Hypoglycemia Overrides Amylin-Mediated Regulation of Gastric Emptying in Rats

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Amylin, a 37–amino acid peptide hormone co-secreted with insulin, potently governs the rate of gastric emptying. Hypoglycemia, in the absence of agents such as amylin, is reported to accelerate gastric emptying. We asked whether hypoglycemia had a similar action on gastric emptying in the presence of amylin. In preliminary experiments using a phenol red gavage technique in fasted SD rats, we showed that insulin administration accelerated gastric emptying in a dosage-dependent manner. This acceleration was totally prevented by coadministration of glucose in dosages that prevented a change in plasma glucose, indicating that insulin per se did not affect gastric emptying. The effect on gastric emptying of hypoglycemia induced by a 5 mU/min insulin infusion (t = 5–90 min) was assessed in conscious rats continuously infused with amylin (50 pmol · kg^{-1} · min^{-1}; t = –30 to 90 min). Gastric emptying was indicated by the appearance in plasma of label from 3-*O*-methyl-[^{3}H]glucose gavaged at t = 0 min. Label appearance was markedly inhibited in rats preinfused with amylin (84% reduced vs. saline controls at t = 30 min), indicating amylin inhibition of gastric emptying. In amylin-treated rats that were subsequently infused with insulin, gavaged label abruptly appeared in plasma when plasma glucose had fallen to 2.1 ± 0.1 mmol/l (at $t \cong 45$ min), consistent with a reversal by hypoglycemia of amylin's inhibition of gastric emptying. These data support the idea of a central "fail-safe" mechanism whereby hypoglycemia can override the slowing of gastric emptying by amylin. *Diabetes* 47:93–97, 1998

Despite the large variations in the carbohydrate density of test meals, the rate at which glucose is released from the stomach in human adults appears to be regulated close to 2 kcal/min (1). This rate is approximately equal to the maximal rate of glucose disposal elicited by physiological postprandial insulin concentrations in insulin-sensitive humans (2). Nutrient release from the stomach thus appears to be tightly regulated, and is likely to be an important element in the regulation of plasma concentrations of glucose and other nutrients.

Amylin, a peptide hormone cosecreted with insulin from pancreatic β-cells in response to nutrient stimuli (3), is likely to contribute to the physiological regulation of nutrient release from the stomach. Amylin potently slows gastric emptying (4); blockade of endogenous amylin with a selective amylin antagonist accelerates spontaneous gastric emptying in rats (5), supporting the interpretation that endogenously secreted amylin tonically restrains gastric emptying. The absence of the gastric actions of endogenous amylin could account for the acceleration of gastric emptying reported in amylin-deficient IDDM patients (6–8) and amylin-deficient diabetic BB rats (4,9), an animal model of type 1 diabetes. Of the several hormones proposed to participate in nutrient-mediated feedback control of gastric emptying (i.e., cholecystokinin, glucagon, glucagon-like peptide 1, gastric inhibitory peptide, pancreatic polypeptide, and amylin), amylin was the most potent inhibitor of gastric emptying (10).

Hypoglycemia is reported to accelerate gastric emptying. We investigated whether hypoglycemia also had this effect when gastric emptying was slowed by amylin. This question has potential clinical importance for insulin-treated diabetes, since several agents that slow nutrient absorption, including pramlintide (a human amylin analog), glucagon-like peptide 1, cholecystokinin, and α-glucosidase inhibitors, have been proposed as therapies. Insulin treatment carries a risk of hypoglycemia, for which oral carbohydrate is the standard treatment. Treatments that delay nutrient absorption, including those that work by delaying gastric emptying, could present a hazard in hypoglycemia, since they would interfere with efforts to restore plasma glucose by oral supplementation.

In the present study, we examined the effect of insulin-induced hypoglycemia on gastric emptying, and the effect of hypoglycemia on amylin-mediated inhibition of gastric emptying in conscious rats. We found that there was accelerated gastric emptying during insulin-induced hypoglycemia, but not during hyperinsulinemic normoglycemia. We further found that the action of amylin that delays gastric emptying was reversed by hypoglycemia. Some of the findings in this study have been previously reported (11).

