CONTROL OF INSULIN SECRETION BY CHOLINERGIC SIGNALING IN THE HUMAN PANCREATIC ISLET

Judith Molina,¹ Rayner Rodriguez-Diaz,¹,²,³ Alberto Fachado,² M. Caroline Jacques-Silva,² Per-Olof Berggren,²,³,⁴ and Alejandro Caicedo¹,²,⁵,⁶

From the ¹Division of Endocrinology, Diabetes and Metabolism, Department of Medicine, University of Miami Miller School of Medicine, Miami, FL 33136, USA; ²Diabetes Research Institute, University of Miami Miller School of Medicine, Miami, FL 33136, USA; ³The Rolf Luft Research Center for Diabetes & Endocrinology, Karolinska Institutet, Stockholm, SE-17177, Sweden; ⁴Division of Integrative Biosciences and Biotechnology, WCU Program, University of Science and Technology, Pohang, 790-784 Korea; ⁵Department of Physiology and Biophysics, Miller School of Medicine, University of Miami, Miami, FL 33136, USA; ⁶Program in Neuroscience, Miller School of Medicine, University of Miami, Miami, FL 33136, USA.

Corresponding authors: Per-Olof Berggren, per-olo%berggren@ki.se, and Alejandro Caicedo, acaicedo@med.miami.edu.

Division of Endocrinology, Diabetes and Metabolism, Department of Medicine 1580 NW 10th Avenue, Miami, FL33136
Phone: (305) 243 6025; Fax: (305) 243 7268; acaicedo@med.miami.edu

Word count: 4,160

Short running title: Cholinergic signaling in human pancreatic islets
ABSTRACT

Acetylcholine regulates hormone secretion from the pancreatic islet and is thus crucial for glucose homeostasis. Little is known, however, about acetylcholine (cholinergic) signaling in the human islet. We recently reported that in the human islet acetylcholine is primarily a paracrine signal released from alpha cells rather than primarily a neural signal as in rodent islets. Here we demonstrate that the effects acetylcholine produces in the human islet are different and more complex than expected from studies conducted on cell lines and rodent islets. We found that endogenous acetylcholine not only stimulates the insulin-secreting beta cell via the muscarinic acetylcholine receptors M3 and M5, but also the somatostatin-secreting delta cell via M1 receptors. Because somatostatin is a strong inhibitor of insulin secretion, we hypothesized that cholinergic input to the delta cell indirectly regulates beta cell function. Indeed, when all muscarinic signaling was blocked, somatostatin secretion decreased and insulin secretion unexpectedly increased, suggesting a reduced inhibitory input to beta cells. Endogenous cholinergic signaling therefore provides direct stimulatory and indirect inhibitory input to beta cells to regulate insulin secretion from the human islet.
Acetylcholine, a classical neurotransmitter that also functions as a non-neuronal paracrine signal, activates muscarinic receptors that play a key role in maintaining many metabolic functions, including glucose homeostasis. There is strong evidence that cholinergic mechanisms are important for function and survival of the endocrine pancreas, the islet of Langerhans (1). Activation of muscarinic receptors leads to improved insulin secretion from pancreatic islets (2-7). Because the muscarinic M3 receptor has been shown to play a critical role in maintaining blood glucose homeostasis in mouse models, approaches aimed at enhancing signaling through beta cell M3 receptors have been proposed as selective pharmacologic intervention points in the treatment of diabetes (5; 8).

It is not always possible, however, to extrapolate structural or functional information from rodent studies to the human situation (9-15). Indeed, recent findings indicate that in the human islet acetylcholine is primarily a paracrine signal released from the glucagon-producing alpha cell rather than primarily a neuronal signal as in the mouse islet (16). In light of these striking species differences, it is likely that muscarinic signaling affects human islet function in ways not predicted by studies in rodents. There is evidence that genetic variations in the M3 receptor are associated with early-onset type 2 diabetes and the acute insulin response in Pima Indians (17), but few studies have investigated cholinergic mechanisms in human islets (16; 18-20). Thus, the cellular responses activated by acetylcholine in human islets remain mostly unknown, in particular those produced in endocrine cells other than beta cells.

To understand the effects of acetylcholine in the human islet we systematically investigated the molecular and functional expression of muscarinic receptors. Using immunohistochemistry, RT-PCR, Western blots, and functional recordings of
cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and hormone secretion we identified muscarinic receptors in the different endocrine cells of the islet. Our results indicate that human beta cells express the muscarinic receptors M3 and M5, whereas human delta cells express M1. Activation of these receptors by endogenously released acetylcholine regulates hormone secretion in a complex manner. Endogenous acetylcholine not only stimulates beta cell function directly by activating M3 and M5 receptors, but also recruits delta cells by activating M1 receptors and somatostatin secretion, which in turn inhibits beta cell function. Our results suggest that in the human islet endogenous acetylcholine provides direct stimulatory as well as indirect inhibitory input to beta cells to regulate insulin secretion.
RESEARCH DESIGN AND METHODS

Pancreatic islets. We obtained human pancreatic islets from deceased donors from the Human Islet Cell Processing Facility at the Diabetes Research Institute, University of Miami Miller School of Medicine, or from the Integrated Islet Distribution Program (IIDP) of the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, and the Juvenile Diabetes Research Foundation (JDRF).

Insulin, glucagon, and somatostatin secretion. A high-capacity, automated perifusion system was used to dynamically measure hormone secretion from pancreatic islets (Biorep Perifusion V2.0.0, Miami, FL). A low pulsatility peristaltic pump pushed HEPES-buffered solution (mM: 125 NaCl, 5.9 KCl, 2.56 CaCl₂, 1 MgCl₂, 25 HEPES, and 0.1% BSA; pH 7.4; and a perifusion rate of 100 µL/min) through a column containing 100 pancreatic islets immobilized in Bio-Gel P-4 Gel (BioRad, Hercules, CA).

Except otherwise stated, glucose concentration was adjusted to 3 mM for all experiments. Stimuli were applied with the perifusion buffer. The perifusate was collected in an automatic fraction collector designed for a 96 well plate format. The columns containing the islets and the perifusion solutions were kept at 37°C, and the perifusate in the collecting plate was kept at < 4°C. Perifusates were collected every minute. Insulin and glucagon release in the perifusate was determined with the human or mouse Endocrine LINCOplex Kit following manufacturer’s instructions (Linco research, St. Charles, MO). Somatostatin secretion was determined using a fluorescent somatostatin enzyme-linked immunoassay kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA). Thapsigargin was purchased from Invitrogen (Carlsbad, CA, USA), the muscarinic toxins
MT3 and MT7 from Peptide Institute, Inc. (Osaka, Japan), and all other drugs from Tocris Bioscience (Ellisville, MO, USA).

