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Human Pancreatic Acinar Cells Do Not Respond to Cholecystokinin

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Abstract: Pancreatic secretion can be influenced by cholecystokinin (CCK) either directly via actions on acinar cells or indirectly via actions on nerves. The presence and functional roles of CCK receptors on human pancreatic acinar cells remains unclear. In the current study human pancreatic acini were isolated and then treated with CCK-8, gastrin and/or carbachol. Functional parameters were measured including intracellular [Ca2+] and amylase secretion. It was observed that human acini did not respond to CCK agonists but did respond to carbachol with robust increases in functional parameters. Adenoviral-mediated gene transfer of CCK1 or CCK2 receptors to the human cells resulted in cell responses to CCK agonists. In order to determine the reason for the lack of responsiveness of the human acini, expression of receptor mRNAs was determined using quantitative RT-PCR and localized by *in situ* hybridization. mRNA levels for CCK1 receptors were ~30 times lower than those of CCK2 receptors, which were ~10 times lower than those of m3 Ach receptors as measured by quantitative PCR. Neither CCK1 nor CCK2 receptors were localized in adult human pancreas by *in situ* hybridization. These results indicate that human pancreatic acinar cells do not respond directly to CCK receptor activation and this is likely due to an insufficient level of receptor expression.

A major physiological function of cholecystokinin (CCK) in human physiology is the stimulation of pancreatic enzyme secretion, a phenomenon that has been known for nearly 60 years (Harper & Raper 1943). However, the mechanisms involved in this response in man remain controversial (Niederau *et al.* 1994; Owyang 1996; Adler 1997). Confusion has arisen due to basic differences between the mechanisms that exist in commonly utilized rodent animal models and those that exist in man. Administration of CCK leads to a rapid increases in pancreatic secretion in both rodents and man (Wank 1995). In rodents it appears that two mechanisms are likely involved, one indirect and one direct. The indirect mechanism involves the binding of CCK to CCK1 receptor expressed in afferent neurones that influence pancreatic secretion by way of a vagal-vagal loop with the final mediator being acetylcholine acting at m3 muscarinic cholinergic receptors (m3 AchR) (Owyang 1996; Adler 1997). This mechanism has also been reported to exist in man (Owyang 1996). The direct mechanism involves the binding of CCK to CCK1 receptors that are expressed in pancreatic acinar cells leading to an increase in intracellular Ca2+ and secretion of digestive enzymes that can be observed *in vitro* (Jensen *et al.* 1989). It is unclear whether this direct mechanism exists in man as the presence and identity of CCK receptors on human pancreatic acinar cells remains controversial despite several studies (Miller 1996).

Our laboratory has evaluated the presence and identity of CCK receptors in pancreatic acinar prepared from normal human pancreas with a focus on their functional roles (Ji *et al.* 2001). As reviewed here, these results show that human pancreatic acinar cells do not respond significantly to CCK or gastrin and suggest that this lack of responsiveness is related to a low level of receptor expression. These data therefore support the conclusion that the stimulatory effects of CCK on pancreatic secretion in humans are mediated exclusively through an indirect pathway.

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Materials and Methods

Materials. Amylase 3 reagent (4,6-Ethylidene (G7)-p-nitrophenyl (G1)-alpha-D-Maltoheptaoside) was obtained from Sigma (St. Louis, MO, USA). Fura 2-AM was purchased from Molecular Probes (Eugene, OR, USA). Collagenase (CLSPA grade) was purchased from Worthington Biochemicals (Freehold, NJ, USA). Sulfated CCK-8 was obtained from Bachem Bioscience Inc. (Torrance, CA, USA). I-Cycler real-time PCR detection system were obtained from Bio-Rad (Hercules, CA, USA). Trizol reagent was from Gibco BRL (Grand Island, NY, USA). The RNeasy® kit and DNase were products of Qiagen (Valencia, CA, USA). SYBR green I® nucleic acid stain was from FMC Bioproducts (Rockkland, ME, USA). ECL® chemiluminescent reagent and Hybond® Nylon membranes were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Primers were synthesized at the University of Michigan DNA core.

Recombinant adenoviruses encoding CCK receptors. The recombinant adenovirus encoding the rat CCK1 receptor has been previously described (Ji *et al.* 2000). The current study utilized a recombinant adenovirus encoding the human CCK2 receptor produced by a similar strategy.

