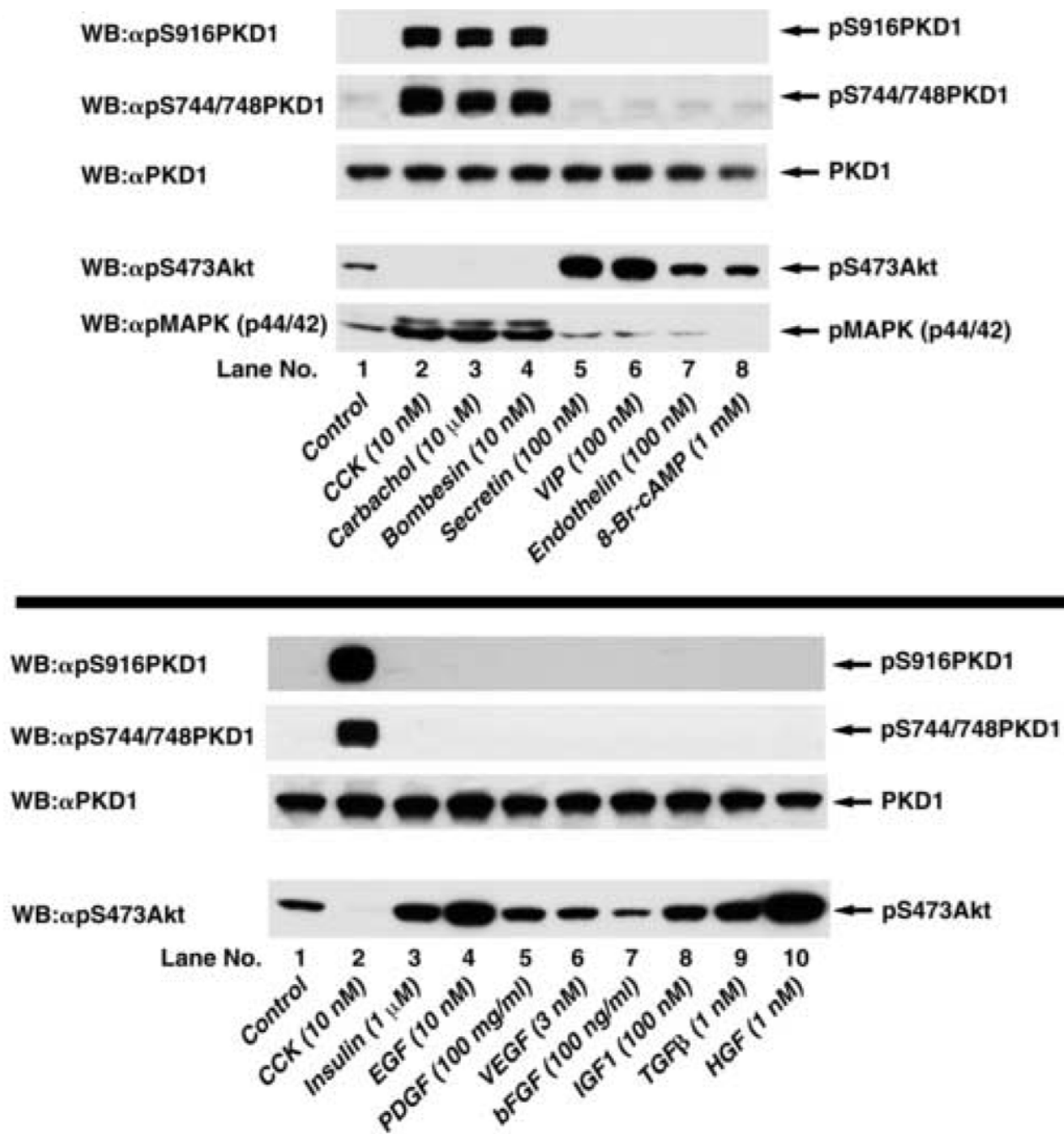


Fig 2. Ability of various pancreatic secretagogues and growth factors to stimulate PKD1 S916 and S744/748 phosphorylation in rat pancreatic acini

Top: Rat pancreatic acini were treated with no additions or with 10 nM CCK, 10 μM carbachol, 10 nM bombesin, 100 nM secretin or 100 nM VIP for 5 min, with 100 nM endothelin for 10 min, or with 1 mM 8-Br-cAMP for 30 min. *Upper 3 panels:* membranes were analyzed using either anti-pS916 PKD1 Ab (panel 1) or anti-pS744/748 PKD1 Ab (panel 2). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 3). *Lower 2 panels:* For positive controls for stimulation by pancreatic secretagogues, membranes were analyzed using anti-phospho Akt and anti-phospho MAPK Ab (panels 4, 5). The bands were visualized using chemiluminescence. Results from a representative experiment

of 6 experiments are shown. **Bottom:** Rat pancreatic acini were treated with no additions, with 10 nM CCK for 5 min as positive control or with 10 nM EGF for 2.5 min, with 1 μ M insulin or 100 ng/ml PDGF for 5 min, with 100 ng/ml bFGF or 1 nM HGF for 10 min, with 3 nM VEGF or 100 nM IGF1 for 15 min or with 1 nM TGF β for 60 min. *Upper 3 panels:* membranes were analyzed using either anti-pS916 PKD1 Ab (panel 1) or anti-pS744/748 PKD Ab (panel 2). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 3). *Lower panel:* For positive controls for stimulation by growth factors, membranes were analyzed using anti-phospho Akt Ab (panel 4). The bands were visualized using chemiluminescence. Results from a representative experiment of 4 experiments are shown.