To stimulate muscarinic receptors we used acetylcholine at a concentration of 10 µM because it was the lowest concentration at which we reliably stimulated hormone secretion. We did not use higher concentrations (e.g. 100 µM) to avoid saturating responses that could hamper pharmacological studies with antagonists and modulators. Also, our studies suggest that the local concentration of acetylcholine may be in the range of 10 µM because inhibiting acetylcholine degradation with physostigmine produced insulin responses that were similar to those elicited by 10 µM acetylcholine (16).

Working with human islet preparations, we could not afford conducting pilot studies to establish thorough concentration-response relationships for all drugs tested. We used concentrations that are approximately an order of magnitude higher than published affinity constants of agonists and antagonists for human muscarinic receptors. Because many of the drugs show a narrow range of specificity, we took care not to use concentrations that start affecting other receptors or produce unspecific effects.

**Determination of cytoplasmic free Ca$^{2+}$ concentration.** Imaging of $[\text{Ca}^{2+}]_i$ was performed as described elsewhere (21). Islets or dispersed islet cells were immersed in HEPES-buffered solution. Glucose was added to give a final concentration of 3 mM. Islets or dispersed islet cells were incubated in Furap2 AM (2 µM; 1 hour) and placed in a closed small-volume imaging chamber (Warner Instruments, Hamden, CT, USA). Stimuli were applied with the bathing solution. Islets loaded with Furap2 were alternatively excited at 340 and 380 nm with a monochromator light source (Cairn Research Optoscan Monochromator, Cairn Research Ltd, Faversham, UK). Images were
acquired with a Hamamatsu camera (Hamamatsu Corp, Japan) attached to a Zeiss Axiovert 200 microscope (Carl Zeiss, Jena, Germany). Changes in the 340/380 fluorescence emission ratio were analyzed over time in individual islets and dispersed cells using MetaFluor imaging software. Peak changes in the fluorescence ratio were measured to compare response profiles between endocrine cells.

To identify endocrine cells we transduced whole human islets with adenoviral constructs that allow islet expression of either GFP or td Tomato under the control of rat insulin promoter-1 (-410/+1 bp), rat glucagon promoter (-775/+7 bp) or human somatostatin promoter (-719/+258 bp), thus color-coding beta, alpha and delta cells, respectively. The rat insulin-1 promoter/GFP-cassette was described earlier (22). The glucagon promoter (-773/+35 bp) was amplified by PCR from genomic rat DNA using 5’-TCCTTCTGTTGAATGCGAG-3’ as the upstream primer and 5’-TTTGAGTGTGTTCTGCGCC-3’ as the downstream primer. A HindIII-site was created by exchanging G versus an A at position +7 bp, which allowed generation of pGlcg.GFP and pGlcg.tdTomato. The human somatostatin promoter was subcloned from pLightSwitch.SST (SwitchGearGenomics, Molano Park, CA). The tdTomato cDNA was subcloned from pRSET.tdTomato. The respective expression cassettes consisting of promoter-GFP-bGHpA or promoter-tdTomato-bGHpA were first subcloned into pENTR1A entry vectors (Invitrogen AB, Lidingö, Sweden), adenoviruses were generated by employing the ViraPower Adenoviral Expression System (Invitrogen AB, Lidingö, Sweden). Human whole islets were also transfected with an adenoviral construct driving expression of DsRed2 under the control of the somatostatin promoter (pAAV-fSST-RFP).
pAAV-fSST-RFP was a gift of Edward Callaway (Addgene plasmid #22913). Three to five days after virus transduction, we dispersed islets into single cells and imaged $[\text{Ca}^{2+}]_i$.

By using identified islet cells and $[\text{Ca}^{2+}]_i$ imaging we were able to establish a stimulation protocol that differentiates between the different endocrine cells (Supplementary Fig. 1). Responses to KCl depolarization were unique to endocrine cells and served to tell them apart from other contaminating cells from the pancreas (e.g. acinar cells). Beta cells typically responded to KCl depolarization and to acetylcholine, but not to kainate or GABA. Alpha cells responded to KCl depolarization and to kainate, but not to acetylcholine or GABA. In addition to responding to KCl depolarization, to kainate, and to acetylcholine, delta cells were the only ones that responded to GABA with increases in $[\text{Ca}^{2+}]_i$. We exposed dispersed cells to these stimuli in random order at the end of the experiment to avoid altering the responses to muscarinic drugs. Using this protocol it was no longer necessary to transfecot cells to identify alpha and beta cells, and indeed for all results shown on dispersed alpha and beta cells we did not use virus transduction. However, we still had to use virus transduction to find the very scarce delta cells.

To unmask activities of M2 and M4 receptors that do not couple to increases in $[\text{Ca}^{2+}]_i$ and do not increase hormone secretion we challenged $[\text{Ca}^{2+}]_i$ and hormone responses to acetylcholine or changes in glucose concentration with receptor-specific antagonists.

**Immunohistochemistry.** Blocks of human pancreas (0.5 cm$^3$) were fixed in 4% paraformaldehyde for 4 hours, cryoprotected in sucrose, and cut on a cryostat (20 µm). Pancreatic biopsies from healthy donors were obtained from the Human Islet Cell
Processing Facility at the Diabetes Research Institute, University of Miami Miller School of Medicine. After a rinse with PBS –Triton X-100 (0.3%), sections were incubated in blocking solution (PBS-Triton X-100 and Universal Blocker Reagent; Biogenex, San Ramon, CA). Thereafter, sections were incubated for 24 hours (20° C) with primary antibodies diluted in blocking solution. Antibodies used included rabbit antibody to muscarinic M1 receptor (AB5164, Millipore) (23), rabbit antibody to CHRM1 (HPA014101, Sigma, validated by the Human Protein Atlas), rabbit antibody to muscarinic M2 receptor (AB5166, Millipore) (24), rabbit antibody to muscarinic M3 receptor (AB9453, Millipore, discontinued), rabbit antibody to CHRM3 (HPA024106, Sigma, validated by the Human Protein Atlas), mouse antibody to M4 (MAB1578, Millipore) (25; 26), rabbit antibody to muscarinic M5 receptor (AB9454, Millipore, discontinued) (27), rabbit antibody to CHRM5 (HPA013172, Sigma, validated by the Human Protein Atlas), rat antibody to somatostatin (MAB354, Millipore), mouse antibody to glucagon (G2654-5, Sigma), and guinea pig antibody to insulin (A0564, Dako). Antibodies against the same receptor produced similar staining patterns, indicating specificity. Immunostaining was visualized by using Alexa Fluor conjugated secondary antibodies (1:500 in PBS; 12 hours at 20° C; Invitrogen). Cell nuclei were stained with DAPI. Slides were mounted with ProLong Anti Fade (Invitrogen). In control experiments, we incubated primary antibodies with corresponding control peptide at a ratio of 50 µg antigenic peptide/ 1 µg antibody at room temperature for 5 h.

