Vol. 179, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1991 Pages 1-9

CO-SECRETION OF AMYLIN AND INSULIN FROM CULTURED ISLET β-CELLS: MODULATION BY NUTRIENT SECRETAGOGUES, ISLET HORMONES AND HYPOGLYCEMIC AGENTS

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Received July 10, 1991

Amylin is a pancreatic islet β-cell peptide hormone which modulates carbohydrate metabolism in skeletal muscle and liver, and could contribute to impaired insulin sensitivity in Type II diabetes. Here we report the first description of amylin secretion from isolated β-cells. We measured amylin secretion from HIT T15 β-cells exposed to glucose, arginine, glucagon, somatostatin, tolbutamide, glyburide, or metformin. With the exception of glucagon at concentrations above 1μM, all compounds induced parallel, dose-dependent changes in secretion of amylin and insulin. We conclude that: 1) insulin and amylin are co-secreted from islet β-cells; (2) nutrient secretagogues and peptide modulators exert direct effects on β-cells to alter amylin and insulin secretion; (3) most modulators of islet β-cell secretion alter amylin and insulin in parallel, but differential secretion can occur; and (4) the HIT cell line is a useful model in which to study amylin metabolism. 1991 Academic Press, Inc.

Amylin is a recently discovered 37 amino acid pancreatic islet protein (1) that is reported to be co-secreted with insulin (2). It was isolated, purified, and sequenced from islet amyloid, and appears to be the major component of the extracellular deposits which are widespread in the islets of patients with non-insulin-dependent diabetes mellitus (NIDDM) (3). Amylin is similar in sequence to the major peptide component of insulinoma amyloid, islet amyloid polypeptide (IAPP) (4). The association of amyloid deposits with NIDDM, and the findings that amylin reduces insulin action in isolated muscle and in intact animals support the proposal that excess amylin action is a pathogenic mechanism in insulin resistant states, including obesity, impaired glucose tolerance, and NIDDM (1,5,6).

Localization of amylin mRNA (7) and presumably amylin protein (8) by cytochemical techniques indicates that most of the islet amylin is in β-cells, *i.e.* is co-localized perhaps with insulin in the same secretory granules. Additionally, at least in rats, there appear to be amylin containing δ-cells, which are known to secrete somatostatin (K.L. Luskey, personal communication). In view of the possible pathologic role of islet amyloid, which could reflect inappropriate secretion of amylin (1), and the proposed endocrine role of amylin in fuel metabolism (1,5,6) and in the pathogenesis of insulin resistant states, it will be important to elucidate the mechanisms controlling amylin secretion.

Previous studies have measured amylin secretion from intact perfused pancreases (2) and isolated whole islets (9), but no study using isolated β-cells has been reported. In order to analyze

0006-291X/91 $1.50

the direct responses of β-cells, distinct from any influence of other islet cell types, we employed the continuous HIT-T15 (HIT) cell line (10) as a model of β-cell function. These cells remain responsive to the nutrient secretagogues glucose and arginine, and to the potential paracrine modulators somatostatin and glucagon. Thus we reasoned that the HIT cell line might be a fruitful system for investigating amylin secretion.

In the present work, a radioimmunoassay suitable for monitoring HIT cell amylin secretion was developed and the secretion of amylin and insulin were measured during 60 minute static incubations with different concentrations and combinations of nutrient and paracrine secretagogues. In addition, the responses to compounds from two classes of hypoglycemic agents used in the treatment of NIDDM, sulphonylureas and biguanides, were evaluated. In general, rates of amylin secretion paralleled rates of insulin secretion, although high concentrations of glucagon did result in a differential secretory response. We discuss the physiologic and pathophysiologic implications of these findings, and comment on the measured molar ratios of secreted amylin and insulin in HIT cells and other preparations.

MATERIALS AND METHODS

Cell culture. HIT cells (American Type Tissue Collection, Rockville, MD) were subcultured weekly in Ham's F-12 medium (Irvine Scientific, Santa Ana, CA) containing 10% horse serum (Gemini Bioproducts, Calabasas, CA), 2.5% fetal bovine serum (FBS; Gemini), 2 mM L-glutamine, 10^5 IU penicillin G/l, 100 μg streptomycin/l, and 10 mM glucose. Cells were maintained at 37°C and 5% CO_2/95% humidified air, and medium was replaced every 2 to 3 days. All experiments were performed with cells from passages 62 to 71.

