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Alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cell function in humans

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Acetylcholine is a neurotransmitter that has a major role in the function of the insulin-secreting pancreatic beta cell[1,2]. Parasympathetic innervation of the endocrine pancreas, the islets of Langerhans, has been shown to provide cholinergic input to the beta cell in several species[1,3,4], but the role of autonomic innervation in human beta cell function is at present unclear. Here we show that, in contrast to the case in mouse islets, cholinergic innervation of human islets is sparse. Instead, we find that the alpha cells of human islets provide paracrine cholinergic input to surrounding endocrine cells. Human alpha cells express the vesicular acetylcholine transporter and release acetylcholine when stimulated with kainate or a lowering in glucose concentration. Acetylcholine secretion by alpha cells in turn sensitizes the beta cell response to increases in glucose concentration. Our results demonstrate that in human islets acetylcholine is a paracrine signal that primes the beta cell to respond optimally to subsequent increases in glucose concentration. Cholinergic signaling within islets represents a potential therapeutic target in diabetes[5], highlighting the relevance of this advance to future drug development.

Acetylcholine is crucial for pancreatic beta cell function. It stimulates insulin secretion by increasing the cytoplasmic free Ca^{2+} concentration, $[Ca^{2+}]_i$, via inositol phosphate production and enhancing the effects of Ca^{2+} on exocytosis via protein kinase C in beta cells[1] (**Supplementary Fig. 1**). Muscarinic receptors found in pancreatic beta cells are essential for maintaining proper insulin secretion and glucose homeostasis in mice[2]. Cholinergic agonists have been reported to restore defective glucose-stimulated insulin secretion[6,7]. In humans, variations in the gene that encodes the muscarinic receptor M3 are associated with increased risk for early-onset type 2 diabetes[8]. It is generally believed that acetylcholine is released during feeding from parasympathetic nerve endings in pancreatic islets[1,3]. The consensus is that the endocrine pancreas is richly innervated by the autonomic nervous system[1,3,4,9], with studies based on the cholinesterase technique revealing dense parasympathetic innervation in cat, rat, rabbit and human islets[10–13]. Human pancreatic islets, however, have not been examined for the presence of prototypical cholinergic markers such as vesicular acetylcholine transporter (vAChT) or choline acetyltransferase (ChAT).

Cells and nerve fibers capable of vesicular release of acetylcholine express vAChT[14]. We performed immunohistochemistry on mouse and human pancreatic sections and found that mouse islets were densely innervated by vAChT-immunoreactive nerve fibers. These fibers formed a plexus with numerous axonal varicosities predominantly innervating beta cells (**Fig. 1**, **Supplementary Fig. 2** and **Supplementary Video 1**). By contrast, although many nerve fibers were immunostained for vAChT in the exocrine regions of the human pancreas, few if any fibers could be seen inside human islets (**Fig. 1b**). Instead, many endocrine cells were strongly vAChT immunoreactive (**Fig. 1b** and **Supplementary Video 2**). Western blots confirmed the specificity of the vAChT staining and further showed that human islets express ChAT and choline transporter 1 (ChT1, **Fig. 1d**). Because our experiments were conducted with isolated islets in which severed nerve fibers had degenerated after ≥ 2 d in culture[15,16], we were able to rule out the contribution of neuronal elements to the western blots and to the physiological experiments described below. Furthermore, we consistently found vAChT, ChAT and ChT1 mRNA expression in human islets that was comparable to or higher than that in the brain (**Fig. 1e–g**). vAChT mRNA levels correlated with ChAT mRNA levels, as expected for gene products that share a common gene locus and regulatory elements for gene transcription[17] (**Fig. 1h**). We thus conclude that human islet cells express the defining components of the cholinergic phenotype.