Confocal images (pinhole = airy 1) of randomly selected islets (2-3 islets per section, minimum 3 sections per human specimen) were acquired on a confocal laser-
scanning microscope (Leica SP5) with a 63x objective (HCX PL APO 63x/1.4 NA Oil lambda blue) at 1024 x 1024 pixel resolution.

**Determination of somatostatin secretion with biosensor cells.** We examined somatostatin secretion using Chem-1 cells expressing the somatostatin receptor 3 coupled to the promiscuous G protein Gα15 to increase [Ca\(^{2+}\)]\(_i\) via the InsP\(_3\) signaling cascade (Millipore, Billerica, MA; Supplementary Fig. 2). Somatostatin biosensors reliably responded to low concentrations of somatostatin (threshold ≈ 10 nM), making them highly sensitive somatostatin detectors. Somatostatin biosensor responses were measured using [Ca\(^{2+}\)]\(_i\) imaging. We loaded somatostatin biosensors with the [Ca\(^{2+}\)]\(_i\) indicator Fura-2 and plated them on cover slips in a perfusion chamber. Individual human islets were placed on top of this layer of biosensor cells. Somatostatin secretion was examined in biosensors cells located immediately downstream of the islet in recordings lasting at least 20 minutes to be able to detect rhythmic behavior. Somatostatin secretion was examined by inspecting recordings for increases in [Ca\(^{2+}\)]\(_i\) in biosensor cells. Changes in the amount of secreted somatostatin were quantified by measuring the area under the curve of [Ca\(^{2+}\)]\(_i\) responses in the biosensor cells during defined time intervals. We expressed data as average traces (± SEM) of the [Ca\(^{2+}\)]\(_i\) responses of the biosensor cells.

We established that biosensor cells responded only to somatostatin and not to other substances. Biosensor cells for somatostatin did not respond to changes in glucose from 3 mM to 11 mM, from 11 mM to 3 mM, or to neurotransmitters such as acetylcholine (10 µM), GABA (100 µM), serotonin (10 µM), muscimol (100 µM), kainate (100 µM), epinephrine (10 µM), or KCl (30 mM). These stimuli did not themselves either elicit biosensor responses or alter the ability of biosensors to respond to
somatostatin. We conducted these controls by $[\text{Ca}^{2+}]_i$ imaging of biosensor cells plated at the same density and in the absence of islets. Crucially, the antagonist cyclosomatostatin (10 µM) completely blocked somatostatin receptors on the somatostatin biosensors and eliminated responses generated from islets.

**Western blotting.** Immunoblot analysis was carried out by standard methods with the antibodies used for immunohistochemistry (1:600). Human islet samples were run in parallel with whole normal brain lysate (NB820-59177, Novus Biologicals), which gave bands at similar molecular weights. For unknown reasons, M3 receptor bands for islet lysates were visible at a higher molecular weight than those for brain lysates.

**RT-PCR.** RNA was extracted from human brain or from human islets using RiboPure™ Kit (Ambion, Austin, TX) and cDNA was prepared using High-Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). PCR reactions were run in duplicate using Taqman gene expression assays (Applied Biosystems, Foster City, CA) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Relative quantification (RQ) of gene expression was done based on the equation $RQ=2^{-\Delta Ct} \times 10,000$, where $\Delta Ct$ is the difference between the Ct value (number of cycles at which amplification for a gene reaches a threshold) of the target gene and the Ct value of the ubiquitous housekeeping gene GAPDH.

**Statistical analyses.** For statistical comparisons we used Student’s $t$-test. Throughout the manuscript we presented data as average ± s.e.m.
RESULTS

Acetylcholine elicits diverse responses in endocrine cells of the human islet. We examined hormone secretion from human islets and found that applying acetylcholine (10 µM) stimulated secretion of insulin and somatostatin, but not that of glucagon (Fig. 1A-C). We further performed \([\text{Ca}^{2+}]_i\) imaging in identified single isolated human islet cells. Acetylcholine elicited \([\text{Ca}^{2+}]_i\) responses in beta and delta cells but not in alpha cells (Fig. 1D and E). These results indicate that acetylcholine activates receptors that couple to increases in \([\text{Ca}^{2+}]_i\), and hormone secretion in beta and delta cells. Our results further show that acetylcholine did not stimulate glucagon secretion, either directly or indirectly via other cells within the islet. Importantly, given that somatostatin strongly inhibits insulin and glucagon secretion (28; 29), acetylcholine may affect hormonal output from the islet indirectly by activating somatostatin secretion.

Human beta cells express functional M3 receptors. We next sought to identify the muscarinic receptors mediating the effects of acetylcholine in the human islet. Acetylcholine acts on 5 different muscarinic receptors termed M1-5, which differ in signaling pathways and pharmacological properties (30). In general, activation of M1, M3, M5 receptors increases \([\text{Ca}^{2+}]_i\) via PLC, whereas activation of M2 and M4 receptors leads to a reduction in cAMP levels. To obtain comprehensive evidence for the presence of the different muscarinic receptors we studied their molecular and functional expression in human islets using a variety of approaches.

We first focused on the expression of M3 receptors because they play a major role in mouse beta cells. Immunohistochemistry, Western blotting, and RT-PCR experiments
showed that M3 receptors were present in human islets (Fig. 2). M3 receptor expression was exclusively localized to beta cells (Fig. 2D-H). Application of acetylcholine induced increases in [Ca^{2+}]_{i} in beta cells that were blocked by atropine (~ 95% reduction), indicating that the effects of acetylcholine were mediated by muscarinic receptors, not nicotinic receptors (Fig. 2I and J). The M3 receptor-specific antagonist J104129 (50 µM) (31), but not the M1 receptor-specific antagonist MT7 (20 nM) (32), reduced [Ca^{2+}]_{i} responses to acetylcholine (Fig. 2J). [Ca^{2+}]_{i} responses to acetylcholine in beta cells decreased when intracellular Ca^{2+} stores were depleted with thapsigargin (1 µM) and were not affected by the absence of extracellular [Ca^{2+}], indicating that acetylcholine activated signaling pathways leading to Ca^{2+} release from intracellular stores (Fig. 2L and M). Insulin secretion stimulated by acetylcholine was also inhibited in the presence of J104129 (Fig. 2K), which is in line with previous results (16). We conclude that beta cells in human islets express M3 receptors. These receptors are not present in alpha or delta cells.

