Galanin inhibits caerulein-stimulated pancreatic amylase secretion via cholinergic nerves and insulin

Savio G. Barreto,1 Charmaine M. Woods,1 Colin J. Carati,2 Ann C. Schloithe,1 Surendra R. Jaya,1 James Toulou,1 and Gino T. P. Saccone1

Departments of 1General and Digestive Surgery and 2Anatomy and Histology, Flinders Medical Centre and Flinders University, Adelaide, South Australia, Australia

Submitted 2 March 2009; accepted in final form 29 May 2009

Barreto SG, Woods CM, Carati CJ, Schloithe AC, Jaya SR, Toulou J, Saccone GTP. Galanin inhibits caerulein-stimulated pancreatic amylase secretion via cholinergic nerves and insulin. Am J Physiol Gastrointest Liver Physiol 297: G333–G339, 2009. First published June 4, 2009; doi:10.1152/ajpgi.00078.2009.—Pancreatic exocrine secretion is affected by galanin, but the mechanisms involved are unclear. We aimed to determine the effect and elucidate the mechanism of action of exogenous galanin on basal and stimulated pancreatic amylase secretion in vitro. The effect of galanin on basal-, carbachol-, and caerulein-stimulated amylase secretion from isolated murine pancreatic lobules was measured. Carbachol and caerulein concentration-response relationships were established. Lobules were coincubated with galanin (10−12 M to 10−7 M), carbachol (10−6 M), or caerulein (10−10 M). Lobules were preincubated with atropine (10−3 M), tetrodotoxin (10−5 M), hexamethionium (10−5 M), or diazoxide (10−7 M and 10−4 M) for 30 min followed by incubation with caerulein (10−10 M) alone or combined with galanin (10−12 M). Amylase secretion was expressed as percent of total lobular amylase. Immunohistochemical studies used the antigen retrieval technique and antisera for galanin receptor (GALR) 1, 2, and 3. Carbachol and caerulein stimulated amylase secretion in a concentration-dependent manner with maximal responses of two- and 1.7-fold over control caerulein stimulated amylase secretion in a concentration-dependent manner. Galanin significantly reduced basal amylase secretion by ~80%. Galanin did not affect caerulein-stimulated amylase secretion but had no effect on carbachol-stimulated or basal secretion. Atropine and tetrodotoxin pretreatment abolished the caerulein-stimulated amylase secretion, whereas hexamethionium had no significant effect. Diazoxide significantly reduced caerulein-stimulated amylase secretion by ~80%. Galanin did not affect caerulein-stimulated amylase secretion in the presence of hexamethionium or diazoxide. Glucose-stimulated amylase secretion was also inhibited by galanin. Immunohistochemistry revealed islet cells labeled for GALR2. These data suggest that galanin may modulate caerulein-stimulated amylase secretion by acting on cholinergic nerves and/or islet cells possibly via GALR2 to regulate insulin release.

pancreatic exocrine secretion; pancreatic lobules

GALANIN IS A 29-AMINO-ACID, COOH terminally amidated peptide that was first isolated by Tatamoto from the porcine intestinal mucosa (47). The 29-amino-acid structure is common to most species, except humans, where it exists as a 30-amino-acid molecule with no amidation at the NH 2 terminus. Galanin is involved in the regulation of numerous physiological functions, particularly in the central nervous and cardiovascular systems, and acts via three receptors designated as galanin receptor (GALR) 1, 2, and 3 (12).

Immunoreactivity for galanin has been localized in pancreatic nerves and also in a subset of islets (4, 7, 12). Ahren et al. (3) were the first to study the effect of galanin on pancreatic exocrine secretion in isolated rat pancreatic acini. They found that, although galanin did not affect basal amylase secretion, it weakly inhibited carbachol- and CCK octapeptide-induced amylase secretion. Subsequent investigations using a variety of preparations and experimental conditions produced conflicting findings. Galanin had no effect on amylase release in some systems (3, 15, 25), whereas in others inhibitory (3, 15, 21, 25, 38, 39, 50) or stimulatory effects were reported (50).

