Dose-Response for Glucagonostatic Effect of Amylin in Rats

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Glucagon secretion from pancreatic α cells is inhibited by insulin from β cells. Amylin is a partner hormone to insulin cosecreted in response to nutrient stimuli, which, like insulin, inhibits β-cell secretion. We investigated whether amylin also inhibits α-cell secretion of glucagon in response to infused L-arginine. Rat amylin (1.2, 3.6, 12, 36, or 120 pmol/kg/min; calculated plasma concentration, 13, 47, 195, 713, and 2,950 pmol/L, respectively; n = 7, 8, 6, 4, and 7) or saline (n = 23) was infused into anesthetized male Harlan-Sprague-Dawley rats during hyperinsulinemic-euglycemic clamps, which were used to equalize the influences of glucose and insulin on glucagon secretion. Plasma glucose and insulin concentrations and mean arterial pressures were not different between amylin- and saline-treated rats during a 10-minute 2-mmol L-arginine infusion delivered during the clamps. Plasma glucagon measurements taken during and after the arginine challenge showed that compared with saline infusions, amylin administration dose-dependently suppressed the glucagon response to arginine by a maximum of 62% (incremental area under the curve [AUC] 0 to 60 minutes) with a plasma amylin EC_{50} of 18 pmol/L ± 0.3 log units. These data indicate that amylin potently inhibits arginine-stimulated glucagon secretion.

THE SECRETION of pancreatic islet hormones is now well established to be regulated by at least four key influences: plasma levels of key nutrients such as glucose and amino acids, the autonomic nervous system, circulating hormones such as the incretins, and islet hormones themselves.[1]

Much investigation has established that β-cell secretion is promoted by glucagon, whereas β-cell products, insulin[2] and amylin,[3] reportedly reduce insulin secretion. Insulin inhibits pancreatic α-cell secretion of glucagon,[4] a so-called "glucagonostatic" effect. Increased α-cell secretion of glucagon at low plasma glucose concentrations may partly result from the loss of a paracrine inhibitory action from glucose-mediated insulin secretion.[5] Exaggerated glucagon secretion in many insulin-dependent diabetic patients may in part reflect the loss of a restraining influence of insulin on pancreatic α cells.[6] Interestingly, glucagon-like peptide-1, a powerful incretion for insulin secretion, reduces glucagon secretion.[7]

Thus far, there are no reports of amylin's action on glucagon secretion in intact animals. Early in vitro studies reported no effect of amylin on glucagon secretion.[8,9] In the present study, we examined whether amylin influences arginine-evoked secretion of glucagon in anesthetized rats. Amylin administration can change plasma glucose and insulin levels, both of which can affect glucagon secretion. To standardize the insulin and glucose influences on glucagon secretion, we used a hyperinsulinemic-euglycemic clamp technique. During amylin infusion at different rates, we observed dose-dependent reductions in the increment of plasma glucagon evoked by a standard infusion of L-arginine. Steady-state plasma amylin concentrations at different amylin infusion rates were measured in parallel experiments, enabling the derivation of a concentration-response relationship for the glucagonostatic effect of amylin identified in the present study. Part of this investigation has been reported in a preliminary communication.[10]

MATERIALS AND METHODS

Animals and Surgical Procedures

Adult male Harlan-Sprague-Dawley rats (age, 92 ± 2 days; body weight, 356 ± 3 g) were housed at 22.8° ± 0.8°C with a 12-hour light/dark cycle. All experiments were performed during the light cycle. The animals were fasted for approximately 20 hours before experimentation. They were anesthetized with 5% halothane, maintained at 2% during surgery and at 0.7% to 1% thereafter. Tracheotomy and cannulation of the right femoral artery and saphenous vein were performed, and body temperature was controlled with a thermoregulator (model 73A; YSI, Yellow Springs, OH) that switched a heated operating table. The femoral arterial line, perfused with heparinized saline (10 U/mL), was used for blood sampling and was also connected to a pressure transducer to monitor blood pressure (Spectramed P23XL transducer with model 13-4615-58 amplifier; Gould, Cleveland, OH). The venous line was used for infusions: rat amylin (lot ME0316; American Peptide, Sunnyvale, CA) or 0.15 mol/L NaCl; and insulin (Humulin-R; Eli Lilly, Indianapolis, IN) and 10% D-glucose (diluted from 50% Dextrose, lot C252312; Nestle & Baxter, Colombus, OH).