**Human beta cells express functional M5 receptors.** Immunohistochemical, Western blotting, and RT-PCR results further indicated that the muscarinic receptor M5 was strongly expressed in human islets (Fig. 3). Like M3 receptors, M5 receptors were localized to beta cells (Fig. 3D-H). Because there are no specific agonists or antagonists for M5 receptors we relied on positive allosteric modulators to reveal the presence of functional M5 receptors (33; 34). As described above, the M3 receptor antagonist J104129 inhibited [Ca^{2+}]_{i} responses in beta cells to acetylcholine, but not completely (Fig. 2J and K). We found that the remaining [Ca^{2+}]_{i} response after blocking M3 receptors with J104129 was increased in the presence of the M5 receptor-specific allosteric
modulator VU 0238429 (10 µM; Fig. 3I and J) (33). Similarly, insulin responses to acetylcholine could be amplified by modulating the M5 receptor with VU 0365114 (10 µM), another allosteric modulator of M5 receptors (Fig. 3K and L) (34). VU 0365114 (10 µM) further increased insulin secretion at basal glucose concentration (3 mM) in the absence of exogenous acetylcholine, indicating that endogenous acetylcholine stimulated M5 receptors in beta cells. The insulin response to VU 0365114 was blocked by atropine, and VU 0365114 did not alter somatostatin secretion in response to acetylcholine or at basal glucose concentration (Fig. 3M). These results show that human beta cells, but not delta cells, express functional M5 receptors.

**Human delta cells express functional M1 receptors.** Immunohistochemistry, Western blotting, and RT-PCR experiments revealed that M1 receptors are expressed in human islets (Fig. 4). In contrast to the expression pattern of M3 and M5 receptors, few cells were immunostained for M1 receptors (Fig. 4A). These cells were somatostatin secreting delta cells, and no other cell type within the human islet expressed M1 receptors (Fig. 4D-H). When we inspected delta cells with [Ca²⁺]ᵢ imaging we found that responses to acetylcholine were abolished by atropine, indicating that responses were mediated by muscarinic receptors (Fig. 4I and K). The M1-specific antagonist MT7 (20 nM) (32) inhibited [Ca²⁺]ᵢ responses elicited by acetylcholine (Fig. 4J and L). These responses were only partially inhibited by MT7 most likely because this toxin fails to elicit a complete inhibition of the receptor (35). Somatostatin secretion was also affected by application of MT7, with an initial, short-lived increase followed by a prolonged inhibition of secretion (Fig. 4M). These changes in somatostatin secretion occurred in the absence of exogenously applied acetylcholine, indicating that endogenously released
acetylcholine was activating M1 receptors in delta cells at basal glucose concentration (3 mM).

**Expression of M2 and M4 receptors in human islets.** M2 and M4 receptors were elusive to molecular and functional characterization. Although both were detected in RT-PCR experiments, M2 receptor immunostaining was absent in the human islet, and immunostaining for M4 receptors gave inconsistent results. Furthermore, we could not determine any changes in hormone secretion or $[\text{Ca}^{2+}]_i$ using the M2 antagonist AF-DX116 (100 nM) (36) and the M4 antagonist MT3 (10 nM) (37) in the absence or presence of acetylcholine or at different glucose concentrations.

**Direct and indirect effects of endogenous acetylcholine on insulin secretion.** Because beta and delta cells express different muscarinic receptors, we sought to understand how endogenously released acetylcholine affects hormone output from the human islet. Using biosensor cells to detect somatostatin secretion in real time (Supplementary Fig. 2) we found that somatostatin secretion was inhibited by atropine (10 µM) and stimulated by blocking acetylcholine degradation with physostigmine (30 µM), indicating that somatostatin secretion was strongly dependent on endogenous cholinergic input (Fig. 5A and B). Perifusion assays of hormone secretion showed similar effects on somatostatin secretion in the presence of atropine and physostigmine at basal glucose concentration (3 mM; Fig. 5C and D). Preincubation with vesamicol (10 µM), a blocker of vesicular acetylcholine transporter (vAChT) that depletes alpha cells of releasable acetylcholine (16), abolished the inhibition of somatostatin secretion by atropine (Fig. 5D), suggesting that acetylcholine released from alpha cells stimulates somatostatin release.
Because somatostatin is a strong inhibitor of insulin secretion (28; 29), we investigated if endogenous acetylcholine could affect insulin secretion indirectly via delta cells. Applying the broad muscarinic antagonist atropine (10 µM) to block cholinergic input increased insulin secretion at basal glucose concentration (3 mM; Fig. 5E and F), suggesting that under basal conditions the net effect of endogenous acetylcholine is a decrease in insulin secretion. This inhibition likely involves the delta cell and somatostatin secretion because direct endogenous cholinergic input to the beta cell would stimulate insulin secretion. Because preincubation with vesamicol abolished atropine-induced increases in insulin secretion and decreases in somatostatin secretion (Fig. 5D-F), it is likely that acetylcholine derived from alpha cells inhibits insulin secretion by stimulating somatostatin secretion.

*In vivo*, alpha cells become activated at periods of approximately 10 minutes (38). To imitate this pattern *in vitro* and to periodically stimulate acetylcholine secretion from alpha cells we used a protocol in which glucose concentrations were varied every 10 minutes from low (3 mM) to high concentrations (11 mM) (16). Under these circumstances, applying the M3 receptor-specific antagonist J104129 (50 nM) decreased the amount of secreted insulin, confirming previous results (Supplementary Fig. 3) (16). However, in the presence of the broad muscarinic antagonist atropine (10 µM) insulin secretion increased (Fig. 5G and H). These results indicate that endogenously released acetylcholine stimulates insulin secretion directly by activating M3 receptors in beta cells and inhibits insulin secretion indirectly by activating M1 receptors and somatostatin secretion from delta cells.
DISCUSSION

We have demonstrated that acetylcholine activates M3 and M5 muscarinic receptors in beta cells and M1 muscarinic receptors in delta cells to modulate hormone secretion from the human pancreatic islet (Fig. 6). Delta cells and somatostatin secretion, in particular, are strongly influenced by endogenously released acetylcholine. We previously showed that endogenous acetylcholine primes beta cells to respond optimally to subsequent increases in glucose concentration (16). Here we show that endogenously released acetylcholine activates delta cells to secrete somatostatin, a major inhibitor of insulin secretion. Thus, intra-islet cholinergic signaling provides positive and negative signaling pathways regulating insulin secretion. The net effects of endogenous acetylcholine on insulin secretion likely depend on the spatial and temporal patterns of acetylcholine secretion from neural or paracrine sources as well as on the biophysical properties of the different muscarinic receptors. The presence of multiple muscarinic receptors on different endocrine cells in the human islets has important implications for the use of cholinergic agents to intervene in diabetes.

