Diabetogenic Effects of Salmon Calcitonin Are Attributable to Amylin-Like Activity

Andrew A. Young, Ming-Wei Wang, Bronislava Gedulin, Timothy J. Rink, Richard Pittner, and Kevin Beaumont

During the development of synthetic calcitonins for therapeutic use in bone disease, a "diabetogenic" (hyperglycemic) effect was observed, particularly with salmon calcitonin. The effect was attributed by some to inhibition of insulin secretion. We have recently reported high-affinity (28 pmol/L) amylin-binding sites in certain areas of rat brain, and found that these sites also bind salmon but not rat calcitonin with comparable high affinity. Rat amylin and salmon calcitonin have been determined to have significant structural homology. In vitro and in vivo studies indicate that rat amylin can exert calcitonin-like effects on osteoclasts and on plasma calcium. Here we report that salmon calcitonin mimics the actions of rat amylin on skeletal muscle glycogen metabolism in vitro; it stimulates glycogenolysis and inhibits incorporation of radiolabeled glucose into glycogen (50% effective concentration [EC_{50}], 0.4 ± 0.11 nmol/L log and 8.4 ± 0.05 nmol/L log, respectively). In anesthetized rats, salmon calcitonin, like rat amylin, rapidly increases plasma lactate concentration, followed by a slower increase in glucose concentration. Like amylin, salmon calcitonin also inhibits the insulin response to 2 mmol infused glucose (insulin increments suppressed by 52% and 57% at 10 minutes for salmon calcitonin and amylin). Other shared actions, such as suppression of appetite, stimulation of renin secretion, inhibition of gastric acid secretion, and inhibition of gastric emptying, further affirm our proposal that the exogenous peptide, salmon calcitonin, is a mimic of endogenous amylin in the rat.

THE CALCITONINS, a family of 32–amino acid peptides secreted from C cells in the thyroid gland of mammals and the ultimobranchial organs of fish, have many biologic actions. The best-known and therapeutically exploited action is a hypocalcemic effect associated with inhibition of osteoclast function. These effects underlie the use of certain calcitonins in the treatment of bone disease with decalcifying lesions, including Paget's disease of bone and osteoporosis.[1] Teleost-fish calcitonins and their analogs are more potent in mammals for many biologic actions than are the native hormones.[2] Effective doses are cheaper to produce, so teleost calcitonins have frequently been used therapeutically.

One of the many actions of salmon calcitonin observed early in its development for therapeutic use was the elevation of plasma glucose, a "diabetogenic" action.[3-5] This action has commonly been attributed to inhibition of insulin secretion,[4,6,7] an action salmon calcitonin shares[3] with mammalian forms of the alternate calcitonin gene product, calcitonin gene–related peptide (CGRP),[8,9] as well as with mammalian amylins.[10,11] Further functional similarity among these peptides is illustrated by the observation that CGRP[12] and amylin[13] both exhibit hypocalcemic activity in vivo and exhibit a calcitonin-like action on osteoclasts in vitro, albeit less potently than the calcitonins.[14] The structural similarity between rat amylin, rat CGRPα, rat calcitonin, and salmon calcitonin is shown in Fig 1.

Amylin is the most recently discovered of these peptides.[15] It is primarily synthesized in and secreted from pancreatic β cells along with insulin.[16] Human and rat amylins were found to influence carbohydrate metabolism, first in isolated skeletal muscles and then in whole animals. In isolated soleus muscle, rat amylin dose-dependently inhibits insulin-stimulated incorporation of radiolabeled glucose into glycogen,[17,18] reduces total glycogen content,[19] and releases lactate from muscle.[20] These effects may be mainly attributed to activation of glycogen phosphorylase[21] via cyclic adenosine monophosphate.[20] In rats, amylin promotes a rapid increase in plasma lactate, followed by a slower elevation of plasma glucose.[22] The hyperglycemic response is associated with increased endogenous glucose production, apparently as a consequence of the increased availability of lactate as a gluconeogenic substrate.[23] The mechanism of this amylin-induced hyperglycemia is different from and synergistic with that of glucagon,[23] which increases plasma glucose primarily by liberation of hepatic glycogen stores. Mammalian CGRPs share several of these metabolic actions both in vitro and in vivo, with similar or somewhat less potency than rat amylin.[24]

During an investigation of receptors for these peptides, we discovered high-affinity amylin binding in membranes from circumscribed areas of rat brain, most concentrated in the *nucleus accumbens*.[25] The K_d of rat amylin competing with Bolton-Hunter labeled rat ^{125}I-amylin at this site was 27 pmol/L. Interestingly, these binding sites also have high affinity (~30 pmol/L) for salmon calcitonin and high affinity for CGRP (~80 pmol/L for CGRPβ and ~200 pmol/L for CGRPα), but only low affinity (K_d ~10 μmol/L) for rat calcitonin.[25]

The study reported here was designed to investigate the possibility that salmon calcitonin was an amylin agonist by comparing, both qualitatively and by dose-response relationships, the actions of salmon calcitonin and rat amylin on glycogen metabolism in isolated soleus muscle and on plasma lactate and glucose response in 18-hour–fasted, anesthetized rats. Because the CGRPs and, to a lesser extent, the amylins are vasoactive peptides, we also monitored heart rate and blood pressure following injections of salmon calcitonin and rat amylin in anesthetized animals. The ability of salmon calcitonin, glucagon, or a combination of the peptides to restore glucose levels in fasted animals

From Amylin Pharmaceuticals, San Diego, CA.
Submitted December 1, 1994; accepted March 9, 1995.
Address reprint requests to Andrew A. Young, MD, PhD, Amylin Pharmaceuticals, 9373 Towne Centre Dr, San Diego, CA 92121.

0026-0495/95/4412-0013$03.00/0

Fig 1. Sequence alignment of rat calcitonin, salmon calcitonin, rat amylin, and rat CGRPα. There are several shared features (C-terminal amide, Cys^2-Cys^7 and Cys^1-Cys^7 disulfide linkages), as well as segments of sequence identity, indicated by the surrounding boxes. Note that salmon calcitonin has homology with rat amylin in some segments where there is little homology with rat calcitonin.

made hypoglycemic with insulin infusion was also examined. We also reexamined, in a way that enabled comparison with amylin, the reported observations that salmon calcitonin reduces the insulin secretory response to infused glucose. We found that salmon calcitonin, but not rat calcitonin, was a potent mimic of amylin action in the rat, and propose that these actions may largely underlie its reported diabetogenic actions. Some of these results have been previously reported in preliminary form.[26]

MATERIALS AND METHODS

In Vitro Soleus Muscle Assay and ^{14}C-glycogen and Total Glycogen Determination