Galanin has also been shown to influence pancreatic islet secretion, most notably, inhibition of insulin secretion (4). The mechanism of the inhibitory action of galanin on insulin secretion has been postulated to be via direct modulation of the islet cell membrane potential (5), inhibition of the dihydropyridine-sensitive voltage-dependent L-type channels (22), reduction of intracellular cyclic AMP levels (14, 31, 33), and inhibition of islet exocytosis by acting in a very late stage in the stimulus-secretion coupling (42).

Insulin, in turn, is known to stimulate pancreatic exocrine secretion (34). The existence of an islet-exocrine portal system had been postulated by Henderson (20) in which the blood from the islets bathes the acinar cells. In addition, in insulin deficiency (diabetes mellitus), the pancreatic exocrine tissue shows reduced response to hormonal stimulation (10, 45, 48). The contribution of the endocrine system to pancreatic exocrine secretion could thus assume significance in understanding the mechanism of galanin on pancreatic exocrine secretion.

The aims of the present study were to determine the effect of galanin on basal and stimulated (carbachol and caerulein) pancreatic amylase secretion in the mouse pancreatic lobule preparation and to clarify its mechanism of action. Because galanin is a neurotransmitter present in pancreatic neurones, a lobule preparation was used for these studies.

MATERIALS AND METHODS

Lobule preparation. Pancreatic lobules were prepared on the basis of previously published methods (16, 30). Swiss strain mice (25–30 g) were euthanized by cervical dislocation, and the pancreata were harvested (6 mice per preparation). The combined pancreata were weighted, rinsed in oxygenated cold modified Krebs solution supplemented with 1.5% dextran and protease inhibitors (Dex-Krebs buffer) (41), and transferred to a Petri dish on ice where it was finely minced with scissors. The minced tissue was then transferred to a 50-ml container where it was washed two to three times in the ice-cold oxygenated Dex-Krebs buffer (15–20 ml) and then finally resuspended in 30 ml of ice-cold oxygenated Dex-Krebs buffer. Approximately 40 mg wet weight of pancreatic lobule preparation suspended in 0.5 ml of Dex-Krebs buffer was added to each well of a 24-well cell
culture plate (Apogen, Roskilde, Denmark). Wells contained varying concentrations of galanin, caerulein, and carbachol alone and in combinations (see below). Plates were placed in the airight containers previously gassed with 5% carbon dioxide-95% oxygen and incubated for 60 min at 37°C in a shaking water bath. Control incubations consisted of lobules incubated in Dex-Krebs buffer alone. In other experiments, the lobules were preincubated with atropine (10^-5 M), tetrodotoxin (10^-5 M), hexamethonium (10^-5 M and 10^-6 M), or diazoxide (10^-4 M and 10^-7 M) for 30 min before the addition of other peptides, and the incubations continued for a further 60 min. All incubations were performed in duplicate. Lobules were sampled separately at time 0 to determine amylase release before the experimental period. These values were then subtracted from the final values in each experiment to derive the net amylase release for the incubation period.

To determine total amylase content, the lobules were transferred to 1.5-ml centrifuge tubes on ice following the incubation period and centrifuged in a microfuge at 14,000 revolution/min for 30 s at 4°C. The supernatant was aspirated and used to measure amylase release. Lysis buffer (0.1 M) consisting of 10^-1 M sodium phosphate buffer, pH 7.8 containing 0.1% SDS, was added to the pellet, and the tube was frozen and then thawed. The thawed pellet was then homogenized manually using a pestle, and the homogenate was centrifuged at 14,000 revolution/min for 10 min at 4°C. The supernatant (lysate) was transferred to another 1.5-ml centrifuge tube. The pellet was rehomogenized and recentrifuged as outlined above, and this supernatant was combined with the previous one and stored at -20°C before amylase assay (16, 30).