Our results reveal that the expression pattern of muscarinic receptors in the human islet is different from what has been described in cell lines and rodent islets (3; 39-41). While M3 receptors play a major role in both human and mouse beta cells, we now show that human beta cells also express M5 receptors, which are barely detectable in rodent islets (3; 39). In the human islet, M1 receptors were confined to delta cells and were not expressed in beta cells, as studies on rodent islets had suggested (3; 39; 42). Also in contrast to what has been reported for rodent alpha cells, human alpha cells did not express muscarinic receptors that couple to increases in $[\text{Ca}^{2+}]_i$, or glucagon secretion (43-
45). None of the muscarinic agonists and antagonists tested affected alpha cell responses or glucagon secretion, even under conditions that would unmask receptors such as M2 and M4 that are coupled to decreases in cell activity. Thus, the effects of acetylcholine in human islets could not have been predicted from studies in rodents.

To understand how acetylcholine may influence hormone secretion from the human islet it is important to consider human islet cell architecture, the position of the different endocrine cell types with respect to one another, and circulatory flow. Endocrine cells of different types intermingle more in the human islet, such that most beta cells face alpha cells, delta cells, or both (10). This close association enables paracrine interactions that may not be possible in the mouse islet (46). An additional consequence of human islet cytoarchitecture is that blood flows through regions with heterogeneous cell populations. This rules out a hierarchy in the sequence in which the different endocrine cells are perfused. Thus, endocrine cells in the human islet are positioned to be able to influence mutually via paracrine signaling using the interstitial space or the vascular route. Given the cellular arrangement in the human islet, paracrine signaling molecules released by any endocrine cells can readily reach the other cell types. This is likely the case of acetylcholine, which we recently found to be secreted as a paracrine signal within the human islet (16).

In light of these findings, models for islet cholinergic signaling now should feature cholinergic input primarily from endocrine alpha cells in human islets, and be distinguished from cholinergic input in mouse islets, which originates mainly from parasympathetic innervation (16; 47; 48). The local paracrine source of acetylcholine makes sense in the context of a cellular arrangement that promotes paracrine interactions.
In human islets, most beta cells are directly exposed to alpha cell secretions, whereas in mouse islets paracrine signals derived from alpha cells would affect only a minority of beta cells. It is also likely that in the human islet paracrine acetylcholine readily reaches delta cells. This proximity is important because acetylcholine is rapidly degraded in the extracellular space. Thus, within the human islet, paracrine acetylcholine can act on endocrine cells of different types and activate a combination of diverse muscarinic receptors. Given the variety of receptors, target cells, and sources for acetylcholine, it is not surprising that the effects of endogenous cholinergic signaling could not be mimicked by exogenous acetylcholine. Indeed, applying exogenous acetylcholine activated both insulin and somatostatin secretion, but blocking endogenous cholinergic signaling with atropine paradoxically increased insulin secretion.

What is the possible role of paracrine acetylcholine in the control of insulin secretion during fluctuations in plasma glucose concentrations? Because alpha cells are activated at glucose concentrations that inhibit insulin secretion, it seems improbable that acetylcholine derived from alpha cells affects beta cell function. It is important, however, to keep in mind that acetylcholine activates signaling cascades that last for tens of minutes (49). Thus, even if secreted exclusively during glucose nadirs, acetylcholine can potentiate beta cell responses to forthcoming rises in glucose concentration. An alternative proposal is that acetylcholine is released independently of glucagon in response to Ca\(^{2+}\) entry via L-type Ca\(^{2+}\)-channels at elevated glucose (50). That acetylcholine and glucagon do not appear to be stored in the same granules suggest that independent release is possible (16). Not to neglect is a conceivable contribution from a subgroup of delta cells, which also express vesicular acetylcholine transporter and
therefore should be capable of vesicular release of acetylcholine (16). A cholinergic input from delta cells would potentiate insulin secretion while beta cells are being stimulated at elevated glucose.

Given the multiple sources for acetylcholine and the various muscarinic receptors, the net effects of cholinergic signaling in the islet will be the sum of many activities that may be constantly fine-tuned under different physiological conditions. The different muscarinic receptors have different desensitization, internalization and downregulation properties that may affect the net effects of acetylcholine in the islet (51; 52). A selective downregulation of M1 receptors in delta cells after prolonged exposure to acetylcholine, for instance, could shift the balance to a point where cholinergic signaling now promotes insulin secretion. It is also likely that changes in glucose concentration or diabetic conditions affect cholinergic signaling. Because the circumstances in the human islet will be very different from those in rodent models and because experiments are more difficult to conduct in human beings, addressing these possibilities experimentally may require establishing research models in which mice are transplanted with human islets (53; 54).

More than 10 years ago, Gilon and Henquin thoroughly reviewed an impressive amount of data on cholinergic mechanisms in the islet (1). In view of recent findings, however, it seems that we are just starting to understand cholinergic signaling in the human islet. Despite decades of research, the identity of muscarinic receptors mediating the effects of acetylcholine had remained elusive. The present study represents an effort to determine the role of muscarinic receptors that couple to an increase in activity in islet endocrine cells, namely M1, M3, and M5 receptors. Although still elusive in our hands, the possibility of negative regulation involving M2 or M4 receptors is intriguing and
warrants exploring further the role of M2 and M4 receptors.
ACKNOWLEDGMENTS

This work was funded by the Diabetes Research Institute Foundation (DRIF); NIH grants R56DK084321 (A.C.), R01DK084321 (A.C.); the Juvenile Diabetes Research Foundation; the Swedish Research Council; the Novo Nordisk Foundation; the Swedish Diabetes Association; the Family Erling-Persson Foundation; the Skandia Insurance Company Ltd; Strategic Research Program in Diabetes at Karolinska Institutet; the Berth von Kantszow’s Foundation; VIBRANT (FP7-2288933); the Knut and Alice Wallenberg Foundation; Funds of Karolinska Institutet, Diabetes and Wellness Foundation; the Stichting af Jochnick Foundation; and the World Class University program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R31-2008-000-10105-0).

The authors thank Kevin Johnson, Lily Barash, and Yuan Liu for technical assistance.

