CCK1 and CCK2 Receptors Are Expressed on Pancreatic Stellate Cells and Induce Collagen Production

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The gastrointestinal hormone cholecystokinin (CCK) can induce acute pancreatitis in rodents through its action on acinar cells. Treatment with CCK, in combination with other agents, represents the most commonly used model to induce experimental chronic pancreatitis. Pancreatic stellate cells (PSC) are responsible for pancreatic fibrosis and therefore play a predominant role in the genesis of chronic pancreatitis. However, it is not known whether PSC express CCK receptors. Using real time PCR techniques, we demonstrate that CCK1 and CCK2 receptors are expressed on rat PSC. Interestingly both CCK and gastrin significantly induced type I collagen synthesis. Moreover, both inhibit proliferation. These effects are comparable with TGF-β-stimulated PSC. Furthermore, the natural agonists CCK and gastrin induce activation of pro-fibrogenic pathways Akt, ERK, and Src. Using specific CCK1 and CCK2 receptor (CCK2R) inhibitors, we found that Akt activation is mainly mediated by CCK2R. Akt activation by CCK and gastrin could be inhibited by the PI3K inhibitor wortmannin. Activation of ERK and the downstream target Elk-1 could be inhibited by the MEK inhibitor U0126. These data suggest that CCK and gastrin have direct activating effects on PSC, are able to induce collagen synthesis in these cells, and therefore appear to be important regulators of pancreatic fibrogenesis. Furthermore, similar to TGF- β , both CCK and gastrin inhibit proliferation in PSC.

Acute and chronic pancreatitis are responsible for a significant morbidity and mortality. Although studies have shown that pancreatic acinar cells activate the mechanisms leading to inflammation and organ destruction in acute pancreatitis (1), another cell type, called pancreatic stellate cells (PSC),³ play a pivotal role in the process leading to pancreatic fibrosis and chronic pancreatitis (2). These fibroblast-like cells represent only a minority of cells in the normal pancreas. Growth factors, *G*-protein-coupled receptors, and toxins activate PSC, leading to proliferation, migration, and production of extracellular matrix constituents including collagen, thereby inducing the organ changes characteristic of chronic pancreatitis (3–8). These cellular effects are mediated by central signal transduction cascades including Akt, ERK, and Src. The gastrointestinal hormone cholecystokinin (CCK) was among the first gastrointestinal hormones discovered. CCK binds to two receptors (CCK1R and CCK2R) that are expressed in many tissues, including the exocrine pancreas (9). The stimulation of CCK receptors by the natural agonists CCK (comparable affinity for CCK1R and CCK2R) and gastrin (1000-fold higher affinity for CCK2R) regulate many physiological and pathophysiological processes (9). Most notably, CCK induces experimental acute pancreatitis in rodents, a process mediated by the CCK1R expressed on pancreatic acinar cells. The pathophysiology of CCK-induced acute pancreatitis and the underlying signal transduction pathways have been extensively studied in whole animals and isolated acinar cells in the last two decades (10). Repeated administration of CCK, or its agonist cerulein, was used in many studies to induce different aspects of chronic pancreatitis, including activation of PSC and fibrosis. In these studies, it had been assumed that chronic pancreatitis is the result of repeated organ inflammation caused by multiple inductions of acute pancreatitis by CCK. Moreover, some studies found that patients suffering from chronic pancreatitis have higher plasma CCK levels than healthy controls (11-13) and this observation was the rationale of a clinical study of CCK-antagonist loxiglumide in chronic pancreatitis (14). Despite these experimental and clinical findings pointing to a role of CCK in chronic pancreatitis, surprisingly, no study has considered the possibility of a direct effect of CCK or CCK agonists on PSC. Even the expression of CCK receptors has not been studied on PSC. To systemically analyze the possible direct role of CCK and CCK receptors in the pathophysiology of chronic pancreatitis, we studied the expression of CCK1R and CCK2R on PSC, as well as the effect of stimulation of these receptors on collagen production, proliferation, and on pro-fibrogenic signal transduction pathways.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (150–250 g) were obtained from Charles River Laboratories (Sulzfeld, Germany). Akt Ser(P)⁴⁷³, anti-caspase 3, Src family (Tyr⁴¹⁶), ERK (Thr²⁰²/ Tyr²⁰⁴), U0126, and ELK-pS383 were from Cell Signaling Technology (Beverly, MA). Anti-goat horseradish peroxidase (HRP) conjugate, anti-actin, anti-p21 Ser¹⁴⁶, anti-p21, anti-p27, and anti-rabbit HRP-conjugate antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p27 Ser¹⁰ was from Abcam (Cambridge, UK). Collagen antibody was from Southern Biotech (Birmingham, AL). α -SMA antibody was from Dako (Denmark). Chamber slides and anti-PY20 antibody were purchased from BD Biosciences (San Jose, CA). L-364,718 and LY288513 were from Tocris (Ellisville, MO). Wortmannin,



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³ The abbreviations used are: PSC, pancreatic stellate cell; CCK, cholecystokinin; α-SMA, smooth muscle actin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide.