Carbamoylcholine chloride (carbachol), atropine sulphate, hexamethonium bromide, BSA, diazoxide, protease inhibitor cocktail, trypsin-chymotrypsin inhibitor, and SDS were purchased from Sigma-Aldrich (St. Louis, MO). Tetrodotoxin was purchased from Alomone Laboratories (Jerusalem, Israel), and galanin (porcine) and caerulein (porcine) and caerulein were purchased from American Peptide (Sunnyvale, CA).

Preliminary experiments were performed to establish the time course and concentration range of carbachol, caerulein, and galanin for subsequent concentration-response studies. Amylase secretion was linear over a 30–90-min incubation period, and 60 min was selected for subsequent concentration-response studies. Amylase secretion was expressed as percentage of total lobule amylase per well. The net amylase release (per 60 min) was then calculated by subtracting the preexperimental amylase activity (i.e., time 0 samples, expressed as percentage of total) from the amylase released per well over the experimental period. For graphical presentation, the data are expressed at percentage of control group amylase activity (lobules incubated in Dex-Krebs only), and the n value refers to the number of preparations.

GALR immunohistochemistry. Mouse pancreas were harvested (n = 4), fixed in 10% buffered formalin, and then processed using standard paraffin histology. Sections (5 μm) were dewaxed in xylene (2 washes for 3 min each), rehydrated in 100% alcohol (2 washes for 3 min each), followed by water (2 washes for 3 min each). Endogenous peroxidase activity was blocked by washing sections in 0.3% hydrogen peroxide solution in PBS (1.5 × 10^-1 M NaCl in 10^-2 M sodium phosphate buffer, pH 7.3), followed by washing with PBS (2 washes for 5 min each). Preliminary studies found high-temperature antigen retrieval was required for these antibodies. Slides were placed in target retrieval solution pH 9 (DakoCytomation, Glostrup, Denmark), brought to the boil using the microwave, and then gently boiled for 10 min at 10% power, then left in the solution to cool for 30 min. Sections were washed with PBS (2 washes for 5 min each). Sections were then placed in a humid box, and 10% normal goat serum (Sigma-Aldrich, Castle Hill, NSW, Australia) in PBS was applied for 30 min. The primary antisera (rabbit anti-mouse GALR1, 2, and 3; Alpha Diagnostics International, San Antonio, TX) was then applied in 10% normal goat serum in PBS and left in the humid box overnight at room temperature. Slides were washed with PBS (2 washes for 5 min), and EnVision+ Dual Link System-HRP (DakoCytomation) was applied. Slides were washed again with PBS (2 washes for 5 min), and the liquid diaminobenzidine substrate-chromogen (DakoCytomation) was applied for 5 min and then washed with distilled water. The sections were counterstained with hematoxylin and dehydrated, and the coverslip was mounted with DePeX mounting medium (Gurr microscopy materials, BDH Chemicals, Kilsyth, Victoria, Australia). Sections were viewed using an Olympus Bx50 microscope and images captured using the software program QCapture (QImaging, Burnaby, BC, Canada). Preliminary studies revealed that consistent positive staining of islets was only obtained with the GALR2 antisera.

Statistical methods. Data are presented means ± SE. Statistical analysis was performed using SPSS version 11.5 (SPSS, Chicago, IL). ANOVA was used to test for concentration-response relationships, and the Mann Whitney U-test analysis was used for other comparison. P < 0.05 was regarded as significant.

