The Constitutive Secretory Pathway Is a Major Route for Islet Amyloid Polypeptide Secretion in Neonatal but Not Adult Rat Islet Cells

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Islet amyloid polypeptide (IAPP or amylin) is a normal secretory product of the pancreatic β-cell that is cosecreted with insulin and is the major constituent of islet amyloid deposits in individuals with type 2 diabetes or insulinomas. We have previously reported that glucose stimulates IAPP, but not insulin secretion, from neonatal rat β-cells when regulated secretion is prevented by use of calcium-free media, suggesting that IAPP secretion occurs via a constitutive secretory pathway. To directly test this hypothesis, we examined the effects of 2 substances—brefeldin A (BFA) and cycloheximide (CHX)—that are predicted to selectively block constitutive secretion on the release of IAPP-like immunoreactivity (IAPP-LI) and immunoreactive insulin (IRI) from neonatal rat islet cell monolayer cultures. When regulated release was prevented by use of calcium-free media, glucose-stimulated IAPP-LI release was nearly abolished by blocking constitutive release with 10 μg/ml BFA (mean ± SD: 8.7 ± 7.7 vs. 29.3 ± 14.3 pmol/l; $n = 5$; $P < 0.05$), an inhibitor of constitutive vesicle formation. Similarly, calcium-independent, glucose-stimulated IAPP-LI secretion was markedly suppressed when new protein synthesis was blocked by administration of 20 μg/ml CHX (4.6 ± 2.1 vs. 29.5 ± 14.0 pmol/l; $n = 5$; $P < 0.005$). Secretion of IRI was low in the absence of calcium, and neither BFA nor CHX had any further effect. When calcium was added to the incubation media to allow regulated secretion of both IRI and IAPP-LI, both BFA (47.7 ± 8.7 vs. 80.7 ± 10.3 pmol/l; $P < 0.001$) and CHX (37.3 ± 5.8 vs. 73.3 ± 6.2 pmol/l; $n = 5$; $P < 0.0001$) inhibited glucose-stimulated IAPP-LI secretion by ~40%, but again had no inhibitory effect on IRI secretion. These data indicate that ~40% of glucose-stimulated IAPP-LI release occurs via a constitutive secretory pathway in neonatal rat islet cells. By contrast, in adult rat islets, glucose-stimulated IAPP-LI release was almost abolished in the absence of calcium (86 ± 3% inhibition; $P < 0.05$) and unaffected by addition of BFA (275 ± 28 vs. 205 ± 89 pmol/l; NS) or CHX (160 ± 20 vs. 205 ± 89 pmol/l; NS), suggesting that constitutive secretion of IAPP does not occur in mature β-cells. Collectively, these data suggest that a significant proportion of glucose-stimulated IAPP secretion from neonatal, but not adult, rat islet cells occurs via a constitutive secretory pathway. Diabetes 49:1477–1484, 2000

Islet amyloid polypeptide (IAPP or amylin), a 37–amino-acid peptide, is the major constituent of islet amyloid deposits that occur in insulinomas and type 2 diabetes (1–3). IAPP is synthesized primarily in the pancreatic β-cell, where it is colocalized with insulin in secretory granules (4,5) and is released along with insulin in response to stimulation by β-cell secretagogues such as glucose (6,7). The physiological function of IAPP has not been determined, although effects on insulin action (8) and secretion (9), gastric emptying (10), and food intake (11) have all been suggested. Further, it is unknown why IAPP forms islet amyloid deposits in type 2 diabetes and insulinomas. The presence of an amyloidogenic region in the midportion of the molecule (amino acids 24–28) is thought to be necessary for amyloid formation (12,13), but it is apparently not sufficient because normal humans do not usually develop islet amyloid (14). We have therefore proposed that defects in processing, sorting, and/or secretion of IAPP by the β-cell may be present in insulinomas and type 2 diabetes and lead to its deposition as islet amyloid (15).

In most studies examining IAPP secretion in vitro, IAPP and insulin are found to be stored and coreleased by β-cells in a relatively constant proportion (6,16). However, under certain experimental conditions, the secretion of IAPP and insulin have been found to be dissociated. For example, production of hyperglycemia by administration of dexamethasone (17–20) or repeated injection of glucose (19) into rats has been found to produce a disproportionate increase in IAPP synthesis and secretion relative to insulin. In addition, using neonatal rat islet cell monolayer cultures, we found that glucose-stimulated secretion of insulin could be completely inhibited by epinephrine or removal of calcium from the incubation medium, but glucose-stimulated IAPP release was only partially inhibited by these agents (21). Our findings suggested that some component of glucose-stimulated IAPP release—but not insulin release—from neonatal rat β-cells cannot be inhibited by epinephrine or removal of calcium.