TGF- β RI kinase inhibitor VIII, and PI3K γ inhibitor II were from Calbiochem (Nottingham, UK). Iscove was from Biochrome AG (Berlin, Germany). SDS and ammonium sulfate were from Bio-Rad. Tris, acrylamide (40%), and glycin were from Carl Roth (Karlsruhe, Germany). Protein ladder was from Fermentas (Burlington, Canada). Agarose G, BCA protein assay, and SuperSignal West Dura were from Fisher Scientific. Films were from Amersham Biosciences. Donkey anti-goat Alexa Fluor 488, donkey anti-mouse Alexa Fluor 596, rabbit anti-goat Alexa Fluor 596, donkey anti-mouse Alexa Fluor 488, Hanks' balanced salt solution, FCS, PenStrep, NEAS, Dulbecco's PBS, and ETDA were from Invitrogen. Cell culture dishes were from Sarstedt (Nümbrecht, Germany). Protease, HEPES, benzamidine, orthovanadate, leupeptin, PMSF, and aprotinin were from Sigma. Protran nitrocellulose membranes were from Whatman. DNase and collagenase were from Roche Applied Science. CCK1R and CCK2R antibodies were from Everest Biotech (Oxfordshire, UK).

Animal Care—Animals were cared according to the guidelines and under supervision of the local animal welfare board.

Tissue Preparation—Pancreatic stellate cells were isolated using intraductal injection of enzyme solution followed by cell isolation and concentration of stellate cells by gradient centrifugation as reported previously (15, 16). Briefly, the common bile duct was intubated via the papilla using a 24-French syringe and 8 ml of an enzyme mixture (800 mg/liter of collagenase P, 400 mg/liter of Protease, and 200 mg/liter of DNase). Pancreases were excised injected with enzymes and cut into pieces. The tissue was disrupted by re-suspension in new enzymes and incubation at 37 °C for 10 min. Finally, cells were isolated using a 150- μ m mesh. Isolated cells were subjected to Histodenz gradient centrifugation as described previously to isolate pancreatic stellate cells. Stellate cells were cultured for 7 days, then subjected to stimulants as described.

Stellate Cell Lysis—After incubation with stimulants and/or inhibitors, stellate cells were lysed in lysis buffer (0.5 mm NaF, 0.2 mm EDTA, 10% glycerol, 10 mm benzamidine, 0.2 mm sodium orthovanadate). Lysates were centrifuged at $10,000 \times g$ for 15 min at 4 °C, and protein concentration was measured using the BCA reagent. Equal amounts of samples were analyzed by SDS-PAGE and Western blotting.

Immunoprecipitation—After cell lysis, equal amounts of samples were incubated overnight with agarose G and 4 μ g of the specific antibody diluted in lysis buffer. Then samples were centrifuged, washed three times, and analyzed by SDS-PAGE and Western blotting.

Western Blotting—Western blotting was performed as described previously.

Whole cell lysates were subjected to SDS-PAGE using Tris glycine gels. After electrophoresis, proteins were 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 room temperature for 1 h. Membranes were incubated overnight with primary antibody, washed three times in washing buffer (50 mM Tris/HCl, pH 8.0, 2 mM CaCl₂, 80 mM NaCl, 0.05% Tween 20) for 5 min, and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Membranes were washed again three

times in washing buffer for 5 min, incubated with chemiluminescense detection reagents and finally exposed to film. The intensity of the protein bands was measured using ImageJ analysis. When reprobing was necessary, membranes were incubated in Stripping buffer (Pierce) for 30 min at room temperature, washed twice for 10 min in washing buffer, blocked for 1 h in blocking buffer at room temperature, and re-probed as described above.

Immunocytochemistry-To detect CCK1R and CCK2R in isolated PSC, cells were grown on glass chamber slides, fixed with acetone, blocked in 2% rabbit-PBS, and immunostained. The sequence was 1:500 CCK1R and CCK2R, and rabbit anti-goat Alexa Fluor 488 at 1:200. α -SMA was counterstained using donkey anti-mouse Alexa Fluor 596. To detect the effects of CCK and gastrin on PSC, cells were grown on glass chamber slides. After serum starvation, cells were stimulated for 24 h with no additions, 1 nM CCK, or 1 μM gastrin (stimulants were replaced after 12 h). Then cells were fixed in acetone and immunostained. Staining sequence for collagen type I was goat anti-collagen I (1:50) and donkey anti-goat Alexa Fluor 488 (1:200). Staining for the α -SMA sequence was mouse anti- α -SMA (1:100) and donkey anti-goat Alexa Fluor 594 (1:200). Nuclei were counterstained with bisbenzimide. Pictures were taken using the IPLab3 software. For the collagen experiments we applied the same exposure time and magnification.

Immunohistology—To detect CCK1R and CCK2R in rat pancreatic tissue, we snap froze pancreatic tissue in liquid nitrogen. 5- μ m slides were then fixed in acetone, blocked in 2% rabbit PBS, and immunostained. Staining sequence was 1:500 CCK1R and CCK2R, and 1:200 for α -SMA. Secondary antibodies were rabbit anti-goat Alexa Fluor 596 for the CCK receptors and donkey anti-mouse Alexa Fluor 488 for α -SMA. Nuclei were counterstained using bisbenzimide.

All micrographs were taken using a Leica DM LB fluorescence microscope equipped with a Retiga I300 camera. We acquired the pictures using the IPLab3 software.