RESULTS

Carbachol, caerulein, and galanin concentration-response relationships. Amylase secretion from control lobule incubation was 8.2 ± 0.8% of the total amylase content (n = 5). Caerulein produced a concentration-dependent increase in amylase secretion peaking at 10^-10 M (13.9 ± 1.6% of total), and amylase secretion was less with higher caerulein concentrations (Fig. 1). Carbachol produced a sigmoidal concentration-response curve with maximal secretion induced by 10^-6 M (16.5 ± 0.8% of total) and a plateau at higher concentrations (Fig. 1). Galanin did not significantly affect basal amylase secretion (Fig. 1).
Effect of galanin on stimulated secretion. Galanin did not significantly affect the amylase secretion evoked by $10^{-6}$ M carbachol (Fig. 2A). In contrast, galanin ($10^{-12}$ M) significantly inhibited the caerulein-evoked amylase secretion by 84% ($P < 0.03$) although this effect was not evident at higher concentrations of galanin tested (Fig. 2B). The effective concentration of galanin was used in subsequent experiments.

Caerulein stimulates amylase secretion: effects of atropine, tetrodotoxin, and hexamethonium with and without galanin. Caerulein ($10^{-10}$ M) stimulated amylase secretion to ~150% of control. Atropine ($10^{-5}$ M) preincubation completely abolished the caerulein response, and coincubation with galanin had no further effect (Fig. 3A). Similarly, preincubation with tetrodotoxin ($10^{-5}$ M) completely abolished caerulein-stimulated amylase secretion, and coincubation with galanin had no
further effect (Fig. 3A). In contrast, preincubation with hexamethonium (10⁻⁵ M and 10⁻⁶ M) did not significantly affect caerulein-stimulated amylase secretion. The addition of galanin in the presence of hexamethonium resulted in a significant inhibition of the caerulein-evoked response, which was comparable to that produced by galanin alone (P < 0.04, Fig. 3B). Amylase secretion from control lobules was 12.4 ± 0.4% of total (n = 6).

Caerulein stimulated amylase secretion: effect of diazoxide with and without galanin. Caerulein (10⁻¹⁰ M) stimulated amylase secretion to 162% of control in these experiments. Diazoxide pretreatment (10⁻⁷ M and 10⁻⁴ M) significantly inhibited caerulein-stimulated amylase secretion (P < 0.02) (Fig. 4, A and B). Addition of galanin (10⁻¹² M) had no further effect. Diazoxide preincubation had no significant effect on carbachol-stimulated amylase secretion (Fig. 4B). Amylase secretion from control lobules was 13.9 ± 0.4% of total.

Glucose stimulated amylase secretion. Glucose stimulated amylase secretion to 180 ± 9% of control (n = 5). Coincubation with galanin (10⁻¹² M) significantly reduced the glucose-stimulated amylase secretion to 105 ± 3% of control (n = 5; P < 0.05).

Immunohistochemical studies showed that mouse pancreas contains a subset of islets that were immunoactive for GALR2 (Fig. 5). No specific labeling was noted in the acinar regions.

**DISCUSSION**

We have demonstrated that galanin inhibits caerulein- and glucose-stimulated, but not basal or carbachol-stimulated, amylase secretion from isolated mouse pancreatic lobules. The caerulein stimulation of amylase secretion is blocked by atropine and tetrodotoxin, implying mediation by cholinergic neurons. The caerulein response was also blocked by diazoxide, which indicates complete dependence on insulin secretion. These data demonstrate for the first time that caerulein-stimulated amylase secretion in lobules involves a combined neural-paracrine mechanism. Galanin inhibited the caerulein-stimulated amylase secretion acting on the cholinergic nerves and/or insulin secretory cells. Galanin also inhibited glucose-stimulated amylase secretion. Immunohistochemistry revealed a subset of islet cells labeled for GALR2. On balance, these data suggest that galanin modulates the caerulein-evoked response by acting on cholinergic nerves and islet cells possibly via GALR2 to regulate insulin secretion.