RESEARCH DESIGN AND METHODS

Animals. Male HSD rats were housed at 22.7 ± 0.8°C in a 12:12 h light:dark cycle and fed and watered ad libitum (Diet LM-485; Teklad, Madison, WI). Rats were fasted for 20–22 h before experiments.

Gastric emptying measurement. Gastric emptying was assessed using two different methodologies. The phenol red dye gavage technique, described in detail below, was used to study insulin and glucose dependence of gastric emptying 20 min after gavage in rats with and without prior amylin administration. Although this method could capture dosage-related effects on gastric emptying, it could not easily capture time-related effects. It was therefore necessary to use a different method to monitor gastric emptying dynamically. To assess the effect of progressive hypoglycemia against the background of a constant amylin effect, we frequently measured the appearance in plasma of gavaged 3-*O*-methylglucose (3-*O*-MG), as detailed below.

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3-*O*-MG, 3-*O*-methylglucose.

Measurement of gastric emptying using dye recovery (phenol red gavage). This method, a modification of a dye-recovery method originally described by Scarpignato et al. (12), has previously been described in detail (4). Briefly, conscious rats were gavaged with 1.5 ml of an acaloric gel containing 1.5% methyl cellulose (M-0262; Sigma, St. Louis, MO) and 0.05% phenol red indicator. Twenty minutes after gavage, rats were anesthetized using 5% halothane; then their stomach was exposed and clamped at the pyloric and lower esophageal sphincters using artery forceps, removed, and opened into an alkaline solution, which was made up to a fixed volume. Stomach dye content was derived spectrophotometrically from this solution by absorbance at λ = 560 nm. To compensate for the loss of ~11% of the dye, which appeared to bind irrecoverably to the gut luminal surface, stomach contents remaining 20 min after gavage were expressed as a percentage of contents recoverable from control rats killed immediately after gavage in the same study. Thus percentage gastric contents remaining = (absorbance at 20 min)/(absorbance at 0 min) × 100.

Effect of insulin on gastric emptying. In this set of studies, gastric emptying was assessed by the phenol red method in 51 rats. Animals were injected subcutaneously with saline or recombinant human insulin (Humulin-R; Lilly, Indianapolis, IN), but not glucose, at insulin dosages of 0 (saline alone), 0.1, 1, 10, or 100 µg (n = 16, 9, 11, 8, and 7, respectively) immediately before gavage. Data obtained from these studies contributed to a raw insulin dosage-response analysis, which looked at the effects of insulin on gastric emptying, regardless of its effects on plasma glucose.

Effect of insulin on gastric emptying in euglycemic rats. In measurements of gastric emptying in 46 rats, subcutaneous insulin injections were accompanied by subcutaneous injections of 50% D-glucose to counteract the insulin-induced hypoglycemia and thus determine any effect of insulin that was unrelated to its hypoglycemic action. The 10-µg dosages of insulin were accompanied by 200-, 300-, and 400-mg s.c. injections of glucose; 100-µg dosages of insulin were accompanied by the same glucose dosages plus a 500-µg dosage. Blood was collected while rats were being killed 20 min after gavage, and plasma glucose was immediately assayed by immobilized enzyme chemistry (glucose-oxidase, 2300 Stat; YSI, Yellow Springs, OH). Only data from animals with a plasma glucose concentration ≥ 4.5 mmol/l as measured 20 min after injection/gavage were included in this euglycemic insulin dosage-response analysis.

Effect of pretreatment with amylin on glucose concentration-response for gastric emptying. A wide range of plasma glucose concentrations was produced in rats injected with either saline (n = 97) or 1 µg rat amylin (n = 53) immediately before phenol red gavage. Different glycemic levels, ranging from normoglycemia to profound hypoglycemia, were induced with various subcutaneous or intravenous dosages of recombinant human insulin up to 100 µg. Gastric dye retention at 20 min versus plasma glucose measured at the same time point were plotted for rats pretreated with amylin or saline. To simplify the plot, gastric-emptying data were sorted into quantiles of n = 5 by plasma glucose concentration only, irrespective of the insulin/glucose combination producing the resulting plasma glucose concentration.