Preparation of acini and infection with virus. Specimens of normal human pancreas were obtained from 6 patients undergoing resection of pancreatic tumours at the University of Michigan Hospital, or from 3 organ donors provided by the Michigan Transplantation Society with no differences noted between acini prepared from these two groups. All human subjects were adults and males and females were treated equally. All experiments were conducted with permission of the University of Michigan Internal Review Board for

use of Human Materials. Acini were prepared by a modification of the methods previously described for the preparation of rodent acini (Ji *et al.* 2000 & 2001). The acini were suspended in HEPES-Ringer buffer (HR) containing 1% bovine serum albumin. The pH was adjusted to 7.4 and equilibrated with 100% O2 before use. In some experiments the acini were infected with virus encoding the human CCK2 or rat CCK1 receptor essentially as previously described (Ji *et al.* 2000).

Analysis of amylase secretion. Amylase secretion in response to CCK-8, gastrin or carbachol was measured as described previously (Ji *et al.* 2000). Results were expressed as a percentage of initial acinar amylase content released.

Analysis of intracellular Ca2+levels. Analysis of intracellular Ca2+ concentration was conducted using ratio metric imaging of Fura-2-loaded cells as described previously (Ji *et al.* 2000 & 2001).

RT-PCR. Standard RT-PCR was conducted using total RNA prepared from human pancreatic acini and whole pancreas isolated using TriZol reagent (Gibco, Grand Island, NY, USA). RNA was purified by digestion for 15 min. with DNase and recovery of RNA using an RNeasy kit (Qiagen, Valencia CA, USA). Reverse transcription was conducted for 45 min. at 48° from 70 ng purified total RNA in a 25 ul volume of Access RT-PCR reaction mixture (Promega, Madison, WI, USA) followed by 40 cycles of standard PCR (30 sec. denaturation at 94°, 1 min. annealing at 56° and 1.5 min extension at 68°). All PCR products were verified by sequencing. Quantitative RT-PCR was conducted by previously published methods (Higuchi *et al.* 1993; Ji *et al.* 2001). SYBR green I was used to monitor the PCR products on the I-Cycler thermal cycler and IQ real-time PCR detection system (BioRad, Hercules, CA, USA). Primers designed for the human CCK1 receptor (Genebank accession L13605) were: forward 5′ TGC GGA CGG TCA CCA ACA TCT T 3′ and reverse 5′GCA CAG GAA GAA GAG GAC CAC GAT3′. Those for the CCK2 receptor (Genebank accession L08112) were: forward 5′GTG ACA GCG ACA GCC AAA GCA G 3′ and reverse 5′CGA GGC GTA GCT CAG CAA GTG A 3′. Those for the m3 Ach receptor (Genebank accession NM–000740) were: forward 5′ATC GGT CTG GCT TGG GTC ATC TC 3′ and reverse 5′AGC GGC CAT ACT TCC TCC TGT TG 3′s. Those for insulin (Genebank accession XM–006400) were: forward 5′AAG AGG CCA TCA AGC ACA TCA CTG 3′ and reverse 5′GGT TCA AGG GCT TTA TTC CAT CTC 3′. Standard curves were prepared using known amounts of plasmids bearing full-length cDNAs and were linear over 5 log units with correlation coefficients of greater than 0.98. The estimate of cell numbers was based upon the value of RNA per pancreatic acinar cell (65 pg) previously measured in the rat (Swift *et al.* 1984).

In situ *hybridization*. *In situ* hybridization assays were performed with slight modifications of previously described methods (Panoskaltsis-Mortari *et al.* 1995) as previously described (Ji *et al.* 2001).

Results

Human pancreatic acinar cells do not respond functionally to cholecystokinin or gastrin.

To test whether activation of CCK receptors directly causes human pancreatic acini to secrete digestive enzymes, we isolated acini from portions of normal human pancreas surgically removed during pancreatic cancer resection or from organ donors. Isolated acini appeared intact with obvious polarity and viability was indicated by exclusion of the vital dye trypan blue (data not shown). These acini were then treated with CCK, gastrin, or as a positive control, with the acetylcholine analogue, carbachol. Treatment with neither CCK-8 nor gastrin caused any increase in amylase secretion even at supraphysiologic concentrations of 100 nM (fig. 1).

In contrast, the isolated acini responded to stimulation with carbachol in a concentration-dependent manner with maximal stimulation of 6.4±0.3 % total amylase per 30 min., (n=3) with 1 mM carbachol.