ELISA-Indirect ELISA was performed essentially as described by Kordes et al. (35). Stellate cells were grown in 12-well plates until confluence. After serum starvation, cells were stimulated for 24 h in serum-free medium containing $50 \,\mu g/ml$ of ascorbic acid (Sigma) with no additions, $2 \,ng/ml$ of TGFβ1 (Peprotech, Hamburg, Germany), 1 nM CCK, and 1 μ M gastrin. Stimulants were replaced after 12 h. The supernatants were collected and diluted (1:4) in PBS. 50 μ l/wells were used to coat Microtiter plates (Nunc Maxisorp, Langenselbold, Germany) overnight at 4 °C. Plates were blocked with 5% milk powder in PBS/T for 1 h. Plates were washed and incubated with anti-type I collagen for 2 h. Subsequently, the secondary anti-goat HRP antibody was used. Unbound secondary antibody was washed away and 100 μ l/well of tetramethylbenzidine substrate (TMB Plus, Kementec, Taestrup, Denmark) were added. Enzymatic reaction was stopped by adding 50 μ l/well of 0.2 N H₂SO₄. Plates were read on a standard plate reader at 540 nm. Purified type I collagen (Serva, Heidelberg, Germany) served as the control.





B Immunofluorescence

CCK1R



C Immunocytochemistry



CCK2R

CCK2R



FIGURE 1. **CCK2 and CCK1 receptors are expressed on rat PSC.** *A*, rat PSC were cultured for 7 days, RNA was isolated, transcribed, and samples submitted to RT-PCR. Negative controls (genomic DNA and water) were included. Expected product sizes are 93 (*CCK2R*) and 118 bp (*CCK1R*). A representative experiment of a total of 3 independent experiments is shown. *B*, rat pancreatic tissue was snap frozen, fixed in acetone, and 5- μ m sections were immunostained. Shown is a merged image of the CCK1R/CCK2R-stained tissue (*red*), counterstained with α -SMA (*green*). Magnification, \times 200. *C*, rat PSC were cultured, trypsinized, and plated on glass chamber slides. Cells were acetone fixed and immunostained for CCK1R/CCK2R and α -SMA. Magnification, \times 200.

Thymidine Uptake—Rat PSC were isolated and 50,000 cells/ well were seeded on 12-well plates. When reaching 60% confluence, cells were serum starved and then stimulated for 24 h in serum-free medium containing 2 μ Ci/ml of [³H]thymidine (PerkinElmer Life Sciences) with no additions, 2 ng/ml of TGF, 1 nM CCK, and 1 μ M gastrin. After 24 h cells were harvested (Brandel Harvester, Unterföhring, Germany) and [³H]thymidine incorporation was measured (Hidex Plate Chameleon, Straubenhardt, Germany). α -SMA as a tissue-specific marker was used to confirm localization on PSC. Snap frozen rat brain sections served as positive control for the antibodies.

CCK Causes Collagen Production in PSC—Upon stimulation by growth factors and several G protein-coupled receptors, PSC have been reported to produce extracellular matrix proteins including type I collagen, leading to pancreatic fibrosis, a constant feature of chronic pancreatitis. To date TGF- β has been the most potent fibrogenic stimulus described in PSC. To assess

MTT Assay-Rat PSC were isolated and cultured. Cells were trypsinized and 100,000 cells/well were seeded in 6-well plates. When reaching subconfluence, cells were serum-starved and stimulated with no additions, 2 ng/ml of TGF, 1 nM CCK, or 1 µM gastrin for 24 h. Stimulating factors were replaced after 12 h. Thereafter, 100 μ g/ml of MTT (Sigma) were added and cells were incubated for 2 h. Supernatants were removed and 250 µl/well of DMSO (Sigma) was added. Reaction products were transferred to microtiter plates and read in a standard microplate reader at 570 nm.

Statistical Analysis—Data are presented as mean \pm S.E. and were analyzed using the Student's *t* test for unpaired data using the Prism software (GraphPad). *p* values <0.05 were considered significant.

RESULTS

Rat PSC Express CCK1 and CCK2 *Receptors*—The presence of mRNA for CCK1 and CCK2 receptors was assessed by RT-PCR in PSC cultured for 7 days. According to the manufacturer of the primers used, specific bands of 118 (CCK1R) and 93 bp (CCK2R) were expected. As shown in Fig. 1A, expression of CCK1R mRNA was found in PSC (*lane 1*), but absent in the genomic DNA (lane 2) and negative control using water (lane 3). Similarly, CCK2R mRNA was found in PSC (lane 4), whereas the corresponding controls using genomic DNA (lane 5) or water (lane 6) did not show a corresponding band. We conclude that mRNA for both CCK1R and CCK2R is expressed in rat PSC.

Furthermore, the presence of both receptors was confirmed by immunohistology (Fig. 1*B*) and immunocytochemistry (Fig. 1*C*).





FIGURE 2. **Effect of CCK and gastrin on collagen type I synthesis in rat PSC.** *A*, rat PSC were cultured, serum-starved, and stimulated without additions, or with 1 nm CCK or 1 μ M gastrin for 24 h (stimulants were replaced every 12 h) and lysed. Lysates were analyzed by Western blot for procollagen type I. Western blots were scanned and densitometry was performed. The values represent the mean \pm S.E. of 6 experiments and are expressed as percent of the pre-treatment values. (*, p < 0.05 versus control). *B*, rat PSC were cultured, serum-starved, and stimulated without additions, or with 2 ng/ml of TGF, 1 nm CCK, or 1 μ M gastrin for 24 h (stimulants were replaced every 12 h). Indirect ELISA was performed using cell culture supernatants. The values represent the mean \pm S.E. of 5 experiments and are expressed as percent of the pre-treatment value. (*, p < 0.05 versus control). *C*, rat PSC were cultured, trypsinized, and plated on glass chamber slides. Serum-starved cells were stimulated without additions, or with 1 nm CCK or 1 μ M gastrin (stimulants were replaced every 12 h) for 24 h. Then cells were acetone fixed and immunostained for collagen type I and α -SMA. Micrographs were taken by using same exposure time and magnification (\times 200).