Male Harlan-Sprague-Dawley rats of approximately 200 g mass were fasted for 4 hours before killing by decapitation. The gastrocnemius muscle was reflected out from the posterior aspect of the tibia, and the soleus, a small, 15 to 20-mm long, 1-mm thick, flat muscle, was stripped clear and split into equal-sized lengths. After being kept for a short period in physiological saline, muscles were added to 50-mL Erlenmeyer flasks containing 10 mL of a pregassed Krebs-Ringer bicarbonate buffer containing (in millimolars) NaCl 118.5, KCl 5.94, $CaCl_2$ 2.54, $MgSO_4$ 1.19, KH_2PO_4 1.19, $NaHCO_3$ 25, and glucose 5.5, recombinant human insulin (Humulin-R; Eli Lilly, Indianapolis, IN), and one of the following: (1) synthetic rat amylin (lot no. ZG485; Bachem, Torrance, CA), (2) synthetic salmon calcitonin (lot no. QG407,408; Bachem), or (3) synthetic rat calcitonin (lot no. QH127; Bachem). Muscles were assigned to different flasks so that the four muscle pieces from each animal were evenly distributed among different assay conditions. Incubation media were gassed by gently blowing carbogen (95% O_2, 5% CO_2) over the surface while they were continuously agitated at 37°C in an oscillating water bath. The pH of gassed media at 37°C was verified as being between 7.1 and 7.4. After a half-hour preincubation, 0.5 μCi [U-^{14}C]glucose was added to each flask for a further 60 minutes. Each muscle piece was then rapidly removed, trimmed of tendons, blotted, frozen in liquid N_2, weighed, and then stored at −20°C for subsequent determination of total and ^{14}C-glycogen. To determine ^{14}C-glycogen, frozen muscle specimens were placed in a vial with 1 mL 60% (wt/vol) potassium hydroxide and digested at 70°C for 45 minutes under intermittent vigorous agitation. Dissolved glycogen was precipitated onto the walls of the vial by addition of 3 mL absolute ethanol and overnight cooling at −20°C. After centrifugation for 30 minutes at 2,000 × *g*, the supernatant was gently aspirated, the glycogen again washed with ethanol and centrifuged, the ethanol aspirated, and the precipitate dried under vacuum to avoid ethanol quenching during scintillation counting. The remaining glycogen was redissolved in 1 mL water and 4 mL scintillation fluid and counted for ^{14}C. In an extra parallel step, to measure total muscle glycogen, pellets were neutralized with 0.2 mL perchloric acid and resuspended in 1 mL acetate buffer, and the glycogen was digested with 18.5 U/mL amyloglucosidase (EC 5.3.2.1.3 from *Aspergillus niger*, Sigma A7420; Sigma Chemical, St Louis, MO) for at least 15 minutes at 23°C. The supernatants were assayed for glucose in an analyzer using D-glucose oxidase–immobilized enzyme chemistry (model 2300-STAT; YSI, Yellow Springs, OH). Purified rabbit liver glycogen (Sigma G4011) was used as a calibration standard.

Glycogen Phosphorylase a Activity

Soleus muscle strips from rats fasted for 18 hours were preincubated in 20-mL polyethylene scintillation vials containing 2 mL Krebs-Ringer bicarbonate buffer with 0.1% fatty acid–free bovine serum albumin for 30 minutes before use. Salmon calcitonin was then added as indicated and incubations continued for 10 minutes, after which each muscle was weighed and frozen as described earlier. Glycogen phosphorylase *a* activity was determined as described previously,[27,28] from the rate of the reverse reaction, glucosyl incorporation into glycogen from glucose-1-phosphate, as indicated by measuring inorganic phosphate release.[29] Muscle pieces were sonicated in 1 mL ice-cold buffer containing 100 mmol/L NaF, 20 mmol/L EDTA, 50 mmol/L Trizma, and 0.5% glycogen (mussel) at pH 6.5. A 0.2-mL aliquot of sample was added to 0.2 mL incubation buffer containing 300 mmol/L NaF, 50 mmol/L glucose-1-phosphate, 10 mmol/L caffeine, and 2% glycogen at pH 6.1 for determination of glycogen phosphorylase *a* activity. Reactions continued at 30°C for 20 minutes until termination by addition of 0.1 mL trichloroacetic acid (20%). Inorganic phosphate level was then measured using the ammonium molybdate method.[29] Results are expressed as nanomoles of inorganic phosphate produced per minute per milligram wet weight of tissue. Total glycogen phosphorylase (*a* + *b*) activity, indicated by the activity seen after addition of adenosine monophosphate, was not affected by salmon calcitonin (results not shown).

Amylin/Salmon Calcitonin/Rat Calcitonin Dose-Responses

Rat amylin, salmon calcitonin, and rat calcitonin dose-response curves were generated using muscles added to media containing 7.1 nmol/L recombinant human insulin (except for phosphorylase determinations) and either synthetic rat amylin, synthetic rat calcitonin, or synthetic salmon calcitonin added to result in final (nominal) concentrations that increased in a half-log progression between 10 pmol/L and 1 μmol/L. Rate of labeling of the extracted muscle glycogen by [U-^{14}C]glucose was expressed as a percentage of the rate obtained in the presence of 7.1 nmol/L insulin but neither amylin nor calcitonin. Dose-response curves were fitted to a four-parameter logistic model using a least-squares iterative routine (ALLFIT, version 2.7; National Institutes of Health, Bethesda, MD) to derive EC_{50}s. Curves fitted to amylin, salmon calcitonin, and rat calcitonin dose-responses were con-

strained to share the same maxima and minima. Since EC_{50} is log-normally distributed, it is expressed as the mean ± SEM of the logarithm.

In Vivo Studies

Animals. Male Harlan-Sprague-Dawley rats (N = 109; weight, 350 g) were housed at 22.7 ± 0.8°C in a 12-hour light/dark cycle (experiments were performed during the light cycle) and fed and watered *ad libitum* (Diet LM-485; Teklad, Madison, WI). Animals were deprived of food for 20 hours before preparation and experimentation as follows.

Surgery/instrumentation. Anesthesia was induced with 5% halothane, maintained at 2% during surgery and at 0.8% to 1% during metabolic recordings. Tracheotomy and cannulation of the right femoral artery and saphenous vein were performed. The femoral arterial line was connected to a pressure transducer (Spectramed P23XL transducer with Model 13-4615-58 amplifier; Gould, Cleveland, OH) and perfused with heparinized saline (2 U/mL) at 3.0 mL/h. All chronically infused agents were added to this infusate. The venous line was used for acute (bolus) injections. A four-limb electrocardiogram was monitored via an ECG/biotach amplifier (Model 13-4615-65A; Gould), and heart rate was derived. Colonic temperature was measured using a thermistor probe and controller (Model 73A; YSI), which provided closed-loop control of core temperature by switching a heated operating table. Signals for heart rate, mean arterial pressure, and colonic temperature were periodically sampled and stored with 12-bit precision at 1 Hz using a computerized data acquisition system (DT2801A A/D converters, DataTranslation, Marlboro, MA; AST Premium 386 computer, AST Research, Irvine, CA; and Labtech Notebook software, Laboratory Technologies, Wilmington, MA).

Analyses. Following a 1.5-hour postsurgical stabilization period, 120-μL arterial samples were collected into heparinized capillaries at intervals indicated by data points in the figures, and the separated plasma was analyzed immediately for glucose and lactate using immobilized-enzyme chemistries (glucose oxidase and L-lactate oxidase, Analyzer model 2300-STAT; YSI). In the intravenous glucose-challenge experiments, 250-μL arterial samples were collected for additional plasma insulin measurement. Insulin concentration was determined using a commercial kit (INCStar Insulin-^{125}I #06130; INCStar, Minneapolis, MN) with a sensitivity (20% full signal) of 28 pmol/L (4 μU/mL) and interassay coefficients of variation of 8.5% to 10% and intraassay coefficients of variation of 6.4% to 10.9%. The kit used rat insulin standards and cross-reacted 100% with human and porcine insulins.