Stimulation of hormone secretion. All reagents were of analytical grade or better unless otherwise stated. Five days before each study, cells were subcultured at 0.26 x 10^6 cells/0.8 ml medium/well in 24-well tissue culture plates (Corning Glass Works, Corning, NY). Medium was replaced on day 3. On day 5, cells were washed twice at 37°C with 1.0 ml Krebs-Ringer buffer (KRB) of the following composition (in mM): NaCl, 119; KCl, 4.74; $CaCl_2$, 2.54; $MgSO_4$, 1.19; KH_2PO_4, 1.19; $NaHCO_3$, 25; *N*-2-hydroxy-ethylpiperazine-*N'*-2-ethanesulfonic acid, 10; and protease-free bovine serum albumin (BSA; Cohn fraction V; Sigma Chemical Co., St. Louis, MO), 1 g/l at pH 7.4. After washing, 1.0 ml KRB was added and plates were incubated (30 min, 37°C) in equilibrium with air. Cells were washed twice more prior to addition of 0.5 ml KRB containing the stated concentrations of test substance(s). Glucagon stock solutions contained 300 TIU aprotinin/l (affinity purified; Sigma). After a 60 min incubation with test substance(s), supernatants were centrifuged (5 min, 500 x g, 5°C; GPKR centrifuge, Beckman Instruments, Inc., Palo Alto, CA) to remove cell debris, then stored in polypropylene test tubes (Brinkman Instrument Co., Westbury, NY) at -20°C until analyzed by radioimmunoassay. Supernatants were analyzed for insulin and amylin content within one week of collection.

Three wells per plate were randomly selected for cell quantitation. Cells were incubated with trypsin, 75 mg, and Na_2EDTA, 30 mg, in 150 ml normal saline (8 min, 37°C), then counted in a Coulter Counter (Model Zf, Coulter Electronics, Hialeah, FL). Cell densities averaged 0.64 ± 0.18 x 10^6 cells/ well (mean ± S.D.) in 13 experiments.

Intracellular amylin, insulin and protein contents. HIT cells (10^7 cells/ml) were sonicated in 30% acetonitrile/0.2 M HCl for 20 seconds on ice then incubated overnight at 4°C. Suspensions were centrifuged at 15,000 rpm, 4°C for 5 minutes to remove debris and supernatants were analyzed for amylin and insulin content by radioimmunoassay. Acetonitrile did not interfere with either assay at concentrations below 0.6% (vol/vol). Levels of insulin extracted by this procedure were comparable with those extracted with acidified ethanol. Total protein content was measured in cell suspensions incubated in 0.1 N NaOH for 1 hour at 37°C using the Bradford reagent and BSA standards (11).

Hormone content in β-cell culture supernatants. Immunoreactive insulin content in supernatants was measured using a commercial radioimmunoassay (IM.78; Amersham, Arlington Heights, IL). Human insulin standards were prepared in KRB. Hamster insulin in supernatants co-diluted with human standards in the range 0.50 to 1.70 pmol/ml (results not shown).

The radioimmunoassay for immunoreactive amylin was developed in our laboratory. Assay diluent was phosphate-buffered saline (PBS; of the following composition, in mM: NaCl, 137; KCl, 2.68; KH_2PO_4, 1.47; Na_2HPO_4, 6.65) containing Triton-X 100, 0.1% (vol/vol), NaN_3, 0.01% (wt/vol), and gelatin, 0.1% (vol/vol; teleostean, 45% aqueous solution, (wt/vol), Sigma) at pH 7.4. All incubations were performed at 4°C. ^{125}I-[Tyr-37]-synthetic human amylin was iodinated by the chloramine-T method and purified by reverse phase chromatography on a C18 Sep-Pak (Waters Associates, Milford MA). Specific activity was approximately 500 mCi/mg.

