Mechanism of Galanin's inhibitory action on pancreatic enzyme secretion: modulation of cholinergic transmission – studies in vivo and in vitro

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Abstract

This study examined the inhibitory mechanism of galanin, a 29 amino acid polypeptide on pancreatic enzyme secretion in anaesthetised rats, isolated pancreatic acini, and lobules. Urethane anaesthetised rats with pancreatic fistulas pretreated with 3-0-methylglucopyranose (500 mg/kg/h) were stimulated with an intravenous bolus of 2-deoxyglucose (2-DG) (75 mg/kg). Maximal amylase secretion was mean (SEM) 274 (19)% of basal secretion. Atropine (150 µg/kg/h) and galanin (10 nmol/kg/h) almost completely inhibited 2-DG stimulated amylase secretion suggesting an inhibition of cholinergic transmission. To further test this possibility this study investigated the effect of galanin on carbachol and cholecystokinin stimulated amylase release from isolated pancreatic acini. Galanin did not suppress carbachol or cholecystokinin stimulated amylase release, indicating that galanin inhibits exocrine secretion by indirect mechanisms. The cholinergic pathway was assessed by using pancreatic lobules containing intrapancreatic neurons. Veratridine, a sodium channel activator, dose dependently stimulated amylase release. Veratridine (100 µM) stimulated amylase release by 411 (10)% of basal secretion. Atropine (1 µM) or tetrodotoxin (1 µM) almost completely blocked veratridine stimulated amylase release. Galanin (1 µM) significantly inhibited veratridine stimulated amylase release with a maximal inhibition of 50% ($p < 0.05$). In addition, when lobules were incubated with [³H]-choline, galanin significantly ($p < 0.05$) inhibited veratridine stimulated release of newly synthesised [³H]-acetylcholine. Thus galanin inhibits pancreatic secretion by inhibiting cholinergic transmission. These studies show that galanin inhibits rat pancreatic enzyme secretion by an indirect mechanism by reducing cholinergic transmission.

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norepinephrine, serotonin, γ -amino butyric acid, vasopressin, cholecystokinin (CCK) and acetylcholine.^{2–5} The localisation and the coexistence with these neurotransmitters strongly suggests that galanin plays an important part in the modulation of neural transmission. In the pancreas galanin immunoreactive nerve fibres have been detected in several species, only the intrapancreatic abundance and distribution varies slightly among them.^{6–9} In the rat galanin immunoreactive nerve fibres are located around blood vessels and scattered in the exocrine pancreatic parenchyma, occasionally in the islets.⁸ In the dog pancreas on the other hand, galanin immunoreactive nerves are most abundant in the islets.⁶ Galanin immunoreactivity has not been detected in the intrapancreatic neurons of the dog, therefore Dunning *et al* suggested an extrinsic origin of the galanin immunoreactive nerves. In contrast with dogs, in the human pancreas galanin immunoreactive nerves are mainly distributed in the exocrine tissue including intrapancreatic ganglia.⁹ According to their results Shimosegawa *et al* suggested an intrinsic origin of galanin immunoreactivity and speculated that in the human pancreas galanin is a likely neurotransmitter in the parasympathetic postganglionic nerve system.

Mixed pancreatic nerve stimulation releases galanin into the venous effluent of dogs inhibiting insulin and somatostatin secretion and modestly stimulating glucagon output.¹⁰ In addition, galanin has been shown to inhibit the secretion of other gastrointestinal hormones, gut motility, and exocrine pancreatic secretion.^{3,11,12} The mechanism, however, through which galanin acts to inhibit exocrine pancreatic secretion is unclear. In the brain and the myenteric plexus galanin has been shown to inhibit cholinergic transmission.^{13–15} On the other hand, galanin weakly inhibited amylase release from isolated rat pancreatic acini suggesting a direct effect on the acini.¹⁶

Therefore, in this study we investigated the hypothesis that galanin inhibits pancreatic enzyme secretion by presynaptic modulation of acetylcholine release. To test this hypothesis we examined the effect of galanin (a) on 2-deoxyglucose (2-DG) stimulated amylase release in anaesthetised rats, (b) on CCK8 and carbachol stimulated amylase release from isolated pancreatic acini devoid of neural elements, (c) on the sodium channel activator veratridine stimulated amylase release from pancreatic lobules contain-

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Galanin, a 29 amino acid peptide, was first isolated from porcine intestinal mucosa by Tatemoto *et al* using a method that detects its C-terminal amide structure.¹ The name was derived from its N-terminal amino acid glycine and the C-terminal amino acid alanine. Galanin is widely distributed in the neurons of the central nervous system and the gut and coexists with

ing neural elements, and (d) on veratridine stimulated acetylcholine release.