Numerical methods. For dose-response studies, sigmoid curve–fitting was performed using ALLFIT (described earlier) to obtain 50% effective doses (ED_{50}s). The indicator response in the in vivo studies was the incremental area under the glucose or lactate concentration profiles relative to baseline, integrated (trapezoidal integral) over the 2 hours following peptide administration. Baseline was defined as the mean of three measurements made in the 30 minutes preceding peptide administration. The same derivations were used to quantify glucose responses in hypoglycemic rats and insulin responses to intravenous glucose. Pairwise comparisons were made using Student's t test, with $P = .05$ as the level for significance. Data throughout are expressed as the mean ± SEM.

Treatment Groups for In Vivo Studies

Intravenous bolus injections. Male Harlan-Sprague-Dawley rats prepared as detailed earlier and allowed a 1.5-hour postsurgical stabilization period were administered as an intravenous bolus one of the following: (1) vehicle only, 0.1 mL 0.15-mol/L saline (n = 6; age, 71 ± 4 days; weight, 328 ± 14 g; fasted 19.4 ± 0.8 hours); (2) synthetic rat amylin, 100 μg in 0.1 mL 0.15-mol/L saline (n = 7; age, 110 ± 2 days; weight, 310 ± 7 g; fasted 20.0 ± 0.7 hours); (3) synthetic salmon calcitonin, 100 μg in 0.1 mL 0.15-mol/L saline (n = 5; age, 95 ± 6 days; weight, 363 ± 5 g; fasted 20.0 ± 0.6 hours); and (4) synthetic rat calcitonin, 100 μg in 0.1 mL 0.15-mol/L saline (n = 6, age, 79 ± 1 days; weight, 346 ± 12 g; fasted 20.8 ± 0.3 hours).

Intravenous dose-response studies. Animals were treated as just described, except that the administered dose (all administered in 0.1 mL saline) ranged from 0.01 to 1,000 μg for amylin (molecular weight, 3,917.9; 8.5 pmol/kg to 850 nmol/kg; n = 23) and from 0.01 to 100 μg for salmon calcitonin (molecular weight, 3,429.7; 9.7 pmol/kg to 97 nmol/kg; n = 19). There were three to seven experiments at each dose level for each compound.

Comparison with glucagon in hypoglycemic rats. Animals prepared as above were started on a 0.1-U plus 0.5-U/h primed/continuous infusion of recombinant human insulin (Humulin-R) that continued until the end of the experiment. After 2 hours of insulin infusion, when plasma glucose had declined to a steady level of 3.2 ± 0.3 mmol/L, animals were administered an intravenous bolus containing one of the following: (1) vehicle only, 0.1 mL 0.15-mol/L saline (n = 5; age, 79 ± 5 days; weight, 318 ± 10 g; fasted 20.9 ± 0.5 hours); (2) synthetic salmon calcitonin (Bachem lot no. ZG407), 100 μg (29.2 nmol) in 0.1 mL 0.15-mol/L saline (n = 5; age, 72 ± 2 days; weight, 340 ± 11 g; fasted 21.1 ± 0.2 hours); (3) glucagon for injection USP (lot no. 4MC51D, Eli Lilly; containing glucagon 1 mg and lactose 49 mg constituted into 1 mL aqueous solution of 1.6% glycerin and 0.2% phenol) 100 μg (28.7 nmol) (n = 4; age, 87 ± 1 days; weight, 326 ± 8 g; fasted 19.9 ± 0.7 hours); or (4) combined glucagon/salmon calcitonin injection, 100 μg of each (n = 5; age, 72 ± 2 days; weight, 329 ± 18 g; fasted 21.0 ± 0.2 hours).

Insulin secretory responses. Anesthetized animals prepared as above were administered a 2-mmol (5.2 mmol/kg) intravenous glucose challenge over 2 minutes. This was performed during a continuous infusion of (1) vehicle only, 0.15-mol/L saline infused at a rate of 0.5 mL/h (n = 18; age, 94 ± 5 days; weight, 388 ± 5 g; fasted 21.8 ± 0.7 hours); (2) synthetic rat amylin, 70 pmol/kg/min in 0.15-mol/L saline infused at a rate of 0.5 mL/h (n = 6; age, 86 ± 5 days; weight, 378 ± 4 g; fasted 22.4 ± 1.6 hours); or (3) synthetic salmon calcitonin (Bachem lot no. ZG407), 70 pmol/kg/min in 0.15-mol/L saline infused at a rate of 0.5 mL/h (n = 6; age, 86 ± 1 days; weight, 381 ± 4 g; fasted 23.3 ± 0.8 hours). Infusion of the peptides began 60 minutes before the glucose challenge and continued for 60 minutes after the challenge.

RESULTS

Isolated Soleus Muscle

The effect of rat amylin, rat calcitonin, or salmon calcitonin on insulin-stimulated incorporation of [U-^{14}C]glucose into glycogen in the isolated, stripped soleus muscle is shown in Fig 2A. Similar to previous reports,[18,25] rat amylin inhibited the incorporation of label into glycogen with an EC_{50} of 8.4 nmol/L in this preparation. Salmon calcitonin inhibited labeling of soleus muscle glycogen with an EC_{50} of 0.4 nmol/L. Rat calcitonin was nearly 1,000-fold less potent than salmon calcitonin, with an EC_{50} of 376 nmol/L. Rat amylin and salmon calcitonin also inhibited labeling of muscle glycogen in the absence of insulin (data not shown), indicating that the effect of each, although functionally antagonizing the insulin response, was independent of it.

Incubation with rat amylin or salmon calcitonin dose-dependently depleted total (unlabeled) muscle glycogen, such that not greater than 6% to 14% of control levels remained after 60 minutes' exposure to concentrations greater than 10 nmol/L (Fig 2B). The EC_{50} for depletion of total muscle glycogen by rat amylin was 1.0 nmol/L, and by salmon calcitonin, 0.4 nmol/L. Salmon calcitonin dose-dependently activated glycogen phosphorylase in soleus muscle with an EC_{50} of 1.4 nmol/L ± 0.5 log units (Fig 2C).

In addition to quantifying a glycogenolytic action of rat amylin in soleus muscle, these data describe a previously unreported activation of glycogen phosphorylase and glycogenolytic action of salmon calcitonin in muscle.

Intravenous Injections

When injected as a 100-μg intravenous bolus into fasted anesthetized rats, rat amylin, salmon calcitonin, and rat calcitonin each caused a rapid elevation of plasma lactate and a slower elevation of plasma glucose (Fig 3A and B). For these effects at this dose, salmon calcitonin was most effective, rat amylin was next most effective, and rat calcitonin was least effective.

