Role of GRPergic neurons in secretin-evoked exocrine secretion in isolated rat pancreas

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The importance of intrapancreatic noncholinergic excitatory neurons in pancreatic exocrine function has been proposed because atropine does not completely block pancreatic secretion induced by vagal stimulation (8, 19) or intrapancreatic neuronal excitation (17, 23, 31). In a recent study (22), we demonstrated that activation of intrapancreatic neurons by electrical field stimulation (EFS) results in a further increase in secretin-induced pancreatic exocrine secretion of the isolated rat pancreas. Because the EFS effect was not completely inhibited by atropine in the study, we also suspected that noncholinergic neurons in the pancreas might be involved in the intrapancreatic neuron-mediated stimulation of secretin-induced pancreatic exocrine secretion.

In addition to cholinergic neurons, the pancreas includes neurons containing peptides that stimulate pancreatic exocrine secretion (15, 18, 29). Among these neurons, stimulatory roles of vasoactive intestinal polypeptide (VIP)-containing neurons in pancreatic exocrine secretion have been well studied in the pig (7) and the rat (19) using electrical vagal stimulation and immunoneutralization of VIP. There are also a few reports suggesting that GRP-containing neurons in the pancreas may play a stimulatory role in pancreatic exocrine secretion. Neurons containing GRP have been recognized in the pancreas of several mammalian species (4, 11, 18). Exogenous GRP stimulates pancreatic enzyme secretion (1, 12, 30). Electrical vagal stimulation increases exocrine secretion as well as GRP output in the porcine pancreas (9, 12). However, it has been reported that a GRP antibody insufficiently reduces the pancreatic exocrine response to electrical vagal stimulation in the pig (9), although GRP antagonists successfully inhibit the response in the pig and the rat (9, 19). Thus the stimulatory role of intrapancreatic GRP-containing neurons in pancreatic exocrine secretion has not been clearly shown.

Therefore, the present study was undertaken to investigate whether GRP-containing neurons in the pancreas could participate in intrapancreatic neuron-mediated stimulation of secretin-induced pancreatic exocrine secretion. An isolated perfused rat pancreas model was used in this study to eliminate possible influences of extrinsic nerves and hormones on pancreatic exocrine secretion.

MATERIALS AND METHODS

Experimental animal preparation. Male Sprague-Dawley rats, weighing 250–300 g, were anesthetized with an intraperitoneal injection of 2% urethan (Sigma, St. Louis, MO) at a dose of 0.7 ml/100 g body wt after a 24-h fast with free access to water. The rats were killed by an intravenous overdose injection of urethan after isolation of the pancreas.

Preparation of totally isolated, vascularly perfused pancreas. The isolated perfused rat pancreas was prepared according to a method described previously (21). In brief, the abdominal aorta was carefully dissected and cannulated with PE-50 tubing (Clay Adams, Parsippany, NJ) just above the celiac artery and then tightly ligated just below the superior mesenteric artery. The pancreatic duct was cannulated at the
duodenal end with PE-10 tubing (Clay Adams) to collect pancreatic secretion. The portal vein was also cannulated with Tygon microbore tubing (Fisher Scientific, Pittsburgh, PA) to drain perfusate. The isolated pancreas was perfused with modified Krebs-Henseleit solution (pH 7.4, 305 mosmol/kg H2O) through the celiac axis and the superior mesenteric artery at a flow rate of 1.2 ml/min using a multistaltic pump (Buchler, Kansas, MO). The perfusate contained 0.1% bovine serum albumin (Sigma), 3% Dextran T-70 (Sigma), and 5.6 mM glucose (Sigma) and was continuously oxygenated with 95% O2-5% CO2. The pancreas was isolated with the duodenum but separated from other neighboring organs and tissues and then placed in a temperature-controlled experimental chamber at 37°C. The chamber was also continuously supplied with Krebs-Henseleit solution at a flow rate of 0.35 ml/min and oxygenated. After an equilibration period of 30 min, pancreatic juice was continuously collected in 15-min samples throughout the entire period of the experiment.

Effects of EFS on secretin-induced pancreatic exocrine secretion. Synthetic porcine secretin (Peninsula, Belmont, CA) at a concentration of 12 pM was intra-arterially infused into the isolated pancreas for 60 min. To excite intrapancreatic neurons, EFS was carried out using biphasic square waves (15 V, 2 ms, and 8 Hz) as described previously (22, 23). EFS was applied to the isolated pancreas for 45 min from 15 min after secretin infusion began until the end of the experiment. A rabbit anti-GRP serum (10 µl/ml; titer of 1:66,000) with or without atropine (2 µM; Sigma) was added in the secretin-containing perfusion solution from 60 min before EFS until the end of the experiment.