J.M. designed experiments, researched data, and edited the manuscript. R.R.-D. researched data and reviewed and edited the manuscript. A.F. and M.C.J.-S. researched data. P.-O.B. and A.C. conceived and designed experiments, analyzed data, and wrote the paper. A.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
REFERENCES


24. Disney AA, Aoki C: Muscarinic acetylcholine receptors in macaque V1 are most frequently expressed by parvalbumin-immunoreactive neurons. J Comp Neurol 2008;507:1748-1762


27. Strang CE, Renna JM, Amthor FR, Keyser KT: Muscarinic acetylcholine receptor localization and activation effects on ganglion response properties. Invest Ophthalmol Vis Sci 2010;51:2778-2789


32. Bradley KN, Rowan EG, Harvey AL: Effects of muscarinic toxins MT2 and MT7, from green mamba venom, on m1, m3 and m5 muscarinic receptors expressed in Chinese Hamster Ovary cells. Toxicon 2003;41:207-215


34. Bridges TM, Kennedy JP, Cho HP, Breininger ML, Gentry PR, Hopkins CR, Conn PJ, Lindsley CW: Chemical lead optimization of a pan G(q) mAChR M(1), M(3), M(5)
positive allosteric modulator (PAM) lead. Part I: Development of the first highly
selective M(5) PAM. Bioorg Med Chem Lett 2010;20:558-562

Life Sci 2005;76:1547-1552

muscarinic autoreceptor subtype in rat striatum as m2 through a correlation of in vivo
microdialysis and in vitro receptor binding data. J Pharmacol Exp Ther 1995;273:273-
279

of muscarinic toxin 3 in functional assays of cloned and native receptors. J Pharmacol
Exp Ther 1999;288:164-170

38. Lang DA, Matthews DR, Peto J, Turner RC: Cyclic oscillations of basal plasma

functional characterization of muscarinic receptor subtypes in insulin-secreting cell lines

40. Miguel JC, Abdel-Wahab YH, Mathias PC, Flatt PR: Muscarinic receptor subtypes
mediate stimulatory and paradoxical inhibitory effects on an insulin-secreting beta cell
line. Biochim Biophys Acta 2002;1569:45-50

42. Balakrishnan S, Mathew J, Antony S, Paulose CS: Muscarinic M(1), M(3) receptors function in the brainstem of streptozotocin induced diabetic rats: their role in insulin secretion from the pancreatic islets as a function of age. Eur J Pharmacol 2009;608:14-22


46. Caicedo A: Paracrine and autocrine interactions in the human islet: more than meets the eye. Semin Cell Dev Biol 2013;24:11-21


FIGURE LEGENDS

FIG. 1. Acetylcholine stimulates beta and delta cells, but not alpha cells in human islets. Acetylcholine (10 µM) increased insulin (A) and somatostatin (C), but not glucagon secretion (B). Responses to KCl depolarization (KCl, 30 mM) are used as reference (n = 3 islet preparations). Horizontal lines in all graphs denote drug application. D: Traces of [Ca$^{2+}$]$^i$ responses to acetylcholine (ACh) and KCl depolarization (30 mM) in beta, alpha, and delta cells. E: Quantification of results as in (D) showing the fractions of endocrine and exocrine cells responding to acetylcholine (black portion of the bar; results pooled from n = 4 islet preparations).

FIG. 2. Human beta cells express functional M3 muscarinic receptors. M3 muscarinic receptors were present in human islets as detected by confocal microscopy of immunostained human pancreatic sections (A), Western blotting of lysates from five human islet preparations (B), and RT-PCR in human islets (I, n = 5) and brain (B) as a control (C). Molecular weight markers were run in parallel (shown is the 82 kDa marker). Scale bar = 20 µm. D-H: Confocal images of human pancreatic sections showing islets immunostained for M3 receptor (D-F, green), glucagon (D, red), somatostatin (soma, E, red), or insulin (G, red). Scale bars = 10 µm, in D applies to E, in F to G and H. I: Traces of [Ca$^{2+}$]$^i$ responses in beta cells showing that responses to acetylcholine (10 µM) were inhibited in the presence of atropine (10 µM). J: Quantification of results as in I shows that peak responses to acetylcholine (Δ 340/380) were inhibited by atropine, the M3 receptor-specific antagonist J104129 (50 nM), but not by the M1 receptor-specific antagonist MT7 (20 nM) (n = 4 islet preparations; Student’s t-test, P < 0.05). K:
Perifusion assay of insulin secretion showing that increases in insulin secretion induced by acetylcholine were inhibited by J104129 (50 nM). L and M: Quantification of [Ca\(^{2+}\)]\(_i\) responses to acetylcholine in the presence of thapsigargin (L) and in nominal 0 [Ca\(^{2+}\)] (M) (n = 16 cells from 3 preparations; Student’s t-test, P < 0.05).

FIG. 3. Human beta cells express functional M5 muscarinic receptors. M5 muscarinic receptors were present in human islets as detected by confocal microscopy of immunostained human pancreatic sections (A), Western blotting of lysates from five human islet preparations (B), and RT-PCR in human islets (I, n = 5) and brain (B) as a control (C). Molecular weight markers were run in parallel (shown is the 60 kDa marker). Scale bar = 20 μm. D-H: Confocal images of human pancreatic sections showing islets immunostained for M5 receptor (D-F, green), glucagon (D, red), somatostatin (soma, E, red), or insulin (G, red). Scale bars = 10 μm, in D applies to E, in F to G and H. I: Traces of [Ca\(^{2+}\)]\(_i\) responses in beta cells showing responses to acetylcholine alone (10 μM, left), in the presence of J104129 (50 nM, middle), and in the presence of J104129 and the M5 receptor modulator VU 0238429 (10 μM, right). J: Quantification of results as in I shows that peak responses to acetylcholine (Δ340/380) in the presence of J104129 were amplified by VU 0238429 (n = 9 cells from 3 preparations; Student’s t-test, P < 0.05). K: Perifusion assay of insulin secretion showing that increases in insulin secretion induced by acetylcholine were amplified by VU 0365114, an allosteric modulator of M5 receptors (10 μM). L: Quantification of results as in K shows that VU 0365114 increased insulin secretion stimulated by acetylcholine (n = 4 preparations; Student’s t-test, P < 0.05). M: Quantification of perifusion assays of somatostatin secretion shows that VU 0365114 did not alter somatostatin secretion stimulated by acetylcholine (n = 3 preparations).
FIG. 4. Human delta cells express functional M1 muscarinic receptors. M1 muscarinic receptors were present in human islets as detected by confocal microscopy of immunostained human pancreatic sections (A), Western blotting of lysates from five human islet preparations (B), and RT-PCR in human islets (I, n = 5) and brain (B) as a control (C). Molecular weight markers were run in parallel (shown is the 64 kDa marker). Scale bar = 20 µm. D-H: Confocal images of human pancreatic sections showing islets immunostained for M1 receptor (D-F, green), insulin (ins, D, red), glucagon (E, red), or somatostatin (soma, G, red). Scale bars = 10 µm, in D applies to E, in F to G and H. I and J: Traces of [Ca^{2+}]_i responses in delta cells showing that responses to acetylcholine (10 µM) were inhibited in the presence of atropine (10 µM, I) and in the presence of the M1 receptor-specific antagonist MT7 (20 nM, J). K and L: Quantification of results as in I and J shows that peak responses to acetylcholine (Δ 340/380) were inhibited by atropine (K) and MT7 (L) (n > 12 cells from 3 preparations; Student’s t-test, P < 0.05). M: Perifusion assay of somatostatin secretion showing that basal somatostatin secretion at 3 mM glucose concentration was inhibited by MT7 (20 nM; n = 3 preparations).