To localize the expression of vAChT to particular cell types within the human islet, we performed multiple immunostaining on human pancreatic sections (**Fig. 2**). We found that most (~80%) vAChT-labeled cells were immunoreactive for glucagon (**Fig. 2b**). Few vAChT cells expressed somatostatin or insulin (**Fig. 2a**,**b**). More than 60% of glucagon-labeled alpha cells were strongly immunoreactive for vAChT (**Fig. 2a**). Most alpha cells (>90%) were also immunoreactive

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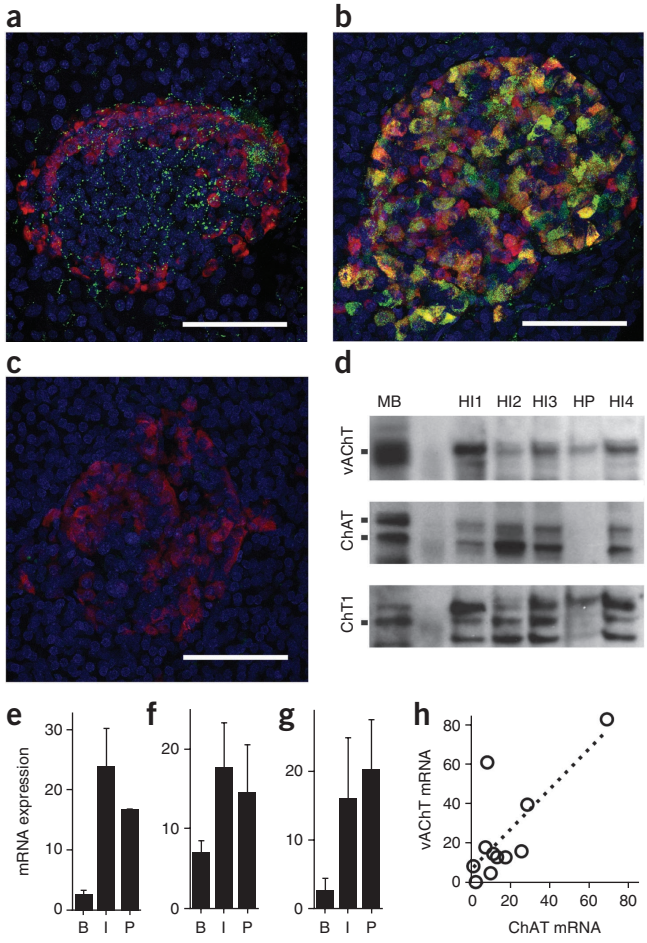


Figure 1 Endocrine cells in human pancreatic islets express cholinergic markers. (**a**) Z-stack of confocal images of a mouse pancreatic section showing an islet immunostained for vesicular acetylcholine transporter (vAChT, green) and glucagon (red). (**b**) Z-stack of confocal images of a human pancreatic section showing vAChT immunostaining in islet cells. Merge of glucagon and vAChT immunostaining appears yellow. (**c**) Z-stack of confocal images of a human pancreatic section showing lack of vAChT staining in human islets after preincubation with control peptide. Scale bars, 50 µm (**a**–**c**). (**d**) Western blotting analyses of lysates from four separate human islet preparations (HI1–HI4) and human pancreatic exocrine tissue (HP), with mouse brain (MB) as a positive control. Specific bands were seen in human islet lysates for vAChT (~70 kDa; top), for choline acetyltransferase (~63 kDa and ~68 kDa; middle) and for ChT1 (~68 kDa; bottom). A molecular marker was run in parallel (second lane). (**e**–**g**) vAChT (**e**), ChAT (**f**) and ChT1 (**g**) mRNA expression in brain (B, $n = 4$), human islets (I, $n = 12$) and human pancreas (P, $n = 3$). Data represent means ± s.e.m. (**h**) vAChT mRNA levels were associated with ChAT mRNA levels ($r^2 = 0.57$; slope significantly different from 0, $P < 0.01$).

for ChAT (**Fig. 2**). Within the alpha cell, vAChT staining did not overlap with glucagon staining and appeared confined to distinct compartments (**Fig. 2c**). We examined the colocalization of vAChT and glucagon immunofluorescence[18] and found a Pearson's correlation coefficient significantly smaller than that of C peptide and insulin colocalization and closer to that of the clearly segregated nuclear DAPI and glucagon staining (**Fig. 2d**). These findings concur with studies showing that in neuroendocrine cells, vAChT localizes preferentially to synaptic-like microvesicles and is excluded from hormone granules[19,20]. Furthermore, human alpha cells have been reported to possess secretory vesicles of different sizes[21]. To determine whether acetylcholine and glucagon are stored in different secretory granules, however, would require electron microscopy studies.