Measurement of onset of gastric emptying using 3-*O*-MG gavage. The appearance of gavaged-labeled glucose in plasma to indicate amylin-mediated inhibition of gastric emptying in rats has been previously described (4). The method is based on the observation that negligible amounts of label appear in plasma when gastric emptying is prevented by pyloric ligation (13), but that tracer appears rapidly in plasma after it reaches absorptive sections of the gut.

Measurement of gastric emptying using 3-*O*-MG during amylin infusion and insulin-induced hypoglycemia. Male HSD rats (340–370 g) were cannulated via the left common carotid artery and external jugular vein 4–5 days before the study. Venous cannulae were used for infusions, and the arterial cannulae were used for sampling of plasma glucose concentration and tritium activity. Rats were infused with either rat amylin or saline vehicle alone, 30 min before gavage (t = –30 min). The amylin infusion of 50 pmol · kg^{-1} · min^{-1} was continued until 90 min after gavage. This infusion rate of amylin in this preparation has been shown to result in plasma amylin concentrations of ~1 nmol/l (14). At t = 0, all rats were gavaged with 1 ml water containing 5 µCi 3-*O*-MG; unlabeled glucose was not included in the gavage. Plasma samples for glucose and tritium were taken at 5-min intervals after gavage. In some amylin-infused rats, an infusion of 5 mU/min of recombinant human insulin (Humulin-R; Lilly) began 5 min after gavage. Thus there were three treatment groups: *1*) saline (infusion beginning at t = –30 min) + saline (infusion beginning at t = 5 min), n = 6; *2*) amylin (infusion beginning at t = –30 min) + saline (infusion beginning at t = 5 min), n = 6; and *3*) amylin (infusion beginning at t = –30 min) + insulin (infusion beginning at t = 5 min), n = 5. Plasma glucose was measured by a glucose oxidase analyzer, as described above. Plasma tritium activity in 10-µl plasma samples diluted with 1 ml water and 4 ml scintillation cocktail (Ecolite; ICN, Costa Mesa, CA) was measured in a β-counter (1209 Rack-beta; LKB-Wallac, Gaithersburg, MD).

Statistical analysis. Pair-wise statistical analyses were performed using Student's t test, and general effects were tested using one-way analysis of variance (Instat v2.0; GraphPad Software, San Diego, CA) with $P < 0.05$ as the level of significance. Dosage-response curves for gastric emptying were fitted to a four-parameter logistic model using a least-squares iterative routine (Prizm v3.0; GraphPad Software). Comparison of rates of gastric emptying events during progressive hypoglycemia used the Mantel-Haenszel (log-rank) test typically used for survival curve analysis (Prizm v3.0; GraphPad Software).

RESULTS

Effect of insulin on gastric emptying. When insulin was injected subcutaneously prior to gavage at dosages sufficient to cause hypoglycemia, there was a marked acceleration of gastric emptying (Fig. 1). The 10- and 100-µg dosages of insulin resulted in plasma glucose concentrations of 2.17 ± 0.1 and 1.83 ± 0.11, respectively, vs. 6.17 ± 0.51 mmol/l in saline-treated controls (both $P < 0.001$). Gastric contents 20 min after gavage were more than fourfold reduced, to 8.6 ± 2.8 and 8.6 ± 3.0, respectively, vs. 36.8 ± 4.9% in saline-treated controls ($P < 0.01$ and $P < 0.001$, respectively).

