Supporting Information

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SI Experimental Procedures

Calculation for ACh Secretion by PSCs. From the data depicted in Fig. 2*A*, the amount of ACh secreted by human PSCs can be estimated on the basis of the calculated mean basal secretion of ACh by PSCs (without neostigmine) which was 120.26 pM/million cells (equivalent to 12 μM/g PSC lysates protein). We have previously shown that an amount of 1 million PSCs corresponds to approximately 10 μg cellular protein. Thus, 120.26 pM/million cells is equivalent to 120.26 pM/10 μg lysates protein, which is equivalent to 12 μM/g protein.

Rat and Human PSC Isolation. Rat and human PSCs were isolated by density gradient centrifugation using a method developed by our group (1). Human pancreatic tissue was obtained from patients undergoing a pancreatic resection for benign pancreatic conditions. Briefly, excised pancreatic tissue was digested with proteases and DNase and the digest centrifuged through a 11.4% wt/vol Nycodenz gradient for 20 min at 1,400 × *g*. Stellate cells were harvested and resuspended in Iscove's modified Dulbecco medium containing 10% FBS, 4 mM glutamine and penicillin/streptomycin 1% vol/vol. Cells were cultured at 37 °C in a humidified 5% CO_2/air atmosphere. Purity of freshly isolated PSCs was assessed by dual staining of cells for the presence of lipid droplets in the cytoplasm and for GFAP. Upon culture on plastic, activated PSCs were identified by immunostaining for α-SMA as described previously (1). Fig. S1 shows a high-power view of freshly isolated PSCs staining positive for GFAP (red, arrows) and lipid droplets (green, arrowheads). All cells examined had abundant lipid droplets, ruling out the possibility of neural cell contamination in the freshly isolated PSC preparations.

Assessment of ChAT, VAChT, and Synaptophysin Expression by PSCs.
Western blotting. Equal amounts of protein from cell lysates (50 μg for ChAT, 100 μg for VAChT) were subjected to gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with polyclonal goat anti-ChAT (1:1,000; Chemicon) or polyclonal goat anti-VAChT antibody (1:1,000; Sigma) overnight at 4 °C. HRP-coupled rabbit anti-goat antibody was used as a secondary antibody and bands were detected by ECL. Rat brain homogenate was used as a positive control.
Immunofluorescence. Human PSCs on coverslips were washed twice with PBS solution and fixed with 4% paraformaldehyde for 10 min. Cells were incubated with polyclonal goat anti-VAChT (1:1,000) or polyclonal goat anti-ChAT (1:1,000) or polyclonal rabbit anti-human synaptophysin (1:200) overnight at 4 °C. After three washes with PBS solution, cells were incubated with the appropriate Alexa Fluor secondary antibody for 30 min. Coverslips were then mounted and imaged by confocal microscopy.

Assessment of Synaptic-Like Vesicles by EM. EM was used to detect the presence of synaptic-like vesicles in PSCs. Human PSCs grown on coverslips were rinsed three times with PBS solution and then fixed with PBS solution containing 4% paraformaldehyde and 0.25% glutaraldehyde for 20 min. Cells on coverslips were osmicated (1% OsO_4/0.1 mol/L sodium cacodylate buffer), dehydrated in an ethanol gradient to 100%, and embedded in Spurr resin. Ultrathin sections parallel and transverse to the growth surface were obtained and viewed on a Phillips CM10 transmission electron microscope.

LC-MS/MS. For highly specific and sensitive measurement of ACh, we used C18 reverse-phase LC coupled to tandem MS. Serially diluted ACh was used to construct a standard curve. All standards and samples received a uniform aliquot of isotope-labeled ACh internal standard to correct for variations in extraction efficiency. ACh was separated by liquid chromatography using an Accela pump (Thermo Scientific). Samples (10 μL) were injected onto a 1-mm internal diameter 80-Å, 4-μm, 250-mm length Synergy Hydro-RP C18 column purchased from Phenomenex. A binary LC solvent system was used consisting of solvent A (20 mM ammonium formate containing 15 mM HFBA) and solvent B (methanol). A flow rate of 100 μL/min was used with an initial 1-min equilibration (100% solvent A), followed by a 1-min gradient to 75% solvent B. Isocratic separation at 75% solvent B (7 min) was used followed by an equilibration at initial conditions (100% solvent A) for 14 min. Total run time per sample was 22 min. The column flow was directed into a TSQ Quantum Access bench-top mass spectrometer (Thermo Scientific), equipped with an electrospray source used in positive ion mode. A capillary voltage of 5,000 V and capillary temperature of 300 °C were used. The Quantum device was operated in selected reaction monitoring mode, selecting parent ions at *m*/*z* values of 146 and 162 for ACh and $^{16}H_2$-ACh internal standard, respectively, and monitoring the respective CID fragment ions at *m*/*z* values of 87.2 and 94.2, respectively. Argon was used as collision gas and collision energy of 14 V. Spectra were accumulated for 0.5 s. All spectra were processed and area integrated using XCalibur software (version 2.07). Peak area ratios of ions of 87.2/94.2 were used for quantification, together with an ACh calibration curve consisting of six duplicate standards in the 0–400 fmol/10 μL (on column) concentration range.