Our immunohistochemical results suggested that in human alpha cells acetylcholine is packaged in secretory vesicles for exocytotic release. We therefore examined human islets for acetylcholine secretion using cellular biosensors, namely Chinese hamster ovary (CHO) cells expressing the muscarinic receptor M3 (**Fig. 3**). We monitored acetylcholine secretion from human islets in real time by recording $[Ca^{2+}]_i$ responses from biosensors loaded with the $[Ca^{2+}]_i$ indicator Fura-2 and placed in apposition to isolated human islets (**Fig. 3a**). The biosensors showed large responses to KCl-mediated depolarization (25 mM) of human islets (**Fig. 3d**), indicating that acetylcholine release was induced from excitable islet cells and ruling out a contribution from exocrine tissue. Stimulation with kainate (100 µM) or lowering the glucose concentration from 16 mM to 3 mM, which are both alpha cell–specific stimuli[16,22,23] (**Supplementary Fig. 3**), induced strong acetylcholine release, as measured by large $[Ca^{2+}]_i$ responses in the biosensors (**Fig. 3c**,**d**). By contrast, increases in the glucose concentration from 3 mM to 16 mM did not elicit acetylcholine secretion (**Fig. 3c**,**d**). Biosensor responses could be blocked by the muscarinic antagonist atropine (5 µM), and none of the stimuli used, including KCl depolarization, induced responses in biosensors in the absence of human islets (**Fig. 3b**). This confirmed that the $[Ca^{2+}]_i$ responses were elicited by acetylcholine released from islet cells. We obtained similar results using an enzymatic assay to detect acetylcholine release (**Fig. 3e**). Because acetylcholine was released in response to treatments known to specifically stimulate alpha cells and not in response to increased glucose concentration, which stimulates beta and delta cells, we conclude that human alpha cells secrete acetylcholine.

We further used the biosensor assay to detect acetylcholine release from mouse islets. For these experiments, we cultured mouse islets for 4 d after isolation to eliminate neural elements, the same time as for human islets. Acetylcholine secretion could not be recorded from mouse islets stimulated with 25 mM KCl or 100 µM kainate (0 out of 26 mouse islets responded versus 17 out of 75 human islets; $P = 0.0052$, Fisher's exact test), consistent with the lack of vAChT immunostaining in mouse endocrine cells (**Fig. 1a**).

What is the role of alpha cell–derived acetylcholine for islet function? Studies have shown that exposure to cholinergic agonists sensitizes beta cells to subsequent stimuli, increasing insulin secretion[1,24]. Given that in human islets most beta cells are closely associated with alpha cells[25], we hypothesized that alpha cells release acetylcholine to prime neighboring beta cells. To test this hypothesis, we first examined whether cholinergic agonists induce insulin responses in human beta cells. At low glucose concentration (3 mM), acetylcholine and the muscarinic agonist oxotremorine elicited concentration-dependent insulin release from isolated human islets, indicating that activation of muscarinic receptors can induce insulin secretion at basal glucose concentrations (**Fig. 4a**,**b**).

To infer the role of acetylcholine as a paracrine signal we manipulated endogenous levels of acetylcholine. Applying the acetylcholinesterase inhibitor physostigmine (30 µM) at 3 mM glucose increased insulin secretion in isolated islets cultured for 4 d (**Fig. 4c**). These insulin responses to physostigmine were strongly inhibited by vesamicol, a selective inhibitor of vAChT that blocks acetylcholine transport into vesicles and depletes cells of releasable acetylcholine (**Fig. 4d**). We also found that physostigmine-induced increases in insulin secretion were partially inhibited by the M3 antagonist J-104129 (**Fig. 4e**).