Euglycemic Clamp Procedures

Following 60 minutes of stabilization after surgery, a primed/continuous infusion of insulin (12 mU + 120 mU/h) was begun 60 minutes before L-arginine administration (designated $t = -60$ minutes) and followed with a variable infusion of 10% D-glucose to maintain plasma glucose at approximately 6 mmol/L. The variable rate of glucose infusion was based on changes in arterial plasma glucose measured every 5 to 10 minutes using an immobilized enzyme chemistry glucose/lactate analyzer (model 2300-STAT; YSI). At −50 minutes, saline was infused (n = 23) or synthetic rat amylin dissolved in saline was infused at 1.2 (n = 7), 3.6 (n = 8), 12 (n = 6), 36 (n = 4), or 120 (n = 7) pmol/kg/min. At 0 minutes, 2 mmol L-arginine in 0.15 mol/L saline was infused over 10 minutes in each animal. The amylin/saline infusions and glucose clamps continued until 120 minutes after administration of L-arginine.

Analyses

Arterial samples were collected into heparinized Natelson capillary tubes at the time points indicated in Fig 1 for measurement of glucose, lactate, glucagon, and insulin levels. The total blood volume drawn over 4 hours from each animal was 2.8 mL, and the volume of saline infused over the same period was approximately 8 mL. For glucagon assays, 100 μL plasma was mixed with 1 μL protease-inhibitor cocktail that contained EDTA (50 μg), elastinal (2.5 μg), leupeptin (0.05 μg), and antipain (0.2 μg). After storage at −20°C for less than 1 week, plasma was assayed for insulin using the Incstar Rat Insulin-I^{125} RIA (kit 06130; Incstar, Stillwater, MN) and for glucagon by radioimmunoassay

From Amylin Pharmaceuticals, San Diego, CA
Submitted April 4, 1996; accepted July 17, 1996.
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0026-0495/97/4601-0013$03.00/0

(kit 07-152101; ICN Biomedicals, Costa Mesa, CA). For the insulin radioimmunoassay, sensitivity was 17 pmol/L, intraassay coefficient of variation (CV) 4.9%, and interassay CV 5.3% to 7.0%. For the glucagon radioimmunoassay, sensitivity was 8.3 pmol/L, intraassay CV 2.4%, and interassay CV 5.9% to 8.3%.

Dose-Concentration Relationship

Glucagon secretion is stimulated by hypovolemia.[11] To minimize blood withdrawal, plasma amylin concentrations resulting from different amylin infusion rates were determined in separate parallel experiments in similarly treated halothane-anesthetized Harlan-Sprague-Dawley rats. Synthetic rat amylin was infused at 1.2 (n = 4), 12 (n = 6), 120 (n = 4), 1,200 (n = 8), or 12,000 (n = 7) pmol/kg/min (0.1, 1, 10, 100, or 1,000 μg/h) for 3 hours, and samples were taken at 30-minute intervals during the infusion. Steady-state plasma amylin concentrations were measured using a two-site immunoenzymometric method and have been published separately.[12]

Numerical Methods

Pairwise statistical analyses were performed using Student's *t* test routines or, when all responses were compared with saline controls, Dunnet's multiple-comparisons test (Instat v2.0; GraphPad Software, San Diego, CA). Results are reported as the mean ± SEM, and *P* less than .05 is used as the level of significance (two-tailed tests). Plasma glucagon responses are plotted as the arginine-induced increment (Δglucagon) above baseline, defined as the mean of values for the prior 30 minutes. Since plasma glucagon responses were essentially complete within 60 minutes, they were quantified for statistical analysis as the trapezoidal area under the Δglucagon-time curve for the 60 minutes following arginine administration (ΔAUC60). Analyses of amylin-mediated lactemic responses used the Δlactate integrated for the full 3 hours of amylin infusion (ΔAUC180). EC_{50} values were obtained using four-parameter iterative least-squares fitting in Prism version 2.5 (GraphPad Software).

RESULTS

Amylin Effects on Glucagon Responses to L-Arginine

The increment in plasma glucagon in response to arginine infusion is plotted in Fig 1. The peak Δglucagon of 160 ± 11 pmol/L in saline-infused rats was observed 20 minutes after starting the arginine infusion. Peak Δglucagon was significantly reduced by 47% to 67% at the three highest amylin infusion rates ($P < .05$ to .01, Dunnet's multiple-comparisons test). Integrated glucagon responses (ΔAUC60) were significantly reduced by 30% to 62% at all but the lowest infusion rate ($P < .05$ to .01).