This represented an increase of approximately 6 times of the basal amylase secretion (1.1±0.08 % total amylase per 30 min., n=3). That the acini had low levels of basal secretion and responded to carbachol supported the conclusion that the isolated acini were functionally intact. To distinguish between a lack of response due to insufficient CCK receptors versus a lack of CCK receptor related signaling molecules such as G-proteins, we next expressed human CCK2 receptors in acini using adenoviral mediated gene transfer as described previously (Ji *et al.* 2000). Infected acini were incubated for 4 hr, as this amount of time was previously shown to allow a level of expression of receptors in acini isolated from receptor deficient mice that was equivalent to that observed in the control mice (Ji *et al.* 2000). Acini infected with the receptor coding adenovirus responded to CCK-8 with a concentration dependent release of amylase that was similar in magnitude to that stimulated by carbachol (fig. 1). Infection with a control adenovirus had no effect on the responsiveness of the acini to CCK analogues (data not shown). These data suggested that the isolated acini were healthy and the lack of response of the isolated acini was not due to a lack of intracellular signaling mechanisms.

Acinar cell secretion mediated by CCK is triggered by an increase in intracellular Ca2+ concentration. The ability of secretagogues to increase intracellular Ca2+ is highly sensitive and more proximal to receptor activation than is secre-

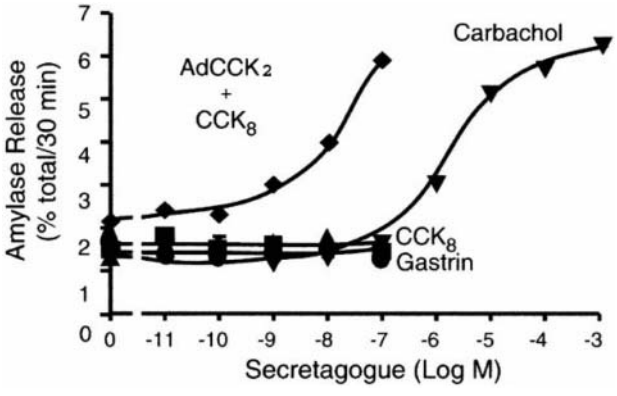

Fig. 1. Human pancreatic acini secrete amylase in response to carbachol but not to CCK-8 or gastrin. Isolated human pancreatic acini either uninfected, or infected for 4 hr with an adenovirus expressing the human CCK2 receptor, were incubated with increasing concentrations of CCK-8, gastrin or carbachol at 37° for 30 min. The concentration of amylase released into the medium was measured using colorimetric reagent and was expressed as a percentage of initial acinar amylase content. Data shown are means of three separate experiments (reproduced from Ji *et al.* 2001 with permission).

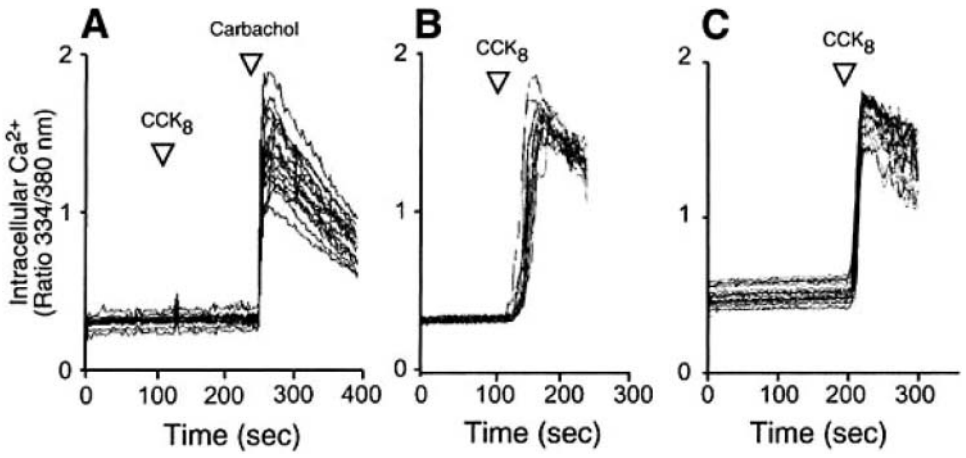

Fig. 2. Human pancreatic acini increase their level of intracellular Ca2+ in response to carbachol but not to CCK-8. Measurement of emitted fluorescence allowing an estimation of intracellular Ca2+were performed with fura2-loaded acini using an Attofluor digital imaging system. At the times indicated CCK-8 (100 nM) or carbachol (1 mM) were introduced. A. Freshly prepared acini were treated with CCK-8 and then carbachol. B. Acini that were infected with the adenovirus bearing human CCK2 receptor for 4 hr were treated with CCK-8 (100 nM). C. Acini that were infected with the adenovirus bearing rat CCK1 receptor for 4 hr were treated with CCK-8 (100 nM). Data shown are typical and were representatives of 4 separate experiments. (reproduced from Ji *et al.* 2001 with permission).

