Selective amylin inhibition of the glucagon response to arginine is extrinsic to the pancreas

R. A. SILVESTRE,1 J. RODRÍGUEZ-GALLARDO,1 C. JODKA,2 D. G. PARKES,2
R. A. PITTNER,2 A. A. YOUNG,2 AND J. MARCO1

1Clínica Puerta de Hierro and Department of Physiology, Universidad Autónoma de Madrid,
28035 Madrid, Spain; and 2Amylin Pharmaceuticals Inc., San Diego, California 92121

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Selective amylin inhibition of the glucagon response to arginine is extrinsic to the pancreas. Am J Physiol Endocrinol Metab 280: E443–E449, 2001.—Amylin, a peptide hormone from pancreatic β-cells, is reported to inhibit insulin secretion in vitro and in vivo and to inhibit nutrient-stimulated glucagon secretion in vivo. However, it has been reported not to affect arginine-stimulated glucagon secretion in vitro. To resolve if the latter resulted from inactive peptide (a problem in the early literature), those experiments were repeated here with well-characterized peptide and found to be valid. In isolated perfused rat pancreas preparations, coperfusion with 1 nM amylin had no effect on glucagon output stimulated by decreasing glucose concentration from 11 to 3.2 mM or on glucagon suppression caused by increasing glucose from 3.2 to 7 mM. Amylin at 100 nM had no effect in isolated islets in which glucagon secretion was stimulated by exposure to 10 mM arginine, even though glucagon secretion in the same preparation was inhibited by somatostatin. In anesthetized rats, amylin coinfusion had no effect on glucagon secretion stimulated by insulin-induced hypoglycemia. To reconcile reports of glucagon inhibition with the absence of effect in the experiments just described, anesthetized rats coinfused with rat amylin or with saline were exposed sequentially to intra- and hypoglycemia. Amylin inhibited arginine-induced, but not hypoglycemia-induced, glucagon secretion in the same animal. In conclusion, we newly identify a selective glucagonostatic effect of amylin that appears to be extrinsic to the isolated pancreas and may be centrally mediated.

AMYLIN IS A Pancreatic β-cell hormone cosecreted with insulin in response to nutrients, including amino acids (19). Intravenous infusion of amylin into rats, at rates resulting in physiological plasma concentrations, inhibited arginine-stimulated glucagon secretion by up to 70% (7). A similar glucagonostatic effect was observed with the amylin analog pramlintide in rats (1, 5), and pramlintide administration inhibited the exaggerated postprandial glucagon surges observed in diabetic patients (18).

In a previous study that appeared initially to contradict those results, pharmacological concentrations (500–750 nM) of rat amylin in the perfused rat pancreas had no effect on arginine-stimulated glucagon secretion (29). Because many preparations of amylin peptide commercially available at the time of that study had reduced or undetermined biological activity (15), we repeated those experiments using material established to be biologically active in isolated soleus muscle. We confirm in the present report that well-characterized rat amylin did not affect arginine-stimulated glucagon secretion in the isolated perfused pancreas or in isolated islets. This result caused us to further explore and identify those conditions where a suppression by amylin of glucagon secretion did or did not occur.

Amylin replacement therapy is proposed for use in diabetic patients treated with insulin, who are thereby susceptible to insulin-induced hypoglycemia. Glucagonostatic effects of amylin were therefore examined in the presence of low glucose concentrations in vivo and in vitro. Additionally, we examined its effects in vitro against peptidergic [vasoactive intestinal peptide (VIP)] and cholinergic (carbachol) glucagonotropic stimuli. Finally, to reconcile an absence of amylin effect under some circumstances with equally robust data showing an effect under other circumstances, we tested amylin’s effects in rats in which both arginine and hypoglycemia were sequentially employed to stimulate glucagon secretion. The selective suppression of nutrient-stimulated glucagon secretion we identify here is discussed in relation to a proposed central (extrapancreatic) mode of action. Some of the data reported herein have been presented in preliminary communications (22, 30).

MATERIALS AND METHODS

Isolated perfused pancreas. Male Wistar rats (200–225 g body wt) fed ad libitum from our inbred colony were used as donors. Animals were maintained in accordance with the

Address for reprint requests and other correspondence: J. Marco, Clínica Puerta de Hierro, Universidad Autónoma de Madrid, San Martín de Porres, 4 28035 Madrid, Spain (E-mail: jmarco@hpth.insalud.es).