FIG. 5. Effects of endogenous acetylcholine on hormone secretion from the human islet. A: Pulsatile secretion of somatostatin as measured by somatostatin biosensor cells was inhibited by atropine (10 µM) at 1 mM glucose concentration (representative of 4 experiments from 3 islet preparations). B: Somatostatin secretion as measured by somatostatin biosensor cells increased in the presence of the cholinesterase inhibitor physostigmine (30 µM) at 3 mM glucose concentration (representative of 4 experiments from 3 islet preparations). C and D: Perifusion assays showing increases in somatostatin secretion in the presence of physostigmine (30 µM) or KCl (30 mM; n = 3 preparations).
(C) and decreases in somatostatin secretion in the presence of atropine (10 µM; n = 4 preparations) (D). 
E and F: Perifusion assay of insulin secretion showing an increase in insulin secretion in the presence of atropine (10 µM; n = 3 preparations). (F) is a closeup of (E). In D-F, the red trace shows secretion in the presence of vesamicol, a blocker of vesicular acetylcholine transporter (10 µM). Glucose concentration was changed from 3 mM to 11 mM at the beginning of the experiment. G: Perifusion assay showing that atropine (10 µM) amplified insulin responses to repeated increases in glucose concentration to 11 mM (11G). A control experiment with untreated islets was run in parallel (black trace). Representative of four experiments. H: Quantification of results as in G show significant increases in insulin secretion in the presence of atropine (n = 4 preparations; one sample t-test to compare the actual mean to a theoretical mean of 100%, P < 0.05).

FIG. 6. Proposed model for paracrine cholinergic signaling in the human islet. Acetylcholine is released from alpha cells and activates M1 receptors on delta cells and M3 and M5 receptors on beta cells. Acetylcholine stimulates insulin secretion directly, but at the same time provides inhibition via somatostatin secretion from delta cells. The net effect of acetylcholine on insulin secretion likely depends on the proximity of the different cells, on the pharmacological properties of the different receptors, and on the glucose concentration. Not to neglect is additional input from cholinergic innervation.
SUPPLEMENTARY DATA

SUPPLEMENTARY FIG. 1. Scheme showing how endocrine cell types were identified after dispersion using imaging of \([\text{Ca}^{2+}]_i\). A: \([\text{Ca}^{2+}]_i\) responses to KCl depolarization, kainate and GABA allow distinguishing beta, alpha, and delta cells in dispersed islet preparations. Stimuli are applied in random order at the end of the experiment. B: Traces of \([\text{Ca}^{2+}]_i\) responses of 2 beta cells (blue), 2 alpha cells (green), and 2 exocrine cells (grey) showing responses to acetylcholine (10 \(\mu\)M) in beta cells and exocrine cells but not alpha cells. Alpha cells responded to kainate (100 \(\mu\)M).

SUPPLEMENTARY FIG. 2. Characterization of somatostatin biosensor cells. A: Schematic of the biosensor cell approach. Responses in the somatostatin biosensor cells were recorded by loading cells with Fura-2 and imaging cytoplasmic \([\text{Ca}^{2+}]_i\). B: Dose-response relationship of somatostatin biosensors cells expressing the somatostatin receptor 3 coupled to the promiscuous G protein G\(\alpha\)15 (mean \(\pm\) SEM from 9 biosensor cells). Somatostatin-28 was used as agonist. C: Somatostatin-28 (100 nM) reliably elicits \([\text{Ca}^{2+}]_i\) responses in somatostatin biosensor cells (mean \(\pm\) SEM from 11 biosensor cells). Shaded portion of the trace shows prolonged application of somatostatin-28. D: \([\text{Ca}^{2+}]_i\) responses to somatostatin-28 (1 \(\mu\)M) are completely blocked by the somatostatin receptor antagonist cyclosomatostatin (10 \(\mu\)M). Cyclosomatostatin was present throughout the shaded portion of the trace (mean \(\pm\) SEM from 11 biosensor cells).

SUPPLEMENTARY FIG. 3. Blocking M3 receptors reduces insulin responses to changes in glucose concentration. Perifusion assay showing that the M3 receptor
antagonist J-104129 (50 nM) reduced insulin responses to multiple increases in glucose concentration to 11 mM (11G).
FIG. 1. Acetylcholine stimulates beta and delta cells, but not alpha cells in human islets. Acetylcholine (10 µM) increased insulin (A) and somatostatin (C), but not glucagon secretion (B). Responses to KCl depolarization (KCl, 30 mM) are used as reference (n = 3 islet preparations). Horizontal lines in all graphs denote drug application. D: Traces of [Ca2+]i responses to acetylcholine (ACh) and KCl depolarization (30 mM) in beta, alpha, and delta cells. E: Quantification of results as in (D) showing the fractions of endocrine and exocrine cells responding to acetylcholine (black portion of the bar; results pooled from n = 4 islet preparations).