Dose-responses for plasma lactate and glucose increases, determined over a 0.1-μg to 1-mg range of intravenous bolus doses, are shown in Fig 3C and D. ED_{50}s for the hyperglycemic effects of amylin and salmon calcitonin were similar (10.8 and 4.4 μg, respectively). ED_{50}s were not determinable for the increase in plasma lactate, since no clear plateau (maximal response) was defined. However, with intravenous doses greater than 1 μg, salmon calcitonin was more effective than amylin in elevating plasma lactate concentration.

Intravenous injections of amylin 100-μg into fasted anesthetized rats were associated with a transient decrease in

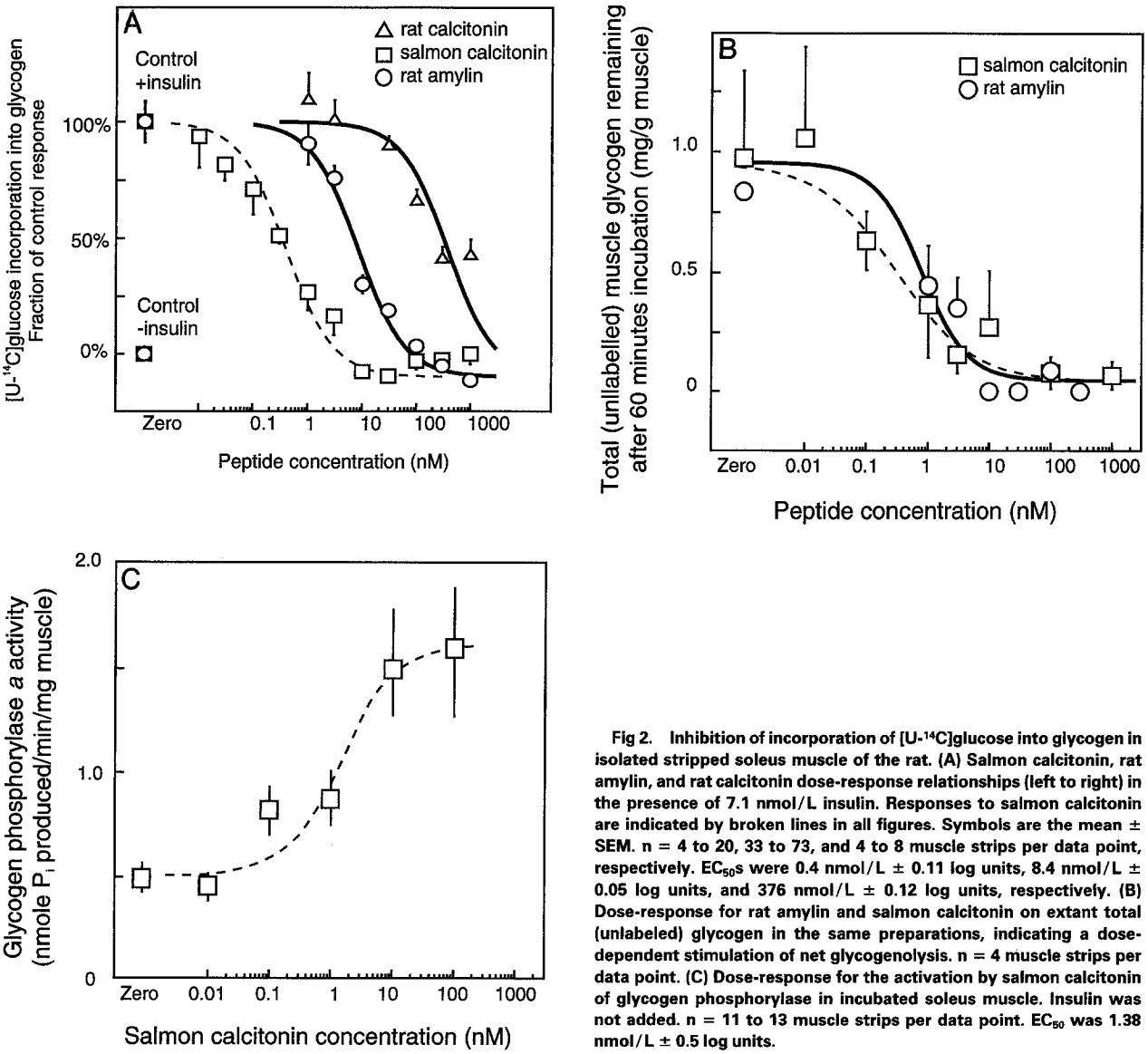

Fig 2. Inhibition of incorporation of [U-^{14}C]glucose into glycogen in isolated stripped soleus muscle of the rat. (A) Salmon calcitonin, rat amylin, and rat calcitonin dose-response relationships (left to right) in the presence of 7.1 nmol/L insulin. Responses to salmon calcitonin are indicated by broken lines in all figures. Symbols are the mean ± SEM. n = 4 to 20, 33 to 73, and 4 to 8 muscle strips per data point, respectively. EC_{50}s were 0.4 nmol/L ± 0.11 log units, 8.4 nmol/L ± 0.05 log units, and 376 nmol/L ± 0.12 log units, respectively. (B) Dose-response for rat amylin and salmon calcitonin on extant total (unlabeled) glycogen in the same preparations, indicating a dose-dependent stimulation of net glycogenolysis. n = 4 muscle strips per data point. (C) Dose-response for the activation by salmon calcitonin of glycogen phosphorylase in incubated soleus muscle. Insulin was not added. n = 11 to 13 muscle strips per data point. EC_{50} was 1.38 nmol/L ± 0.5 log units.