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guidelines established by the European Union (86/609). After anesthesia of the rat with pentobarbital sodium (50 mg/kg ip), the pancreas was dissected and perfused in situ according to the procedure of Leclercq-Meyer et al. (14) as adapted in our laboratory (28). Effluent samples were collected from the portal vein, without recycling, at 1-min intervals (flow rate, 2 ml/min) and frozen at −20°C until the time of assay. The perfusion medium consisted of a Krebs-Henseleit buffer [in mM: 115 NaCl, 4.7 KCl, 2.6 CaCl₂, 1.19 H₂KPO₄, 1.19 MgSO₄·7H₂O, and 24.9 HNaCO₃ (gas phase 95% O₂-5% CO₂; pH 7.4)] supplemented with 4% (wt/vol) dextran T-70 (Pharmacia LKB Biotechnology, Uppsala, Sweden), 0.5% (wt/vol) Cohn fraction V bovine albumin (Sigma Chemical, St. Louis, MO), and glucose (3.2, 5.5, or 11 mM; Sigma). Rat amylin (Amylin Pharmaceuticals, San Diego, CA) was dissolved in 0.9% NaCl containing 0.1% bovine albumin (Cohn fraction V). This solution (17.8 nM) was prepared daily, immediately before experiments, and when added to the perfusate, the final concentration was 1 nM amylin. After a 35-min equilibration period, baseline samples were collected for 5 min, and at time 0, normal saline with or without amylin was infused through a sidearm cannula. Glucagon secretion was stimulated by infusing 5 mM L-arginine hydrochloride (Sigma), 1 nM VIP (Peninsula Laboratories Europe), or 50 μM carbachol (Sigma) and by decreasing perfusate glucose concentration from 11 to 3.2 mM. Integrity of the isolated pancreas preparation was verified by measuring inhibition of glucagon secretion while increasing perfusate glucose concentration from 3.2 to 7 mM. Glucagon was analyzed by RIA (4). Anti-glucagon serum (Unger’s O4A) was kindly donated by R. H. Unger (Health Sciences Center, University of Texas, Dallas, TX). All samples for each series of experiments were analyzed within the same assay.

For all analyses, results are expressed as means ± SE. Integrated hormone responses were calculated using the trapezoidal method as the integrated area of the curve, for specified epochs, above or below the mean preinfusion level (average of all the baseline levels for each perfusion). The normal distribution of data was tested with the Kolmogorov-Smirnov test (27). Pairwise comparisons were performed using Student’s t-test for unpaired samples. P < 0.05 was used as the level of statistical significance throughout.

Isolated islets. As a further test of potential direct effects of amylin on pancreatic islets, these were isolated from male Lewis rats (Harlan Sprague Dawley, Indianapolis, IN) weighing between 150 and 200 g. Islets of Langerhans were isolated from whole pancreas by use of a method of collagenase digestion originally described by Kostianovsky et al. (12) and modified by Lakey et al. (13). Briefly, the pancreas was dissected free of fat, cleaned, and placed into 5 ml of collagenase-P (1.8 mg/ml; Boehringer Mannheim) plus DNase (0.1 mg/ml), minced, and incubated at 37°C for 20 min. Digested pancreas was then shaken vigorously and washed in HEPES buffer, and islets were separated on a Ficoll gradient. Selected islets were then hand picked and cultured in RPMI/FCS (10%) for 3–4 days at 37°C until experimentation. After being washed in RPMI media for 1 h and then incubated in Hanks’ BSS buffer containing 3 mM glucose for 1 h, triplicate groups of 15 islets were then incubated at 37°C in fresh Hanks’ buffer for a further hour of treatment, after which media were collected and frozen at −70°C until assayed for glucagon. Separate treatments consisted of addition of the following to buffer medium: 3 mM D-glucose (control), 3 mM D-glucose plus 10 mM L-arginine, 3 mM D-glucose plus 10 mM L-arginine plus 100 nM somatostatin, 3 mM D-glucose plus 10 mM L-arginine plus 100 nM rat amylin.