Administration of amylin leads to a rapid increase in plasma lactate in rats.[13] This action, which appears to result from lactate release from muscle[14] following amylin-stimulated, cyclic adenosine monophosphate–mediated glycogenolysis,[15] can be used to indicate biological activity of the peptide. In the present experiments, plasma lactate was significantly increased, but at only the two highest amylin infusion rates ($P < .01$ each). That is, there were doses at which amylin inhibited glucagon secretion but did not affect plasma lactate concentration.

Amylin Dose-Concentration Relationship

Plasma amylin concentrations during and after amylin infusion at different rates are published elsewhere.[12] Steady-state plasma amylin is related to the infusion rate by the expression, $[\text{amylin}] = 10^{(\log \text{inf rate}) \times 1.18 + 1.024}$, where [amylin] is in pmol/L and, infusion rate is in picomoles per kilogram per minute.

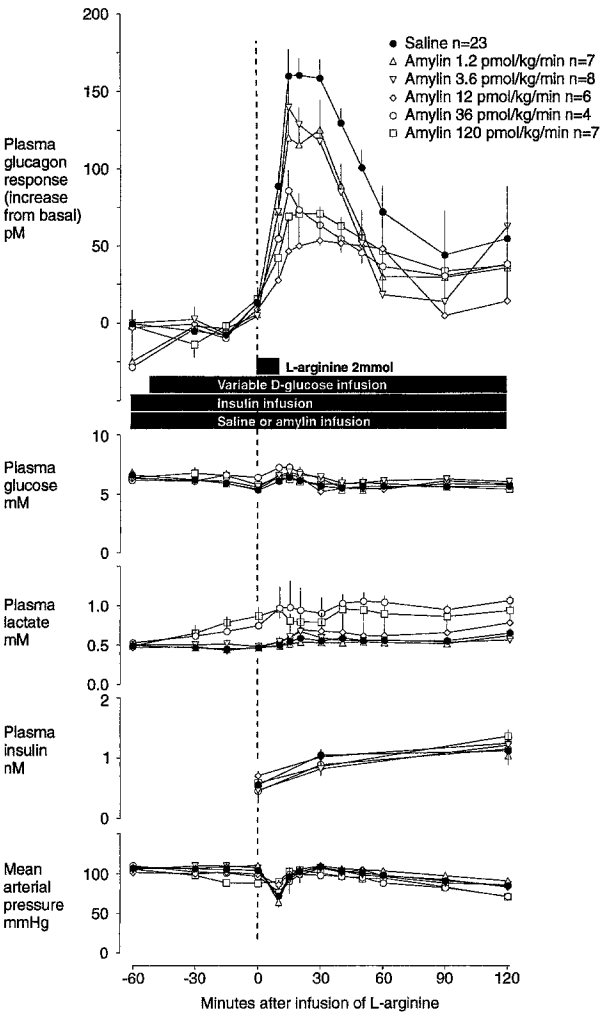

Fig 1. Glucagon response (change in plasma concentration from baseline), plasma glucose, lactate, and insulin concentrations, and mean arterial pressure after L-arginine administration in rats. Timing of infusions of insulin and glucose for the hyperinsulinemic-euglycemic clamp procedure, L-arginine challenge, and amylin/saline infusions are shown as solid bars. Integrated glucagon responses (ΔAUC60) and integrated lactate responses (ΔAUC180) were significantly different between treatment groups ($P < .0001$ by 1-way ANOVA); glucose and insulin concentrations and mean arterial pressures were not different. Results are the mean ± SEM. Amylin infusion rates of 1.2, 3.6, 12, 36, and 120 pmol/kg/min relate to absolute infusion rates (per rat) of 0.1, 0.3, 1, 3, and 10 μg/h. Baseline glucagon concentrations were 82 ± 36 pmol/L (mean ± SD).

Plasma amylin concentrations predicted by this formula were used to construct concentration-response curves. For these analyses, a plasma amylin concentration of 5 pmol/L, typical of that reported for fasting rats (4.9 ± 0.2 pmol/L[16]), was used for saline-infused animals.