One hundred µl of human synthetic amylin standard (50 to 1200 pg/ml KRB; Bachem, Torrance, CA; mol. wt. human amylin = 3904 Da) or unknown sample was combined with 100 µl polyclonal rabbit antiserum specific for human amylin (1:20,000, Peninsula Laboratories, Belmont, CA; no detectable cross-reactivity with human insulin, glucagon, somatostatin, pancreatic polypeptide, or calcitonin gene-related peptide-2 (CGRP-2); 0.1% cross-reactivity with rat CGRP-1) in 12 x 75 mm polystyrene tubes (Sarstedt, Hayward, CA). Standards and unknowns were assayed in triplicate or duplicate respectively. After an 18 hr incubation, 100 ml of tracer containing 20,000 dpm was added, and samples were incubated for an additional 18 h. To ensure complete immunoprecipitation of amylin:antibody complexes, 100 µl of rabbit gamma globulin (0.35 mg/ml, Scantibodies Laboratory, Santee, CA) and 100 µl of goat-anti-rabbit gamma globulin (0.50 mg/ml, purified grade; Scantibodies) were added. After a 15 min incubation, 1.0 ml 2% polyethylene glycol (wt/vol; mol. wt. = 8 kDA; Sigma) in assay buffer was added, and samples were centrifuged (30 min, 1500 x g, 5°C). Samples were decanted and pellets counted on a gamma counter (model 1277, LKB Wallac, Turku, Finland).

Data are expressed as the percentage change in pmol of hormone secreted/ 10^6 cells, relative to controls (absence of test substance) or as pmol of amylin secreted/pmol of insulin secreted (A/I ratio).

Statistical analysis and data handling. Results are expressed as the mean ± SEM of n replicate independent experiments. Glucose response curves for amylin and insulin secretion were compared using a 4-parameter logistic equation (ALLFIT program, National Institutes of Health, Bethesda MD). Statistical analyses were performed using the Statview 512+ program (BrainPower, Inc., Calabasas, CA). Secretion in the presence and absence of test substance(s) was compared by analysis of variance using Dunnett's test for comparison of multiple experimental groups with a control. Correlations between amylin and insulin secretion were determined by linear regression analysis. Significance was set at $P < 0.05$ unless otherwise stated.

RESULTS

Amylin radioimmunoassay. Fig. 1 shows a typical standard curve for the displacement of ^{125}I-[Tyr-37]-human amylin by unlabeled human amylin. The amylin radioimmunoassay had a

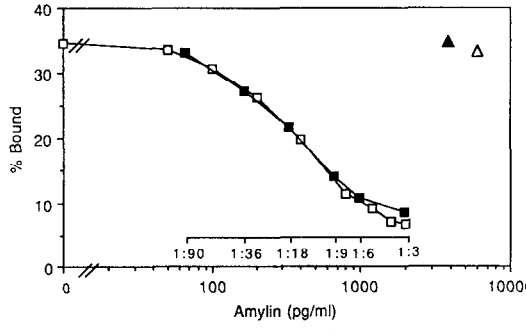


Fig. 1. Amylin Radioimmunoassay. Displacement of ^{125}I-[Tyr 37]-human amylin by increasing amounts of human amylin (□) and hamster amylin (■) are shown above. Hamster amylin in HIT cell supernatant was diluted in KRB as indicated. Each point represents the mean of triplicate (human) or duplicate (hamster) determinations. Glucagon (▲; 5 µM) and somatostatin (△; 100 nM) had no effect on the displacement curve.