whether CCK or gastrin can stimulate collagen type I production in PSC (Fig. 2) and to compare its action to TGF- β we performed Western blot analysis and indirect ELISA upon stimulation with CCK and gastrin (Western blot, ELISA) and TGF- β (ELISA). After 24 h of stimulation, CCK (1 nM) caused a 136.3% increase (p < 0.05) in procollagen type I production. Similarly, gastrin (1 μ M) caused a 136% (p < 0.05) increase. TGF- β caused an increase in collagen production of 149%. Furthermore, to demonstrate that collagen type I production was specifically induced in PSC, we performed immunocytochemistry for collagen type I and α -SMA (Fig. 2, *right panel*). These results demonstrate that CCK and gastrin induced collagen production by rat PSC, and that collagen synthesis was comparable, and not statistically significantly different from TGF- β stimulated collagen production.

CCK and Gastrin Inhibit Proliferation in PSC—Some profibrogenic factors like TGF- β are also known to inhibit proliferation of PSC. To assess if CCK and gastrin have similar effects in inhibiting proliferation we performed thymidine uptake and MTT assays. After 24 h of stimulation TGF- β inhibited proliferation by 15.91%, CCK by 18.1%, and gastrin by 14.45% over controls (Fig. 3*A*). These results demonstrate that CCK and gastrin have similar effects to TGF- β in inhibiting proliferation of PSC. To elucidate if apoptosis or cell cycle arrest were involved in inhibition of proliferation upon stimulation, we performed caspase-3 assays, as well as p21 and p27 analysis. The missing cleavage of caspase-3 and the up-regulation of p21 and p27 suggest that G_1 cell cycle arrest, not apoptosis, is the likely mechanism involved. To address the question whether the up-regulation of p21 and p27 was due to an increase in transcriptional level or phosphorylation, we performed qPCR and Western blot analysis. As shown in Fig. 3C, p21 up-regulation is in part due to an increase in the transcriptional level and in part due to phosphorylation. Unlike p21, p27 is mostly regulated by phosphorylation (Fig. 3D).

Effect of CCK and Gastrin on Central Pro-fibrogenic Signal Transduction Pathways in Rat PSC—CCK has been reported to activate multiple signal transduction pathways including Akt, ERK, and Src in rat pancreatic acini 1 (7, 24). These central signal transduction pathways mediate activation of PSC and pancreatic fibrogenesis (5, 16, 25, 26). We analyzed whether CCK can activate Akt, ERK, and Src signal transduction pathways in rat PSC. Subsequently, we characterized the time

course of this effect using a CCK concentration that caused maximal Akt activation (1 nM). CCKs effect on Akt was maximal at 2.5 min (3.8 \pm 1.6-fold increase), significant at 5 min $(2.3 \pm 1.2$ -fold increase) with values returning to baseline after 10 min (Fig. 4A). CCK also $(1.7 \pm 0.8$ -fold) increased ERK phosphorylation after 2.5 min, with significant stimulation (1.8 \pm 0.06-fold, p = 0.0018) after 5 min, stimulation slightly above baseline (1.4 \pm 0.8-fold) after 10 min, and values comparable with baseline thereafter (Fig. 4A). Moreover, CCK caused significant stimulation (1.4 \pm 0.3-fold, p < 0.05) of Src family kinases after 2.5 min with maximal stimulation (1.7 \pm 0.3-fold, p = 0.014) at 5 min and stimulation slightly above baseline at 10 and 30 min (Fig. 4A). These data demonstrate that 1 nM CCK caused significant stimulation of the fibrogenic signal transduction pathways of Akt, ERK, and Src in rat PSC. Similar to CCK, gastrin activated Akt, ERK, and Src family kinases in rat PSC (Fig. 4A). Gastrin $(1 \mu M)$ caused maximal Akt stimulation at 2.5 min, significant stimulation at 5 and 10 min with values returning to baseline after 30 min (Fig. 4A). Similarly, gastrin $(1 \mu M)$ caused maximal ERK phosphorylation after 2.5 min with values returning to baseline after 3 min (Fig. 4A). Gastrin had a more complex effect on activation of Src family kinases with moderate stimulation after 2.5 and 5 min, no significant stimulation at 10 min, and strong stimulation at 30 min (Fig. 4A). These data