tion. Therefore, we tested whether or not human acinar cells would respond to cholecystokinin analogues by an increase in this parameter using fluorometric analysis of Ca2+ levels with the sensitive dye, Fura-2. Isolated human pancreatic acinar cells did not respond to CCK-8 (fig. 2) or gastrin (data not shown) even at concentrations of 100 nM. However, the same acini subsequently responded to treatment with carbachol (1 mM) with a robust increase in intracellular calcium. Furthermore, acini infected with adenovirus bearing either the human CCK2 or rat CCK1 receptor gained the ability to respond strongly to CCK-8 (100 nM) (fig. 2) or gastrin (data not shown).

Human pancreatic acinar cells do not express high levels of CCK receptors

To investigate CCK receptor gene expression in human pancreatic acinar cells, we utilized RT-PCR to determine the presence of mRNA for CCK1 and CCK2 receptors and, for comparison, the m3 Ach receptor. Standard RT-PCR indicated the presence of minor levels of message for the CCK1 receptor and more abundant levels of CCK2 receptor and m3 Ach receptor mRNA (fig. 3). To quantitatively compare the expression levels of the receptors we utilized quantitative real-time RT-PCR. Standard curves were de-

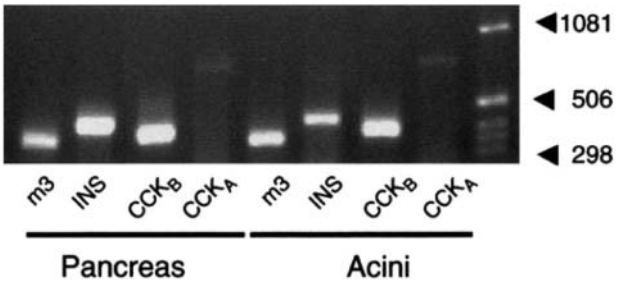

Fig. 3. RT-PCR amplifies both CCK1 and CCK2 receptor mRNA from total RNA prepared from whole pancreas and isolated acini. RNA was prepared from pancreatic acini and from whole pancreas. RT-PCR was performed using 70 ng DNase-purified RNA and the results shown are representatives of 3 independent experiments. That the bands amplified represented the expected genes was verified by sequencing (modified from Ji *et al.* 2001 with permission).

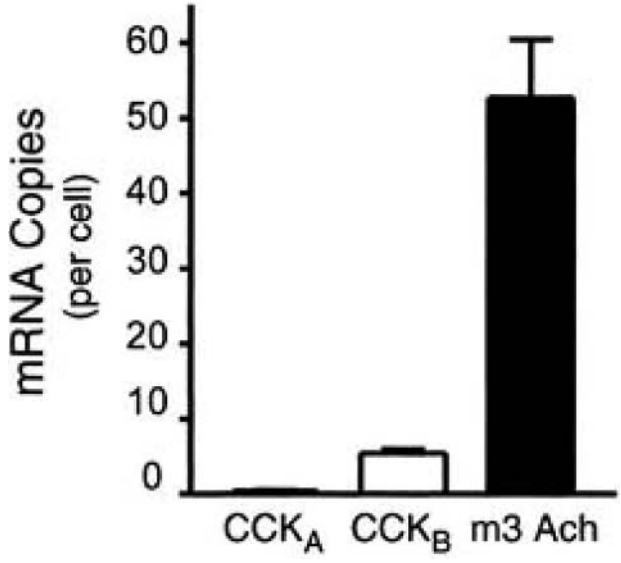

Fig. 4. Quantitative real-time RT-PCR indicates that CCK receptor mRNAs are expressed at low levels in human acinar cells. Real-time quantitative RT-PCR was conducted using SYBR green I® to monitor the PCR products with an I-Cycler real RT-PCR detection system. Quantitation of mRNA expression was conducted using standard curves for human CCK1, CCK2, and m3 Ach receptors and using 65 pg of total RNA per acinar cell (Swift *et al.* 1984) as an estimate of the cell numbers (reproduced from Ji *et al.* 2001 with permission).