Amylin Concentration-Response for Glucagonostatic Effect

Integrated glucagon responses (ΔAUC60) from Fig 1 were combined with plasma amylin concentrations predicted for each of the different amylin infusion rates to yield the concentration-response relationship shown in Fig 2a. The EC_{50} for the glucagonostatic effect of amylin measured in this way was 18 pmol/L ± 0.3 log units. In contrast, the EC_{50} for the hyper-

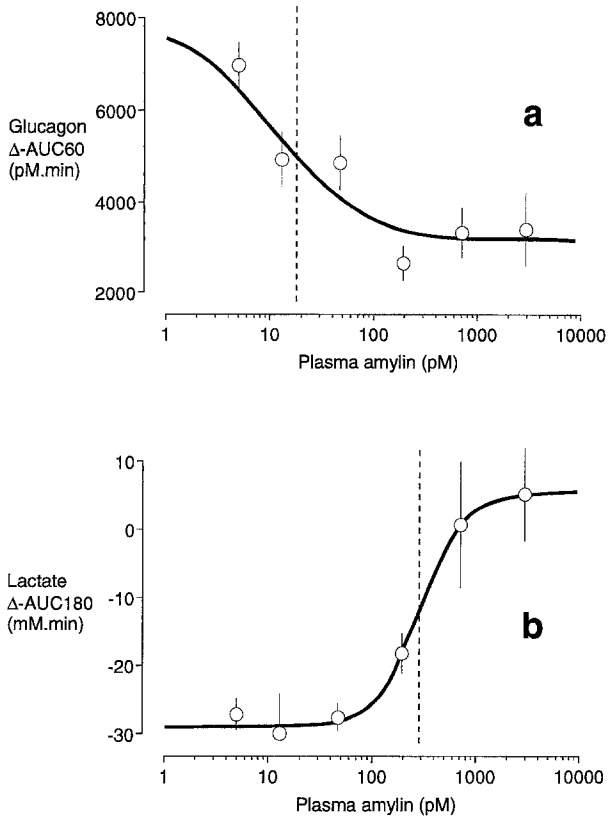

Fig 2. Concentration-responses for glucagonostatic and lactemic effects of intravenous amylin infusions in rats. (a) Glucagonostatic effect: the glucagon response was quantified as the integral of the increment in glucagon concentration for 60 minutes after arginine administration (ΔAUC60). (b) Lactemic effect: the plasma lactate response to amylin, which was unaffected by arginine administration, was quantified as the integral of the increment in lactate concentration for the 180 minutes after starting amylin infusion (ΔAUC180). Plasma amylin concentrations were calculated from the published[12] relationship between the steady-state concentration and infusion rate in this animal model. The calculated EC_{50} for amylin's glucagonostatic effect was 18 pmol/L ± 0.28 log units, and for the hyperlactemic effect, 288 pmol/L ± 0.04 log units (shown as broken lines).

lactemic effect of infused amylin (lactate ΔAUC180) was 288 pmol/L ± 0.04 log units (Fig 2b).

Plasma glucose concentrations and insulin concentrations for the 60 minutes following arginine infusion during euglycemic clamps (Table 1) did not differ between treatment groups (P = .36 and .59, respectively, by one-way ANOVA). The glucose infusion rate required to maintain euglycemia from 0 to 60 minutes in the glucose clamp did not differ between treatment groups (P = .36). Neither was mean arterial pressure over 60 minutes different between treatment groups (P = .77).

DISCUSSION

The present results show that in experimental conditions that minimized differences in plasma glucose concentration, plasma insulin concentration, or mean arterial pressure, the stimulation of glucagon secretion by L-arginine in anesthetized rats was dose-dependently inhibited by exogenous amylin. Significant effects were seen at amylin infusion rates that increased the basal plasma concentration by approximately 40 pmol/L, comparable to excursions of amylin concentrations reported in fed animals.[16]

In the present study, to address whether amylin affected glucagon secretion, we used L-arginine as an α-cell secretagogue stimulus. However, the α cell also responds to changes in glucose and insulin concentrations, both of which can be altered by amino acid infusions and amylin infusions. Amino acid infusions increase the endogenous production of glucose and also directly stimulate the secretion of insulin. Amylin infusions stimulate endogenous production of glucose.[13] The mechanism of amylin-mediated glucose production appears to involve an increase in the availability of lactate as a gluconeogenic substrate (Cori cycling).[17] Amylin appears to directly inhibit insulin secretion, as described earlier. At high intravenous doses, amylin can also decrease blood pressure,[18] which, through sympathoadrenal activation, could stimulate glucagon secretion.[19]