We hypothesized that this epinephrine- and calcium-independent component of glucose-stimulated IAPP release observed in neonatal rat islet cells was due to release of this

peptide via the constitutive secretory pathway. In the present study, we directly tested this hypothesis by examining IAPP and insulin secretion in the presence of 2 agents that specifically block constitutive secretion. Brefeldin A (BFA) is a compound that collapses the Golgi apparatus, thus preventing release of constitutive secretory vesicles from the trans-Golgi network (22,23). Cycloheximide (CHX) inhibits new protein synthesis, arresting the bulk flow process by which the constitutive pathway is driven (24,25). We also immunostained neonatal rat islet cell cultures to determine whether β-cells were the likely source of constitutively secreted IAPP. Finally, to determine whether constitutive release of IAPP is also observed in mature β-cells, we also examined the effects of calcium-free media, BFA, and CHX on IAPP and insulin release from islets isolated from adult rats.

RESEARCH DESIGN AND METHODS

Materials. Arginine, isobutylmethylxanthine (IBMX), carbachol, BFA, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO), CHX from Calbiochem (San Diego, CA), somatostatin (Ala^{5}Trp^8) from Bachem (Torrance, CA), and cyclic somatostatin from Peninsula (Belmont, CA).

Cell preparation and culture. Monolayer cultures of neonatal rat pancreatic islet cells were prepared as previously described (6). Before the experiments, cells were cultured for 3–5 days in medium consisting of 45% National Cancer Tissue Center 135, 45% medium 199 (Gibco, Grand Island, NY), and 10% fetal calf serum supplemented with 16.7 mmol/l glucose and 50 μg/ml gentamicin.

Adult rat islets were isolated from pancreases of male Wistar rats (250–300 g) by a collagenase digestion technique adapted from previous methods (26). In brief, the pancreatic duct of pentobarbital-anesthetized rats was retrogradely cannulated (PE50 tubing) at Vater's ampulla, and 15–20 ml ice-cold collagenase (0.25 mg/ml; Type XI; Sigma) was slowly injected. The inflated pancreas was excised and incubated at 37°C without shaking for 20–25 min. The pancreas was then triturated through a plastic 10-ml pipette and the digested tissue washed 2–3 times by centrifugation in 50 ml ice-cold Hanks' balanced salt solution. After dithizone staining, islets were hand-picked under a dissecting microscope into a 24-well plate (100 islets/well) and cultured for 48 h in RPMI containing 11.1 mmol/l glucose, 10% fetal calf serum, and antibiotics.

Islet cell secretion experiments. Experiments were performed on neonatal rat islet cell monolayer cultures after 3–5 days of culture. After a 2-h preincubation in Krebs-Ringer bicarbonate buffer plus 0.1% BSA (KRBB-BSA) and 1.67 mmol/l glucose, the islet cells were incubated for 2 h in 1 ml KRBB-BSA containing the desired concentration of glucose (1.67 or 16.7 mmol/l), calcium (0 or 2 mmol/l), BFA, or CHX. The incubation medium was then collected and the cells extracted in 1.0 ml acid-ethanol. Media and cell extracts were centrifuged, and the supernatants were stored at –20°C until assayed. Experiments performed on adult rat islets were identical except that the volume of the incubation medium was 0.6 ml.

Radioimmunoassays. Immunoreactive insulin (IRI) was measured by a previously described radioimmunoassay (RIA) (27). IAPP-like immunoreactivity (IAPP-LI) was measured by an RIA as previously described (16), with minor modifications. The antiserum (Peninsula) was raised in a rabbit against synthetic rat IAPP and cross-reacts <0.1% with insulin and calcitonin gene-related peptide. Synthetic rat IAPP (Peninsula) was used as standard. ^{125}I-IAPP was prepared by a lactoperoxidase iodination technique and was purified by reverse-phase high-performance liquid chromatography using an acetonitrile gradient of 28–38% over 30 min. On this gradient, unlabeled IAPP elutes before 2 radioactive peaks (peak 1 and peak 2). Radiolabeled IAPP from peak 1 was used in all assays. Intra- and interassay variations of this IAPP radioimmunoassay are 13% and 14%, respectively, and the minimum detectable concentration is 2.8 pmol/l.