FIGURE 3. Effect of CCK, Gastrin and TGFbeta1 on proliferation of PSC. A, thymidine uptake: 50,000 cells/well were seeded, stimulated for 24 h, and [3 H]thymidine incorporation measured. The values represent the mean \pm S.E. of 5 experiments and are expressed as percentage of the control. *, p < 0.01 versus control. MTT assay, rat PSC were isolated and cultured for 7 days. Then 100,000 cells/well were seeded, stimulated, and the MTT assay performed. The values represent the mean \pm S.E. of 5 experiments and are expressed as percentage of the control.*, p < 0.01 versus control. B, rat PSC were cultured, serum starved, and stimulated for 24 h with no additions, ow with 2 ng/ml of TGF, 1 nm CCK, 1 μm gastrin, 100 μm cisplatin, and then lysed. Lysates were analyzed by Western blot (WB) for p21, p27, and caspase 3. β -Actin served as loading control. Shown is a representative experiment. C, rat PSC were cultured, serum starved, and stimulated for 24 h with no additions, or with 2 ng/ml of TGF, 1 nM CCK, and 1 µM gastrin. Cells were lysed/harvested and immunoprecipitations (*IP*) were performed using anti-p21 Ser¹⁴⁶. Western blots were then analyzed for anti-p21 Ser¹⁴⁶. RNA from harvested cells was isolated, reverse transcribed, and real time PCR for p21 mRNA was performed. The values indicated represent the mean ±S.E. of 4 experiments and are expressed as percentage of the control. (*, p < 0.05 versus control). D, rat PSC were cultured, serum starved, and stimulated for 24 h with no additionsm or with 2 ng/ml of TGF, 1 nM CCK, and 1 μ M gastrin. Cells were lysed/harvested and Western blots were performed using anti-p27 Ser¹⁰. β -Actin served as loading control. RNA from harvested cells was isolated, reverse transcribed, and real time PCR for p27 mRNA was performed. The values indicated represent the mean \pm S.E. of 4 experiments and are expressed as percentage of the control.

demonstrate that, similar to CCK, gastrin stimulated Akt, ERK, and Src in rat PSC.

CCK and Gastrin Modulate Akt Activation in a Dose-dependent Manner in Rat PSC-We have recently shown that CCK1R induced a biphasic modulation of Akt activity in rat pancreatic acini depending on the CCK concentration (18). Furthermore, recent publications have shown an important role of Akt in PSC activation (6, 19). Phosphorylation of Akt at serine 473 has been shown to closely correlate with Akt activity in numerous studies (20, 21). Therefore, we examined the effect of different doses of CCK on Akt Ser473 phosphorylation in rat PSC (Fig. 4B). Doses as low as 1 pM CCK caused a significant increase in Akt Ser473 phosphorylation. This response was maximal at 1 nm (2.36 \pm 0.45-fold increase) and then decreased to be just slightly above control at 1 μ M. These results suggest that CCK has a complex effect on Akt activation in rat PSC: low doses of CCK induce significant Akt activation, whereas higher doses of CCK partially reverse this activation. To assess the effect of CCK2R on Akt activation in rat PSC, we studied the effect of the CCK2R-preferring agonist gastrin on Akt serine 473 phosphorylation (Fig. 4B). Gastrin (0.1 nm) caused a significant (p < 0.01)increase in Akt Ser⁴⁷³ phosphorylation and this effect was maximal at 1 μ M (2.25 \pm 0.40-fold). These data suggest that CCK2R stimulated Akt activation in rat PSC.

CCKs Action on Collagen Production, Akt Activation Was Mediated by Both Receptors but Primarily by the CCK2R in Rat PSC—Although CCK1R binds CCK with high affinity (K_d in the nanomolar range) and gastrin with low affinity (K_d in the micromolar range), the CCK2R had almost equal affinity for gastrin and CCK (for review, see Ref. 22). To determine whether CCK and gastrin effects are mediated by CCK1R or CCK2R, we used L364 and LY288513 as specific CCK1R and CCK2R inhibitors, respectively (for





FIGURE 4. **Effect of CCK and gastrin on central fibrogenic signal transduction pathways in rat PSC.** *A*, rat PSC were cultured for 7 days and stimulated without additions (*control*) with 1 nm CCK (*left panels*) or with 1 μ m gastrin (*right panels*) and then lysed. Lysates were analyzed by Western blot (*WB*) for pAkt, pERK, and pSrc. Blots were stripped and analyzed for actin. Shown is a representative experiment of a total of 3 experiments. *B*, rat PSC were cultured for 7 days and treated with CCK (*left panel*) and gastrin (*right panel*) for 5 min. Proteins were analyzed for phosphorylated Akt by Western blot. Blots were stripped and analyzed for actin. Shown are representative results of a total of 6 experiments.

review of CCKR inhibitors, see Ref. 9). The CCK1R inhibitor L364 caused a moderate, but significant (p < 0.001) inhibition of Akt phosphorylation by 1 nM CCK (Fig. 5A, second versus fourth lane) and had a similar effect on Akt phosphorylation by 1 μ M gastrin (Fig. 5A, seventh versus ninth lane). CCK2R inhibitor LY288513 strongly inhibited (p < 0.0001) Akt phosphorylation caused by 1 pM CCK (Fig. 5A, second versus third lane) and almost completely suppressed the effect of 1 µM gastrin on Akt phosphorylation (Fig. 5A, seventh versus eighth lane). A combination of both inhibitors inhibited Akt phosphorylation to a greater and statistically significant extent than the inhibition of one receptor alone, showing clearly that both receptors are involved (Fig. 5A, *fifth* and *tenth lane*). The same inhibitors were used to evaluate CCK1 and -2 receptor inhibition on collagen synthesis and proliferation (Fig. 5B). Similar to the results on Akt activation inhibition of CCK2R leads to a greater decrease of collagen synthesis than inhibition of CCK1R; simultaneous inhibition of both receptors reduces collagen production back to unstimulated levels, whereas proliferation raises production to unstimulated levels. These results clearly show that the inhibitory effect of CCK and gastrin is mediated through CCK1 and CCK2 receptors.