Materials and methods

MATERIALS

The following substances were purchased: trypsin (type II-S), soybean trypsin inhibitor (SBTI) (type I-S), 2-deoxyglucose, 3-0-methylglucopyranose, taurocholate, veratridine, tetrodotoxin, physostigmine, hemicholinium, atropine sulphate, urethane from Sigma Chemical (Deisenhofen, Germany), N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), bovine serum albumin (fraction V) from E Merck (Darmstadt, Germany), collagenase from Bayer Diagnostic (München, Germany), minimal Eagle's amino acid supplement from Gibco/BRL (Life Technologie, Eggenstein, Germany), rat galanin from Peninsula Laboratories (Heidelberg, Germany), and [methyl-³H] choline chloride from Amersham (Braunschweig, Germany).

METHODS

In vivo pancreatic secretion studies

Male starved Sprague-Dawley rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany), weighing between 200–250 g, were anaesthetised with urethane (1.3 g/kg body weight). Body temperature was maintained at 38°C with a heating pad. The pancreas was exposed through a midline abdominal incision and the common bile pancreatic duct was cannulated at the sphincter of oddi with a tapered polyethylene tip attached to silastic tubing. A second cannula was placed into the duodenum, slightly above the sphincter for continuous infusion of a trypsin bile acid solution (2 mg trypsin, dissolved in 50 mmol/l NaHCO₃, 78 µmol/l sodium taurocholate, rate of infusion 1 ml/h). This amount was sufficient to suppress CCK release.¹⁷ For intravenous infusions a cannula was inserted into the external jugular vein and the incisions covered with a moist gauze.

The diverted bile pancreatic juice was collected in small vials for 15 minute periods, the volume determined, and amylase measured using an auto-analyser (Eppendorf ACP 5040, Hamburg, Germany) according to the method of Kruse-Jarres *et al.*¹⁸ After a two hour basal period, atropine (150 µg/kg/h) or galanin (10 nmol/kg/h) was infused intravenously for the remainder of the experiment; control rats received saline.¹² Thirty minutes after the start of galanin infusion, rats were pretreated with 3-0-methylglucopyranose (500 mg/kg/h) for 30 minutes and stimulated with a bolus injection of 2-deoxyglucose (2-DG; 75 mg/kg).¹⁹ 2-DG stimulates pancreatic secretion by cholinergic central stimulation caused by cytoglucoopenia in the nuclei of the solitary tract.²⁰ 3-0-methylglucopyranose in addition to 2-DG is thought to aggravate the central cytoglucoopenia.¹⁹ After the bolus injection of 2-DG pancreatic secretion was measured for additional 120 minutes. Results were expressed as a percentage of basal amylase output.

Isolated pancreatic acini studies

Isolated rat pancreatic acini were prepared by collagenase digestion, as described previously, to test if galanin directly affects CCK or carbachol stimulated amylase release.²¹ Briefly, pancreatic tissue was incubated in 10 mM HEPES buffered Ringer solution (HR) containing 100 U/ml collagenase, 6 mM glucose, 0.5% bovine serum albumin, 0.1% soybean trypsin inhibitor, and Eagle's minimum amino acid supplement; the digestion buffer was additionally injected into the pancreatic interstitium to permit better digestion of tissue. After 30 minutes' incubation at 37°C under O₂ saturation in a shaking water bath, acini were dispersed with mild shearing forces, passed through a double filter gauze, and purified by sedimentation in 4% bovine serum albumin. Acini were washed three times in HR and added in aliquots to prepared vials containing CCK8 or carbachol in the presence or absence of galanin (1 µM). The vials were incubated under O₂ saturation at 37°C in a shaking water bath for 30 minutes. The reaction was stopped by placing the vials on ice and separating the cells by centrifugation. Amylase released into the medium and total acinar amylase content were measured. All experiments were done in triplicate and amylase released was expressed as a percentage of initial total amylase content.