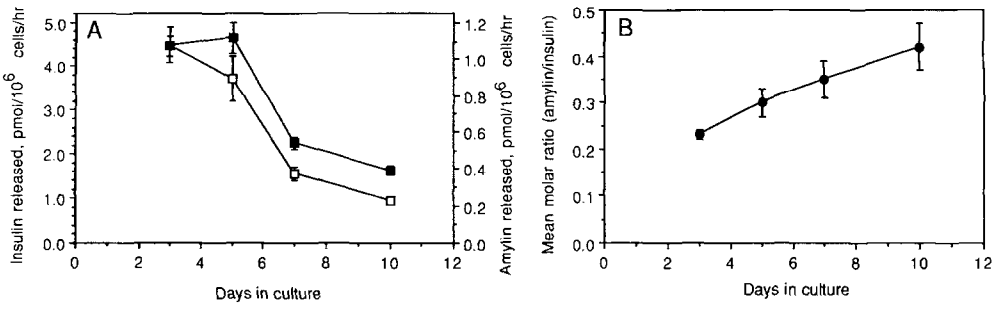


Fig. 2. (A) Glucose-stimulated amylin and insulin release. HIT cells were incubated for 60 minutes with increasing concentrations of glucose. Data are expressed as % of basal amylin (■) or insulin (□) release, which occurred in the absence of glucose. Mean basal amylin and insulin release were 0.120 ± 0.026 and 0.443 ± 0.175 pmol/10^6 cells respectively. Mean ± SEM of 4 replicate experiments. (B) Arginine-stimulated amylin and insulin release. HIT cells were incubated for 60 minutes with increasing concentrations of arginine. Data are expressed as % of basal amylin (■) or insulin (□) release, which occurred in the absence of arginine. Mean basal amylin and insulin release were 0.145 ± 0.035 and 0.369 ± 0.073 pmol/10^6 cells respectively. Mean ± SEM of 7 replicate experiments.

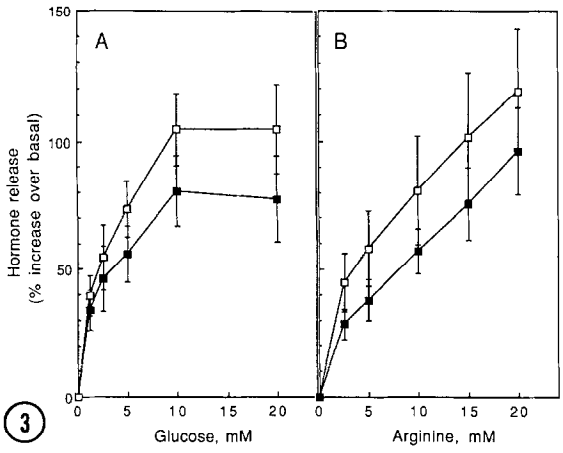
sensitivity of 8 pg amylin/ml and a linear range of 200 to 800 pg/ml. Amylin-like immunoreactivity in HIT cell supernatants co-diluted with human amylin within the linear range. Recovery of human amylin added to HIT cell supernatants was 100 ± 15% within this range. Glucagon (5 μM) and somatostatin (100 nM) had no effect on the displacement curve (Fig. 1). All experimental values were read from the linear portion of the standard curve.

Stimulation of amylin and insulin release by glucose and arginine. HIT cells contained 48.5 ± 18.2 ng amylin and 73.6 ± 27.7 ng of insulin per 10^6 cells; the molar A/I ratio in cell extracts was 0.98 ± 0.08 (mean ± SD, n=3).

Physiological concentrations of glucose stimulated amylin and insulin secretion from HIT cells in a dose-dependent manner (Fig. 2A). The half-maximal responses of both hormones occurred at approximately 3 mM glucose. Maximum secretion, occurring at 10 mM glucose, was 0.296 ± 0.046 and 0.834 ± 0.197 pmol/10^6 cells/hour for amylin and insulin, respectively, or 4.84 ± 0.75 and 20.26 ± 4.79 ng/mg protein/hour, based on a total protein content of 0.233 ± 0.070 mg /10^6 cells. There was a strong correlation between the amounts of amylin and insulin secreted ($r = 0.952$, $P < 0.0001$). The mean A/I ratio was 0.41 ± 0.06 and did not vary significantly within the glucose concentration range studied.

Arginine also stimulated amylin and insulin secretion in a parallel, dose-dependent manner (Fig. 2B). Maximum hormone secretion was not observed under the conditions tested. Changes in amylin and insulin secretion in response to increasing arginine were comparable ($r = 0.926$, $P < 0.0001$). The mean A/I ratio was 0.44 ± 0.02, independent of arginine concentration in the range tested.

Glucose-stimulated secretion was measured in HIT cells cultured for 3, 5, 7 or 10 days (Fig 3A). Secretion in response to 10 mM glucose was maximum for both amylin and insulin on day 5 and tended to decline with longer culture periods. Because the decreases in amylin and insulin secretion were not proportional (*i.e.* insulin secretion decreased more rapidly than amylin secretion), the A/I ratio steadily increased from 0.23 ± 0.01 on day 3 to 0.42 ± 0.05 pmol/10^6