**Regulation of amylase secretion.** Pancreatic amylase secretion is under neurohormonal regulation. The parasympathetic system acting via the vagus is known to regulate pancreatic exocrine secretion (32). Vagal efferent neurotransmission to the pancreas originates from the dorsal motor nucleus of vagus and passes via preganglionic fibers to synapse with the intrapancreatic ganglia. The preganglionic neurotransmission is mediated by acetylcholine acting through the nicotinic and muscarinic receptors, whereas the postganglionic neurotransmission is mediated by acetylcholine acting through only muscarinic receptors (9, 32). On the basis of investigations by Herzig et al. (21) and Flowe et al. (15), galanin appeared to influence amylase secretion in the rat via a neural mechanism. The pancreatic lobule preparation used in the present study, as opposed to the acinar cell preparation that is devoid of the neural component, permits the study of neurally mediated responses. The lobule preparation retains the basic architecture of the acinar pancreas with intact nerves and islets.

**Galanin and amylase secretion.** Previous studies of the action of galanin on amylase secretion have not used mouse pancreatic lobules. The carbachol concentration-response relationship displayed by the mouse pancreatic lobule preparation is similar to that reported with rat lobules (8, 27, 50) and guinea pig lobules (29, 50, 51). It is only in the mouse preparation that a specific action of galanin on the amylase secretion has been observed. These studies show that galanin at a concentration of 10⁻⁴ M elicited an inhibition of the caerulein-evoked response, which was comparable to that produced by galanin alone (P < 0.02). These results are consistent with the view that galanin inhibits amylase secretion in the mouse preparation.
pig pancreatic acini preparations (6). The caerulein concentration-response relationship showed a peak amylase secretion at $10^{-10}$ M and a fall in amylase secretion at higher concentrations. This phenomenon was noted by Scheele et al. (40) and has been attributed to a number of possible factors including deranged acinar cell exocytosis.

We found that galanin had no effect on basal amylase release. This supports previous studies that used rat isolated acinar cells (3, 25) and rat pancreatic lobules (15, 47). Galanin exhibited a variable effect on stimulated secretion depending on the secretagog used. Galanin had no effect on carbachol-stimulated amylase secretion but significantly inhibited caerulein-stimulated secretion. Our finding is at variance to that of Ahren et al. (3), who found that galanin inhibited carbachol-stimulated amylase release from isolated rat pancreatic acini, but supports that of Herzig et al. (21), who showed that galanin had no effect on carbachol-mediated amylase secretion from isolated rat acinar cells. To our knowledge, the effect of galanin on caerulein-stimulated amylase secretion has not been previously reported.

**Carbachol and caerulein-evoked secretion.** Carbachol acts directly on muscarinic receptors (M1 and M3) on the acinar cells to stimulate amylase secretion (19). M3 receptors are also located on insulin-secreting islet cells; however, in the present study, the carbachol-stimulated amylase secretion was not influenced by diazoxide, suggesting that carbachol activation of the acinar cell muscarinic receptors was dominant. In contrast, caerulein, at concentrations comparable to physiological concentrations of CCK, has been postulated to stimulate amylase secretion via mechanisms including CCK-1 receptors (18), atropine-sensitive neural pathways (21, 46), and nicotinic receptors (11). Our findings that caerulein-stimulated amylase secretion is completely abrogated by atropine and tetrodotoxin confirms previous reports that the effect of caerulein on pancreatic exocrine secretion is mediated through cholinergic neural pathways (21, 46). These cholinergic neurons could innervate the islets and acinar cells (4, 32). This finding also suggests that caerulein is not acting directly on CCK receptors on acinar cells. The lack of a significant inhibition of caerulein-stimulated amylase secretion by preincubation with hexamethonium suggests that nicotinic receptors are not involved. A presynaptic site of action of caerulein, however, is not excluded. Overall, these data are consistent with galanin modulating a neural mechanism.