Akt Activation by CCK and Gastrin Are Mediated by PI3K in Rat PSC—Akt activation by growth factors is generally mediated by PI3K in most experimental systems. In rat pancreatic acini, Akt activation by the high affinity state of the CCK1R is mediated by PI3K (18). Therefore, we wanted to study if PI3K mediates Akt activation by CCK and gastrin in rat PSC. The highly specific PI3K inhibitor wortmannin (10 μ M) caused a significant decrease of basal Akt Ser473 phosphorylation in rat PSC (Fig. 6A, second versus first lane) and almost completely suppressed Akt Ser473 phosphorylation caused by CCK and gastrin. These data demonstrate that CCK- and gastrin-stimulated Akt phosphorylation are mediated by PI3K in rat PSC. PI3K consists of two major components: p85, which is the regulatory site and p110, the catalytic domain (27). To study the events involved in CCKand gastrin-mediated PI3K activation, we inhibited the β and γ subunits of G protein-coupled receptor targeting p110 using the PI3K γ inhibitor II. As shown in Fig. 6C no inhibition was seen. On the other hand, inhibition of the Src pathway using the inhibitor PP2 leads to a marked decrease in CCK and gastrin-stimulated p85 activation (Fig. 6B). This result shows that CCK and gastrin activation of the PI3K is regulated by Src-dependent activation of the p85 subunit.

ERK Mediates Activation of Transcription Factor Elk-1 in Response to Gastrin and CCK in Rat PSC—The transcription factor Elk-1 has been reported to mediate ERK-induced c-fos transcription in response to growth factors, leading to cellular growth and proliferation in some cell systems (28, 29). This pathway has been reported to mediate PSC activation by alcohol and acetaldehyde (30). Therefore, we wanted to investigate whether CCK and gastrin cause Elk-1 activation and whether this activation is mediated by ERK. Our experiments show that both CCK and gastrin caused a significant increase in Elk-1 Ser³⁸³ phosphorylation, reflecting activation of Elk-1 (Fig. 7). As reported for pancreatic acini (17), the specific MEK inhibitor U0126 inhibited ERK activation induced by CCK and gastrin (Fig. 7). Moreover, U0126 completely inhibited stimulation of Elk-1 phosphorylation induced by CCK and gastrin (Fig. 7). These data demonstrate that, similar to findings reported in other cells, in rat PSC, transcription factor Elk-1 is a downstream target of the MEK-ERK pathway activated by CCK and gastrin.

Recent studies (31, 32) have shown that beyond its localization in the nucleus, transcription factor Elk-1 can be found in the cytosol. We did not find a cytosolic fraction of Elk-1 in PSC and subsequently no nuclear transition upon stimulation with CCK or gastrin (data not shown).

Pathways Involved in CCK and Gastrin-stimulated Collagen Production and Inhibition of Proliferation—As shown in Fig. 2, CCK and gastrin stimulate collagen production in PSC. Fig. 3 shows the inhibitory effect on proliferation of CCK and gastrin.





FIGURE 5. Effect of CCK1R and CCK2R inhibitor on Akt activation, collagen synthesis, and proliferation mediated by CCK or gastrin in rat PSC. A, rat PSC were cultured for 7 days, serum starved, and preincubated without additions, 1 μ M CCK1R inhibitor L364 for 2.5 min, 10 nM CCK2R inhibitor LY288513, or a combination of both for 5 min. Cells were stimulated without additions (control), 1 nM CCK for 5 min or 1 µM gastrin for 5 min and then lysed. Upper panel, lysates were analyzed by Western blot for Akt $Ser(P)^{473}$. Blots were stripped and analyzed for actin. Shown is a representative experiment of a total of 6 experiments. Lower panel, Western blots (WB) were scanned and densitometry was performed. The values represent the mean \pm S.E. of 6 experiments and are expressed as fold of the maximal stimulation achieved with CCK or gastrin alone (*, p < 0.05). B, ELISA: rat PSC were cultured for 7 days, serum starved, and preincubated with 1 μ M L364 for 2.5 min, or with 10 nM CCK2R LY288513 for 5 min, or with the combination. Cells were stimulated without additions, or with 1 nm CCK or 1 μ m gasrin for 24 h. Stimulants and inhibitors were replaced after 12 h. Indirect ELISA using cell culture supernatants was performed. MTT assay: rat PSC were cultured for 7 days, serum starved, and preincubated with 1 μ M L364 for 2.5 min, with 10 nm CCK2R LY288513 for 5 min, or with the combination. Cells were stimulated without additions, or with 1 nm CCK or 1 μ m gasrin for 24 h. Stimulants and inhibitors were replaced after 12 h. MTT assays were performed. The values represent the mean \pm S.E. of 3 experiments and are expressed as percentage of the control. *, p < 0.05 versus control.

Furthermore, we were able to show that CCK and gastrin stimulate different signaling pathways. Therefore we next wanted to elucidate which pathways are involved in CCK- and gastrinstimulated collagen production and inhibition of proliferation. As shown in Fig. 8, *A* (CCK) and *B* (gastrin), inhibition of Src with the specific inhibitor PP2 leads to a statistically significant decrease in collagen synthesis in PSC, whereas inhibition of MEK and PI3K shows only a trend to decrease collagen production without reaching statistical significance. Fig. 8, *C* (CCK) chronic pancreatitis. To date, the cytokine TGF- β had been the most potent stimulator of collagen synthesis in PSC (35). As shown in Fig. 2, the effect of CCK and gastrin on collagen synthesis was comparable with TGF- β -stimulated collagen production supporting their potential role in development of pancreatic fibrosis. Although expression of other matrix proteins, such as fibronectin, TIMP-1, and MMP has been described in PSC (36), CCK and gastrin were unable to stimulate the production of fibronectin and TIMP1.

and D (gastrin), shows that the same intracellular mechanisms are involved in inhibiting proliferation.