Hypoglycemia-stimulated glucagon secretion in vivo. Male Harlan Sprague-Dawley rats weighing ~350 g and fasted 18–20 h were halothane anesthetized and cannulated via the saphenous vein for intravenous infusion/injection and via the.
femoral artery for sampling analytes and for recording arterial pressure. Mean arterial pressure (Spectramed P23XL transducer and Universal amplifier; Gould, Valley View, OH), heart rate from an electrocardiogram (Biotach; Gould), and colonic temperature were continuously recorded by computer interface (Data Translation, Marlboro, MA). Heparinized (2 U/ml) saline was infused via the arterial line at 3 ml/h from time \( t = -60 \) min. At \( t = -30 \) min, rat amylin at 50 pmol \( \cdot \) kg\(^{-1} \) \( \cdot \) min\(^{-1} \) was added or not added, and at \( t = 0 \) min, insulin was infused intravenously (or not) at 5 mU/min, with initiation of infusion being accompanied by a 200-mU prime in some animals. There were thus five treatment groups: 1) saline at \( t = -30 \) min, saline at \( t = 0 \) min, \( n = 4 \); 2) amylin at \( t = -30 \) min, saline at \( t = 0 \) min, \( n = 4 \); 3) saline at \( t = -30 \) min, insulin at \( t = 0 \) min, \( n = 4 \); 4) amylin at \( t = -30 \) min, insulin at \( t = 0 \) min, \( n = 6 \); and 5) amylin at \( t = -30 \) min, insulin plus prime at \( t = 0 \) min, \( n = 4 \). The purpose of the prime in group 5 was to compensate for an effect of higher amylin doses to increase plasma glucose in nondiabetic rats (36) and to thereby ensure that there was an amylin-infused group that was as hypoglycemic or more hypoglycemic than the insulin-only treatment group (group 3). Samples for glucose and lactate were taken at -30, -15, and 0 min and every 5 min thereafter until \( t = 120 \) min. Samples for glucose were taken at -30, 0, 10, 20, 30, 40, 50, 60, 75, 90, 105, and 120 min and were measured by RIA (Linco Research, St. Charles, MO).

Effects of amylin on sequential arginine- and hypoglycemia-stimulated glucagon secretion. Animals were anesthetized and prepared as described above. Thirty minutes after surgery, saline or rat amylin was infused continuously intravenously at 50 pmol \( \cdot \) kg\(^{-1} \) \( \cdot \) min\(^{-1} \) \( (n = 5–6) \) from \( t = -60 \) min until the end of the experiment \( (t = 240 \) min). Insulin was infused concurrently at 2 mU/min. At \( t = 0 \) min, 2 mmol L-arginine were infused intravenously over 10 min. A variable glucose infusion was used to clamp plasma glucose at 5.67 ± 0.06 mM until \( t = 120 \) min, whereupon glucose infusion was ceased while still continuing insulin administration, allowing plasma glucose to fall and remain low. To match the hypoglycemic profile during this portion of the experiment, amylin-treated rats received an additional 90

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Fig. 2. A: effect of 1 nM rat amylin on glucagon secretion at 11 mM glucose (from 0 to 5 min) and on glucagon secretion stimulated by decreasing perfusate glucose to 3.2 mM (from 5 to 30 min) in the isolated rat pancreas. ○, Saline infusion control experiments \( (n = 6) \); △, amylin infusion experiments \( (n = 6) \). Symbols indicate means ± SE. B: effect of 1 nM rat amylin on glucagon release at 3.2 mM glucose (from 0 to 10 min) and on inhibition of glucagon secretion induced by increasing perfusate glucose concentration from 3.2 to 7 mM \( (10 \) to 25 min) in the perfused rat pancreas. ○, Saline infusion control experiments \( (n = 7) \); △, amylin infusion experiments \( (n = 7) \). Symbols indicate means ± SE.

Fig. 3. Effects of 10 mM L-arginine on glucagon secretion from isolated rat islets in culture and effects of 100 nM somatostatin (SRIF) or 100 nM rat amylin on arginine-stimulated glucagon secretion. Control conditions are 3 mM glucose; other conditions are additions to control. Bars indicate means ± SE.
mU insulin bolus when glucose infusion was stopped at $t = 120$ min to preempt the rise in blood glucose after similar doses of amylin in nondiabetic rats (36). Samples for glucose, amylin, and glucagon were taken at the time points indicated in Fig. 5. Plasma glucose was determined by immobilized oxidase chemistry on a YSI 2300 Stat Plus (YSI, Yellow Springs, OH). Samples for glucagon were determined by RIA (sensitivity 7 pM, interassay variability 7.9%, intra-assay variability 10.9%; ICN Pharmaceuticals, Orangeburg, NY). Other chemicals were obtained from Sigma. Plasma amylin concentrations were determined using a specific two-site immunoenzymometric assay (24).

**RESULTS**

**Isolated perfused pancreas: secretagogues.** Glucagon responses to L-arginine, carbachol, and VIP, shown in Fig. 1, A-C, indicate that the perfused pancreas preparation was functioning. Addition of rat amylin (1 nM) did not significantly modify the glucagon responses to L-arginine (5 mM), carbachol (50 μM), or VIP (1 nM). In these three experiments, amylin was also without effect on unstimulated glucagon release, that is, during the 10-min perfusion period preceding the infusion of secretagogues.