Effect of insulin on gastric emptying when hypoglycemia was prevented by glucose injections. Both the 400-mg of glucose administered to rats receiving 10 µg insulin and the 500-mg dosage of glucose administered to rats receiving 100 µg insulin prevented insulin-induced hypoglycemia (20-min plasma glucose 5.64 ± 0.23 and 5.91 ± 0.26, respectively, vs. 6.17 ± 0.51 mmol/l in saline-treated controls). When hypoglycemia was thus prevented, the insulin injection did not accelerate gastric emptying (Fig. 1, ▲).

Gastric emptying as a function of plasma glucose 20 min after gavage. Data from rats pretreated with either amylin or saline and in which both plasma glucose and gastric emptying were measured are shown as glucose concentration-responses in Fig. 2. Compared with saline-pretreated controls, rats injected with 1 µg amylin had markedly delayed gastric emptying (i.e., essentially total retention of gastric contents) if plasma glucose was greater than ~5 mmol/l, as previously reported (4). In both amylin- and saline-pretreated animals, gastric emptying was accelerated if plasma glucose was lowered by insulin treatment. Concentration-response analysis of the plasma glucose at which gastric retention was 50% of that observed during euglycemia returned EC_{50} of 3.5 ± 0.1 mmol/l in amylin-pretreated rats and 4.1 ± 0.4 mmol/l in saline-pretreated controls (P = 0.2; t test with Welch's correction); that is, the effect of hypoglycemia in accelerating gastric emptying in rats with amylin-mediated inhibition of gastric emptying was similar to the effect in controls. With a plasma glucose concentration of 1.75 mmol/l, there was very little retention of gastric contents (3%).

Effect of continuous amylin infusion on gastric emptying. Plasma glucose concentrations in rats infused with either amylin or saline are shown in Fig. 3*A*. In both groups of rats, there was a small increase in plasma glucose following gavage, consistent with the stress of handling and of this procedure. Thereafter, rats infused with amylin (50 pmol · kg^{-1} · min^{-1}) maintained a plasma glucose of ~7.8 mmol/l, slightly above that of rats infused with saline, and consistent with the hyperglycemic effect of this infusion rate of rat amylin (15) (plasma amylin concentration ~1 nmol/l). Plasma tritium activity from gavaged 3-*O*-MG is shown in Fig. 3*B*. In animals preinfused with saline, an increase in plasma 3-*O*-MG–derived tritium was apparent 5 min after gavage, consistent with the emptying of the stomach that appeared to be complete by 30 min after gavage. In rats preinfused with