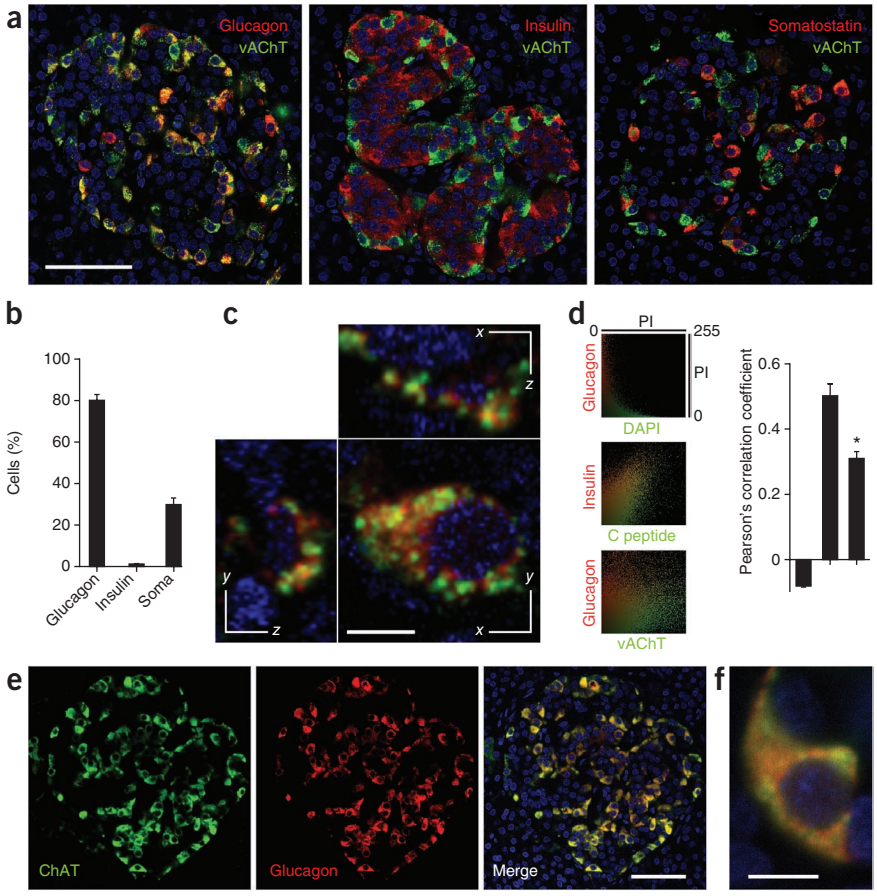


Figure 2 Human alpha cells express vAChT and ChAT. (**a**) Confocal images of human pancreatic sections showing vAChT immunostaining (green) co-stained with glucagon immunostaining (red, left), with insulin immunostaining (red, middle) or with somatostatin immunostaining (red, right). Colocalization appears yellow. (**b**) Quantification of the percentage of vAChT immunostained cells also labeled for glucagon, insulin or somatostatin ($n = 3$ human pancreata). Percentages do not add exactly to 100% because analyses were performed on different sections. (**c**) Glucagon (red) and vAChT immunostaining (green) in alpha cells at high magnification. Shown are three optical planes through an alpha cell. (**d**) Scatter plots of pixel intensities (PI) of glucagon and DAPI immunofluorescence in alpha cells (left, top), insulin and C peptide immunofluorescence in beta cells (left, middle) and glucagon and vAChT immunofluorescence in alpha cells (left, bottom). Bar graph (right) shows the thresholded Pearson's correlation coefficient values for glucagon-DAPI (left column) insulin–C peptide (middle column) and glucagon-vAChT colocalization (right column; $n = 12$ cells, analysis of variance (ANOVA) followed by multiple comparison, $^*P < 0.05$). (**e**) ChAT immunostaining (green, left) in glucagon-labeled alpha cells (red, middle). Colocalization appears yellow (merge, right). (**f**) High magnification confocal image of an alpha cell stained for glucagon (red) and ChAT (green). Scale bars, 50 μm (**a**,**e**) and 5 μm (**c**,**f**). Data represent means ± s.e.m.