Pancreatic lobule studies

Pancreatic lobules containing postganglionic neural elements and islets were prepared by the method of Scheele and Palade.²² Briefly, the pancreas was removed and HR buffer solution injected into the interstitium. Lobules were excised with forceps and scissors. The lobules were washed and transferred into baskets with mesh bottoms allowing the buffer to drain but retain the lobules. The baskets were placed into temperature controlled chambers at 37°C under a constant drip (0.5 ml/min) of HR buffer. The superfusion medium was collected under the basket in small vials. After a 45 minute adjustment period in the chambers five 4 minute basal collections were taken and the lobules were stimulated with the sodium channel activator veratridine (10 µM–500 µM) for eight minutes.²³ Veratridine has no effect on pancreatic acini²⁴; it is known to depolarise existing cells by blocking the inactivation of Na⁺ channels.²⁵ Each stimulation was followed by eight 4 minute collection periods. In the different treatment groups atropine (1 µM) or tetrodotoxin (1 µM), a specific sodium channel blocker, or galanin (1 µM) was already added individually to the superfusion buffer 20 minutes before and throughout the stimulation period. At the end of the experiment, the lobules were homogenised with a Wheaton-Dounce glass-glass homogeniser. Amylase content of the homogenates and the superfusion medium was determined and results are expressed as a percentage of basal fractional release. Baseline secretion is expressed as 100%.

Acetylcholine release studies

Acetylcholine release was measured by the

Figure 1: Effect of atropine and galanin on 2-deoxyglucose (2-DG) stimulated amylase output in anaesthetised rats. After a two hour basal period atropine (150 $\mu\text{g/kg/h}$) or galanin (10 nmol/kg/h) were infused throughout the remainder of the experiment. Rats were pretreated with 3-O-methylglucopyranose for 30 minutes before the bolus injection of 2-DG (75 mg/kg). 2-DG ($\square$), 2-DG + atropine ($\circ$), 2-DG + galanin ($\bullet$). Two way analysis of variance with repeated measures showed a significant inhibitory effect of atropine and galanin over time ($F=8.7$; $p<0.05$; $df\ 1/18$); values are means of eight experiments.

Figure 2: Effect of galanin on cholecystokinin 8 (CCK8) (A) and carbachol (B) stimulated amylase release from isolated pancreatic acini. Control acini ($\square$), acini treated with galanin (1 μM) ($\bullet$). Values are means of seven experiments.

method described by Wu *et al.*²⁶ Pancreatic lobules were depleted of endogenous acetylcholine by electrical field stimulation. Platinum wires were attached to the bottom of a basket, which was placed into a heating chamber under a constant drip. Pulses of 1 ms duration at 4 Hz at 40 volts were given for 15 minutes. The lobules were incubated with 0.2 μM [methyl- ^3H] choline chloride in HR buffer containing 50 μM physostigmine, a cholinesterase inhibitor, at 37°C for 45 minutes in a shaking incubator under O_2 saturation. Afterwards the lobules were washed six times for 10 minutes and transferred into the baskets. Hemicholinium (10 μM) was added to the buffer to prevent released acetylcholine being taken up again. The same perfusion system and experimental design as described above was used in the pancreatic lobule studies. Instead of amylase measurement 1 ml of collected superfusion buffer was added to 9 ml of scintillation fluid (Quickzint 2000; Zinser Analytic, Germany). The lobules were solubilised overnight in 10% sodium dodecyl sulfate. Radioactivity was counted in a Beckman LS-7800 scintillation counter. Counts were expressed as a percentage of basal fractional release. Baseline secretion is expressed as 100%. Veratridine evoked [^3H] efflux has been called [^3H] acetylcholine, because earlier studies showed that virtually all the veratridine evoked release of [^3H] activity from intrapancreatic cholinergic ganglia represents [^3H] acetylcholine.^{27,28}

STATISTICS

Results are expressed as mean (SEM). Data were analysed using analysis of variance with the method of Dunn for multiple contrasts or Student's *t* test where appropriate. Statistical significance was set at $p<0.05$.