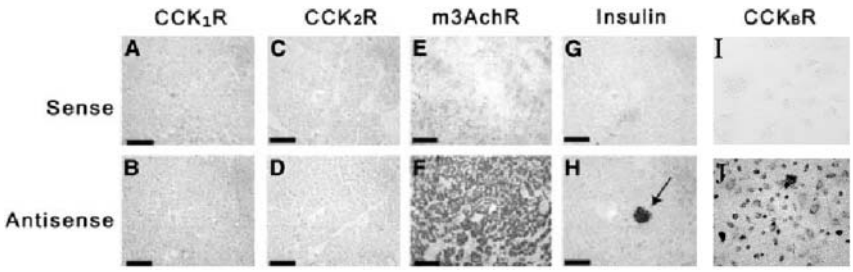

Fig. 5. *In situ* hybridization detects m3 Ach receptor and insulin mRNA but not CCK receptor mRNA in human pancreas. *In situ* hybridization was performed on 10 μm frozen sections of human pancreas using digoxigenin labeled RNA probes for CCK1R (A,B) CCK2R (C,D) m3 AchR (E,F) and insulin (G,H). Sense probes (A,C,E,G) were utilized as negative controls. After incubation with the labeled probes the sections were incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase and detected by the formation of a dark precipitate. No specific localization was observed for CCK1 or CCK2 receptor mRNAs, whereas, m3 AchR mRNA was observed throughout the acinar portion of the pancreas and insulin mRNA was observed localized to islets (arrow). Pancreatic cancer cell line panc-1 transfected with human CCK2 receptor (J) is a positive control for CCK2R probe (modified from Ji *et al.* 2001).

veloped using plasmids bearing cDNAs for the receptors (data not shown). Data from the quantitative RT-PCR allowed the estimation of the amount of message for the specific receptors that was present in the starting RNA samples prepared from isolated human acini (fig. 4). This analysis indicated that CCK1 receptor mRNA was expressed at a very low level, less than one copy per acinar cell. The level of mRNA for the CCK2 receptor was also low at approximately 5 copies per cell. Both CCK receptors genes were expressed at an extent that was far lower than that of the m3 Ach receptor, which was calculated to be approximately 50 copies per cell.

In situ hybridization localizes mRNA for m3 ach receptors and insulin but not CCK receptors in human pancreas

Quantitative RT-PCR studies indicated the presence of small amounts of mRNA for CCK1 and CCK2 receptors in the pancreas. The relative levels of mRNAs for these receptors were not enriched in purified acini, suggesting that they were not highly expressed in pancreatic islets. However, it remained a possibility that this minor level of mRNA was a result of relatively high levels of expression within a non-acinar cell population that co-purified with acini in our isolation procedure. In order to investigate this possibility we conducted in situ hybridization experiments using probes for CCK1, CCK2 and m3 Ach receptors, as well as insulin. Using this approach, it was not possible to identify any specific cells expressing either CCK receptor subtype mRNA within the pancreas (fig. 5 B,D). However, these probes were able to localized CCK receptor RNA in cell lines exogenously expressing the appropriate CCK receptor genes (fig. 5 J). In contrast to the lack of signal for CCK receptors, *in situ* hybridization of sections from the same specimens with a probe for the m3 Ach receptor mRNA indicated abundant expression localized to acinar cells (fig. 5 F). Furthermore, *in situ* hybridization studies with the insulin probe also showed strong and appropriate localization within islets (fig. 5 H).

Discussion

The present results indicate that human pancreatic acinar cells do not respond to physiologic concentrations of either CCK or gastrin in terms of cellular signaling or biological activities that would be expected from our knowledge of the function of these receptors in acinar cells from other species. Because one of the primary criteria for determining whether or not a hormone has a direct physiologic role in regulating a specific cell is that treatment of that cell with concentrations of the hormone likely to occur normally within the organism elicits a functional response, these results suggest that neither CCK nor gastrin should be considered direct physiologic regulators of human pancreatic acinar cells.

A major concern in these types of studies is the functional integrity of the isolated cells. In the present study, the functionality of the isolated acini was indicated by their responsiveness to activation of m3 Ach receptors with carbachol and after gene transfer of CCK receptors. Because the isolated human acini were capable of responding to CCK in the presence of sufficient levels of receptor expression, these data support the hypothesis that lack of sufficient receptor expression explains the lack of response to CCK treatment.