Effects of exogenous GRP on secretin-induced pancreatic exocrine secretion. Synthetic porcine GRP (1-27) (Sigma) was added in a perfusion solution at a concentration of 30, 100, or 300 pM from 15 min after the secretin infusion until the end of the experiment. Atropine (2 µM) was infused into the isolated pancreas from 60 min before infusion of GRP (100 pM) until the end of the experiment.

Production of anti-GRP antiserum and radiimmunoassay of GRP. An anti-GRP antiserum was produced in a New Zealand White rabbit by multiple injections of an antigen prepared by conjugation of synthetic porcine GRP to bovine serum albumin (Buchler, Kansas, MO). The antiserum had a titer of 1:66,000. The effective affinity constant and the total binding sites of the antiserum, determined using the Scatchard equation (26), were 1.07 × 1011 and 0.71 × 1011 M⁻¹, respectively. The heterogeneity index and the average affinity constant, obtained by using Karush’s linear regression analysis and Split’s equation (27), were 0.76 and 0.62 µM, respectively. The antiserum showed a full cross-reactivity with bombesin (Sigma) but not with other gut peptides including CCK. A tracer was prepared by lactoperoxidase (Calbiochem, La Jolla, CA)-catalyzed iodination of synthetic Tyr⁴-bombesin (Sigma) with 125I (Amersham, Amersham, UK), which had a specific radioactivity of 2,644 µCi/nmol as determined by the self-displacement method (28). Radiimmunoassay of GRP was carried out using synthetic bombesin (Sigma) as a standard according to a method described previously (14).

Determination of GRP concentration in portal effluent. Fifteen-minute samples of portal effluent were collected in ice-chilled test tubes during the whole period of the experiment. Tetrodotoxin (1 µM; Sigma) or atropine (2 µM) was added in a perfusion solution from 60 min before EFS began until the end of the experiment. Concentrations of GRP in portal effluents were determined by radiimmunoassay after extraction of GRP with 98% acetone containing 0.35 N acetic acid.

Measurements of pancreatic secretions of fluid and amylase. The flow volume of pancreatic juice was determined by measuring the length of microtube filled by pancreatic juice in 15 min, which had a capacity of 3.8 µl/cm. The activity of α-amylase in pancreatic juice was measured according to a method reported previously (25).

Statistical analysis of data. All results are illustrated as means ± SE. The data were analyzed using Student’s t-test. The difference was considered significant when the P value was <0.05.

RESULTS

Effects of anti-GRP antiserum and atropine on action of EFS in secretin-induced pancreatic exocrine secretion. As shown in Figs. 1 and 2, EFS significantly increased (P < 0.001) secretin-induced pancreatic secretions of fluid and amylase from 12.93 ± 0.69 and 45.10 ± 4.24 U/45 min to 20.14 ± 0.87 and 231.42 ± 16.22 U/45 min, respectively. The anti-GRP antiserum did not significantly reduce the fluid secretory response (12% inhibition), whereas it did significantly suppress (P < 0.001) the amylase secretory response (43% inhibition). When the antiserum and atropine (2 µM) were simultaneously administered, the effects of EFS on the secretin-induced secretions of fluid and amylase were significantly lower (P < 0.001) than the corresponding values obtained without the antiserum and atropine and quite similar to those induced by secretin alone. Thus EFS-enhanced secretin-induced pancreatic exocrine secretion was completely inhibited by simultaneous infusion of the anti-GRP antiserum and atropine. The antiserum did not affect pancreatic exocrine secretion in the basal state.

Fig. 1. Effects of an anti-gastrin-releasing peptide (GRP) antiserum (GRP Ab) and atropine (ATR) on action of electrical field stimulation (EFS) in secretin (S)-induced secretion of fluid from isolated perfused rat pancreas. A: time courses of fluid secretion. B: cumulative fluid secretion over period (45 min) of EFS. Antiserum (10 µl/ml; titer of 1:66,000) and atropine (2 µM) were added in perfusate from beginning to end of experiment. Control, combined action of EFS and secretin. Each value represents mean ± SE of data from 7 (control) or 6 (others) pancreata. Fluid secretion evoked by EFS and secretin is not modified (ND) by antiserum alone, but it is significantly reduced (P < 0.001) to level of secretin alone by simultaneous infusion of antiserum and atropine.
Effects of EFS on GRP release from isolated perfused rat pancreas. The mean concentration of immunoreactive GRP in portal effluent significantly increased (P < 0.01) from a basal level of 1.08 ± 0.08 pM to a peak level of 2.36 ± 0.29 pM during the first 15-min period of EFS, and then it declined gradually (Fig. 3). Tetrodotoxin completely inhibited the EFS-stimulated pancreatic release of immunoreactive GRP, but atropine did not.