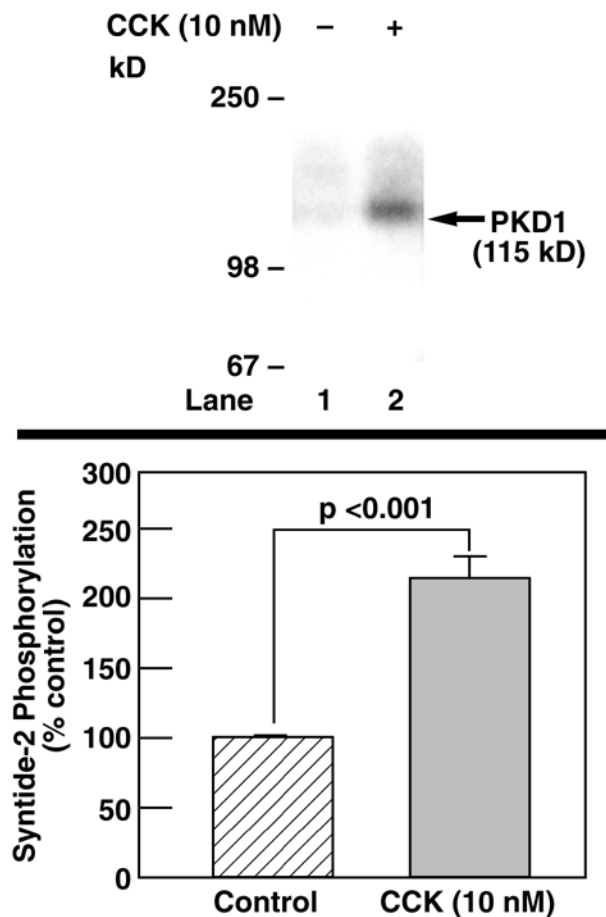


Fig 3. CCK activates PKD1 in rat pancreatic acini

Rat pancreatic acini were treated for 5 min with 10 nM CCK or with no additions and then lysed. For determination of PKD1 autophosphorylation, immunoprecipitates were incubated with kinase buffer containing ^{32}P -ATP and subjected to electrophoresis. Gels were dried and subjected to autoradiography (upper panel). Shown is a representative experiment of 3 others. For assessment of phosphorylation of the exogenous substrate syntide-2, immunoprecipitates were incubated with kinase buffer containing ^{32}P -ATP and syntide-2. The reaction was stopped by adding 0.75% phosphoric acid and spotted onto P81 phosphocellulose squares. Incorporation of ^{32}P was determined by scintillation counting. The values indicated are the means \pm S.E. of 7 independent experiments and are expressed as percent of the pretreatment value.

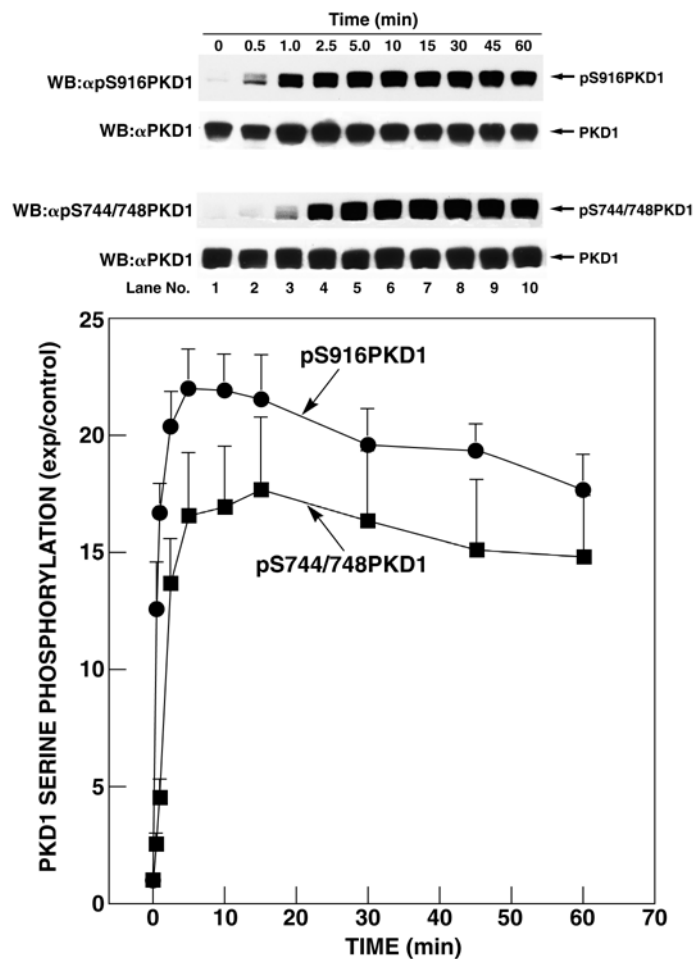


Fig 4. Time course of CCK stimulation of PKD1 S916 and S744/748 phosphorylation in rat pancreatic acini

Rat pancreatic acini were treated with no additions or with 10 nM CCK for the indicated periods of time and then lysed. *Upper 2 panels*: membranes were analyzed using anti-pS916 PKD1 Ab (panel 1). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 2). *Lower 2 panels*: membranes were analyzed using anti-pS744/748 PKD Ab (panel 3). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 4). The bands were visualized using chemiluminescence and quantification of phosphorylation was assessed using scanning densitometry. The *upper part* shows a representative experiment. The values shown in the *bottom part* are the means \pm S.E. of 4 independent experiments and are expressed as fold increases over the pretreatment level (exp/control).