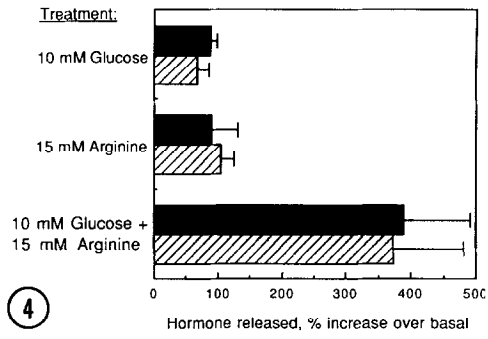


Fig. 3. Amylin and insulin secretion from HIT cells following increasing culture periods. (A) After 3, 5, 7 or 10 days in culture, HIT cells were incubated for 60 minutes with 10 mM glucose. Data are expressed as pmol amylin (■) or insulin (□) released/10^6 cells/hour. Mean ± SEM of 4 replicate wells. (B) Mean molar A/I ratio following increasing culture periods. Cells were treated as described above. Mean ± SEM of 4 replicate wells.

Fig. 4. Arginine enhancement of glucose-stimulated secretion. HIT cells were incubated for 60 minutes with glucose or arginine alone or in combination. Data are expressed as % of basal amylin (cross-hatched bars) or insulin (solid bars) release in the absence of secretagogues. Mean ± SEM of 3 replicate experiments. Mean basal amylin and insulin release were 0.184 ± 0.047 and 0.489 ± 0.070 pmol/10^6 cells respectively.

cells after 10 days in culture ($P<0.001$; Fig. 3B). Maximum secretion coincided with entry of cells into the exponential phase of growth (data not shown).

Potentiation of hormone secretion. Arginine has previously been shown to potentiate glucose-stimulated amylin secretion from isolated, perfused rat pancreases (2). In HIT cells, amylin and insulin secretion in response to the combination of 10 mM glucose and 15 mM arginine were both increased 2.2-fold compared to the sum of the rates in the presence of individual secretagogues (Fig. 4.). The A/I ratio did not change with the combined secretagogues relative to either secretagogue alone (10 mM glucose: 0.32 ± 0.01; 15 mM arginine: 0.41 ± 0.05; 10 mM glucose plus 15 mM arginine: 0.34 ± 0.01, n = 3; P = n.s.).

Glucagon modulation of amylin and insulin secretion. Glucagon dose-dependently enhanced both amylin and insulin secretion from HIT cells incubated in the presence of mixed 1.25 mM glucose plus 1.25 mM arginine (Fig. 5A). At 5 μM glucagon the A/I ratio was significantly reduced; insulin release exceeding amylin release by up to 1.5-fold (Table 1).

Somatostatin modulation of amylin and insulin secretion. Somatostatin suppressed both amylin and insulin release from HIT cells stimulated with 10 mM glucose plus 15 mM arginine (Fig. 5B). Amylin and insulin secretion remained proportionate with increasing somatostatin ($r = 0.991$, $P < 0.0001$). The A/I ratio was 0.36 ± 0.02 over the somatostatin concentration range tested.

Modulation of amylin and insulin release by tolbutamide, glyburide, and metformin. The effects of tolbutamide, glyburide and metformin on glucose-stimulated secretion are compared in Table 2. In the presence of 10 mM glucose the sulphonylureas stimulated an