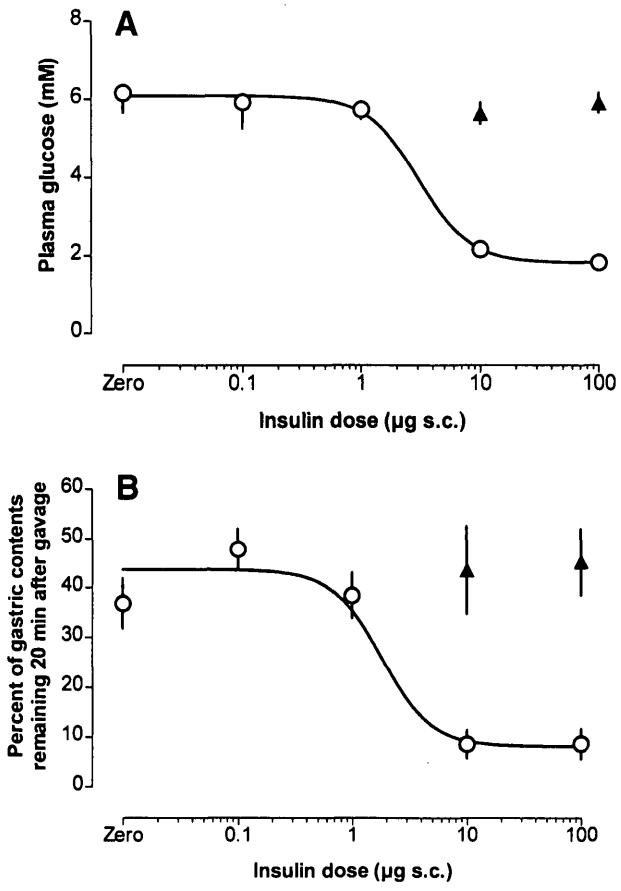

FIG. 1. Insulin dosage-response for plasma glucose (*A*) and rate of gastric emptying (*B*) assessed by measuring gastric contents remaining 20 min after gavage of an acaloric gel containing phenol red dye. Experiments were performed in fasted conscious HSD rats. ○, experiments where insulin only was injected; ▲, experiments where 400 and 500 mg glucose were co-injected with 10 and 100 μg insulin, respectively, to prevent hypoglycemia. Data are means ± SE; *n* = 5–16 rats for each data point.

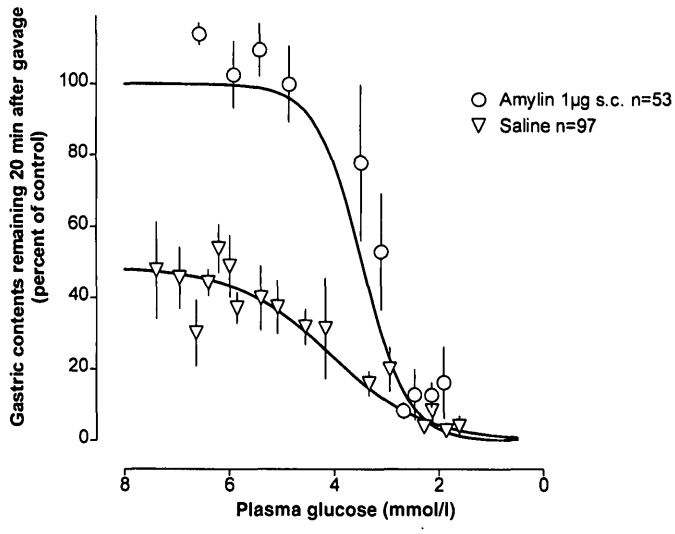

FIG. 2. Plasma glucose concentration responses for gastric retention of phenol red dye 20 min after gavage in amylin- and saline-pretreated rats. Data are means ± SE for gastric emptying after grouping all available gastric emptying:plasma glucose data pairs into *n* = 5 quantiles. The glucose EC_{50} for hypoglycemic acceleration of gastric emptying in amylin-treated rats (○) was 3.5 ± 0.1 mmol/l and was not different from that in saline-treated controls (▽); 4.1 ± 0.4 mmol/l. Plasma glucose is plotted on a reverse scale to indicate a progression into hypoglycemia.

amylin, plasma 3-*O*-MG–derived activity remained low (~16% of activity in control rats at *t* = 30 min). This observation is consistent with the amylin infusion having substantially delayed gastric emptying.

Effect of insulin-induced hypoglycemia on gastric action of amylin. Within 30 min after starting a 5 U/min insulin infusion in amylin-infused rats, the plasma glucose concentration declined to ~2.7 mmol/l (Fig. 3*C*). A comparable decline was observed with insulin in rats preinfused with saline instead of amylin (data not shown). As can be seen in Fig. 3*D*, both groups of rats that were infused with amylin showed initially low plasma 3-*O*-MG levels, consistent with a continuous inhibition of gastric emptying. In rats subsequently infused with a hypoglycemic dosage of insulin, there was an abrupt increase in plasma tritium 45 min after gavage (when plasma glucose had fallen to ~2.1 mmol/l), consistent with the onset of gastric emptying and absorption of labeled 3-*O*-MG across the small intestine (Fig. 3*D*). Thus insulin-induced hypoglycemia at a certain plasma glucose concentration appeared to reverse amylin-mediated regulation of gastric emptying. The onset of gastric emptying, defined in individual rats as a doubling of basal plasma tritium, was an earlier and more frequent event in hypoglycemic than in normoglycemic amylin-infused rats ($P < 0.001$; Mantel-Haenszel log-rank test).