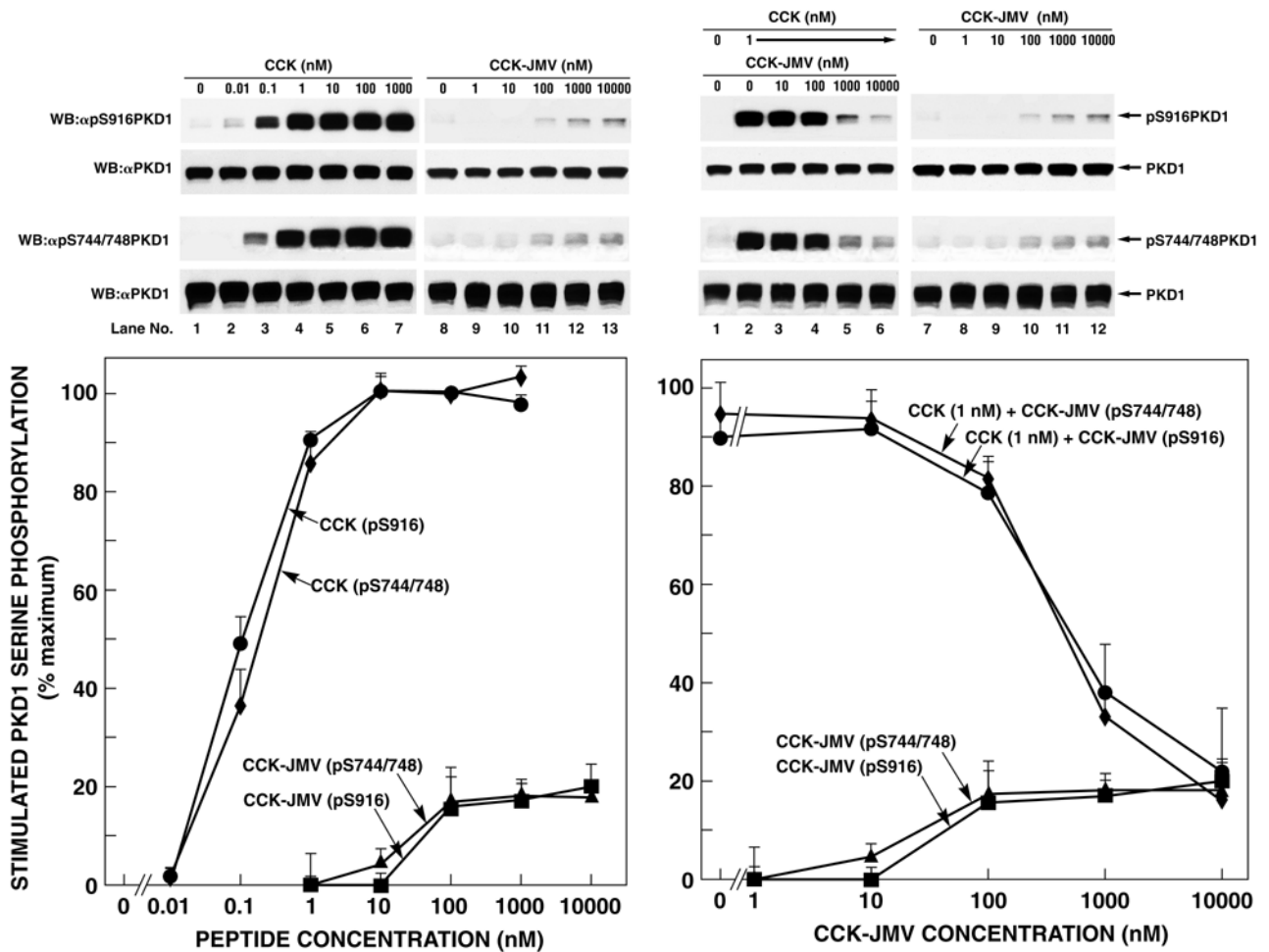
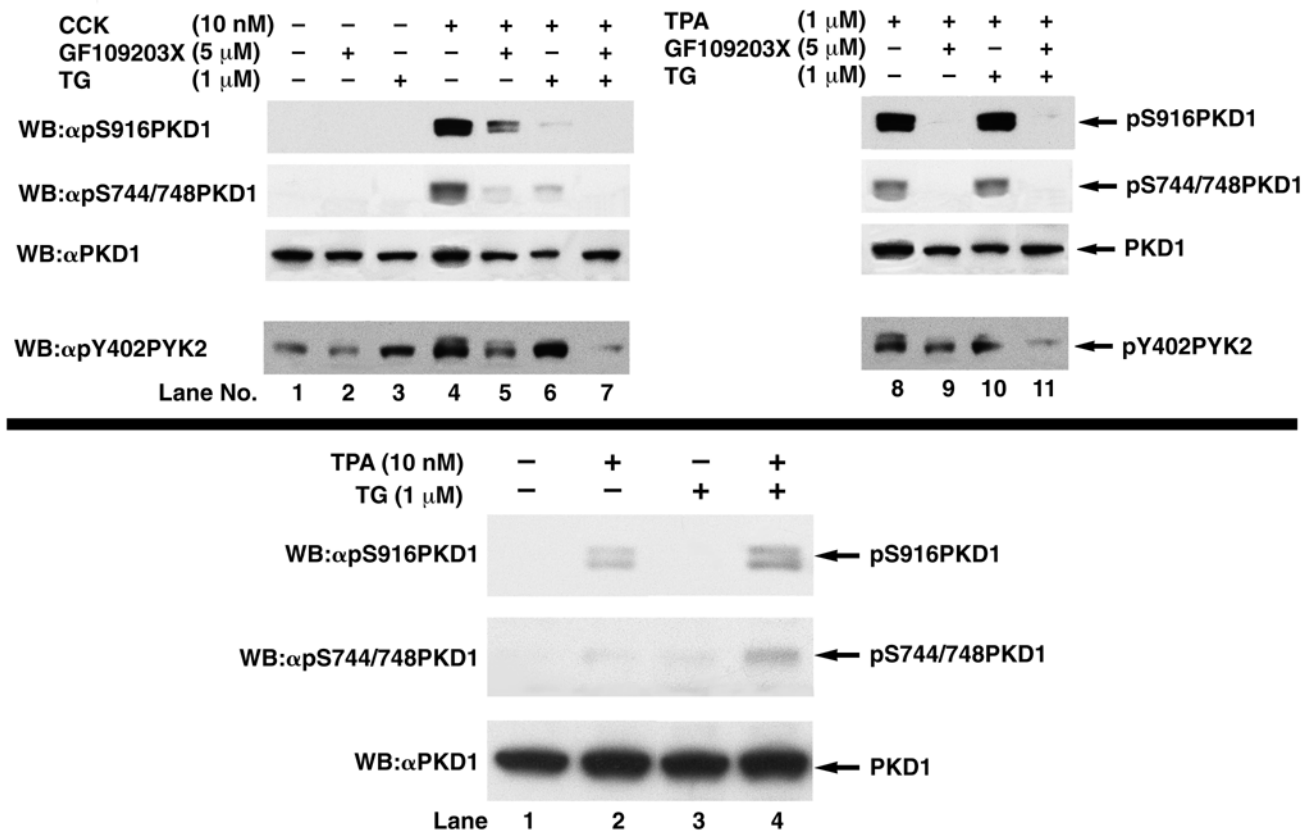


Fig 5. Concentration-dependence of CCK and CCK-JMV stimulation of PKD1 serine 916 and serine 744/748 phosphorylation in rat pancreatic acini

Left: Rat pancreatic acini were treated with no additions, with CCK or CCK-JMV at the indicated concentrations for 5 min and then lysed. *Upper 2 panels:* membranes were analyzed using anti-pS916 PKD1 Ab (panel 1). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 2). *Lower 2 panels:* membranes were analyzed using anti-pS744/748 PKD1 Ab (panel 3). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 4). **Right:** Rat pancreatic acini were treated with no additions or with CCK or/and CCK-JMV at the indicated concentrations for 5 min and then lysed. Whole cell lysates were submitted to SDS-PAGE and transferred to nitrocellulose membranes. *Upper 2 panels:* membranes were analyzed using anti-pS916 PKD1 Ab (panel 1). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 2). *Lower 2 panels:* membranes were analyzed using anti-pS744/748 PKD1 Ab (panel 3). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 4). The bands were visualized using chemiluminescence and quantification of phosphorylation was assessed using scanning densitometry. *The upper part* shows a representative experiment. The values shown in the *bottom part* are the means \pm S.E. of 4 independent experiments and are expressed as percentages of maximal increase caused by 100 nM CCK.

**Fig 6.**

Top: Effect of the PKC inhibitor GF109203X and/or depletion of intracellular calcium with thapsigargin on CCK- (left panel) and TPA- (right panel) stimulated PKD1 S916 and S744/748 phosphorylation in rat pancreatic acini. Rat pancreatic acini were pretreated for with no additions, with GF109203X (5 μM) for 2 h or with thapsigargin (1 μM) in a calcium-free medium (with EGTA 5 μM) for 1 h. Acini were then incubated with no additions (control), with 10 nM CCK for 10 min or with 1 μM TPA for 5 min and then lysed. *Upper 3 panels:* membranes were analyzed using anti-pS916 PKD1 Ab (panel 1) or anti-pS744/748 PKD1 Ab (panel 2). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 3). *Lower panel:* For positive control for calcium-free conditions, membranes were analyzed using anti-phospho PYK2 (Y402) Ab (panel 4). The bands were visualized using chemiluminescence and quantification of phosphorylation was assessed using scanning densitometry. The *upper part* shows a representative experiment of 5 independent experiments. **Bottom:** Effect of an acute increase in intracellular calcium on PKD1 phosphorylation. Rat pancreatic acini were treated with TPA (10 nM) and/or Thapsigargin (1 μM) for 10 min and then lysed. Membranes were analyzed using anti-pS916 PKD1 Ab (panel 1) or anti-pS744/748 PKD1 Ab (panel 2). To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab (panel 3). A representative blot of 3 independent experiments is shown.