DISCUSSION

Our results clearly demonstrate that both CCK1 and CCK2 receptors are expressed in rat PSC. First, using the RT-PCR technique, we found that mRNA for both CCK1R and CCK2R is present in rat PSC. For both PCR, the specificity of the primer-target interaction was confirmed by the presence of a specific band in agarose-gel electrophoresis (Fig. 1). Furthermore, an interaction with possible contaminations of genomic DNA can be ruled out by the absence of a specific band in controls using genomic DNA (Fig. 1A). Furthermore, we were able to show the presence of both receptors using immunohistochemistry and immunocytology, also showing colocalization with α -SMA (Fig. 1, B and C). Most importantly, the data show that both receptors are involved in crucial cellular functions such as collagen synthesis and proliferation. Our data demonstrate for the first time that CCK and gastrin cause a significant increase in collagen I synthesis (Fig. 2). Synthesis of type I collagen contributes to organ fibrosis in the liver and pancreas. Therefore, our finding that CCK receptors have a direct effect on PSC by increasing collagen synthesis is of particular interest, because it demonstrates for the first time that CCK receptors can induce pancreatic fibrosis. CCK serum levels are often elevated in patients with chronic pancreatitis. A direct fibrogenic effect of CCK could be an important mediator of pancreatic fibrosis, suggesting that CCK receptors could be an interesting therapeutic target in patients with





FIGURE 6. **Effect of CCK and gastrin on PI3K and Akt.** *A*, rat PSC were cultured for 7 days and preincubated without additions or with 10 μ M wortmannin for 30 min. After this preincubation, cells were stimulated without additions (*control*) or with 1 nm CCK for 5 min or with 1 μ M gastrin for 5 min, and then lysed. Lysates were analyzed by Western blot (*WB*) for Akt Ser(P)⁴⁷³. Blots were stripped and analyzed for actin. *B*, rat PSC were cultured for 7 days and preincubated without additions or with 10 μ M PP2 for 60 min. After this preincubation, cells were stimulated without additions (*control*), with 1 nm CCK for 5 min or with 1 μ M gastrin for 5 min, and then lysed. Immunoprecipitation (*IP*) was performed using anti-PY20 antibody. Western blots were then analyzed for p85. *C*, rat PSC were cultured for 7 days and preincubated without additions or with 2 μ M PI3K γ inhibitor II for 60 min. After this preincubation, cells were stimulated without additions (*control*), or with 1 nm CCK for 5 min, and then lysed. Immunoprecipitation (*IP*) was performed using anti-PY20 antibody. Western blots were then analyzed for p85. *C*, rat PSC were cultured for 7 days and preincubated without additions or with 2 μ M PI3K γ inhibitor II for 60 min. After this preincubation, cells were stimulated without additions (*control*), or with 1 nm CCK for 5 min or 1 μ M gastrin for 5 min, and then lysed. Western blots were analyzed for Akt Ser(P)⁴⁷³. Blots were stripped and analyzed for actin.

To further evaluate the functional role of CCK and gastrin in PSC, we examined proliferation. Similar to TGF-β, and in contrast to other cytokines like PDGF (35), both CCK and gastrin inhibited proliferation. As shown in Fig. 3, the inhibitory effect is unlikely to be caused by apoptosis. However, accumulation of p21 and p27 upon stimulation with CCK, gastrin, and TGF- β supports the hypothesis that the mechanism involves G_1 cell cycle arrest. p21 is considered one of the most important effector molecules of p53, but is also known to be regulated via many p53 independent pathways. It is known to be regulated on the transcriptional level and to undergo posttranslational modifications, such as phosphorylation (37). p27 is regulated by several different independent mechanisms. Phosphorylation plays a major role: for example, phosphorylation of Ser¹⁰ leads to export from the nucleus and phosphorylation of Thr¹⁸⁷ marks the protein for degradation (37, 38). As shown in Fig. 3, *C* and *D*, p21 is regulated on the transcriptional and protein levels, whereas p27 is regulated by phosphorylation upon stimulation with CCK and gastrin in PSC. To address the question which



FIGURE 7. Effect of MEK inhibitor U0126 on ERK and Elk-1 activation mediated by CCK or gastrin in rat PSC. Rat PSC were cultured for 7 days, serum starved, and preincubated without additions or with 20 μ M U0126 for 60 min. After this preincubation, cells were stimulated without additions (*control*), or with 1 nM CCK for 5 min or 1 μ M gastrin for 5 min, and then lysed. Upper panel, lysates were analyzed by Western blot (*WB*) for pERK and pElk-1. Blots were stripped and analyzed for actin. Shown is a representative experiment of a total of 3 experiments. *Lower panel*, Western blots were scanned and densitometry was performed. The values represent the mean \pm S.E. of 3 experiments and are expressed as fold of the pre-treatment values.

profibrogenic pathways are involved, we investigated the Akt, Src, and ERK pathways.