Isolation of Rat Pancreatic Acini. Rat acini were isolated as described by Haber et al. (2). Pancreata from male Sprague–Dawley rats were digested in Hepes Ringer buffer (0.02 M Hepes, pH 7.4, 4.7 mM KCl, 1 mM $NaHPO_4$, 4 mM NaOH, 0.57 mM $MgCl_2$, 128.5 mM NaCl, 0.011 M glucose, 1.9 mM glutamine) containing collagenase P (65 mg/L) and soybean trypsin inhibitor (50 mg/mL). Following digestion and filtration through 150-μm gauze, the cell suspension was gently layered over a solution of 4% BSA in Hepes Ringer buffer and centrifuged at 480 rpm (Avanti JE Centrifuge, Rotor JS-5.3, Beckman Coulter) for 5 min. The resulting pellet was washed three times in Hepes Ringer buffer containing BSA (0.5% wt/vol) and 2.55 mM $CaCl_2 0.2H_2O$. Cells with viability (assessed by trypan blue exclusion) greater than 95% were used for the coculture experiments described later.

Assessment of CCK-Receptor Expression by PSCs. The presence of CCK-1 and CCK-2 receptors on culture-activated rat and human PSCs (passage 1–3) was assessed by Western blotting and immunofluorescence.
Western blotting. Antibodies specific for CCK1 receptor (rabbit anti-rat CCK1 receptor antiserum AR5 1122) and CCK2 receptor (rabbit anti-rat CCK2 receptor antibody 9262) were provided by Marie-Luise Kruse (Christian-Albrechts-University Kiel, Germany) and Jean Morisset (University of Sherbrooke, Quebec, Canada), respectively. Cell lysates were analyzed by immunoblotting using the aforementioned antibodies at concentrations of 1:10,000 (CCK1) and 1:8,000 (CCK2). HRP-coupled goat anti-rabbit antibody (1:2,000) was used as the secondary antibody. CCK1 and CCK2 receptor bands were detected by ECL. For each receptor, negative controls comprised Western blotting performed with CCK1 and CCK2 receptor antibody solutions that had been pretreated with their respective immunization peptides, thereby leading to preadsorption of active antibody. Briefly, anti-

bodies were incubated with the corresponding peptide (40 μg/mL) for 2 h. The preabsorbed antibodies were then used for Western blotting as detailed earlier.

Immunofluorescence. Human PSCs on coverslips were rinsed twice in PBS solution, fixed for 10 min in acetone at −20 °C and saturated over 10 min at room temperature in sodium borohydride 2 mg/mL. Cells were then permeabilized for 30 min in PBS solution containing Triton X-100 0.4% and 7% normal goat serum, followed by incubation with the primary antibody at room temperature using the following dilutions: CCK1 receptor antiserum, 1:400; CCK2 receptor antibody, 1:1,000 (antibodies as described earlier for Western blot analysis). Cells were incubated with FITC-conjugated goat anti-rabbit antibody (2 μg/mL) for 12 h at room temperature. All antibody dilutions were carried out in PBS solution, Triton X-100 0.4%, and 1.4% normal goat serum. Images were analyzed by confocal microscopy.

Effect of CCK on PSC Functions. To assess the effect of CCK on PSC signaling, we assessed PSC proliferation and α-SMA expression (a marker of PSC activation). MAPK activation was assessed in response to CCK exposure.

PSC proliferation. To assess the effect of CCK8 on PSC proliferation, the rate of cell proliferation was assessed using the Dojindo Cell Counting Kit-8 per the manufacturer's instructions. Briefly, human PSCs were incubated with CCK8 at both a physiological dose (20 pM) and supraphysiological doses (100 pM and 500 pM) for 24 h at 37 °C. For these experiments, we used 2,500 PSCs per well of a 96-well plate in 100 μL of media containing 10% FBS.

α-SMA expression by PSCs. The effect of CCK8 on PSC activation was assessed by measuring αSMA expression by Western blotting as previously described (3). Briefly, human PSCs were incubated with CCK8 (20 pM, 100 pM, or 500 pM) for 24 h and cell lysates were collected.