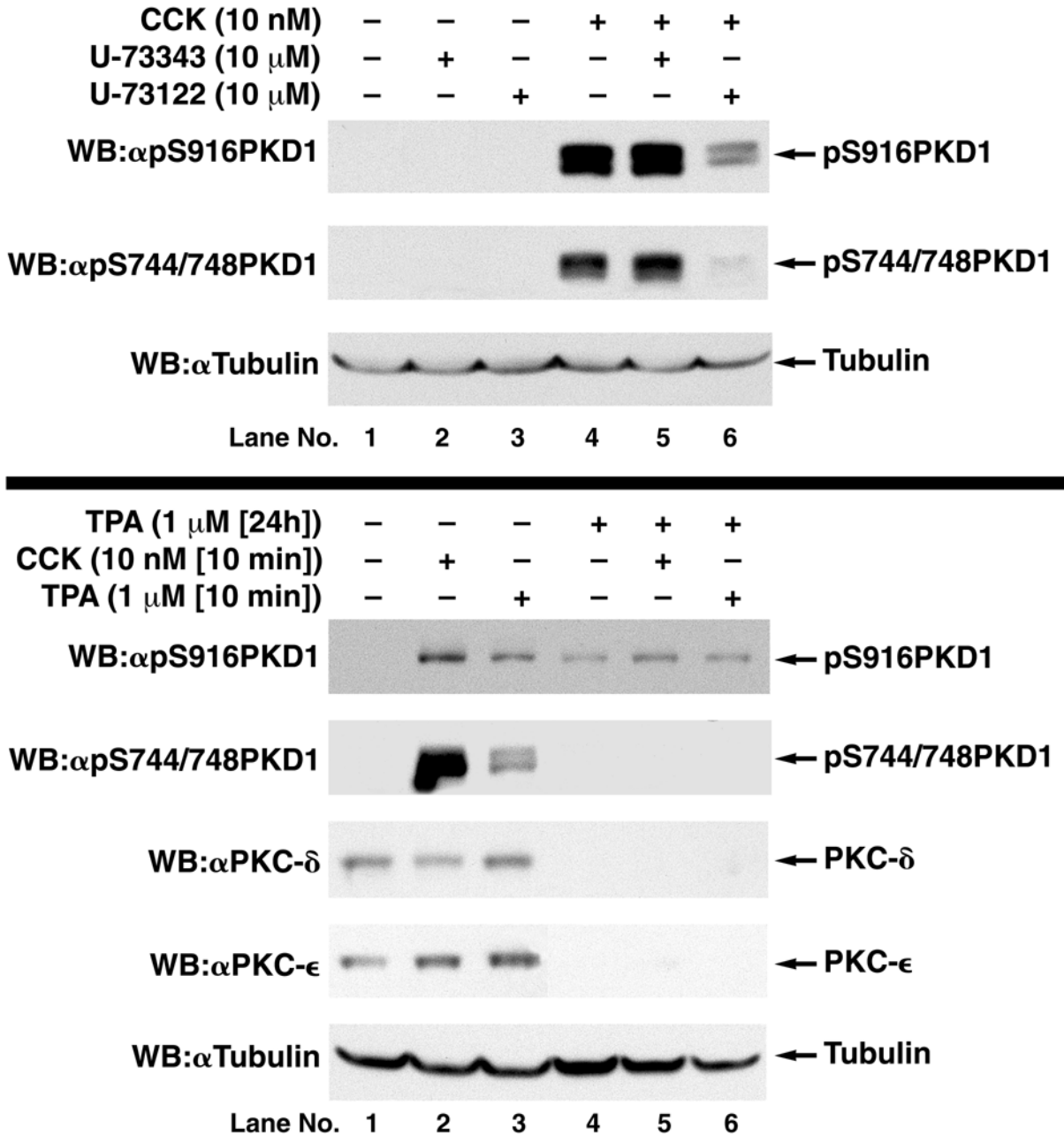


Fig 7.
Top: Effect of the inhibition of phospholipase C on CCK stimulated PKD1 S916 and S744/748 phosphorylation in rat pancreatic acini. Rat pancreatic acini were pretreated for 3 hours with no addition, with the inactive compound U-73343 (10 μ M) or the active compound U-73122 (10 μ M). Subsequently, acini were incubated with no additions (control) or with 10 nM CCK for 10 min and then lysed. *Upper panel:* Membranes were analyzed using anti-pS916 PKD1 Ab. *Middle panel:* Membranes were analyzed using anti-pS744/748 PKD1 Ab. *Lower panel:* To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-Tubulin Ab. The bands were visualized using chemiluminescence and quantification of phosphorylation was assessed using scanning densitometry. A representative experiment of 3 independent experiments is shown. **Bottom:** Effect of PKC down-regulation by 24h TPA

incubation on CCK and TPA stimulated PKD1 S916 and S744/748 phosphorylation in rat pancreatic acini. Rat pancreatic acini were cultured on collagen-coated dishes in Waymouth's Medium with 5% FCS as described in Methods and pretreated with no addition or with 1 μ M TPA for 24 h. Subsequently, acini were incubated with no additions (control), with 10 nM CCK for 10 min or with 1 μ M TPA for 5 min and then lysed. *Panel 1:* Membranes were analyzed using anti-pS916 PKD1 Ab. *Panel 2:* Membranes were analyzed using anti-pS744/748 PKD1 Ab. *Panel 3:* Membranes were analyzed using anti-PKC- δ Ab. *Panel 4:* Membranes were analyzed using anti-PKC- ϵ Ab. *Lower panel:* To verify loading of equal amounts of protein, membranes were stripped and re-blotted with an anti-Tubulin Ab. The bands were visualized using chemiluminescence. A representative experiment of 3 independent experiments is shown.