DISCUSSION

In the present study, we showed that insulin-induced hypoglycemia causes an accelerated emptying of a gavaged acaloric gel from the stomach of conscious rats. These findings support those in human studies in which hypoglycemia was reported to accelerate gastric emptying (16,17). In our studies in rats, the acceleration of gastric emptying after insulin administration was prevented by coadministration of glucose. The acceleration of gastric emptying thus appeared to be a result of the hypoglycemia and not a direct action of insulin. We also found that insulin-induced hypoglycemia reversed the inhibition of gastric emptying induced by infusion of a pharmacological amount of amylin.

The findings in this study indicate the existence of a hypoglycemia-mediated mechanism that can override the physiological effect of amylin in limiting nutrient delivery from the stomach. We also observed, in the same rat preparation, hypoglycemic override of the gastric inhibitory effects of pramlintide (18), a synthetic human amylin analog currently being evaluated in therapy of insulin-treated diabetic patients to help control postprandial hyperglycemia. A similar gastric acceleration with hypoglycemia also occurs in rats in which emptying has been slowed by administration of glucagon-like peptide 1 or cholecystokinin octapeptide (data not shown). A normal or elevated plasma glucose thus appears to be a necessary precondition for peptide-mediated inhibition of gastric emptying to occur.

Because hypoglycemia represents a major threat to the well-being of the organism, such a fail-safe function is teleologically attractive. The acceleration of gastric emptying during hypoglycemia could be viewed as a reflex that facilitates recovery from hypoglycemia by promoting rapid absorption of