**Isolated perfused pancreas: hypoglycemia.** As shown in Fig. 2A, pancreases were initially perfused at a high glucose concentration (11 mM), and after 10 min, this concentration was abruptly lowered to 3.2 mM. This reduction of infusate glucose concentration was associated with a progressive increase in glucagon output from $129 \pm 31$ pg/min at $t = 5$ min to $578 \pm 99$ pg/min at $t = 30$ min ($P < 0.05$). Restoration of the initial 11 mM D-glucose concentration resulted in a prompt decrease in glucagon release to basal values. Coinfusion of amylin from $t = 0$ until $t = 30$ min had no effect on the incremental glucagon response ($4.8 \pm 1$ ng/25 min in control experiments vs. $4.5 \pm 1.3$ ng/25 min in amylin experiments; $P = 0.8$) and no apparent effect on the glucagon secretory pattern.

Figure 2B shows the secretion of glucagon under opposite experimental conditions, where periods of low glucose concentration (3.2 mM) bracketed a period of euglycemia (7 mM). The inhibitory effect of 7 mM glucose concentration on glucagon output was not significantly modified by amylin infusion (incremental area $2.7 \pm 0.4$ vs. $2.4 \pm 0.8$ ng/15 min in control experiments; $P = 0.7$).

**Isolated islets.** Culturing islets in 10 mM arginine resulted in an increase in the rate of glucagon secretion of $15.5 \pm 4.3$-fold over that observed in the presence of 3 mM glucose (designated basal; $P < 0.02$; Fig. 3).
Addition of 100 nM somatostatin to islets cultured in 10 mM arginine reduced the 15.5-fold stimulation of glucagon secretion by 55 ± 21% (not significant (NS)). In contrast, addition of 100 nM rat amylin to buffer containing 10 mM arginine did not reduce glucagon secretion (102 ± 46% of islets treated with 10 mM arginine alone; NS vs. arginine stimulated; Fig. 3).

Hypoglycemia-stimulated glucagon secretion in vivo. In rats preinfused with saline only, insulin reduced plasma glucose from 5.72 ± 0.22 to 2.11 ± 0.11 mM after 60 min of infusion (P < 0.001; Fig. 4A). In rats preinfused with amylin, insulin reduced plasma glucose from 6.39 ± 0.17 to 2.39 ± 0.17 mM after 60 min (P < 0.001). Glucose remained between 1.6 and 2.2 mM from t = 60 to t = 120 min in both groups. In saline-treated rats, insulin-induced hypoglycemia increased plasma glucagon concentration from 262 ± 35 to a peak of 2,141 ± 348 pg/ml compared with an increase from 217 ± 18 to 2,070 ± 273 pg/ml in the amylin-treated rats (Fig. 4A). The area under the curve (AUC) for glucagon (2 h) was not different between groups (saline: 2,115 ± 289 and amylin: 2,173 ± 302 pg·mL⁻¹·h⁻¹; P = 0.91).

To accommodate a glycemic effect that is present at high amylin infusion rates in rats (35), the glucagon response was analyzed as a function of current plasma glucose concentration (Fig. 4B). The nonlinear relationship between plasma glucagon and glucose concentrations showed increasing glucagon output until a glucose concentration passed below ~2 mM, whereupon glucagon secretion began to diminish. This relationship was similar whether or not amylin was being infused; a LOWESS curve was fitted to the glucagon-glucose scatterplot to approximate the center of distribution; in amylin-infused rats, the proportion of all data points above the curve was as great as or greater than the proportion in saline-infused rats (Fig. 4B); that is, amylin did not diminish hypoglycemia-stimulated glucagon secretion in the present study. Mean arterial pressure, falls in which can stimulate glucagon secretion (16), was not different between saline- and amylin-infused groups.