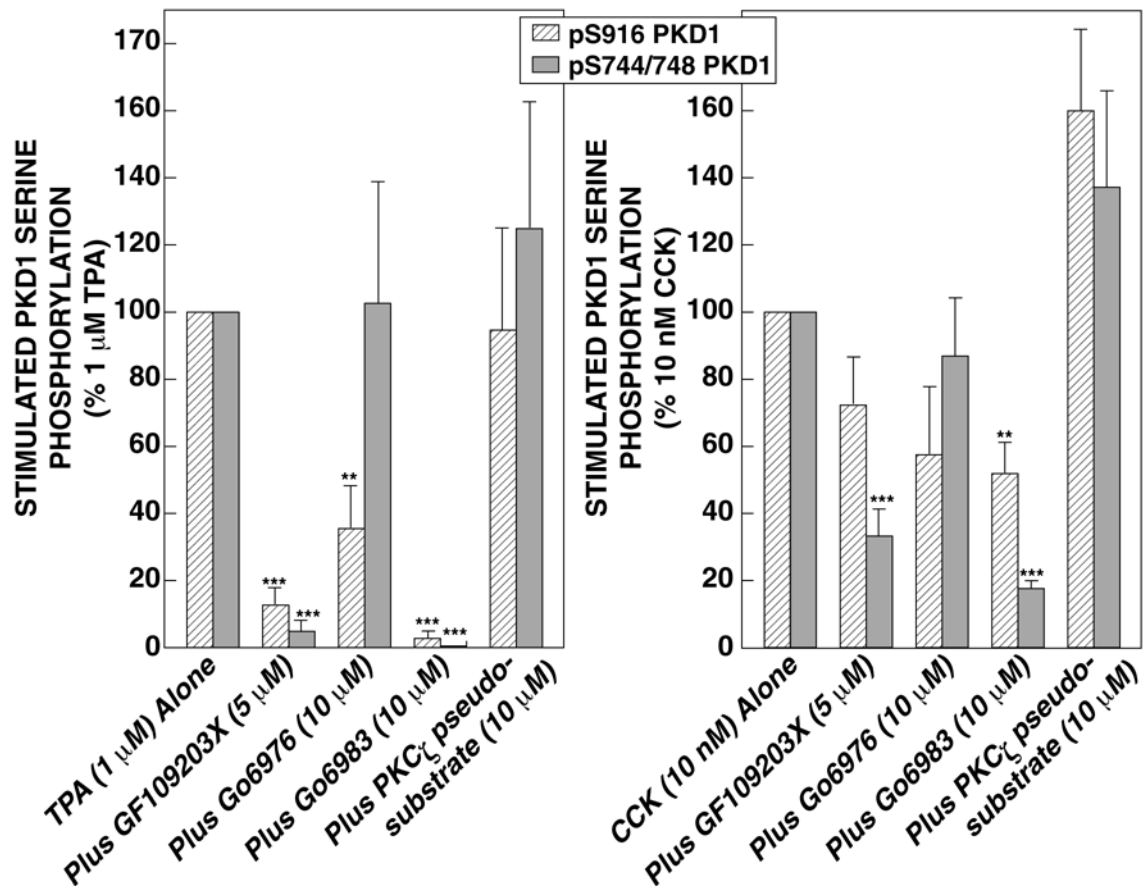
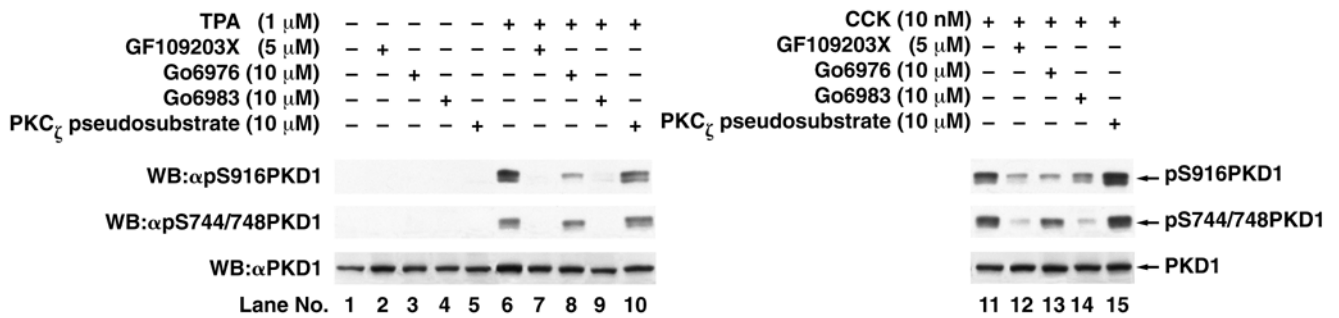


Fig 8. Effect of the inhibition of classical/novel/atypical PKC isoforms on TPA (left panel) and CCK (right panel) stimulated PKD1 S916 and S744/748 phosphorylation in rat pancreatic acini. Rat pancreatic acini were pretreated for with no addition, with GF109203X (5 μ M), Go6976 (10 μ M), Go6983 (10 μ M) for 2 h or with PKC ζ pseudosubstrate inhibitor (10 μ M) for 3 h. Subsequently, acini were incubated with no additions (control), with 10 nM CCK for 10 min or with 1 μ M TPA for 5 min and then lysed. *Upper panel:* Membranes were analyzed using anti-pS916 PKD1 Ab. *Middle panel:* Membranes were analyzed using anti-pS744/748 PKD1 Ab. *Lower panel:* To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-PKD1 Ab. The bands were visualized using chemiluminescence and quantification of phosphorylation was assessed using scanning densitometry. The *upper part* shows a representative experiment. The values shown in the *bottom part* are the means \pm S.E. of 6 independent experiments and are expressed as percentages of maximal increase caused

by 1 μ M TPA or 10 nM CCK. ** and *** indicate $p < 0.01$ and $p < 0.001$ compared to stimulation with CCK or TPA alone.