This antagonist by itself reduced insulin secretion at 3 mM glucose, and this effect was negligible at 11 mM glucose (data not shown). Physostigmine-induced increases in insulin secretion were reduced at 11 mM glucose (**Fig. 4f**). These experiments indicate that acetylcholine is endogenously released at low glucose concentrations to stimulate insulin secretion and that acetylcholine secretion requires vesicular mechanisms. These results are consistent with our immunohistochemical results showing the presence of vAChT in alpha cells.

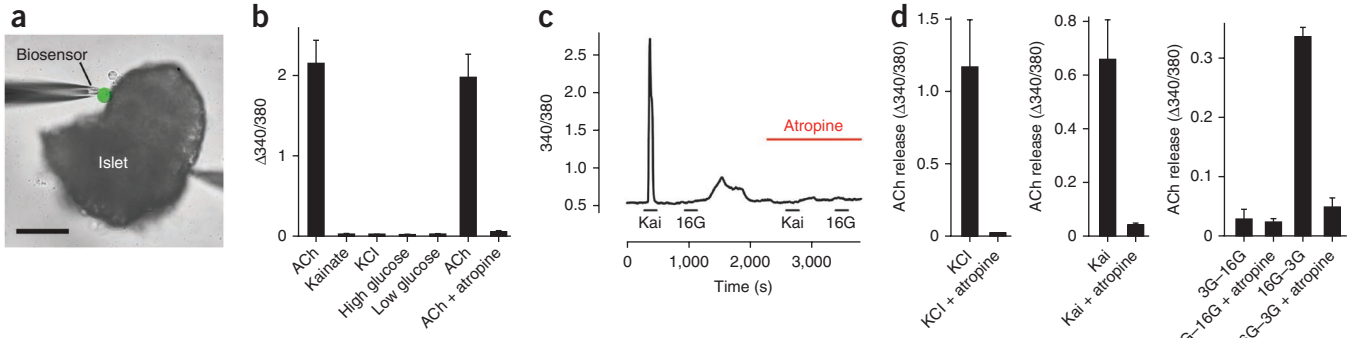
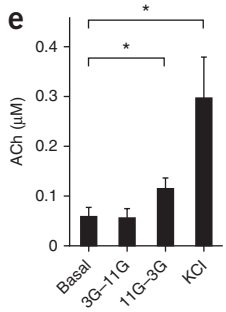


Figure 3 Isolated human islets secrete acetylcholine (ACh) in response to alpha cell–specific stimuli. (**a**) Photomicrograph of an ACh biosensor (colorized green) apposed to an isolated human islet to monitor ACh secretion evoked by stimulation of islet cells. Responses in the biosensor were recorded by loading biosensors with Fura-2 and imaging cytoplasmic $[Ca^{2+}]$. Scale bar, 50 μm. (**b**) $\Delta[Ca^{2+}]_i$ (Δ340/380) responses in biosensors, in the absence of human islets, to direct application of ACh (10 μM), kainate (100 μM), KCl (25 mM), changes in glucose concentration (from 3 mM to 16 mM (high glucose) or from 16 mM to 3 mM (low glucose)) or ACh (10 μM) in the presence of the muscarinic antagonist atropine (5 μM). (**c**) Trace of the 340/380 Fura-2 ratio in an ACh biosensor positioned against the islet as in **a** shows stimulus-induced secretion of ACh from endocrine cells in a human islet. Horizontal lines denote application of kainate (Kai, 100 μM), a transient increase in glucose concentration from 3 mM to 16 mM (16G) or application of atropine (red, 5 μM). (**d**) Summary of data from experiments conducted as those shown in **c**. Bars show means ± s.e.m. for ACh biosensor signals (Δ340/380) in response to stimulation of islets with KCl (25 mM) depolarization ($n = 8$ experiments), kainate (100 μM, $n = 11$), increases in glucose concentration (from 3 mM to 16 mM, 3G–16G) or decreases in glucose concentration (from 16 mM to 3 mM, 16G–3G, $n = 4$). Biosensor responses were blocked by atropine (5 μM). (**e**) ACh release in response to increasing the glucose concentration from 3 mM to 11 mM (3G–11G), lowering the glucose concentration from 11 mM to 3 mM (11G–3G) or to depolarization with KCl (25 mM), as determined with a fluorescent enzymatic assay (see Online Methods; $n = 6$ islet preparations; ANOVA followed by multiple comparison, $^*P < 0.05$). Data represent means ± s.e.m.