Effects of EFS on GRP release from isolated perfused rat pancreas. The mean concentration of immunoreactive GRP in portal effluent significantly increased (P < 0.01) from a basal level of 1.08 ± 0.08 pM to a peak level of 2.36 ± 0.29 pM during the first 15-min period of EFS, and then it declined gradually (Fig. 3). Tetrodotoxin completely inhibited the EFS-stimulated pancreatic release of immunoreactive GRP, but atropine did not.

Effects of exogenous GRP on secretin-induced pancreatic exocrine secretion. As shown in Fig. 4, synthetic porcine GRP given at 30, 100, or 300 pM dose-dependently increased spontaneous pancreatic secretions of fluid and amylase, with correlation coefficients of 0.8353 and 0.7282, respectively. GRP further elevated secretin (12 pM)-induced pancreatic secretions of fluid and amylase dose-dependently, with correlation coefficients of 0.7922 and 0.7514, respectively. The fluid secretion stimulated by a combination of GRP and secretin was not different from the sum of individual values produced by GRP alone and secretin alone. However, the amylase secretion stimulated by a combination of GRP and secretin was not different from the sum of individual values produced by GRP alone and secretin alone. However, the amylase secretion stimulated by a combination of GRP and secretin was greater than the sum of that evoked by individual peptides, and the differences increased dose-dependently. Atropine (2 µM) did not change the GRP (100 pM) effect on secretin-induced pancreatic exocrine secretion, as presented in Table 1.

Table 1. Effects of atropine (2 µM) on combined actions of GRP (100 pM) and secretin (12 µM) in secretions of fluid and amylase from isolated perfused rat pancreas

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>GRP</th>
<th>−Atr</th>
<th>+Atr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid, µl/45 min</td>
<td>12.32 ± 0.69</td>
<td>10.86 ± 0.83</td>
<td>18.67 ± 1.78</td>
<td>16.33 ± 1.21</td>
</tr>
<tr>
<td>Amylase, U/45 min</td>
<td>45.10 ± 4.24</td>
<td>105.40 ± 7.36</td>
<td>217.76 ± 17.67</td>
<td>201.42 ± 15.06</td>
</tr>
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Values are means ± SE from 6 pancreata. Atropine (Atr) was dissolved in a perfusion solution from 60 min before start of gastrin-releasing peptide (GRP) infusion until end of the experiment. S, secretin.
DISCUSSION

In the present study, EFS further increased secretin-induced secretions of fluid and amylase in the isolated perfused rat pancreas, confirming our previous report (22). Immunoneutralization of endogenous GRP by a specific anti-GRP antiserum markedly inhibited the EFS effect on secretin-induced amylase secretion (43% inhibition) but slightly reduced the EFS effect on secretin-induced fluid secretion (12% inhibition). The result is in good agreement with previous observations that a GRP antagonist and desensitization of the pancreas to GRP successfully inhibit pancreatic protein secretion evoked by electrical vagal stimulation in the isolated perfused porcine pancreas (9). In that study (9), however, either Fab fragment molecules or intact molecules of GRP antibody insufficiently reduced the vagally stimulated pancreatic protein secretion. Species differences may be a possible explanation for this discrepancy, but it remains to be explained because neither the characteristics of the two anti-GRP antibodies nor their actions have been compared under the same experimental conditions. Inhibition by a specific bombesin-receptor antagonist of vagally stimulated pancreatic amylase secretion has been also observed in the anesthetized rat (19).

EFS increased the mean concentration of GRP immunoreactivity from the basal level of 1.08 pM to the peak level of 2.36 pM in portal venous effluent of the isolated pancreas. The increase in portal concentration of immunoreactive GRP was completely inhibited by tetrodotoxin. These observations indicate that EFS activates GRPergic neurons in the pancreas to release GRP. It has been shown that ganglia in the rat pancreas contain neurons strongly reacting to an anti-GRP antiserum and that these neurons’ nerve terminals are in contact with pancreatic acini (4, 11). Cholinergic neurons do not appear to be involved in the release of GRP because atropine does not modify the EFS-evoked pancreatic release of GRP in this study. The atropine-resistant release of GRP has been already shown by electrical stimulation of the vagus in the isolated perfused porcine pancreas (12). A totally isolated perfused pancreas model was used in this study, in which the pancreatic circulation was completely separated from the systemic circulation. Thus the results of the present study, a release of GRP by EFS as well as a blocking of the EFS effect by the GRP antibody, provide direct evidence that endogenous GRP released by EFS from intrapancreatic neurons is a neuromodulator that stimulates pancreatic amylase secretion in rats.