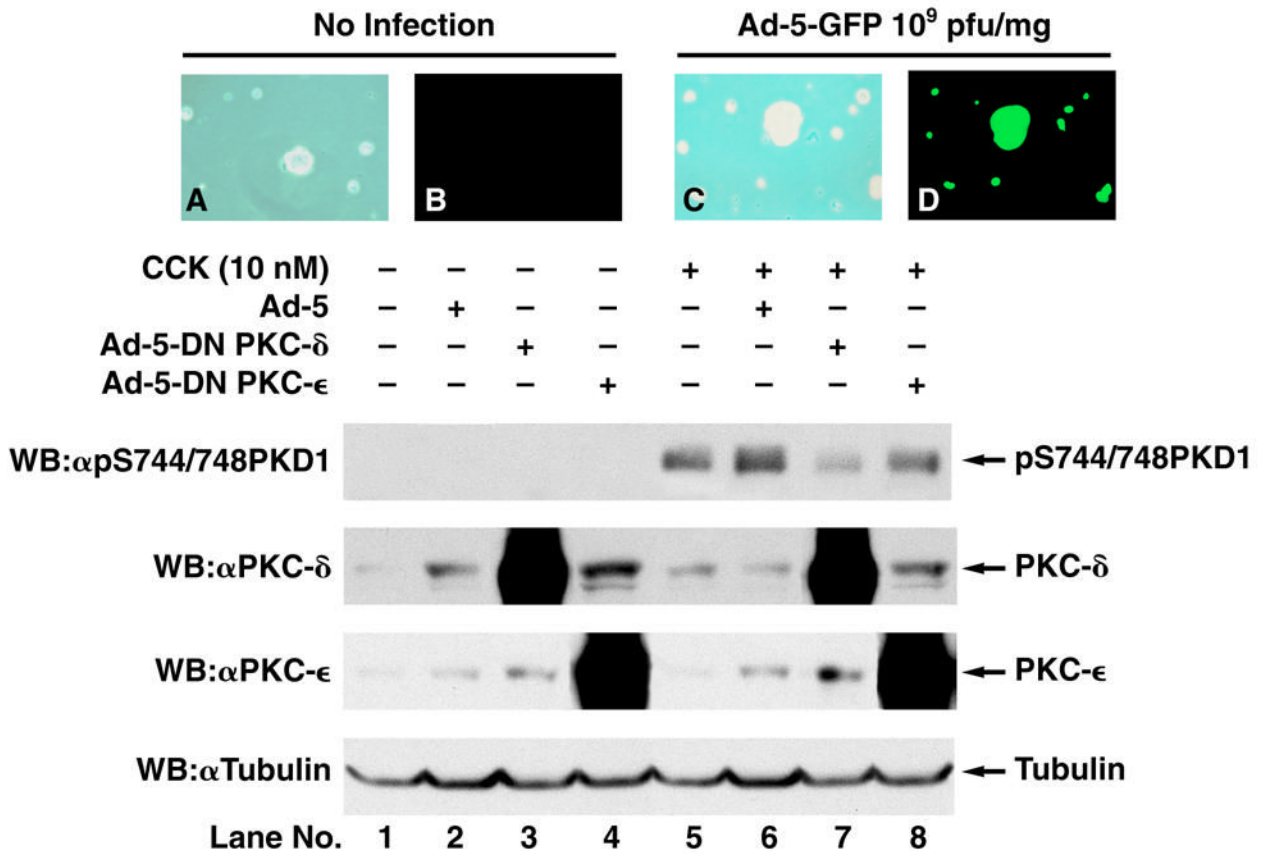
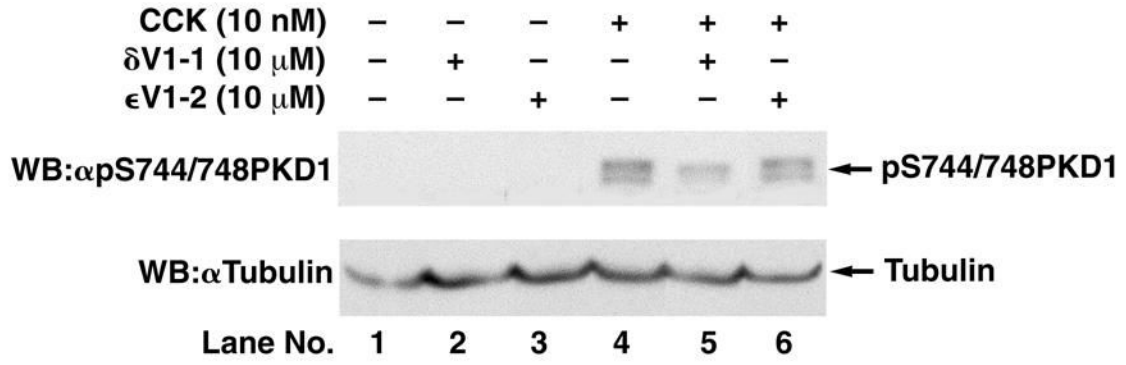


Fig 9. CCK-induced PKD1 phosphorylation is mediated by PKC- δ

Top: Rat pancreatic acini were pretreated for with no addition, with the PKC- δ translocation inhibitor δ V1-1 (10 μ M) or the PKC- ϵ Translocation inhibitor ϵ V1-2 (10 μ M) for 4 h.

Subsequently, acini were incubated with no additions (control) or with 10 nM CCK for 10 min.

Upper panel: Membranes were analyzed using anti-pS916 PKD1 Ab. *Lower panel:* To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-tubulin Ab. These results show a representative experiment of 3 independent experiments. **Bottom:**

Rat pancreatic acini were cultured as described in Methods and infected with no addition (Pictures A,C), with an empty adenoviral vector or with adenovirus encoding GFP (Pictures B,D), DN-PKC- δ or DN-PKC- ϵ . Pictures A/C show phase contrast microscopy and Pictures B/D show fluorescence microscopy after 24h. After 24h, acini for western blot experiments

were incubated with no additions (control) or with 10 nM CCK for 10 min and then lysed. Whole cell lysates were submitted to SDS-PAGE and transferred to nitrocellulose membranes. *Upper panel:* Membranes were analyzed using anti-pS744/748 PKD1 Ab. *Second panel:* Membranes were analyzed using anti-PKC- δ Ab. *Third panel:* Membranes were analyzed using anti-PKC- ϵ Ab. *Lower panel:* To verify loading of equal amounts of protein, membranes were stripped and re-blotted with anti-Tubulin Ab. The bands were visualized using chemiluminescence. These results show a representative experiment of 3 independent experiments.