110x83mm (300 x 300 DPI)
FIG. 2. Human beta cells express functional M3 muscarinic receptors. M3 muscarinic receptors were present in human islets as detected by confocal microscopy of immunostained human pancreatic sections (A), Western blotting of lysates from five human islet preparations (B), and RT-PCR in human islets (I, n = 5) and brain (B) as a control (C). Molecular weight markers were run in parallel (shown is the 82 kDa marker). Scale bar = 20 µm. D-H: Confocal images of human pancreatic sections showing islets immunostained for M3 receptor (D-F, green), glucagon (D, red), somatostatin (soma, E, red), or insulin (G, red). Scale bars = 10 µm, in D applies to E, in F to G and H. I: Traces of [Ca2+]i responses in beta cells showing that responses to acetylcholine (10 µM) were inhibited in the presence of atropine (10 µM). J: Quantification of results as in I shows that peak responses to acetylcholine (Δ 340/380) were inhibited by atropine, the M3 receptor-specific antagonist J104129 (50 nM), but not by the M1 receptor-specific antagonist MT7 (20 nM) (n = 4 islet preparations; Student’s t-test, P < 0.05). K: Perifusion assay of insulin secretion showing that increases in insulin secretion induced by acetylcholine were inhibited by J104129 (50 nM). L and M: Quantification of [Ca2+]i responses to acetylcholine in the presence of thapsigargin (L) and in nominal 0
$[\text{Ca}^{2+}] (M)$ (n = 16 cells from 3 preparations; Student's t-test, $P < 0.05$).
FIG. 3. Human beta cells express functional M5 muscarinic receptors. M5 muscarinic receptors were present in human islets as detected by confocal microscopy of immunostained human pancreatic sections (A), Western blotting of lysates from five human islet preparations (B), and RT-PCR in human islets (I, n = 5) and brain (B) as a control (C). Molecular weight markers were run in parallel (shown is the 60 kDa marker). Scale bar = 20 µm. D-H: Confocal images of human pancreatic sections showing islets immunostained for M5 receptor (D-F, green), glucagon (D, red), somatostatin (soma, E, red), or insulin (G, red). Scale bars = 10 µm, in D applies to E, in F to G and H. I: Traces of [Ca2+]i responses in beta cells showing responses to acetylcholine alone (10 µM, left), in the presence of J104129 (50 nM, middle), and in the presence of J104129 and the M5 receptor modulator VU 0238429 (10 µM, right). J: Quantification of results as in I shows that peak responses to acetylcholine (Δ 340/380) in the presence of J104129 were amplified by VU 0238429 (n = 9 cells from 3 preparations; Student’s t-test, P < 0.05). K: Perifusion assay of insulin secretion showing that increases in insulin secretion induced by acetylcholine were amplified by VU 0365114, an allosteric modulator of M5 receptors (10 µM). L: Quantification of results as in K shows that VU
0365114 increased insulin secretion stimulated by acetylcholine (n = 4 preparations; Student’s t-test, P < 0.05). M: Quantification of perifusion assays of somatostatin secretion shows that VU 0365114 did not alter somatostatin secretion stimulated by acetylcholine (n = 3 preparations).
FIG. 4. Human delta cells express functional M1 muscarinic receptors. M1 muscarinic receptors were present in human islets as detected by confocal microscopy of immunostained human pancreatic sections (A), Western blotting of lysates from five human islet preparations (B), and RT-PCR in human islets (I, n = 5) and brain (B) as a control (C). Molecular weight markers were run in parallel (shown is the 64 kDa marker). Scale bar = 20 µm. D-H: Confocal images of human pancreatic sections showing islets immunostained for M1 receptor (D-F, green), insulin (ins, D, red), glucagon (E, red), or somatostatin (soma, G, red). Scale bars = 10 µm, in D applies to E, in F to G and H. I and J: Traces of [Ca2+]i responses in delta cells showing that responses to acetylcholine (10 µM) were inhibited in the presence of atropine (10 µM, I) and in the presence of the M1 receptor-specific antagonist MT7 (20 nM, J). K and L: Quantification of results as in I and J shows that peak responses to acetylcholine (Δ 340/380) were inhibited by atropine (K) and MT7 (L) (n > 12 cells from 3 preparations; Student’s t-test, P < 0.05). M: Perfusion assay of somatostatin secretion showing that basal somatostatin secretion at 3 mM glucose concentration was inhibited by MT7 (20 nM; n = 3 preparations).
FIG. 5. Effects of endogenous acetylcholine on hormone secretion from the human islet. A: Pulsatile secretion of somatostatin as measured by somatostatin biosensor cells was inhibited by atropine (10 µM) at 1 mM glucose concentration (representative of 4 experiments from 3 islet preparations). B: Somatostatin secretion as measured by somatostatin biosensor cells increased in the presence of the cholinesterase inhibitor physostigmine (30 µM) at 3 mM glucose concentration (representative of 4 experiments from 3 islet preparations). C and D: Perifusion assays showing increases in somatostatin secretion in the presence of physostigmine (30 µM) or KCl (30 mM; n = 3 preparations) (C) and decreases in somatostatin secretion in the presence of atropine (10 µM; n = 4 preparations) (D). E and F: Perifusion assay of insulin secretion showing an increase in insulin secretion in the presence of atropine (10 µM; n = 3 preparations). (F) is a closeup of (E). In D-F, the red trace shows secretion in the presence of vesamicol, a blocker of vesicular acetylcholine transporter (10 µM). Glucose concentration was changed from 3 mM to 11 mM at the beginning of the experiment. G: Perifusion assay showing that atropine (10 µM) amplified insulin responses to repeated increases in glucose concentration to 11 mM (11G). A control experiment with untreated islets was run in parallel (black trace). Representative of four experiments. H: Quantification of results as in G show significant increases in insulin secretion in the presence of atropine (n = 4 preparations; one sample t-test to compare the actual mean to a theoretical mean of 100%, P < 0.05).
FIG. 6. Proposed model for paracrine cholinergic signaling in the human islet. Acetylcholine is released from alpha cells and activates M1 receptors on delta cells and M3 and M5 receptors on beta cells. Acetylcholine stimulates insulin secretion directly, but at the same time provides inhibition via somatostatin secretion from delta cells. The net effect of acetylcholine on insulin secretion likely depends on the proximity of the different cells, on the pharmacological properties of the different receptors, and on the glucose concentration. Not to neglect is additional input from cholinergic innervation.
SUPPLEMENTARY FIG. 1. Scheme showing how endocrine cell types were identified after dispersion using imaging of [Ca2+]i. A: [Ca2+]i responses to KCl depolarization, kainate and GABA allow distinguishing beta, alpha, and delta cells in dispersed islet preparations. Stimuli are applied in random order at the end of the experiment. B: Traces of [Ca2+]i responses of 2 beta cells (blue), 2 alpha cells (green), and 2 exocrine cells (grey) showing responses to acetylcholine (10 µM) in beta cells and exocrine cells but not alpha cells. Alpha cells responded to kainate (100 µM).

51x27mm (300 x 300 DPI)
SUPPLEMENTARY FIG. 2. Characterization of somatostatin biosensor cells. A: Schematic of the biosensor cell approach. Responses in the somatostatin biosensor cells were recorded by loading cells with Fura-2 and imaging cytoplasmic [Ca2+]. B: Dose-response relationship of somatostatin biosensors cells expressing the somatostatin receptor 3 coupled to the promiscuous G protein Gα15 (mean ± SEM from 9 biosensor cells). Somatostatin-28 was used as agonist. C: Somatostatin-28 (100 nM) reliably elicits [Ca2+]i responses in somatostatin biosensor cells (mean ± SEM from 11 biosensor cells). Shaded portion of the trace shows prolonged application of somatostatin-28. D: [Ca2+]i responses to somatostatin-28 (1 µM) are completely blocked by the somatostatin receptor antagonist cyclosomatostatin (10 µM). Cyclosomatostatin was present throughout the shaded portion of the trace (mean ± SEM from 11 biosensor cells).
SUPPLEMENTARY FIG. 3. Blocking M3 receptors reduces insulin responses to changes in glucose concentration. Perifusion assay showing that the M3 receptor antagonist J-104129 (50 nM) reduced insulin responses to multiple increases in glucose concentration to 11 mM (11G).

45x43mm (300 x 300 DPI)