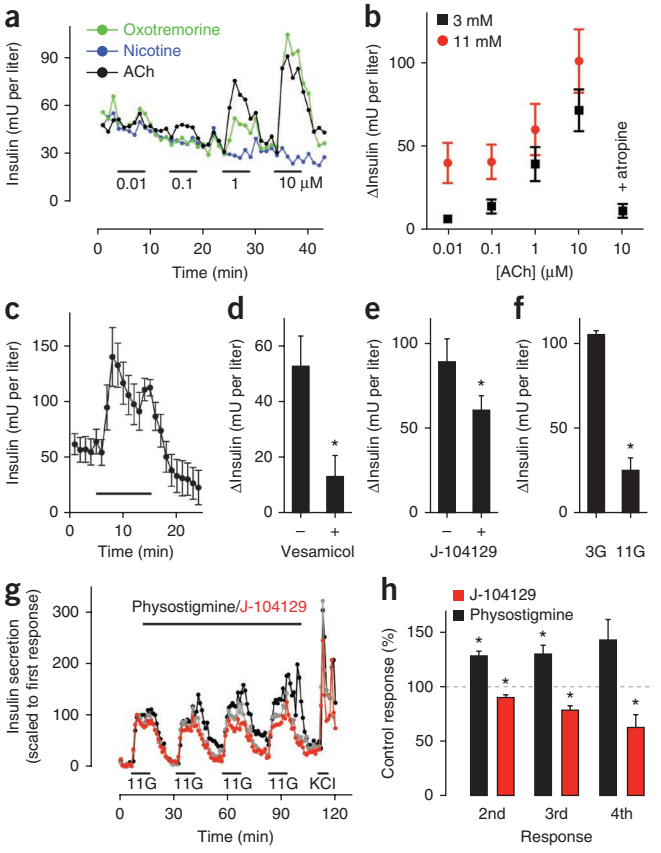


Figure 4 Endogenously released ACh amplifies glucose-induced insulin secretion in human islets. (**a**) Insulin release from human islets elicited by ACh, the muscarinic agonist oxotremorine and nicotine. Horizontal lines denote stimulus application. Representative traces of $n = 3$ islet preparations. (**b**) Summary of data from experiments similar to those shown in **a** but conducted in the presence of low (3 mM) and high (11 mM) glucose ($n = 3$ preparations). (**c**) Insulin secretion elicited by the acetylcholinesterase inhibitor physostigmine (30 μM) at 3 mM glucose ($n = 5$ human islet preparations). (**d**–**f**) Physostigmine-induced increases in insulin secretion (ΔInsulin) in the absence (–) or presence (+) of the vAChT blocker vesamicol (10 μM, **d**), the M3 receptor antagonist J-104129 (50 nM, **e**) or when the experiment was performed at different glucose concentrations (3 mM, 3G; 11 mM, 11G, **f**; Student's *t* test, $P < 0.05$). (**g**) Insulin secretion induced by repeatedly raising glucose from 3 mM to 11 mM in the presence of physostigmine (30 μM) or in the presence of J-104129 (50 nM); representative traces of four experiments. A control experiment with untreated islets was run in parallel (gray symbols). Horizontal lines at bottom denote drug application. 11G indicates 15 min of elevated glucose (11 mM). Islets were stimulated four times with glucose followed by 25 mM KCl. (**h**) Summary of data from experiments such as those shown in **c**. Responses are expressed as percentage of the respective insulin response of control islets (100%, gray dashed line; $n = 4$ preparations for J-104129 treatment; $n = 5$ preparations for physostigmine treatment). One-sample *t* tests were used to compare the actual mean to a theoretical mean of 100% (control; $^*P < 0.05$). Data represent means ± s.e.m.