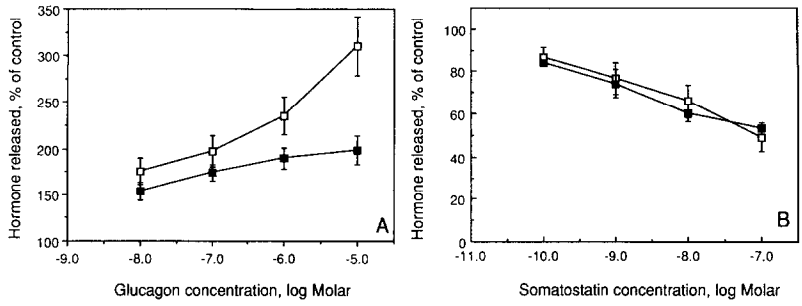


Fig. 5. (A) Glucagon enhancement of amylin and insulin release. HIT cells were incubated for 60 minutes with increasing concentrations of glucagon in the presence of 1.25 mM glucose plus 1.25 mM arginine. Data are expressed as % of amylin (■) or insulin (□) released in response to glucose plus arginine. Mean amylin and insulin release in control wells were 0.367 ± 0.096 and 1.058 ± 0.316 pmol/10^6 cells, respectively. Mean ± SEM of 4 replicate experiments. (B) Somatostatin suppression of amylin and insulin release. HIT cells were incubated for 60 minutes with increasing concentrations of somatostatin in the presence of 10 mM glucose plus 15 mM arginine. Data are expressed as % of amylin (■) or insulin (□) release in response to glucose plus arginine. Mean amylin and insulin released in control wells were 0.528 ± 0.254 and 1.617 ± 0.877 pmol/10^6 cells respectively. Mean ± SEM of 3 replicate experiments.

increase in amylin and insulin secretion. Glyburide was at least 100-fold more potent than tolbutamide, a finding consistent with the known greater potency of second generation sulphonylureas (*e.g.* glyburide) compared to first generation compounds (*e.g.* tolbutamide) (12). Mean molar A/I ratios were 0.17 ± 0.01 and 0.16 ± 0.01 for tolbutamide and glyburide, respectively. The biguanide metformin, in contrast to the sulphonylureas, failed to increase amylin or insulin secretion above basal levels when tested at concentrations up to 100 μM.

DISCUSSION

Amylin and insulin secretion increased in a dose-dependent manner in response to the nutrient secretagogues glucose and arginine, and both were potentiated equally by mixed glucose and arginine.

Amylin secretion decreased in a dose-dependent manner with increasing concentrations of the islet δ-cell/gut hormone somatostatin. The effects of somatostatin to diminish amylin and insulin secretion were equivalent. These results are consistent with a previous report that somatostatin

Table 1. Effects of increasing glucagon concentrations on the mean amylin/insulin (A/I) secretion ratio

Glucagon Concentration (μM)	*n*	Molar A/I Ratio
0.0	14	0.39 ± 0.08
0.01	15	0.34 ± 0.11
0.1	15	0.35 ± 0.13
1.0	15	0.31 ± 0.09
5.0	16	0.26 ± 0.08 †

A/I ratio (mean ± SD) determined for *n* individual wells. Data are taken from experiments described in figure 5A. † denotes significant difference from controls (absence of glucagon); ANOVA, Dunnett's test; P<0.05.

Table 2. Effects of tolbutamide, glyburide, and metformin on glucose-stimulated secretion from HIT cells

Treatment	Concentration	Hormone released (% of control) Amylin	Insulin	A/I Ratio
Tolbutamide	10 μM	126.5 ± 3.8	112.1 ± 6.2	0.14 ± 0.01
	100 μM	142.2 ± 2.5	139.5 ± 8.4	0.19 ± 0.02
Glyburide	0.1 μM	126.7 ± 9.5	158.4 ± 4.1	0.17 ± 0.01
	1.0 μM	141.5 ± 8.5	134.9 ± 7.5	0.15 ± 0.01
Metformin	10 μM	97.9 ± 8.5	98.7 ± 1.9	0.22 ± 0.02
	100 μM	93.4 ± 3.8	94.8 ± 3.7	0.22 ± 0.01

Cells were incubated for 60 minutes with 10 mM glucose in the presence of test substances. Data are expressed as % of amylin or insulin released in the presence of 10 mM glucose alone (control). Mean amylin and insulin released in control wells were 0.422 ± 0.015 and 2.369 ± 0.108 pmol/10^6 cells/hour respectively. Mean ± SEM of 4 replicate wells.

decreased amylin secretion in humans (13) and with the known effect of somatostatin to inhibit insulin secretion (14). It is thought that somatostatin may exert this effect *in vivo* to modulate insulin secretion in a physiologically relevant manner (14). Our results provide evidence that somatostatin could also regulate amylin secretion through paracrine or endocrine mechanisms.

Amylin and insulin secretion from cultured islet β-cells were both stimulated in a dose-dependent fashion by the islet α-cell/gut hormone glucagon, which has previously been shown to exert modulatory effects on insulin secretion from islets (14,15). Our results provide evidence that glucagon directly stimulates amylin secretion from islet β-cells. Glucagon, originating either from pancreatic islet α-cells or the gut, could therefore be a physiological paracrine regulator of amylin secretion in the islet *in vivo*..