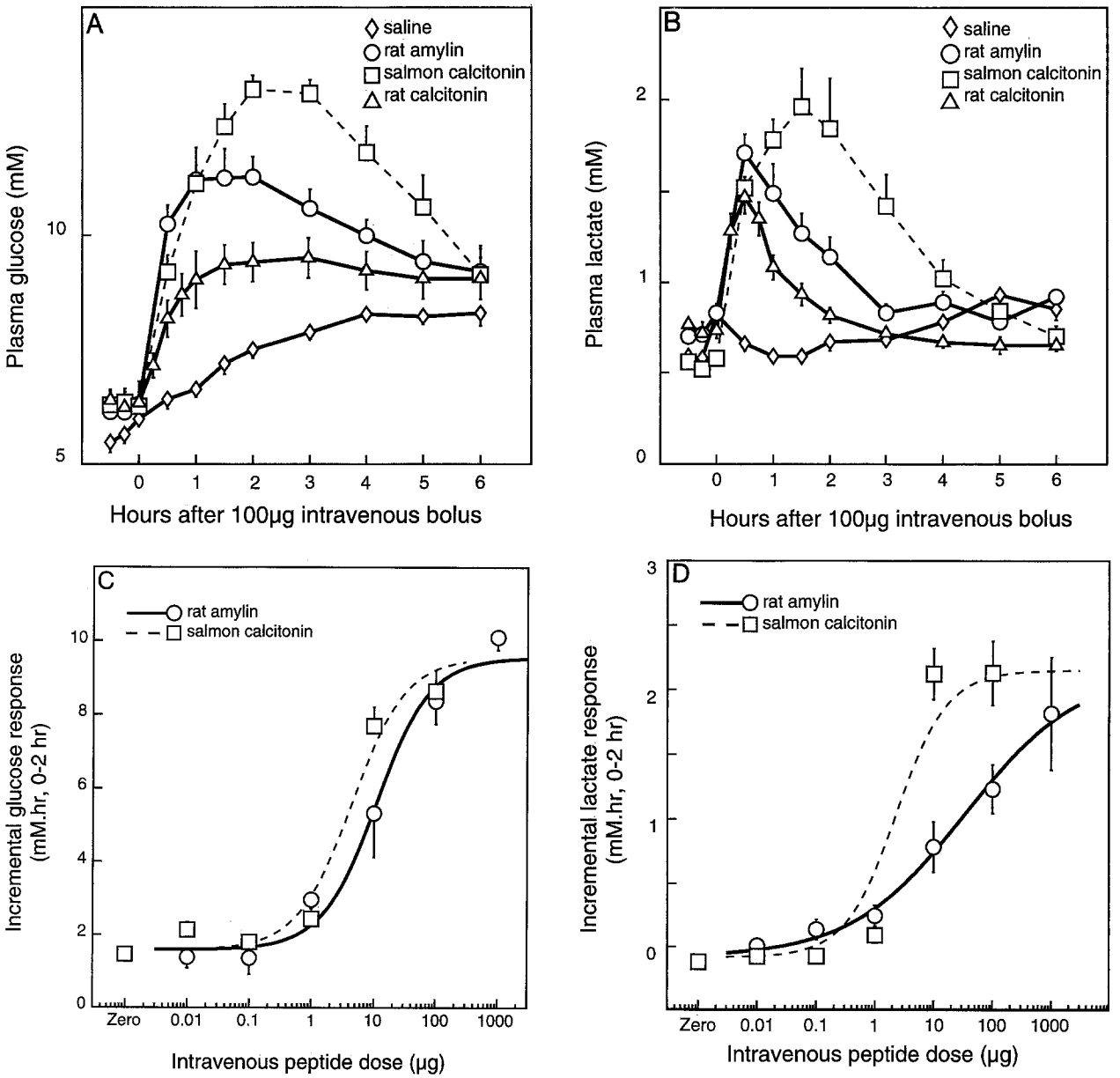

Fig 3. Effects of injection of rat amylin, salmon calcitonin, or rat calcitonin in fasted anesthetized rats. (A) Changes in plasma glucose following injection of 100 μg of either rat amylin (n = 7), salmon calcitonin (n = 5), or rat calcitonin (n = 6), compared with control saline infusions (n = 6). At 120 minutes, salmon calcitonin response > amylin response ($P < .001$), amylin response > rat calcitonin response ($P < .001$), and rat calcitonin response > saline control response ($P < .05$). (B) Effects of the same injections on plasma lactate. (C) Dose-dependency of the hyperglycemic effects of rat amylin and salmon calcitonin. The curves were not different from each other. (D) Hyperlactemic effects of these same injections. Lactate responses to salmon calcitonin were greater than those to amylin at the 10- and 100-μg doses ($P < .01$). Symbols and bars have the same meaning as in Fig 2.

arterial pressure, as previously reported.[22] The same dose of salmon calcitonin did not decrease arterial pressure. The arterial pressure response, quantified as the decrement from baseline integrated over the 30 minutes following bolus intravenous injection, was analyzed as a function of dose. There was no significant effect of salmon calcitonin on arterial pressure at any of the doses administered. The ED_{50} for the hypotensive effect of intravenously administered amylin was 12.8 nmol. In contrast, the ED_{50} for the hypotensive effect of human CGRPα has been previously reported to be 0.29 nmol,[24] consistent with observations in other preparations[30] that CGRP is up to 2 orders of magnitude more potent than amylin in vasodilator activity.

In fasted anesthetized rats made moderately hypoglycemic with a primed/continuous infusion of insulin, salmon calcitonin 100 μg produced a greater glycemic recovery (increment in plasma glucose integrated over 2 hours) than the same dose of glucagon (7.06 ± 0.425 *v* 3.90 ± 0.75

mmol/L · h, $P < .05$, Student's t test). A combined dose of salmon calcitonin 100 μg and glucagon 100 μg was synergistic in that the glycemic recovery, integrated as above, was approximately twice the sum of the separate responses to salmon calcitonin or glucagon (13.9 ± 0.93 mmol/L · h, 95% confidence interval = 11.3 to 16.5, $P < .05$ v summed responses of 7.06 ± 3.90 mmol/L · h; Fig 4A). A similar synergy between rat amylin and glucagon has also been described in an equivalent hypoglycemic model.[23]

Plasma lactate increased in both groups of animals in which salmon calcitonin was administered, ie, alone or in combination with glucagon. As previously reported,[23] lactate did not increase when glucagon was administered alone, despite an increase in plasma glucose. These observations indicate that the increase in lactate is associated with administration of salmon calcitonin, but not necessarily with administration of glucagon or with increases in plasma glucose (Fig 4B). A similar pattern has been observed with regard to administration of rat amylin, glucagon, or amylin plus glucagon.[23]

Insulin Secretory Response

The glycemic response to an intravenous glucose challenge in fasted, anesthetized rats preinfused with rat amylin or salmon calcitonin (5.0 and 5.5 μg/h, respectively) is shown in Fig 5A. Both rat amylin and salmon calcitonin infusions resulted in elevations of plasma glucose relative to controls at various time points throughout the test. At any given time point, the elevation of plasma glucose was greater with salmon calcitonin than with the same dose of rat amylin. The insulin response to glucose challenge is shown in Fig 5B. Despite plasma glucose being higher than in control rats, the insulin response within the first 60 minutes of the challenge was considerably less during infusions of either rat amylin (232 ± 39 v 387 ± 23 pmol/L · h, $P < .01$) or salmon calcitonin (302 ± 45 pmol/L · h, $P < .05$, one-tailed t test). During the second hour, insulin levels were considerably higher in salmon calcitonin–infused rats than in controls, possibly reflecting the persisting hyperglycemia in these animals.

DISCUSSION

The present study describes a novel spectrum of actions of salmon calcitonin on carbohydrate metabolism. This spectrum of actions includes (1) activation of skeletal muscle glycogen phosphorylase and of glycogenolysis, (2) inhibition of insulin-mediated incorporation of radioglucose into soleus muscle glycogen, (3) rapid increase of plasma lactate, (4) increase of plasma glucose when administered in vivo, and (5) synergism with glucagon in evoking these hyperglycemic effects. This spectrum of actions matches that previously reported for rat amylin.[31] There are several reports that salmon calcitonin produces hyperglycemia,[3,32] but with clinical doses, a tachyphylaxis appears to diminish this effect over a few days, so hyperglycemia does not persist as a complication in patients administered the peptide long term.[6,7,32] The pharmacological concentrations used in the present study served to elicit robust measurable