In our previous report (22), atropine inhibited the EFS effects on secretin-induced secretions of fluid and amylase by 28 and 72%, respectively, in the isolated rat pancreas whereas tetrodotoxin completely abolished the EFS effect. The incomplete inhibition by atropine of the effect of intrapancreatic neuronal excitation on spontaneous or secretin-induced pancreatic exocrine secretion has been also demonstrated in the rat (17, 19, 23, 31). In the present study, anti-GRP antiserum inhibited EFS-enhanced secretin-induced pancreatic secretions of fluid and amylase by 12 and 43%, respectively. However, simultaneous infusion of the anti-GRP antiserum and atropine completely reduced the EFS-enhanced secretin-induced secretions of fluid and amylase to the levels induced by secretin alone. Thus EFS did not further elevate secretin-induced pancreatic exocrine secretion when the actions of both intrapancreatic GRPergic and cholinergic neurons were simultaneously blocked. The results strongly indicate that the remaining portion of the EFS effect on secretin-induced pancreatic exocrine secretion, which is not inhibited by atropine, is mainly mediated by intrapancreatic GRPergic neurons in the rat. Besides the GRPergic neuron, the VIPergic neuron (7, 19) and the CCKergic neuron (19) in the pancreas have been suggested to be involved in vagally stimulated pancreatic exocrine secretion.

The roles of the peptidergic neurons in secretin-induced pancreatic exocrine secretion remain to be elucidated in future studies. Extrinsic nerves to the pancreas do not appear to participate in the action of EFS because hexamethonium, a well-known ganglionic block, did not influence the pancreatic response to EFS (22) although it completely blocked vagally stimulated pancreatic secretion (19).

Synthetic porcine GRP, given intra-arterially to the isolated pancreas at concentrations of 30, 100, or 300 pM, dose-dependently increased secretin-induced secretions of both fluid and amylase. Secretin-induced pancreatic secretion was effectively elevated by exogenous GRP when its concentration was >100 pM. Because the peak concentration of immunoreactive GRP in portal effluent during application of EFS was 2.36 pM, it is suggested that the effective concentration of GRP at the action site is 50 times higher than the GRP concentration appears in the circulation. At 100 and 300 pM GRP, fluid secretion was the same as the sum of fluid secretion induced by GRP alone and secretin alone, whereas amylase secretion was greater than the sum of amylase secretion evoked by individual peptides. Thus an additive effect on fluid secretion and a potentiation effect on amylase secretion were observed. These observations, together with the findings that anti-GRP antiserum predominantly inhibits the effect of EFS on secretin-induced amylase secretion, support previous suggestions that GRP is a potent stimulator of enzyme secretion rather than of fluid secretion in the rat pancreas (1, 20). Although it is not understood at the present time how GRP potentiates secretin-induced amylase secretion, we assume that the potentiation may occur because of interactions of intracellular messengers of GRP and secretin because GRP increases intracellular levels of Ca2+ and secretin increases intracellular levels of cAMP (32). It has been reported that the protein secretory response is greater than the additive response to individual secretagogues when a Ca2+-mediated secretagogue is combined with a cAMP-mediated secretagogue (6).

A possible cholinergic role in the pancreatic response to GRP has been suggested because atropine inhibits pancreatic exocrine secretion that is stimulated by bombesin/GRP in the dog (13) and in the pig (12). To
confirm the cholinergic role in the GRP action, we inhibited cholinergic activity in the isolated rat pancreas by atropine in this study. Unexpectedly, atropine did not reduce the effect of GRP on secretin-induced pancreatic exocrine secretion in this study. Thus GRP appears to act independently on intrapancreatic cholinergic tone in the rat pancreas. The observation that atropine is unable to modify bombesin-induced amylase secretion from pancreatic fragments of the rat (5) is also in accordance with our results. One possible explanation for this discrepancy may be species differences, but it remains to be elucidated because of controversial reports. It has been reported that atropine has no effect on bombesin/GRP-induced hyperpolarization of pancreatic acinar cells (24). Furthermore, bombesin/GRP may stimulate pancreatic exocrine secretion by extrapancreatic release of CCK (3, 10), the action of which in the exocrine pancreas is reduced by atropine (16).

In summary, an anti-GRP antiserum effectively reduced the effect of intrapancreatic neuronal excitation on secretin-induced pancreatic amylase secretion only, but simultaneous infusion of the antiserum and atropine completely abolished the neuronal excitation effect on secretions of both fluid and amylase. Excitation of pancreatic neurons resulted in a significant increase in pancreatic release of GRP that was atropine resistant. Exogenous GRP predominantly augmented secretin-induced amylase secretion in a cholinergic-independent manner. Therefore, we conclude that GRPergic neurons in the pancreas play an important role in the neuronal-mediated enhancement of secretin-induced exocrine secretion in the rat.

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