In vivo, the secretion of insulin and glucagon fluctuates constantly with periods of approximately 10 min[26,27]. We hypothesized that these fluctuations allow alpha cells to increase acetylcholine secretion and influence beta cells. We reproduced these hormonal fluctuations *in vitro* by subjecting isolated human islets to an experimental protocol in which we stimulated beta cells and alpha cells intermittently while modulating cholinergic signaling (**Fig. 4g**,**h** and **Supplementary Fig. 3**). When acetylcholine degradation was inhibited with physostigmine (30 μM), insulin release increased during repeated exposure to high glucose (11 mM) (**Fig. 4g**,**h**). Blocking muscarinic receptors with the general antagonist atropine (10 μM) produced variable results, most likely because multiple receptors on different cells were activated (data not shown). By contrast, adding the M3 receptor-specific antagonist J-104129 (50 nM) consistently reduced insulin responses (**Fig. 4g**,**h**). These results show that endogenously released acetylcholine contributed to the enhanced beta cell response by activating M3 receptors. Thus, in the absence of any influence from the autonomic nervous system, endogenously released acetylcholine in human islets is able to sensitize the beta cell to subsequent increases in glucose concentration.

On the basis of our results, we propose that acetylcholine is a paracrine signal secreted by alpha cells in human islets. Our findings showing that alpha cells express vAChT and that alpha-specific stimuli induce acetylcholine secretion indicate that acetylcholine is stored in alpha cells for exocytotic release. In our model, alpha cells release acetylcholine when activated by lowering glucose concentration to prime the beta cell response to a subsequent increase in glucose concentration. Although additional paracrine effects of acetylcholine on other cells within the human islet (for example, delta cells) remain to be investigated, our results suggest that acetylcholine serves as a feed-forward signal to keep the beta cell responsive to future challenges, thus limiting plasma glucose fluctuations. Moreover, the intracellular signaling pathways activated by acetylcholine may promote long-term survival of beta cells[28], thus further suggesting that alpha cell–derived acetylcholine may act as a trophic factor.

This paracrine interaction is only possible because of the unique cytoarchitecture of the human islet, where most beta cells are closely associated with alpha cells[25,29,30]. With beta cells comprising 64% and alpha cells most of the remaining volume in the human islet[31], there is a high probability for a beta cell to be close to an alpha cell. Indeed, most beta cells (70–80%) face alpha cells[25,30] and maintain a strong association even after dispersion of the islet[30]. This cellular arrangement is compatible with the notion that paracrine interactions occur via the interstitial space between endocrine cells, although the vascular route may also be used[32]. Thus, in human islets, alpha cells seem optimally placed to influence beta cells.

Although we cannot rule out a contribution of parasympathetic nervous input, our results suggest that cholinergic innervation of human islets may be sparse. This is consistent with studies showing that the influence of neural input on insulin secretion occurring before the actual absorption of nutrients (cephalic phase) has a relatively minor role in humans[33,34]. Along these lines, vagotomized patients have normal postprandial serum insulin levels[35], and individuals with type 1 diabetes who have undergone pancreas transplantation (and thus have denervated islets) remain euglycemic without therapy[36–38]. Furthermore, it is possible that the reported parasympathetic influence on islet function in human beings may be mediated by peptidergic axons[12]. The human islet may thus be self reliant in terms of cholinergic input. That acetylcholine is a paracrine signal, and not only a neural signal as in rodents, further implies that cholinergic signaling in human islets is activated under circumstances that cannot be modeled with rodent studies, highlighting the importance of species divergence in the pancreatic islet. Because cholinergic signaling pathways have been proposed as intervention points to promote beta cell function and survival[5], our study has major implications for therapies in diabetes mellitus.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