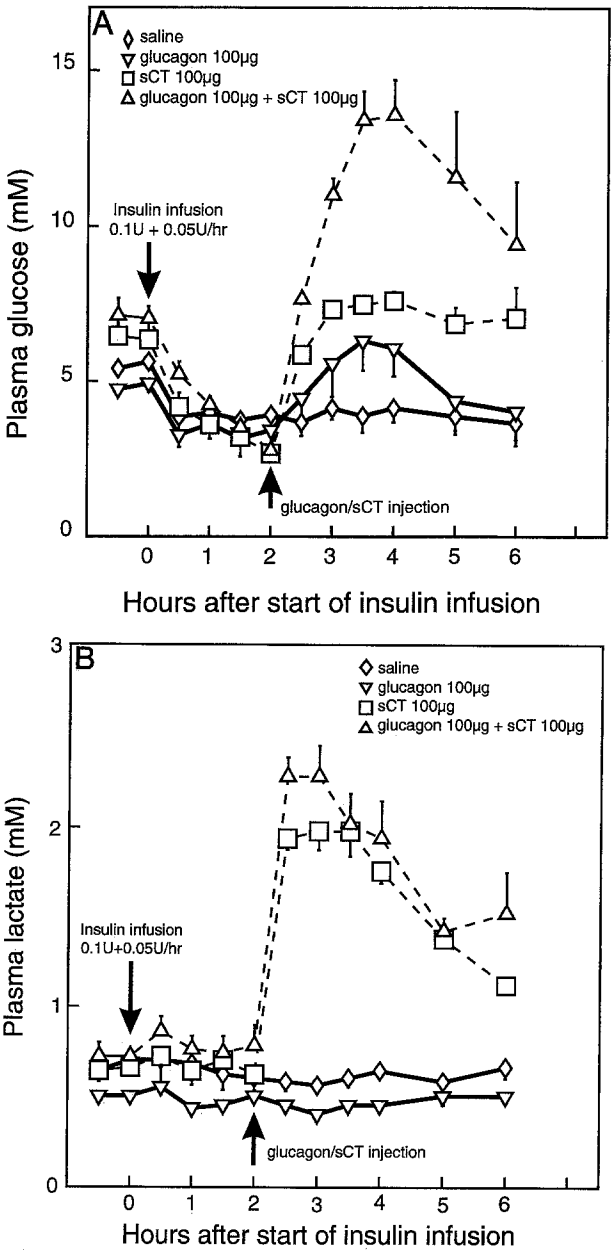

Fig 4. Synergistic effect of glucagon and salmon calcitonin during insulin-induced hypoglycemia. (A) Hyperglycemic response to combined glucagon + salmon calcitonin (measured as the incremental response integrated over the 2 hours after injection) was greater than the response to either peptide alone and greater than the sum of both. A similar synergy between the hyperglycemic effects of rat amylin and glucagon has recently been described.[23] (B) Plasma lactate responses to the same injections. SCT, subcutaneous.

responses to both amylin and salmon calcitonin, enabling comparison of a broad spectrum of actions. This comparison was not designed to address the physiological relevance of these actions.

Several studies, including those using the hyperlactemic clamp technique,[33] support the proposal that the hyperglycemic effects of exogenous amylin are mediated via the Cori

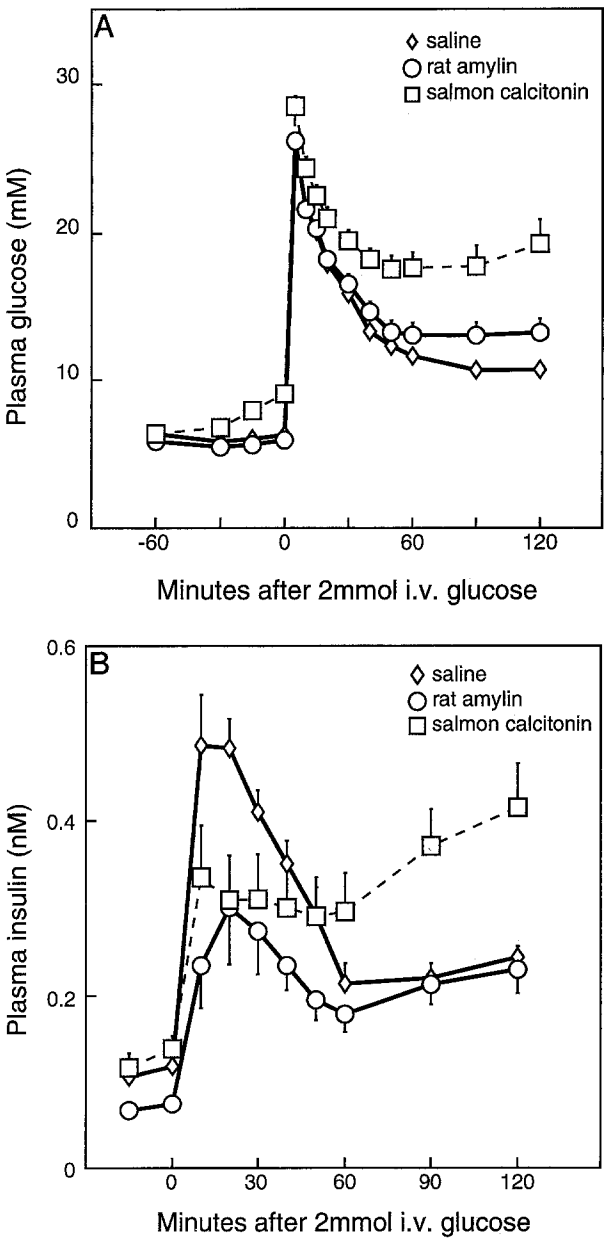

Fig 5. Effect of rat amylin or salmon calcitonin infusions on an intravenous (i.v.) glucose challenge. (A) Effects on glucose concentration. (B) Effects on insulin response to a 2-mmol glucose load infused into fasted anesthetized rats.

cycle.[34] By mobilizing peripheral glycogen stores, amylin increases the availability of lactate for gluconeogenesis. Glucose appearance then follows gluconeogenic conversion and is greater under circumstances of high gluconeogenic efficiency, such as during fasting or following glucagon administration.[23]

We propose that the principal mechanism responsible for the acute hyperglycemic action of salmon calcitonin is analogous to that of amylin just described. This proposal is supported by the unexpected finding that salmon calcitonin and rat amylin both bind with high affinity to sites in the *nucleus accumbens* of rat brain. Affinity for these binding sites has proven predictive of amylin agonism and antagonism in isolated-muscle and whole-animal preparations.[25] Indeed,[8-32] salmon calcitonin effectively blocks the metabolic actions of rat amylin without substantially affecting CGRP receptors.[35]

In its effects on the endocrine pancreas, salmon calcitonin also appears to mimic the actions of amylin. Several studies report that salmon calcitonin inhibits insulin release, as shown in Fig 5 of the present study.[3-6] In fact, before the present study, suppression of insulin secretion appears to have been the favored explanation for the hyperglycemic effect of salmon calcitonin.[4,6,7] Substantial evidence now supports a feedback inhibition of β-cell insulin secretion by amylin. In several preparations, including isolated β-cell lines,[36] isolated islets,[37] isolated perfused pancreas,[10,11] and intact rat,[38] rat amylin inhibits glucose-mediated insulin secretion or glucose-stimulated electrical activity.[39] In the isolated perfused pancreas, rat amylin exhibits an EC_{50} for the inhibition of insulin secretion of less than 75 pmol/L.[11] Further evidence for an effect of amylin on β-cell secretion comes from whole-animal[40] and isolated-islet preparations,[41] in which preinfusion with nonselective and selective amylin blockers amplified the insulin response to nutrient stimuli. In view of amylin's inhibitory effects at the β cell, the finding that salmon calcitonin behaves as a potent amylin agonist can explain the previously observed effects of salmon calcitonin to inhibit insulin secretion.