MAP Kinase Activation. Phosphorylated ERK1/2 and p38 kinase in culture activated human PSCs were assessed by Western blotting of cell lysate proteins, as described previously (3). Briefly, cells were preincubated with serum-reduced (0.1% FBS) culture medium for 3 h at 37 °C. Cells were then incubated with or without CCK8 (20, 100, and 500 pM) for 5 min. To confirm that total MAPK levels were unchanged by the treatments, blots were stripped and reprobed with antibodies to total ERK1/2 and total p38. Densitometry analysis was performed using Bio-Rad Quantity One software.

Materials. All general chemicals were analytical grade and purchased from Sigma Chemical. CCK8, cerulein, and protease type XIV were purchased from Sigma Chemical. Collagenase P was from Roche. Secondary antibodies were purchased from DAKO. Iscove modified Dulbecco medium, DMEM, FBS, and DNase were purchased from Invitrogen. Alexa Fluor secondary antibodies were purchased from Molecular Probes. The BCA protein assay kit was purchased from Pierce. α-SMA antibody was purchased from Sigma Chemical. The ERK1/2 and p38 antibodies were purchased from Cell Signaling.

1. Apte MV, et al. (1998) Periacinar stellate shaped cells in rat pancreas: Identification, isolation, and culture. *Gut* 43:128–133.
2. Haber PS, et al. (2004) Non-oxidative metabolism of ethanol by rat pancreatic acini. *Pancreatology* 4:82–89.
3. McCarroll JA, et al. (2003) Pancreatic stellate cell activation by ethanol and acetaldehyde: Is it mediated by the mitogen-activated protein kinase signaling pathway? *Pancreas* 27: 150–160.

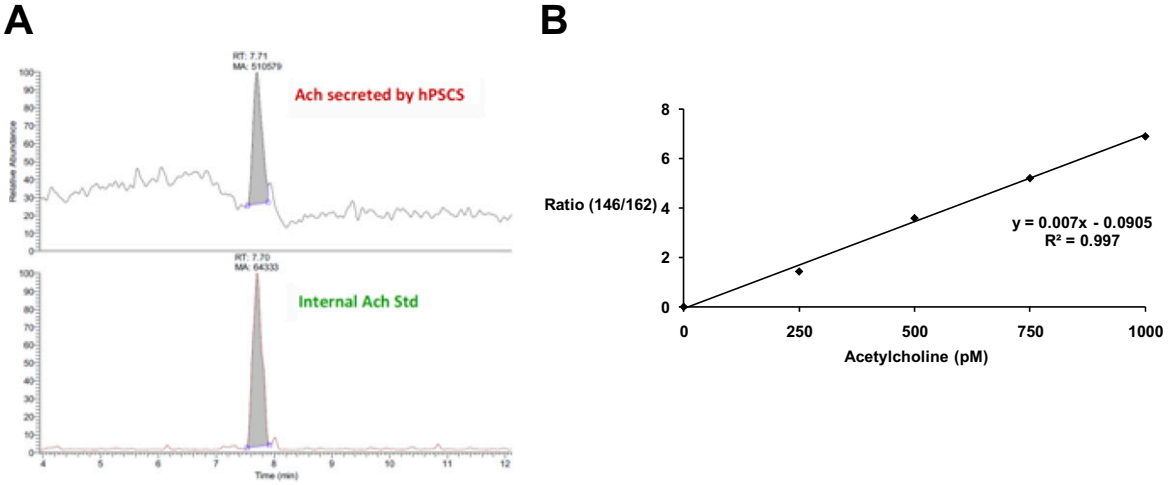

Fig. S1. ACh secretion by PSCs. All PSC secretions were collected after 15 min in Hepes buffer for measurement of ACh. (*A*) Representative LC-MS/MS trace for ACh secreted by culture-activated human PSCs compared with the deuterated ACh internal standard ($^{16}H_2$-ACh). Stable isotope $^{16}H_2$-labeled ACh (parent ion *m/z*, 162) internal standard coeluted with PSC extracted ACh (parent ion *m/z*, 146) and was observed at an LC retention time of 7.7 min. (*B*) Representative ACh standard curve. For each standard (0, 250, 500, 750, 1,000 pM) the *m/z* 146/162 ratio (ACh/$^{16}H_2$-ACh internal standard) was calculated. The *x* axis of the standard curve represents the concentration range of the analyte (ACh) standards, and the *y* axis represents the "instrument response," shown as peak area ratio of the ACh standard relative to its $^{16}H_2$-ACh stable isotope-labeled internal standard.