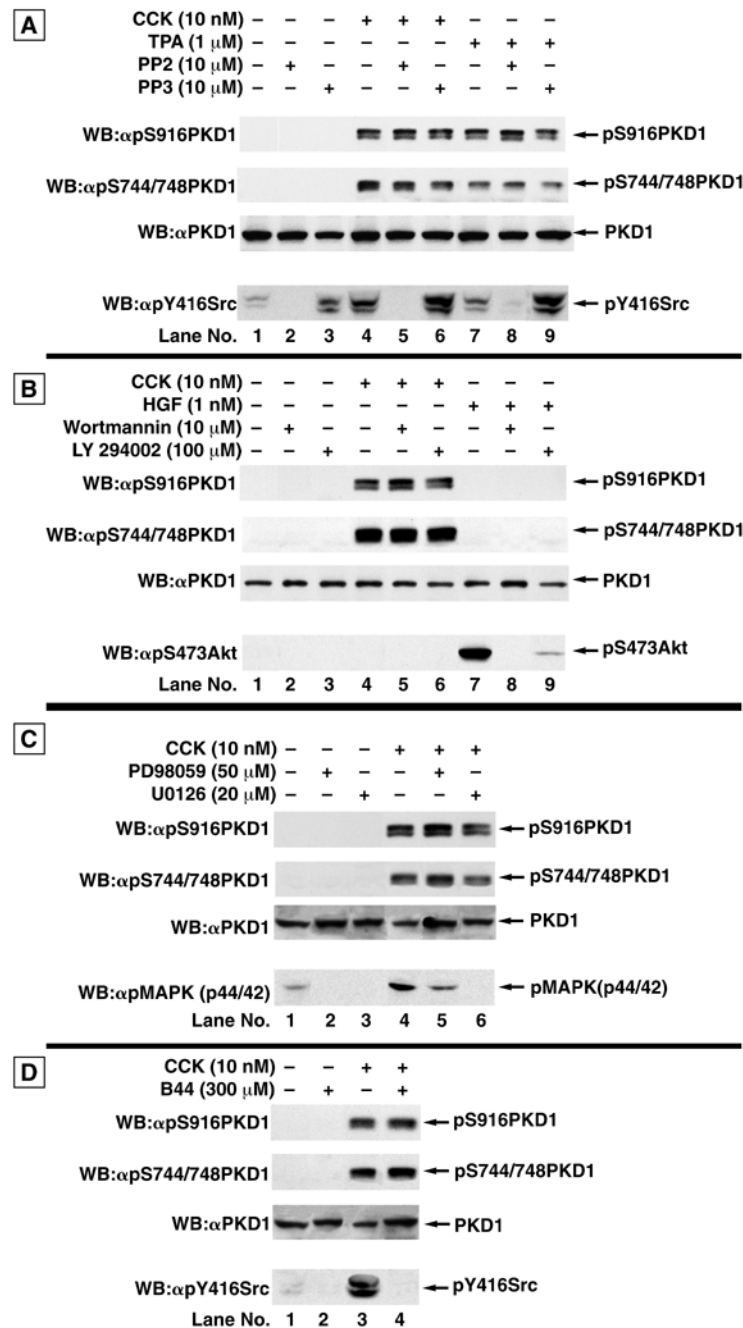


Fig 10. Inhibition of Src (A), PI3K (B), MAPK (C) or tyrosine phosphorylation (D) have no effect on CCK-stimulated PKD1 S916 and S744/748 phosphorylation in rat pancreatic acinar cells
 Rat pancreatic acini were pretreated for with no addition or with PP2 (10 μ M) or PP3 (10 μ M) (A), with LY294002 (100 μ M) for 1 h or with wortmannin (10 μ M) for 30 min (B), with U0126 (20 μ M) for 1 h or PD98059 for 30 min (C) and with B44 (300 μ M) for 1 h. Subsequently, acini were incubated with no additions (control), with 10 nM CCK for 10 min or with 1 μ M TPA for 5 min or with 1 nM HGF for 10 min and then lysed. Whole cell lysates were submitted to SDS-PAGE and transferred to nitrocellulose membranes. *Upper panel:* Membranes were analyzed using anti-pS916 PKD1 Ab. *Second panel:* Membranes were analyzed using anti-pS744/748 PKD1 Ab. *Third panel:* To verify loading of equal amounts of protein, membranes

were stripped and re-blotted with anti-PKD1 Ab. *Lower panel:* to verify that the used inhibitors could influence cellular functions in our experimental model, membranes were analyzed with anti-pY416 Src (A,C), anti-pS473 Akt (B) or anti-pMAPK (C) as positive controls. The bands were visualized using chemiluminescence and quantification of phosphorylation was assessed using scanning densitometry. These results show a representative experiment of 3 others.

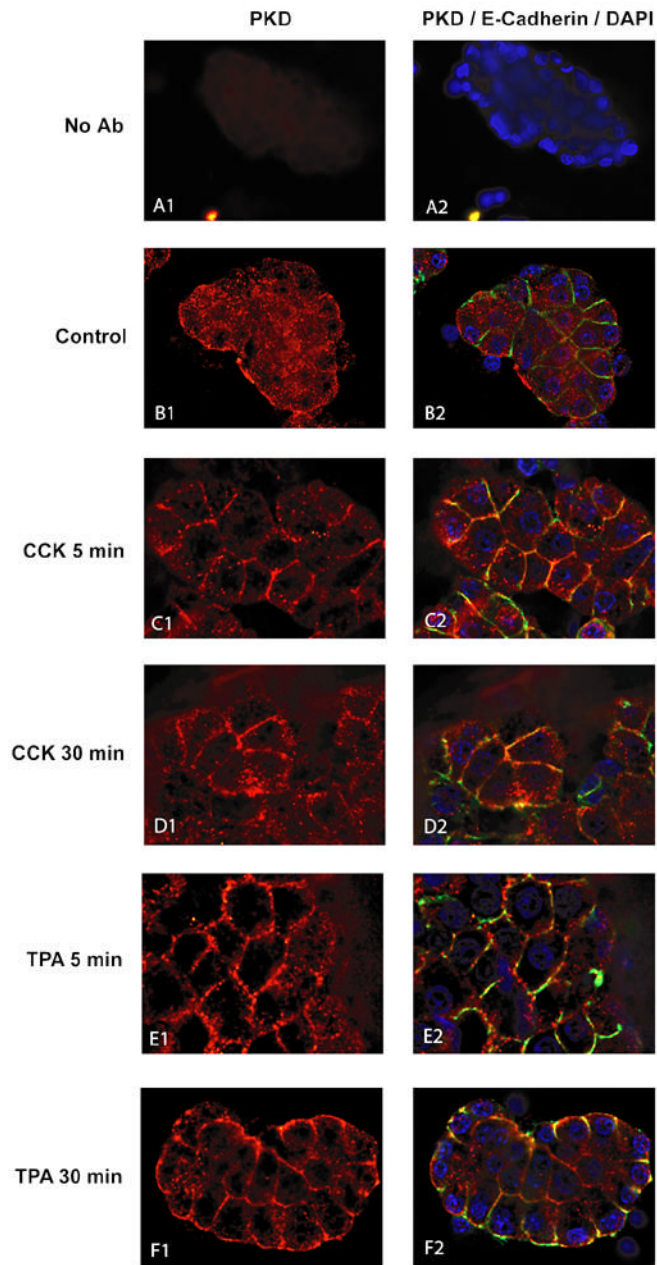


Fig 11. PKD immunofluorescence-cytochemistry in rat pancreatic acini with and without CCK treatment

Pancreatic acini were incubated with no addition, CCK (10 nM) or TPA (1 μ M) for time periods indicated below. After stimulation cells were washed, fixed, permeabilized and transferred onto poly-L-lysine coated glass slides by cyto centrifugation as described in Methods. Cells were then labeled using polyclonal rabbit anti-PKD (Santa Cruz, C-20) and mouse anti E-cadherin primary antibodies. Specific binding was detected using an Alexa Fluor555- and Fluor488-conjugated secondary antibodies so that red staining represents staining for total PKD and green staining represents E-caadherin. Nuclei were counterstained using DAPI (blue). Fluorescent images were collected using a Leica CTR5000 microscope. *Panels A1–2* show acini without the addition of the primary antibodies but with normal isotype-matched serum as a negative control. *Panels B1–2* shows acini treated with incubation buffer only. *Panels*

C1-2 and E1-2 show cells treated for 5 min with 10 nM CCK (panels C1-2) or 1 μ M TPA (panel E1-2). *Panels D1-2 and F1-2* show cells treated for 30 min with 10 nM CCK (panel D1-2) or 1 μ M TPA (panels F1-2). Shown are results of a typical experiment representative of 6 independent experiments. Cells shown are representative of >90% of total cells present.