Results

IN VIVO STUDIES

Basal amylase output was monitored two hours before the start of galanin to ensure a complete suppression of the negative feedback mechanism.¹⁷ Basal amylase output averaged 1544 U/h. Pretreatment with 3-O-methylglucopyranose (500 mg/kg/h) before the bolus injection of 2-DG did not stimulate amylase output (data not shown). Bolus injection of 2-DG (75 mg/kg) raised amylase output by 274 (19)% within 45 minutes (Fig 1). Atropine (150 $\mu\text{g/kg/h}$) blocked the stimulatory effect of 2-DG completely proving that 2-DG acts by cholinergic pathways to stimulate pancreatic enzyme secretion (Fig 1). Also galanin (10 nmol/kg/h) completely inhibited 2-DG-stimulated amylase output suggesting that galanin might affect cholinergic transmission (Fig 1).

IN VITRO STUDIES

Isolated pancreatic acini studies

In isolated pancreatic acini stimulation with various concentrations of CCK8 and carbachol stimulated amylase release in the typical biphasic

Figure 3: Amylase release from pancreatic lobules containing neural elements in response to veratridine, a sodium channel activator. Values are means of at least six experiments.

fashion with an optimal secretion at 10 pM and 5 μ M, respectively (Fig 2). Galanin at 1 μ M did not change the amylase response to CCK8 or carbachol showing that galanin has no direct effect on the pancreatic acini to inhibit amylase secretion and that the inhibitory effect of galanin seen in vivo has to be indirect (Fig 2). Lower doses of galanin were without any effect as well.

Pancreatic lobule studies

In pancreatic lobules the sodium channel activator veratridine depolarises these neurons causing a dose dependent amylase release into the superfusion buffer (Fig 3). The sodium channel activator veratridine (100 μ M) caused a 412 (10)% stimulation of basal amylase release. Tetrodotoxin (1 μ M), a specific sodium channel blocker, abolished amylase secretion stimulated by the highest dose of veratridine used (Fig 4). Atropine (1 μ M) also blocked the effect of veratridine showing that veratridine stimulated amylase release is mediated by a cholinergic pathway. Addition of galanin to the superfusion buffer for 20 minutes caused a 50% inhibition of veratridine (100 μ M) stimulated amylase release providing further evidence that galanin inhibits cholinergic transmission (Fig 5).

To prove this hypothesis, we preincubated

Figure 4: Effect of atropine or tetrodotoxin (TTX) on veratridine stimulated amylase release from pancreatic lobules. Values are means of six experiments. * $p < 0.05$.

Figure 5: Effect of galanin on veratridine stimulated amylase release from pancreatic lobules. Values are means of 11 experiments. * $p < 0.05$.

pancreatic lobules with [methyl- 3 H] choline chloride for 45 minutes, which is synthesised to [3 H]-acetylcholine and measured release of radioactivity from the lobules in response to veratridine. Veratridine (500 μ M) stimulated [3 H]-acetylcholine release by 177 (6)% (Fig 6). Galanin treatment inhibited this stimulation to 114 (3)% providing direct evidence that galanin inhibits pancreatic secretion by an inhibition of cholinergic transmission (Fig 6).

Discussion

Galanin completely inhibited 2-DG stimulated amylase release in anaesthetised rats – a stimulus that is known to act by cholinergic pathways.^{19,20} Yagci *et al* found that galanin potently inhibited bombesin, secretin, and cholecystokinin stimulated pancreatic protein and amylase secretion in the anaesthetised rat.¹² According to the study from Yagci *et al* we used a maximal dose of galanin to test the effect on cholinergically stimulated amylase secretion and did not perform additional experiments with lower concentrations, because our aim was to further elucidate the inhibitory mechanism.