R.R.-D., M.C.J.-S., A.F. and J.M. performed hormone assay experiments and ELISAs; R.D. performed experiments with biosensor cells to detect acetylcholine secretion; R.R.-D. and M.C.J.-S. conducted Amplex assays to measure acetylcholine secretion; R.R.-D. and M.H.A. collected, analyzed and quantified immunohistochemical data, and R.R.-D. performed RT-PCR and western blotting. R.R.-D., R.D., M.H.A., C.R., S.D.R., P.-O.B. and A.C. designed the study, analyzed data and wrote the paper. R.R.-D. and R.D. contributed equally to the study. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Pancreatic islets. We obtained human pancreatic islets from the Human Islet Cell Processing Facility at the Diabetes Research Institute, University of Miami Miller School of Medicine, or from the Islet Cell Resource basic science islet distribution program, Islet Cell Resource Centers Consortium, Division of Clinical Research, National Center for Research Resources, National Institutes of Health. Mouse islets were obtained from C57BL/6 mice killed by exsanguination under general anesthesia. Mouse islet isolation was performed using collagenase digestion followed by purification on density gradients. All experimental protocols using mice were approved by the University of Miami Animal Care and Use Committee.

Determination of acetylcholine secretion with biosensor cells. We adapted real time measurements of acetylcholine secretion from a previously published study[39]. We used Fura-2–loaded CHO cells stably expressing muscarinic M3 receptors[40].

Determination of acetylcholine secretion with Amplex assay. We measured acetylcholine secretion with the Amplex red Acetylcholine Assay Kit (Invitrogen).

Insulin and glucagon secretion. We measured insulin and glucagon secretion as previously described[16,22,23]. We purchased kainate, oxotremorine, physostigmine hemisulfate, vesamicol hydrochloride and J-104129 fumarate from Tocris Bioscience and atropine sulfate and nicotine from Sigma.

Immunohistochemistry. We performed immunostaining as previously described[16,23,25] in sections of >20 human pancreata. Antibodies used included rabbit antibody to vAChT (Synaptic Systems, 139103; control peptide 139-1P), rabbit antibody to vAChT (Sigma, V5387), rabbit antibody to ChT1 (Chemicon, AB5966), mouse antibody to somatostatin (Chemicon, MAB354), mouse antibody to glucagon (Sigma, G2654), guinea pig antibody to insulin (Dako, A0564) and rabbit antibody to C peptide (GeneTex, GTX14181). In control experiments, we incubated primary antibodies with the corresponding control peptide at a ratio of 50 µg antigenic peptide to 1 µg antibody at 22 °C for 5 h.

For ChAT immunostaining we used rabbit antibody to ChAT (Chemicon, AB143), goat antibody to ChAT (Chemicon, AB144P), or rabbit antibody to ChAT (Pierce, OSC00008W). Only antibody AB144P gave reliable results, but it required signal amplification (ABC method followed by tyramide signal amplification). The strong signal obtained after amplification may explain why the proportion of ChAT-stained alpha cells (90%) was higher than that of vAChT-stained alpha cells (60%).

Colocalization studies. We quantified the degree of association of glucagon and vAChT staining within alpha cells with the colocalization macros of Volocity software (PerkinElmer) as previously described[18].

Western blotting. We carried out immunoblot analysis by standard methods with the antibodies used for immunohistochemistry (1 in 1,000).

RT-PCR. We extracted RNA from total human brain (Applied Biosystems/Ambion), total human pancreas (Applied Biosystems/Ambion), or human islets using the RiboPure Kit (Applied Biosystems/Ambion), and we prepared cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). We ran PCR reactions in duplicate using Taqman gene expression assays (Applied Biosystems) in a StepOnePlus Real-Time PCR System (Applied Biosystems). We performed relative quantification (RQ) of gene expression based on the equation $\mathrm{RQ} = 2^{-\Delta C_t} \times 10{,}000$, where ΔC_t is the difference between the C_t value (number of cycles at which amplification for a gene reaches a threshold) of the target gene and the C_t value of the ubiquitous housekeeping gene GAPDH.

Statistical analyses. For statistical comparisons we used Student's *t* test, ANOVA followed by multiple comparisons (Bonferroni) or Fisher's exact test.

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