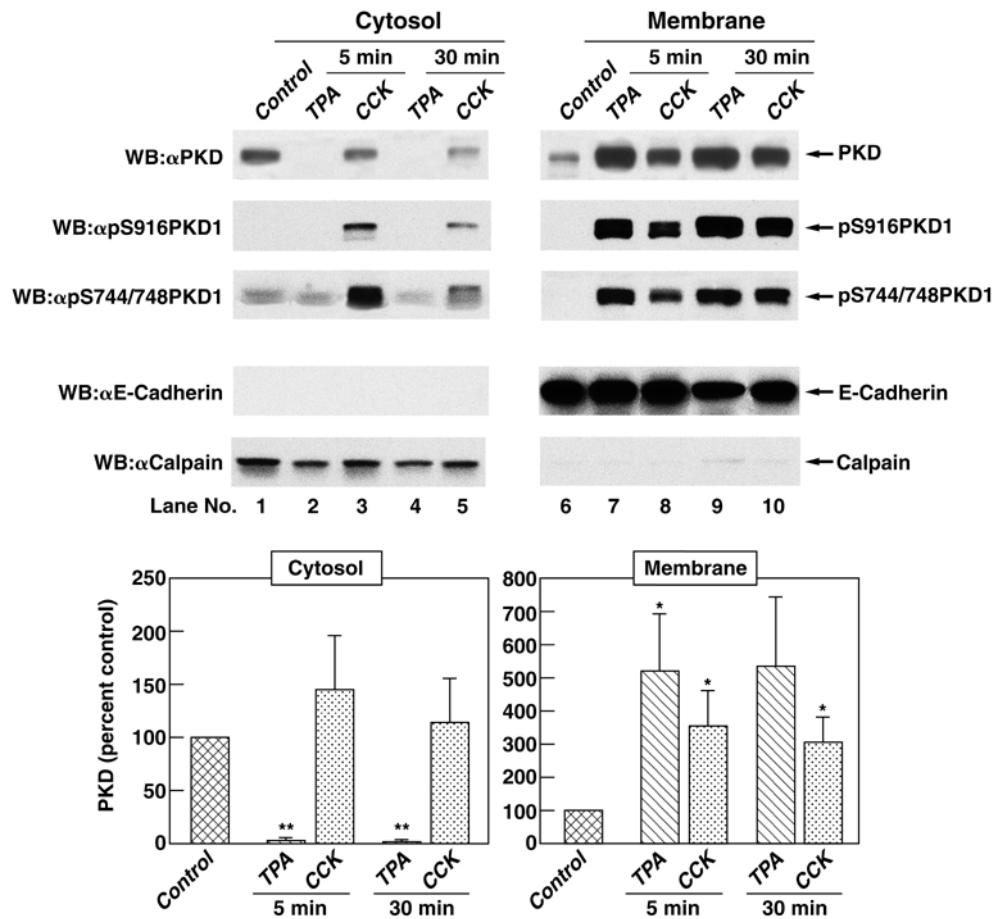


Fig 12. Ability of CCK and TPA to stimulate translocation of total PKD and phospho-PKD to the cell membranes in rat pancreatic acinar cells

Acini were incubated with or without 1 μ M TPA or 10 nM CCK for the time periods indicated. Samples were then processed as described in Methods to obtain subcellular fractions. Lysates from these fractions were submitted to SDS-PAGE and transferred to nitrocellulose membranes. *Upper 3 panels:* membranes were analyzed using anti-PKD Ab, anti pS916PKD1 Ab or anti pS744/748PKD1 Ab. *Lower 2 panels:* to assess the effectiveness of subcellular fractionation, the cytosol and membrane fractions were analyzed using anti-E-Cadherin Ab, a marker for the membrane fraction or anti-Calpain Ab, a marker for the cytosol, fraction. The bands were visualized using chemiluminescence and quantification of phosphorylation was assessed using scanning densitometry. The *upper part* shows a representative experiment. The *lower part* shows mean values \pm S.E. of 8 independent experiments. * and ** indicate $p < 0.05$ and $p < 0.001$, respectively, compared to the control group.

Table 1
Comparison of site-specific phosphorylation of PKD1 in rat pancreatic aciniⁱ.

Variable	pS916	PKD1 serine phosphorylation pS744/748	p
Stimulation by pancreatic secretagogues ⁱⁱ			
Yes	CCK, carbachol, bombesin	CCK, carbachol, bombesin	
No	Secretin, VIP, endothelin	Secretin, VIP, endothelin	
Stimulation by pancreatic growth factors ⁱⁱⁱ			
Yes	-	-	
No	Insulin, EGF, PDGF, VEGF, bFGF, IGF1, TGFβ, HGF	Insulin, EGF, PDGF, VEGF, bFGF, IGF1, TGFβ, HGF	
Stimulation by post-receptor activators			
Yes	TPA	TPA	
No	Ionophore, 8-Br-cAMP	Ionophore, 8-Br-cAMP	
t _{1/2} of CCK ^{iv} (s)	28.9 ± 3.1	103 ± 7.4	0.0031
t _{max} of CCK ^{iv} (s)	525 ± 75	900 ± 122	NS ^v
EC ₅₀ of CCK ^{vi} (nM)	0.14 ± 0.02	0.19 ± 0.05	NS
EC ₅₀ of CCK-JMV ^v (nM) ⁱ	1070 ± 725	119 ± 60	NS
Fold increase with CCK (100nM) ^{vi}	53.4 ± 7.2	11.2 ± 1.3	0.013
Fold increase with CCK-JMV(10 μM) ^{vi}	4.4 ± 0.9	2.5 ± 0.4	NS
CCK stimulation with GF109203X(5 μM) (% CCK alone)	38.0 ± 3.2	13.0 ± 0.8	0.026
CCK stimulation with Thapsigargin (1 μM) (% CCK alone)	4.2 ± 0.8	14.4 ± 1.4	0.032
CCK stimulation with Go6976(10 μM) (% CCK alone)	57.5 ± 20.0	86.9 ± 17.3	NS
CCK stimulation with Go6983(10 μM) (% CCK alone)	51.5 ± 9.1	17.6 ± 2.1	0.011

ⁱ Results are calculated from the data shown in Figures 2–8. For CCK stimulation after pre-incubation with GF109203X, thapsigargin, Go6976 and Go6983, results are expressed as the percentages of the stimulation seen with CCK (10 nM) after pre-incubation in incubation buffer without any additions.

ⁱⁱ Concentrations and incubation times are reported in Figure 2.

ⁱⁱⁱ Concentrations and incubation times are reported in Figure 2.

^{iv} Stimulation with 10 nM CCK as described in Fig. 4.

^v NS: p value not significant (≥0.05).

^{vi} Stimulation with CCK for 5 min as described in Fig. 5.