In the present study, a high concentration of glucagon produced a significant decrease in the molar A/I ratio secreted from β-cells. In contrast to the effects of the other substances studied, glucagon stimulated secretion of insulin to a greater extent than that of amylin. This result is significant, as it indicates that isolated islet β-cells can differentially secrete amylin and insulin. The differential response of amylin and insulin to modulation by glucagon could have importance for future understanding of the interaction between islet hormones in the control of islet function, and also of their relative roles in the regulation of intermediary metabolism. The physiologic significance of this differential effect of glucagon on amylin and insulin secretion is unclear in view of the high glucagon concentration required to observe the effect. However, the concentration of glucagon adjacent to β-cell membranes in the islet *in vivo* could conceivably be of the same order as the levels used in our study. Whether high concentrations of glucagon exert a similar effect on isolated, normal islet β-cells also remains to be established.

The molar ratios of amylin to insulin secreted from HIT cells in these studies (0.14 to 0.44) are consistent with rates of secretion reported from a study using the isolated, perfused rat pancreas (2), but higher than that reported in a third study which used isolated, cultured neonatal rat islet cells, in which the molar ratio was approximately 0.01 (16). Measured recoveries of both amylin and insulin in our system are close to 100%, so A/I ratios reported herein probably accurately reflect the HIT cell system. Differences in the secreted A/I ratio observed between the continuous

HIT cell line on one hand, and cultured primary rat islets on the other, could reflect differences in rates of amylin or insulin secretion between β-cells of different origins. In addition, the molar A/I ratio secreted from HIT cells varied with time in culture, suggesting that culture conditions might contribute to discrepancies in reported values.

The sulphonylureas tolbutamide and glyburide, and the biguanide metformin, are widely used in the clinical treatment of NIDDM to reduce hyperglycemia. Sulphonylureas directly stimulate the β-cell to secrete insulin while biguanides act through incompletely defined mechanisms to increase insulin sensitivity in peripheral tissues. In the present study, tolbutamide and glyburide stimulated release of both amylin and insulin from HIT cells. The magnitude of the insulin responses at 100 μM tolbutamide and 1.0 μM glyburide (1.4-fold increase) were comparable to observations made for insulin secretion using isolated rat islets (12). The main stimulus-response coupling pathway for β-cell secretion is probably mediated through closing of the ATP-sensitive K channel, which is present in β-cell membranes, and thought to be a target for sulphonylurea drugs (17,18). In isolated human β-cells, this channel has a half-maximal sensitivity of 18 μM tolbutamide in whole-cell patches, and similar values have been measured in rodent cells (19). Although the results of the current study do not allow calculation of an EC_{50} for the effect of tolbutamide on amylin secretion, they are not inconsistent with this reported apparent affinity of tolbutamide for the ATP-sensitive K channel.

Metformin did not influence secretion of either insulin or amylin at drug concentrations of up to 100 μM. Previous observations have led to the conclusion that metformin fails to influence insulin secretion in a variety of systems (20).

The stimulation of amylin release by sulphonylureas may have clinical relevance. Islet amyloid could mediate the progression of secondary β-cell failure which frequently complicates sulphonylurea therapy. Excessive secretion of amylin, resulting in high local concentrations of the peptide in the region of the islets, could accelerate amyloid deposition and β-cell dysfunction (5). Our observations could therefore provide a mechanism contributing to the secondary failure associated with sulphonylurea therapy.

The objective of the present study was to evaluate the amylin secretory response of HIT cells to a variety of agents known to regulate insulin secretion. For most modulators, the molar A/I ratio remained constant as secretion rates varied. However, the ratio did vary between different cell preparations, from about 0.14 to 0.44. The quantitative and qualitative descriptions of amylin secretion presented here are largely consistent with previously reported observations derived from the isolated, perfused rat pancreas, and also from studies in whole animals and humans. We conclude that the continuous HIT T15 islet β-cell line is a useful model system for the study of amylin secretion. Further, the relative ease of undertaking studies of gene expression and protein turnover in cultured cells should allow dissection of the regulation of amylin transcription, translation, and processing.

ACKNOWLEDGMENTS

We thank Dr. Molly Stone for advice and support during these studies, Dr. Terry Fieser for development of the amylin radioimmunoassay, and Dr. T.J. Rink for constructive criticism of the manuscript.

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