Our results demonstrate that rat PSC express functional CCK1 and CCK2 receptors (Figs. 4*B* and 5, *A* and *B*). Because numerous studies have found that rat acinar cells express only CCK1R, expression of both CCK1R and CCK2R in rat PSC was unexpected. Previous studies using CCK1R antibodies in rat, pig, and human pancreas (33) or CCK1R and CCK2R autoradiography in human pancreas (35) did not describe expression of CCK1R on fibroblast-like cells. Because PSC represent only a minority of cells in the normal pancreas, single positive PSCs on thin sections might be missed. Therefore, our data demonstrating expression of both CCK1R and CCK2R on rat PSC are compatible with these previous studies.

Our finding that CCK2R can activate PI3K and Akt is consistent with previous studies showing PI3K activation by gastrin in AR42J cells (40) and CCK2R-transfected COS-7 cells (41). Our data suggest that CCK1R could also have an effect on Akt phosphorylation, but studies with specific CCK1R agonists are needed to clearly define that effect.



FIGURE 8. **Pathways involved in CCK and gastrin-stimulated collagen production and inhibition of proliferation.** *A*, rat PSC were cultured, serum starved, and stimulated without additions, or preincubated with wortmannin (30 min, 10 μ mol), PP2 (10 μ mol 60 min), U0126 (20 μ mol 60), and stimulated with 1 nm CCK for 24 h (stimulants were replaced every 12 h). Indirect ELISA was performed using cell culture supernatants. The values represent the mean \pm S.E. of 4 experiments and are expressed as percent of the pre-treatment value. *B*, rat PSC were cultured, serum starved, and stimulated without additions, or preincubated with wortmannin (30 min, 10 μ mol), PP2 (10 μ mol 60 min), U0126 (20 μ mol 60), and stimulated with 1 μ M gastrin for 24 h (stimulants were replaced every 12 h). Indirect ELISA was performed using cell culture supernatants. The values represent the mean \pm S.E. of 4 experiments and are expressed as percent of the pre-treatment value. *C*, *MTT assay*: rat PSC were isolated and cultured for 7 days. Then 100,000 cells/well were seeded, stimulated with 1 nm CCK, and the MTT assay was performed. The values represent the mean \pm S.E. of 5 experiments and are expressed as percentage of the control. *, p < 0.01 versus control. *D*, *MTT assay*: rat PSC were isolated and cultured for 7 days. Then 100,000 cells/well were seeded, stimulated with 1 μ M gastrin, and MTT assay was performed. The values control. *GW*, gastrin + wortmannin.

Our data demonstrate that both CCK and gastrin cause activation of the MEK-ERK pathway in rat PSC, leading to activation of the transcription factor Elk-1. This is compatible with studies showing activation of ERK by CCK in rat pancreatic acini (10) and with studies showing activation of ERK by CCK and gastrin in AR42J pancreas cancer cells (42). Our finding that gastrin and CCK induce significant ERK activation is particularly relevant because PSC activation and proliferation caused by PDGF, one of the strongest known activators of PSC, is mediated by ERK (16, 43). We have shown that both CCK and gastrin cause reproducible activation of Src family kinases (Fig. 4A). This finding is compatible with studies showing activation of Src kinases in rat pancreatic acini by CCK (mediated by CCK1R)(24) and with studies showing activation of Src kinases by gastrin in AR42J and Panc-1 pancreas cancer cells (44-46) as well as in other multiple cells. Activation of Src family kinases by CCK and gastrin in rat PSC is interesting because Src family kinases activate the JAK2-STAT pathway after PDGF stimulation in PSC and thereby could be important regulators of PSC proliferation (26). Furthermore, we were able to show (Fig. 8)

that inhibition of Src reduces collagen synthesis and proliferation in a statistically significant manner, pointing to the Src pathway as a pivotal pathway in CCK- and gastrinstimulated collagen production and inhibition of proliferation.

Chronic pancreatitis is a complex disease and our knowledge of the exact pathophysiologic mechanisms are still incomplete. Recent studies suggest that the development of chronic pancreatitis requires a first episode of pancreatitis (sentinel acute pancreatitis event), which, by the release of different cytokines, triggers activation of immune cells and stellate cells, leading to chronic inflammation, fibrosis, and destruction of normal organ architecture, resulting in loss of organ function (47, 48). In this process, activation of stellate cells is crucial, because these cells have been shown to be responsible for the development of pancreatic fibrosis, which is a constant feature of chronic pancreatitis. In rodent animal models, CCK is routinely used in combination with other agents to induce chronic pancreatitis (23, 49-51). These studies have supposed that the role of CCK in these models was to trigger the sentinel pancreatitis event by interacting with CCK receptors on acinar cells and inducing necrosis of acinar cells (49). Our finding that CCK can

directly activate stellate cells and induce collagen production suggests that the role of CCK is more complex: 1) by its action on acinar cells, it could contribute to the induction of the sentinel pancreatitis event; and 2) by its action on stellate cells, it could serve as an important regulator of pancreatic fibrosis. In conclusion, we report for the first time that rat PSC express CCK1 and CCK2 receptors, that the natural agonists CCK and gastrin induce activation of pro-fibrotic signaling pathways PI3K/Akt, MEK/ERK, and Src, induce activation of the transcription factor Elk-1, and most notably significantly increase synthesis of type I collagen and inhibit proliferation.

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