There are therefore many potential interactions between amino acids, glucose, amylin, and insulin that could confound the interpretation of amylin's influence on arginine-induced glucagon secretion. To avoid such confusion, the known influences of glucose and insulin on α-cell secretion were controlled in the present experiments using the euglycemic-hyperinsulinemic glucose clamp technique.[20] We asked whether amylin-mediated changes in plasma lactate affected the glucagon response to arginine. In the present study, suppression of glucagon secretion occurred at amylin infusion rates of 3.6 and 12 pmol/kg/min, where no change in plasma lactate was observed (Figs 1 and 2). We conclude that amylin suppression

Table 1. Clamp Variables 0 to 60 Minutes After L-Arginine Infusion

Variable	Amylin Infusion Rate, pmol/kg/min (μg/h)					
	0	1.2 (0.1)	3.6 (0.3)	12 (1)	36 (3)	120 (10)
Sample size (n)	23	7	8	6	4	7
Calculated amylin (pmol/L)	5	13	47	195	713	2,951
Glucose (mmol/L)*	5.80 ± 0.12	5.76 ± 0.14	6.25 ± 0.25	5.81 ± 0.19	6.31 ± 0.16	5.99 ± 0.35
Insulin (mmol/L)*	0.90 ± 0.05	0.80 ± 0.08	0.77 ± 0.10	0.95 ± 0.07	0.79 ± 0.16	0.86 ± 0.10
Lactate (mmol/L)	0.54 ± 0.03	0.52 ± 0.04	0.59 ± 0.02	0.61 ± 0.15	0.96 ± 0.20‡	0.87 ± 0.10‡
Glucose infused (μmol)*	507 ± 62	405 ± 81	364 ± 127	400 ± 91	206 ± 93	335 ± 85
Arterial pressure (mmHg)*	98.0 ± 2.0	99.9 ± 1.4	97.0 ± 1.9	99.1 ± 4.7	92.4 ± 5.8	96.3 ± 2.3
Glucagon response, ΔAUC60 (pmol/L · min)	6,985 ± 494	4,934 ± 608	4,860 ± 589†	2,653 ± 382‡	3,171 ± 653‡	3,397 ± 808‡

NOTE. Concentrations are expressed as time-weighted means from times 0 to 60 minutes.

*Not significantly different from saline controls at any dose.

†$P < .05$ *v* saline control.

‡$P < .01$ *v* saline control.

of glucagon secretion is not an indirect consequence of changes in plasma lactate.

An investigation of glucagon secretion in the isolated perfused pancreas did not reveal a glucagonostatic effect of rat amylin.[9] It is possible that amylin-mediated inhibition of glucagon secretion in intact animals could involve extrapancreatic pathways, such as the autonomic nervous system, that are not functional in the isolated perfused pancreas.

Concentration-response analysis identified the plasma amylin concentrations required to suppress glucagon secretion in this in vivo model as being less than 50 pmol/L, close to amylin concentrations reported to circulate normally in rats.[16] It is possible that endogenous amylin could modulate α-cell secretion by an extrapancreatic endocrine mechanism (ie, via the systemic circulation). It is also worth considering that amylin, secreted from the β-cell–rich islet medulla into the local islet portal circulation[21] in high local concentrations, could act directly on α cells as it passes to the islet cortex. Further experiments are required to determine whether amylin's glucagonostatic effects reflect a direct effect on α cells, or whether the inhibition of glucagon secretion involves mechanisms peripheral to the islet.

In summary, the present study reveals a potent and significant effect of systemically administered amylin to suppress secretion of the α-cell hormone, glucagon. This finding adds to the known matrix of modulatory effects of pancreatic islet hormones on the secretion of other islet hormones. The cellular mechanisms of the glucagonostatic effect of amylin and its potential impact on normal metabolic regulation and in the pathophysiology of diabetes are important areas for further study.

ACKNOWLEDGMENT

We gratefully acknowledge the technical assistance of Carolynn Jodka, Deborah Green, and Richard Lawler and the secretarial assistance of Nancy Gallaher-Wakefield.

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