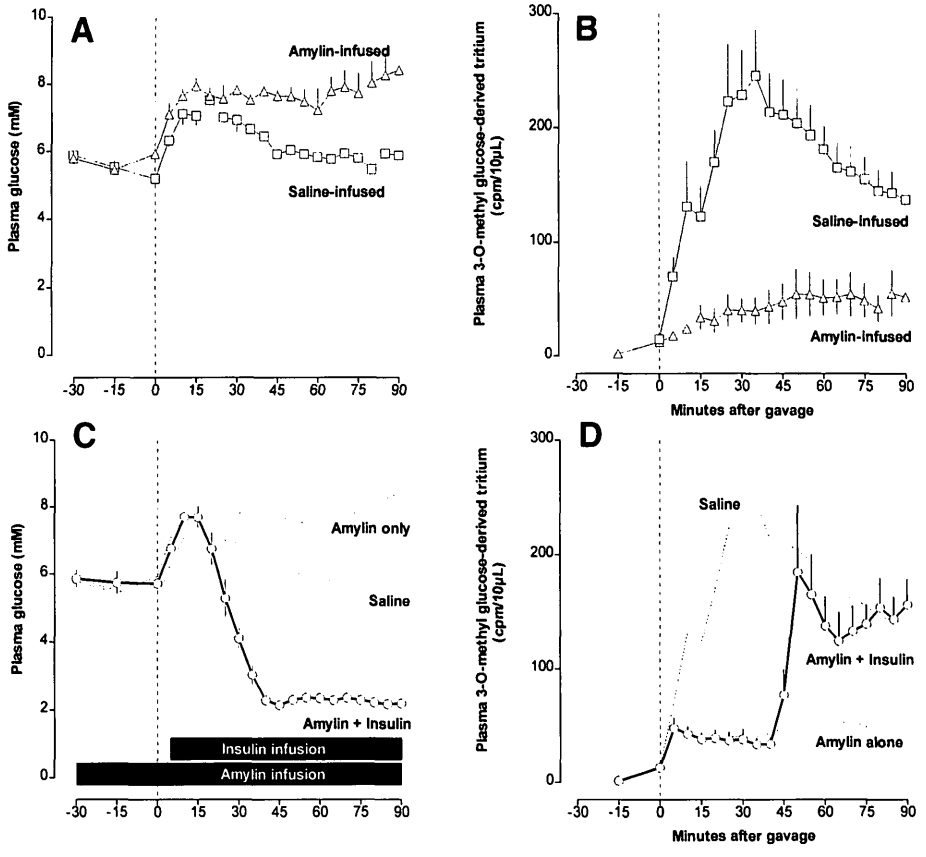

FIG. 3. Plasma glucose (*A*,*C*) and tritium derived from gavaged 3-*O*-MG (*B*,*D*) in conscious fasted rats. *A*: plasma glucose in rats infused with either amylin at 50 pmol · kg^{-1} · min^{-1} (△, *n* = 6) or saline (□, *n* = 6) from *t* = –30 until *t* = 90 min. Rats were gavaged with tritiated 3-*O*-MG at *t* = 0. *B*: plasma tritium derived from labeled 3-*O*-MG. Plasma glucose (*C*) and tritium (*D*) in rats first infused with amylin and then infused with insulin at 5 mU/min (○, *n* = 5) from *t* = 5 until *t* = 90 min. Responses from *A* and *B* are plotted for reference. Data are means ± SE. Periods of infusion for amylin and insulin, where applicable, are indicated by bars in *C*.

ingested carbohydrates. This reflex is yet to be tested in humans, but, if present, could allow us to predict a reduced risk of hypoglycemia in insulin-treated diabetic patients using amylin replacement therapy. A slower rate of gastric emptying, as is reported in patients administered pramlintide (19), could increase by several-fold the fraction of time that a carbohydrate-containing meal is present in the stomach. Through accelerated gastric emptying during impending hypoglycemia, this reserve is now available as part of a counterregulatory defense.

Such a mechanism may help to explain why, despite lowering of glycosylated hemoglobin (20) and other indexes of plasma glucose concentration in diabetic subjects (21), there has been no detectable increase in incidence of hypoglycemia in patients administered pramlintide (20,22). An increase in frequency of hypoglycemic episodes might have been predicted, based on Diabetes Control and Complications Trial data, in which lowering of HbA_{1c} with intensified insulin treatment resulted in two- to sixfold increases in hypoglycemia (23).

What mechanisms could mediate the hypoglycemic override of amylin regulation of gastric emptying? There is some evidence to support the possibility that amylin modulates gastric emptying via central nervous pathways. Control of gastric motility involves pathways interconnecting the nucleus tractus solitarius and the area postrema (24), a circumventricular organ with a leaky blood-brain barrier (allowing neuronal access to circulating amylin) and a high density of amylin receptors (25). Lesioning of areas such as the area postrema may more definitively identify the central site(s) mediating amylin's gastric actions. Glucose-sensitive neurons, including those that are activated by decreases in glucose concentration, have recently been described in both the area postrema and the nucleus tractus solitarius (26). It is possible that these glucose-sensitive neurons, proposed as part of a glucoprotective fail-safe loop (26), may communicate with, or be identical to, those that mediate amylin's gastric actions.

Vagotomy eliminates the ability of amylin to modulate gastric emptying (27), further indicating that the response to amylin is not intrinsic to the stomach, but depends, at least partly, on neural connections. A recent report that hypoglycemic acceleration of gastric emptying is blocked by atropine also implicates the vagus nerve (and hence the central nervous system) in this action (17). Some neurons within the dorsal motor nucleus of the vagus nerve, including those that project axons to the stomach, have also been identified as being glucose responsive (26). Studies are planned to investigate how the gastric emptying response to pramlintide or hypoglycemia is affected in patients with impaired vagal function.

In summary, it appears that amylin and glucose concentrations each influence interacting central structures that control gastric emptying. The plasma glucose concentration has a permissive effect on amylin inhibition of gastric emptying; the plasma glucose concentration at which hypoglycemia accelerated gastric emptying was similar in the presence and absence of exogenous amylin. The present study identified a mechanism that may be an important physiological defense against hypoglycemia and may be relevant in patients being treated with agents that reduce postprandial plasma glucose excursions (e.g., pramlintide) in part by regulating gastric emptying.

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