In isolated pancreatic acini galanin failed to

Figure 6: Effect of galanin on veratridine stimulated 3 H-acetylcholine (3 H-ACh) release from pancreatic lobules. Values are means of nine experiments. * $p < 0.05$.

have any inhibitory action on CCK8 or carbachol stimulated amylase release. By contrast with our findings, Ahren *et al* reported a weak, but significant, inhibitory action on CCK8 and carbachol stimulated amylase release in isolated rat pancreatic acini.¹⁶ They used different concentrations of galanin (100 pM–100 nM) on amylase secretion stimulated by a supramaximal dose of CCK8 (10 nM) and a submaximal dose of carbachol (10 μ M). At 10 nM CCK they saw a 9.7 (0.6)% stimulation of total amylase release. Addition of galanin (100 nM) reduced amylase secretion to 8% only. Carbachol stimulated amylase release by 13.5%; addition of galanin (1 nM) reduced stimulation to 11.25%. The inhibition by galanin seems to be rather weak. To see a direct effect of galanin, we tested the highest concentration of galanin (1 μ M) on various doses of CCK8 and carbachol. Our acini were far more responsive to CCK8 and carbachol with the maximal secretion of 24 (1.3)% at (10 pM) and 21.6 (1.7)% at (5 μ M), respectively (see Fig 2). Interestingly, in the study reported by Ahren *et al* higher doses of galanin (for example, 100 nM v 10 or 1 nM) did not inhibit carbachol stimulated amylase release. To propose a direct effect of galanin on pancreatic acini, specific receptors must exist on the acinar cell as they have been found on hamster β cells and the insulinoma cell line Rin m 5F.^{29,30} Higher concentration of galanin should lead to a higher occupancy of possible galanin receptors on the acinar cells and therefore result in a greater inhibition of amylase secretion. Overall, the weak inhibitory effect of galanin on pancreatic acini reported by Ahren *et al* cannot account for the complete inhibition of 2-DG stimulated amylase secretion seen in vivo. It remains unclear if the reduced responsiveness of their acini accounts for the different findings in isolated pancreatic acini.

Because there was not a direct effect of galanin on pancreatic acini in our preparation we investigated possible indirect mechanisms. Veratridine dose dependently stimulated amylase release in pancreatic lobules by depolarising neural elements in the lobules, which was significantly inhibited by galanin. Proof that galanin indeed inhibits cholinergic transmission from presynaptic cholinergic neurons was provided by the [³H]-acetylcholine study. Galanin significantly inhibited [³H]-acetylcholine release. These results provide direct evidence that galanin inhibits pancreatic enzyme secretion by inhibiting presynaptic cholinergic transmission.

Because galanin did not completely block amylase release, it might be possible that additional inhibitory mechanisms are responsible for the full inhibitory action of this peptide. The secretion of the islet hormones somatostatin, glucagon, and pancreatic polypeptide – all known inhibitors of exocrine pancreatic secretion – are inhibited rather than stimulated by galanin.⁴ On the other hand, insulin secretion is inhibited as well. A recent study showed that anti-insulin serum could block the postprandial exocrine pancreatic secretion,³¹ yet the mechanism of insulin's action on pancreatic secretion is not known. A reduced insulin secretion could account in part for the diminished exocrine secretion in vivo, although the effects of hyper-

glycaemia on pancreatic secretion are contradictory.^{32,33}

The results of this study fit into the general concept of pancreatic enzyme regulation. The importance of the cholinergic nervous system during the intestinal phase of digestion has been well reported.^{34,35} Adler *et al* showed in humans a complete blockade of meal stimulated pancreatic enzyme secretion by atropine, while the potent CCK receptor antagonist loxiglumide caused only a 60% inhibition. Inhibitory peptides like galanin, pancreatic polypeptide, pancreastatin, peptide YY, and neuropeptide Y modulate stimulated pancreatic secretion by reducing acetylcholine release.^{27,28,36,37} The results of this study show that galanin is a functional member of this family. Although the exact significance of galanin in the regulation of pancreatic enzyme secretion is currently unknown, the new powerful receptor antagonist M-15 will provide us with the tool to further elucidate the complex regulation of pancreatic enzyme secretion.³⁸

In summary, this study has shown that galanin is a powerful inhibitor of pancreatic enzyme secretion. Galanin elicits its inhibitory action indirectly, partly by inhibition of acetylcholine release from intrapancreatic neurons.

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