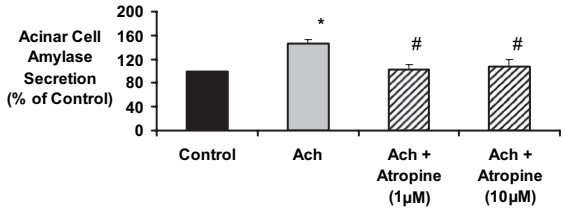

Fig. S2. Rat pancreatic acini were incubated with ACh (100 nM) for 30 min with or without a 5-min preincubation with atropine (muscarinic receptor antagonist, 1–10 μM). ACh significantly stimulated acinar amylase secretion (n = 3 separate cell preparations; $^{*}P < 0.001$) and atropine at both concentrations blocked this increase ($^{\#}P < 0.05$).

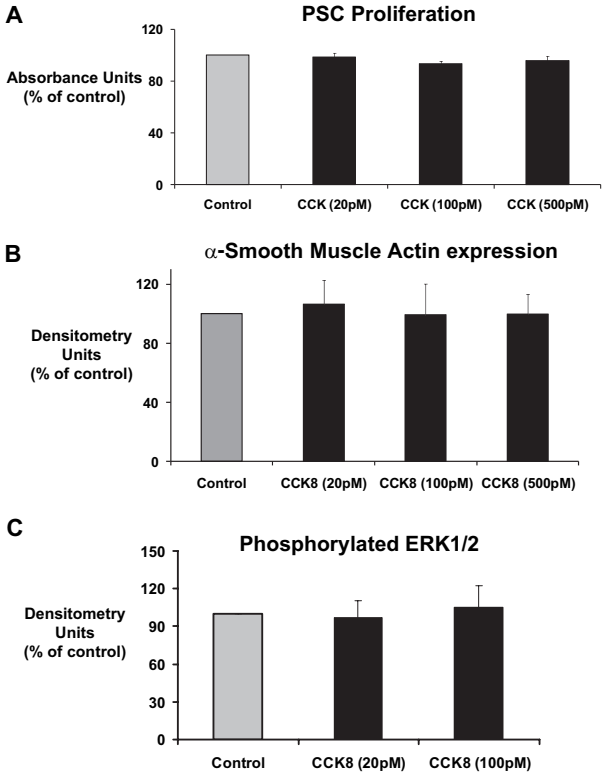

Fig. S3. The effect of CCK8 on proliferation, α-SMA expression and MAP kinase activation by PSCs. (*A*) Human PSCs were incubated with CCK8 (20, 100, and 500 pM) for 24 h and proliferation assessed using the Dojindo Cell Counting Kit-8. CCK8 had no effect on PSC proliferation (n = 3 separate cell preparations). (*B*) Densitometry of Western blots for α-SMA showing that CCK8 had no effect on α-SMA expression in human PSCs. (*C*) Human PSCs were incubated for 5 min with CCK-8 (20 pM and 100 pM) and phosphorylated ERK was assessed by Western blotting. Results show a graph of densitometry analysis of pERK1/2 normalized to total ERK. Incubation of human PSCs with CCK8 had no effect on the activation of ERK1/2 (n = 3 separate cell preparations).

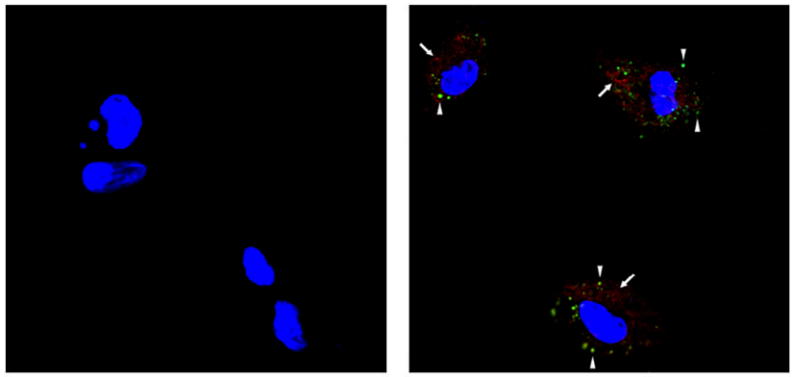

Fig. S4. Expression of GFAP and lipids by quiescent rat PSCs. A representative immunofluorescent confocal micrograph of rat PSCs demonstrates strong positive staining for GFAP (red, arrows) and abundant lipid droplets (BODIPY; green, arrowheads) in the cytoplasm, whereas the negative controls showed no staining (magnification 1,000×). Nuclei were stained with DAPI (blue).