The lack of responsiveness of the human acini to CCK or gastrin in terms of changes in intracellular Ca2+ was particularly striking. Agonist occupancy of CCK1, CCK2, or m3 Ach receptors leads to the activation of phospholipase C and the production of inositol 1,4,5-trisphosphate (IP3) which then activates IP3 receptors and causes the release of intracellular Ca2+. This signaling pathway is exquisitely sensitive. Thus, the lack of responsiveness of the human acinar cells to CCK or gastrin in this assay suggests that these receptors are absent from these cells. Furthermore, these data strongly suggest that neither CCK nor gastrin act as direct physiologic regulators of human pancreatic acinar cells. This is clearly different from what is observed in rodents.

To our knowledge only a single previous study had been published indicating the effects of CCK analogues on biological actions in human acini (Susini *et al.* 1986). This previous study also found that human pancreatic acini did not respond to physiologic concentrations of CCK. However, it was reported that secretion could be stimulated by supraphysiologic concentrations of cholecystokinin analogues. In the current study we did not observe responses even at concentrations of 100 nM CCK, which is approximately 10,000 times the peak serum level of CCK (Liddle *et al.* 1985). The explanation for this difference between the two studies is unclear. However, it is unlikely that responses to extremely high, non-physiologic, concentrations of CCK analogues are biologically relevant.

Although functional responses to CCK or gastrin were not observed in human pancreatic acini, CCK receptor gene expression was detectable by RT-PCR as has been previously reported (Monstein *et al.* 1985). However, the level of receptor mRNA expression in human pancreas has not been previously quantitated, nor has receptor mRNA expression in isolated acini previously been investigated. We utilized quantitative PCR (Higuchi *et al.* 1993) to measure the levels of mRNA expression. Our data indicate that both CCK1 and CCK2 receptor mRNA levels are very low in the human pancreas. CCK1 levels were difficult to detect by standard RT-PCR and were estimated to be less than one copy per acinar cell by real-time quantitative RT-PCR. CCK2 receptor mRNA levels, although higher than CCK1 receptor mRNA levels, were also very low. Compared to the levels of mRNA for m3 Ach receptors, CCK2 receptor message levels were at least 10 times lower. While there is no direct correlation between mRNA levels and protein levels, these data suggest that CCK receptor numbers are likely much lower than m3 Ach receptor numbers. In contrast, in rat pancreatic acinar cells the copy numbers of CCK1 receptor mRNA were estimated to be similar to those observed for the m3 Ach receptor in man (data not shown). Therefore, the likely explanation for the lack of functional responses to CCK and gastrin is that the levels of expression of receptors for these hormones are inadequate. More direct evidence on this issue came from *in situ* hybridization experiments that did not observe CCK2 or CCK1 receptor mRNA localization on human pancreatic acinar cells.

The current data support the hypothesis previously derived from several independent lines of investigation that the regulation of pancreatic secretion in man relies on indirect actions of CCK (Owyang 1996). In man, administration of CCK at concentrations that would not be expected to activate CCK2 receptors elicits a strong secretion response from the pancreas while administration of gastrin causes a very limited response (Valenzuela *et al.* 1976; Cantor *et al.* 1986). Furthermore, CCK1, but not CCK2 antagonists inhibit the stimulatory effects of a meal on pancreatic secretion (Cantor *et al.* 1991 & 1992). Therefore, CCK1 receptors appear to mediate the physiological effects of CCK on human pancreatic secretion. However, utilizing real-time quantitative PCR we found that CCK1 receptor gene expression was extremely low in the pancreas. This low level of expression helps explain the contradictions in the literature concerning the ability to measure CCK1 receptor mRNA in human pancreas. Autoradiographic binding studies have also supported the absence of significant levels of CCK1 receptors in the human pancreas (Tang *et al.* 1996). Finally, in the current study we observed no effects of CCK on signaling or function in isolated human pancreatic acini. Therefore, the evidence is very strong that the effects of CCK on pancreatic secretion are not mediated directly on acinar cells in man. Previously it has been shown that man express CCK1 receptors on afferent neurons (Moriarty *et al.* 1997) and anti-cholinergic drugs block the effects of CCK on human pancreatic secretion (Soudah *et al.* 1992). Thus, human pancreatic secretion is likely regulated exclusively via the indirect effects of CCK on afferent neurones.

In summary, the data presented in this study indicate that neither CCK nor gastrin directly activate cellular responses on pancreatic acinar cells in man. Based upon the results of real-time quantitative RT-PCR and *in situ* hybridization it seems likely that the explanation for this lack of responsiveness is that these receptors are expressed only at very low levels.

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