Several other actions are shared by both rat amylin and salmon calcitonin. Both rat amylin[42,43] and salmon calcitonin[44] dose-dependently suppress appetite following peripheral or intracerebroventricular administration. Both peptides stimulate plasma renin activity.[45-47] Both inhibit gastric emptying[48-50] and gastric acid secretion.[51,52] Both are hypocalcemic[13] and inhibit osteoclast motility.[14] And both peptides activate adenylyl cyclase in skeletal muscle[53,54] and in kidney.[45,55]

One action of amylin[30,56] not shared by salmon calcitonin,[57] exemplified in the present study, is its hypotensive effect. Instead, amylin shares this action with the related peptide, CGRP. The vasoactive effects of CGRP and amylin are likely to be mediated via the $CGRP_1$ receptor, since they can be blocked with $^{8\text{-}37}$hCGRP.[56] The observation that salmon calcitonin produces amylin-like effects on carbohydrate metabolism without changing arterial pressure implies that the receptor system through which such metabolic effects are mediated is separable from the CGRP receptor implicated in vascular control. That is, the data presented here add further to existing evidence[24,25] for amylin receptors that are distinct from CGRP receptors.

For actions reflective of effects on bone, such as hypocalcemia and inhibition of osteoclast motility, human calcitonin is 12 to 30 times more potent than human amylin.[14,58] However, the present study indicates that for actions reflective of effects on carbohydrate metabolism, such as on muscle glycogen metabolism in vitro and on plasma glucose and lactate in vivo, rat amylin is more potent than rat

calcitonin. These results suggest that receptors mediating the effects of rat amylin and rat calcitonin on carbohydrate metabolism and on calcium metabolism are distinguishable.

In summary, we propose that the hyperglycemic effects of salmon calcitonin are the result of mimicry of the metabolic effects of the endogenous mammalian peptide, amylin. In several other actions (listed earlier), salmon calcitonin also behaves as a mimic of amylin. Additionally, differing orders of potency of rat calcitonin, salmon calcitonin, CGRP, and amylin for their effects on blood pressure, carbohydrate metabolism, and calcium metabolism support the idea that these distinct biologic effects are mediated via distinct receptor systems.

ACKNOWLEDGMENT

We gratefully acknowledge the technical assistance of Pamela Klucas, Pamela Smith, Deborah Wolfe-Lopez, Erica Larson, and Stacey Provost. We also gratefully acknowledge the secretarial assistance of Nancy Gallaher-Wakefield.

REFERENCES

1. Azria M: Calcitonin in therapeutic use, in Azria M (ed): The Calcitonins—Physiology and Pharmacology. Basel, Switzerland, Karger, 1989, pp 133-144

2. Guttman S: Chemistry and structure-activity relationship of natural and synthetic calcitonins, in Pecile A (ed): Calcitonin 1980: Proceedings of an International Symposium. Amsterdam, The Netherlands, Excerpta Medica, 1981, pp 11-24

3. Petralito A, Lunetta M, Liuzzo A, et al: Effects of salmon calcitonin on blood glucose and insulin levels under basal conditions after intravenous glucose load. J Endocrinol Invest 2:209-211, 1979

4. Passariello N, Giugliano D, Sgambato S, et al: Calcitonin, a diabetogenic hormone? J Clin Endocrinol Metab 53:318-323, 1981

5. Ziegler R, Bellwinkel S, Schmidtchen D, et al: Effects of hypercalcemia, hypocalcemia and calcitonin on glucose stimulated insulin secretion in man. Horm Metab Res 4:60, 1972

6. Ziegler R, Deutschle U, Raue F: Calcitonin in human pathophysiology. Horm Res 20:65-73, 1984

7. Gattereau A, Bielmann P, Durivage J, et al: Effect of acute and chronic administration of calcitonin on serum glucose in patients with Paget's disease of bone. J Clin Endocrinol Metab 51:354-357, 1980

8. Lewis CE, Clark A, Ashcroft SJ, et al: Calcitonin gene–related peptide and somatostatin inhibit insulin release from individual rat B cells. Mol Cell Endocrinol 57:41-49, 1988

9. Pettersson M, Ahren B, Bottcher G, et al: Calcitonin gene–related peptide: Occurrence in pancreatic islets in the mouse and the rat and inhibition of insulin secretion in the mouse. Endocrinology 119:865-869, 1986

10. Kogire M, Ishizuka J, Thompson JC, et al: Inhibitory action of islet amyloid polypeptide and calcitonin gene–related peptide on release of insulin from the isolated perfused rat pancreas. Pancreas 6:459-463, 1991

11. Dégano P, Silvestre RA, Salas M, et al: Amylin inhibits glucose-induced insulin secretion in a dose-dependent manner—Study in the perfused rat pancreas. Regul Pept 43:91-96, 1993

12. MacIntyre I, Alevizaki M, Bevis PJ, et al: Calcitonin and the peptides from the calcitonin gene. Clin Orthop 217:45-55, 1987

13. Datta HK, Zaidi M, Wimalawansa SJ, et al: In vivo and in vitro effects of amylin and amylin amide on calcium metabolism in the rat and rabbit. Biochem Biophys Res Commun 162:876-881, 1989

14. Towhidul-Alam ASM, Moonga BS, Bevis PJR, et al: Amylin inhibits bone resorption by a direct effect on the motility of rat osteoclasts. Exp Physiol 78:183-196, 1993

15. Cooper GJS, Willis AC, Clark A, et al: Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. Proc Natl Acad Sci USA 84:8628-8632, 1987

16. Ogawa A, Harris V, McCorkle SK, et al: Amylin secretion from the rat pancreas and its selective loss after streptozotocin treatment. J Clin Invest 85:973-976, 1990

17. Leighton B, Cooper GJS: Pancreatic amylin and calcitonin gene–related peptide cause resistance to insulin in skeletal muscle in vitro. Nature 335:632-635, 1988

18. Young AA, Gedulin B, Wolfe-Lopez D, et al: Amylin and insulin in rat soleus muscle: Dose responses for cosecreted noncompetitive antagonists. Am J Physiol 263:E274-E281, 1992

19. Young DA, Deems RO, Deacon RW, et al: Effects of amylin on glucose metabolism and glycogenolysis in vivo and in vitro. Am J Physiol 259:E457-E461, 1990

20. Pittner R, Beaumont K, Young A, et al: Dose-dependent elevation of cyclic AMP activation of glycogen phosphorylase and release of lactate by amylin in rat skeletal muscle. Biochim Biophys Acta 1267:75-82, 1995

21. Young AA, Mott DM, Stone K, et al: Amylin activates glycogen phosphorylase in the isolated soleus muscle of the rat. FEBS Lett 281:149-151, 1991

22. Young AA, Wang MW, Cooper GJS: Amylin injection causes elevated plasma lactate and glucose in the rat. FEBS Lett 291:101-104, 1991