Effects of amylin on sequential arginine- and hypoglycemia-stimulated glucagon secretion. The glucagon response to the L-arginine challenge, integrated over 90 min, was reduced by 45% in the amylin-infused rats (559 ± 71 vs. 1,002 ± 162 pg·mL⁻¹·h⁻¹ in controls, P < 0.05). Within 20 min of the variable glucose infusion being stopped, insulin reduced plasma glucose from 5.67 ± 0.03 to 2.44 ± 0.17 mM (amylin-treated rats) and from 5.67 ± 0.06 to 2.39 ± 0.17 mM (saline-treated rats), and glucose remained between 1.6 and 2.2 mM over the next 100 min. In response to this hypoglycemic challenge, plasma glucagon concentration increased from 205 ± 43 to 2,544 ± 264 pg/ml in amylin-treated rats and from 247 ± 49 to 2,761 ± 382 pg/ml in control rats [glucagon AUC (120–240 min) 3,707 ± 507 vs. 4,401 ± 640 pg·mL⁻¹·h⁻¹, respectively; P = 0.44; Fig 5]; that is, in this animal model, amylin infusion significantly inhibited arginine-stimulated glucagon secretion, consistent with a previous description (7), but...
did not affect hypoglycemia-stimulated glucagon secretion. In amylin-infused animals, mean steady-state plasma amylin concentration was 589 pM ± 29% coefficient of variation. Mean arterial pressure remained between 93 and 106 mmHg throughout the experiment and decreased slightly during administration of L-arginine.

**DISCUSSION**

In our isolated rat pancreas model, 1 mM rat amylin did not modify glucagon release when perfused at low (3.2 mM), normal (5.5 mM), or high (11 mM) glucose concentrations, and it failed to affect the increase in glucagon output that followed the lowering of the perfusate glucose concentration from 11 to 3.2 mM. In the isolated pancreas, 1 mM amylin was also without effect on glucagon secreted in response to established secretagogues such as arginine, VIP, and carbachol, in agreement with previous results obtained with a 500-fold higher amylin concentration (29). In a state of increased α-cell secretory activity (when the pancreas was perfused with 3.2 mM glucose), the suppression of glucagon output induced by augmenting the glucose concentration of the medium (to 7 mM) was not significantly modified by the simultaneous infusion of amylin. In isolated islets, 100 nM amylin did not affect arginine-stimulated glucagon secretion. That is, in neither isolated islets nor isolated pancreas preparations was there an effect of amylin on glucagon secretion. These negative findings in regard to glucagon secretion in isolated preparations contrast with the clear inhibitory effect that amylin has on insulin secretion in similar preparations (2, 3, 11, 20, 23, 26, 31, 33).

In the present study in anesthetized rats, the glucagon response to insulin-induced hypoglycemia was not affected by the infusion of amylin at pharmacological concentrations, but several studies now affirm an effect of physiological and/or therapeutic concentrations of amylin or analogs to inhibit nutrient-stimulated glucagon secretion in rodents and humans (1, 5–7, 18, 21). In an effort to reconcile this apparent dichotomy, arginine and hypoglycemia were used sequentially to stimulate glucagon secretion in the same animal in the present study. In this study, a glucagonostatic action observed after the arginine challenge phase was not present with even supraphysiological concentrations of amylin during hypoglycemic stimulation of glucagon secretion. This result newly identifies a selective glucagonostatic effect of amylin.

Our observations on the lack of effect of amylin on glucagon release from isolated islets disagree with the work of Wang et al. (32) but concur with other data presented here. It appears likely from the present set of experiments and from those reported from similar preparations that effects of amylin agonists to inhibit nutrient-stimulated glucagon secretion are not intrinsic to isolated islets or the isolated perfused pancreas; they are a feature of intact individuals. The present study does not identify what extrapancreatic mechanisms mediate this effect, but plausible explanations could include both humoral and neural pathways. This latter mechanism of action would be consistent with other glucose-dependent glucoregulatory effects of amylin that apparently reside in the area postrema (25) and are overridden by hypoglycemia (34). The autonomic nervous system appears to be implicated differently in hypoglycemia-stimulated than in arginine-stimulated glucagon secretion in normal women (10). It could prove that inhibition of glucagon secretion in response to each of these stimuli differentially involves autonomic mechanisms.

An amylin receptor agonist, pramlintide, is being explored for its utility in treatment of types of diabetic subjects in which exaggerated amino acid-stimulated glucagon secretion and excess glucagon action complicate metabolic control (8, 9, 17). The findings in the present study that this potentially antidiabetic action of amylin does not impede counterregulatory glucagon secretion could be relevant to clinical safety.

In conclusion, the present results demonstrate an effect of exogenous rat amylin to inhibit arginine-induced, but not hypoglycemia-induced, glucagon secretion in intact rats. The absence of any effect of rat amylin on glucagon release from isolated preparations indicates that its selective glucagonostatic effect is extrapancreatic and possibly centrally mediated. The finding that amylin does not modify the increase in glucagon secretion evoked by an abrupt decline in plasma glucose concentration suggests that therapeutic administration of amylin analogs would not suppress the counterregulatory glucagon response elicited by hypoglycemia.

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