**Role for insulin secretion.** Insulin has been shown to influence pancreatic exocrine secretion (10, 20, 35, 45, 48). In vitro and in vivo studies (17, 28) have indicated that caerulein directly stimulates insulin secretion, and other studies have shown that insulin stimulates pancreatic exocrine secretion (4, 24, 34, 44, 49). Our present findings suggest that the action of caerulein on pancreatic amylase secretion in lobules is secondary to its effect on insulin secretion because the caerulein-evoked response is blocked by diazoxide. Diazoxide acts on ATP-dependent potassium channels in insulin-secreting islets cells. In addition, glucose, at a concentration known to stimulate insulin secretion, also stimulated amylase secretion. This is the first direct demonstration that caerulein-stimulated amylase secretion is insulin dependent. The action of insulin on exocrine secretion is believed to be via the islet-acinar portal system. In the lobule preparation used in the present study, blood flow is lacking; however, the close proximity of the acinar and islet cells suggests that diffusion of insulin to surrounding acinar cells is likely.

**Endogenous galanin.** There are two potential sources for endogenous galanin in the pancreas, neural and endocrine. Immunoreactivity for galanin has been localized in pancreatic nerves with fibers present as all part of the pancreas although the density is species dependent (2, 7). Moreover, galanin immunoreactivity has been localized in a subset of islets (7). The pancreatic cells types expressing GALRs have not been elucidated, and the molecular expression of GALR subtypes in the pancreas has received little attention. GALR3 mRNA is expressed in human pancreas (26), and we have demonstrated that the three GALR subtype mRNAs are expressed in the mouse pancreas, with GALR3 the most highly expressed (29).

As galanin modulates caerulein-stimulated, but not basal, amylase secretion, this suggests that the inhibitory effect of galanin is either at the level of the cholinergic innervation (intrapancreatic ganglia or intrinsic neurones) and/or the islet cells (Fig. 6). The pancreatic islets receive significant cholinergic and galaninergic innervation (4). The canine pancreatic islets are known to be associated with a dense network of galanin immunoreactive nerve fibers (14). A role for islets is further supported by our observation that a subpopulation of mouse pancreatic islet cells, but not the surrounding acinar tissue, expresses GALR2 immunoreactivity. Galanin could also act to modulate cholinergic neurotransmission, acting presynaptically, as has been demonstrated in the heart and myenteric plexus (36, 37). Further studies are required to define the site of action and the galanin receptor subtypes involved in the galanin response reported in the present study.

![Diagrammatic representation of postulated mechanism of galanin of inhibition of caerulein-stimulated amylase secretion via insulin secretion](http://ajpgi.physiology.org/)
An islet and/or neural-mediated mechanism may explain the lack of effect of galanin on CCK-stimulated amylase secretion from isolated rat pancreatic acinar cells (21).

The inhibition of galanin of CCK-stimulated amylase secretion has been reported by several investigators using in-vivo preparations (15, 38, 50). Our study represents the first demonstration of this effect with caerulein in a lobule preparation and indicates that direct mediation by the central nervous system is not involved. Exogenous galanin in vivo has also been shown to inhibit 2-deoxyglucose-stimulated amylase secretion (15, 21, 50). 2-Deoxyglucose is known to stimulate vagal nerves that regulate pancreatic amylase secretion via acetylcholine release (34). Thus in vivo galanin could modulate 2-deoxyglucose-induced vagal activity in the central nervous system or in the pancreas. The acetylcholine analog bethanochol selectively acts at M3 on acinar cells to stimulate amylase secretion (23). Galanin has no effect on bethanochol-stimulated amylase secretion (15, 50), and we found that galanin did not affect carbachol-stimulated amylase secretion. Thus this evidence suggests that galanin is unable to block the actions of such agents that act directly at receptors on the acinar cell. In contrast, we have demonstrated that glucose-induced amylase secretion was inhibited by galanin, supporting the insulin-secreting cells as one site of the action of galanin.

In conclusion, our data suggest that caerulein stimulates amylase secretion by a combined neural-paracrine mechanism. Galanin inhibits caerulein-stimulated amylase secretion by acting on cholinergic neurones that regulate insulin secretion and/or on insulin-secreting islet cells. Insulin, in turn, stimulates amylase secretion.

ACKNOWLEDGMENTS

We thank D. Keating for useful discussion.

GRANTS

The work was supported by the Flinders Medical Centre Foundation.

REFERENCES