23. Young AA, Cooper GJS, Carlo P, et al: Response to intravenous injections of amylin and glucagon in fasted, fed, and hypoglycemic rats. Am J Physiol 264:E943-E950, 1993

24. Young AA, Rink TJ, Wang MW: Dose response characteristics for the hyperglycemic, hyperlactemic, hypotensive and hypocalcemic actions of amylin and calcitonin gene–related peptide-I (CGRP-alpha) in the fasted, anaesthetized rat. Life Sci 52:1717-1726, 1993

25. Beaumont K, Kenney MA, Young AA, et al: High affinity amylin binding sites in rat brain. Mol Pharmacol 44:493-497, 1993

26. Rink TJ, Beaumont K, Young AA: Salmon calcitonin potently mimics amylin actions on isolated soleus muscle and in increasing plasma lactate and glucose in rats. Br J Pharmacol 108:318P, 1993 (abstr)

27. Stalmans W, Hers HG: The stimulation of liver phosphorylase *b* by AMP, fluoride and sulfate. A technical note on the specific determination of the *a* and *b* forms of liver glycogen phosphorylase. Eur J Biochem 54:341-350, 1975

28. Hue L, Bontemps F, Hers H: The effects of glucose and of potassium ions on the interconversion of the two forms of glycogen phosphorylase and of glycogen synthetase in isolated rat liver preparations. Biochem J 152:105-114, 1975

29. LeBel D, Poirier GG, Beaudoin AR: A convenient method for the ATPase assay. Anal Biochem 85:86-89, 1978

30. Brain SD, Wimalawansa S, MacIntyre I, et al: The demonstration of vasodilator activity of pancreatic amylin amide in the rabbit. Am J Pathol 136:487-490, 1990

31. Rink TJ, Beaumont K, Koda J, et al: Structure and biology of amylin. Trends Pharmacol Sci 14:113-118, 1993

32. Stracca M, Doepfner W: Hyperglycaemic effect of calcitonin: Development and reversibility of tolerance, in Doepfner W (ed): Calcitonin 1984. Amsterdam, The Netherlands, Excerpta Medica, 1986, pp 48-55

33. Rink T, Gedulin B: Lactate clamp: A new technique for studying carbohydrate metabolism in vivo. Diabetes 42:245A, 1993 (abstr)

34. Young AA: Amylin and its effects on the Cori cycle, in Taking Control in Diabetes. Surrey, UK, Synergy Medical Education, 1993, pp 16-20

35. Beaumont K, Kenney MA, Wolfe-Lopez D, et al: A receptor antagonist selective for amylin over CGRP blocks amylin's metabolic actions. Diabetes 42:215A, 1993 (abstr)

36. Murakami M, Suzuki S, Sato Y, et al: Effects of amylin on insulin secretion from RINm5F cells. Diabetes 39:266A, 1990 (abstr)

37. Ohsawa H, Kanatsuka A, Yamaguchi T, et al: Islet amyloid polypeptide inhibits glucose-stimulated insulin secretion from isolated rat pancreatic islets. Biochem Biophys Res Commun 160:961-967, 1989

38. Fürnsinn C, Leuvenink H, Roden M, et al: Inhibition of glucose induced insulin secretion by amylin in rats in vivo. Diabetologia 35:29A, 1992 (abstr)

39. Wagoner PK, Chen C, Worley JF, et al: Amylin modulates beta-cell glucose sensing via effects on stimulus-secretion coupling. Proc Natl Acad Sci USA 90:9145-9149, 1993

40. Young AA, Carlo P, Rink TJ, et al: $^{8\text{-}37}$hCGRP, an amylin receptor antagonist, enhances the insulin response and perturbs the glucose response to infused arginine in anesthetized rats. Mol Cell Endocrinol 84:R1-R5, 1992

41. Wang Z, Bennet WM, Ghatei MA, et al: Influence of islet amyloid polypeptide and the 8-37 fragment of islet amyloid polypeptide on insulin release from perifused rat islets. Diabetes 42:330-335, 1993

42. Chance WT, Balasubramaniam A, Zhang FS, et al: Anorexia following the intrahypothalamic administration of amylin. Brain Res 539:352-354, 1991

43. Morley JE, Flood JF: Amylin decreases food intake in mice. Peptides 12:865-869, 1991

44. Yamamoto Y, Nakamuta H, Koida M, et al: Calcitonin-induced anorexia in rats: A structure-activity study by intraventricular injections. Jpn J Pharmacol 32:1013-1017, 1982

45. Young AA, Vine W, Carlo P, et al: Amylin stimulation of renin activity in rats: A possible link between insulin resistance and hypertension. J Hypertens 12:S152, 1994 (suppl 3)

46. Clementi G, Rapisarda E, Fiore CE, et al: Effects of salmon calcitonin on plasma renin activity and systolic blood pressure in the rat. Neurosci Lett 66:351-355, 1986

47. Malatino LS, Fiore CE, Foti R, et al: Acute effects of salmon calcitonin in man include stimulation of the renin-angiotensin-aldosterone system. Miner Electrolyte Metab 13:316-322, 1987

48. Brown K, Menius A, Sandefer E, et al: The effects of amylin on changes in plasma glucose and gastric emptying following an oral glucose load in conscious dogs. Diabetes 43:172A, 1994 (abstr)

49. Young AA, Gedulin B, Vine W, et al: Gastric emptying is accelerated in diabetic BB rats and is slowed by subcutaneous injections of amylin. Diabetologia 38:642-648, 1995

50. Jonderko G, Golab T, Jonderko K: Calcitonin suppresses gastric emptying of a radiolabelled solid meal in humans. Br J Clin Pharmacol 24:103-105, 1987

51. Doepfner W: Effects of synthetic salmon calcitonin on gastric secretion and ulcer formation in conscious cats and rats, in Goebell H, Hotz J (eds): Effects of Calcitonin and Somatostatin on the Gastrointestinal Tract and Pancreas. Demeter Verlag, Grafelfing, 1976, pp 60-70

52. Guidobono F, Coluzzi M, Pagani F, et al: Amylin given by central and peripheral routes inhibits gastric acid secretion. Peptides 15:699-702, 1994

53. Kenney MA, Moore CX, Pittner R, et al: Salmon calcitonin binding and stimulation of cyclic AMP generation in rat skeletal muscle. Biochem Biophys Res Commun 197:8-14, 1993

54. Pittner RA, Wolfe-Lopez D, Lupien J, et al: Amylin increases cyclic AMP levels and stimulates glycogenolysis in rat skeletal muscle. Diabetologia 37:117A, 1994 (abstr)

55. Katz AI, Lindheimer MD: Actions of hormones on the kidney. Annu Rev Physiol 39:97-133, 1977

56. Gardiner SM, Compton AM, Kemp PA, et al: Antagonistic effect of human α-calcitonin gene–related peptide [8-37] on the regional hemodynamic actions of rat islet amyloid polypeptide in conscious Long Evans rats. Diabetes 40:948-951, 1991

57. Peguero-Rivera AM, Corder CN: Hemodynamic effects of calcitonin in the normal rat. Peptides 13:571-575, 1992

58. MacIntyre I, Moonga BS, Zaidi M: Amylin, a new calcium-regulating hormone from the pancreas. J Physiol (Lond) 434:78P, 1991