Immunohistochemistry. Neonatal rat pancreatic islet cell monolayers were cultured in chamber slides (LabTek, Campbell, CA), washed in Hanks' balanced salt solution, fixed in 4% paraformaldehyde (in 0.1 mol/l phosphate buffer) for 30 min, and stored in phosphate-buffered saline (PBS) at 4°C before immunostaining by indirect immunofluorescence for IAPP plus either insulin, somatostatin, or glucagon, using previously described techniques (28). The following primary antibodies were used: *1*) IAPP: #8342, raised in rabbits to synthetic rat IAPP; *2*) insulin: monoclonal antibody (Sigma); *3*) glucagon: monoclonal antibody (Sigma); and *4*) somatostatin: monoclonal antibody MS#12 (gift of Dr. J. Ensinck). All antibodies were used at a dilution of 1:500 in 0.05 mol/l PBS, 0.25% Triton-X, and 1.0% BSA, except the somatostatin antibody, which was used at a dilution of 1:250. Double immunostaining was achieved by incubat-

ing the cells with the first primary antibody overnight (4°C), washing in 0.05 mol/l PBS plus 0.25% Triton-X, and then incubating the cells for 60 min with appropriate secondary antibodies conjugated to either fluorescein or rhodamine (Jackson Immunoresearch Laboratories, West Grove, PA), used at a dilution of 1:200. After washing, the cells were incubated with the second primary antibody overnight at 4°C followed by the appropriate secondary antibody (fluorescein or rhodamine conjugated) for 60 min. The cells were then washed, mounted in polyvinyl alcohol, and viewed with a Zeiss Axioplan fluorescent microscope equipped with a Hamamatsu C4880 fast-cooled camera and M2 software from Imaging Research (St. Catharines, ON, Canada). Separate images of the same cells were acquired using a rhodamine filter for IAPP and fluorescein filter for insulin, glucagon, and somatostatin, to allow assessment of the extent of colocalization of IAPP with other islet hormones.

Calculation of regulated and constitutive secretion of IAPP-LI and IRI. If IAPP-LI secretion occurs via both the regulated and constitutive secretory pathways whereas IRI secretion occurs predominantly via the regulated pathway, we predicted that the secreted IAPP-LI/IRI ratio should vary inversely with the degree of stimulation of regulated secretion from the β-cell. To test this prediction, we used the results from the current and previous studies in which IAPP-LI and IRI were measured after a 2-h incubation of neonatal rat islet cells in 16.7 mmol/l glucose plus a range of concentrations of a variety of substances known to affect regulated secretion from the β-cell. Specifically, we analyzed the results of 203 incubations of neonatal rat islet cells in 16.7 mmol/l glucose plus known stimulators (10 μmol/l to 10 mmol/l carbachol, 10 nmol/l to 1 μmol/l isoproterenol, 10 mmol/l arginine, 0.1 mmol/l IBMX) or inhibitors (0.1–100 μmol/l epinephrine, 1–100 nmol/l somatostatin analogs, no calcium) of β-cell–regulated secretion.

Although the parameter of interest was the IAPP-LI/IRI ratio, the actual regression was performed on IAPP-LI as the *y* variable versus IRI as the *x* variable because this relationship appeared to be linear. Because of heteroscedasticity and errors in both the *x* and *y* variables (29), a weighted orthogonal regression technique was used (30). The *x* axis (IRI) was assumed to be a random variable having a measurement error only, estimated at 11% from quality control statistics. The *y* axis (IAPP-LI) was assumed to have both a measurement error, estimated at 3.6 + 74/IAPP-LI%, and biological variation, estimated at 20%. Measurement error was computed from assay quality control statistics, and biological variation was estimated from residuals from a spline approximation (31).

The regression equation is IAPP-LI = β IRI + α where α and β are parameters identified by the regression. Rearranging to express the hormone ratio, the equation becomes: IAPP-LI/IRI = β + α / IRI.

In this form, it is apparent that when IRI is small and constitutive release predominates, the value of the IAPP-LI/IRI ratio increases. Furthermore, when IRI is high and regulated release of both IAPP-LI and IRI predominates, β represents the ratio of IAPP-LI/IRI during regulated secretion and may therefore approximate the ratio of these hormones in secretory granules. Finally, the equation demonstrates that the relationship between IAPP-LI/IRI and the degree of regulated secretion (represented by IRI levels) is curvilinear and is specifically in the form of a hyperbola.

Data and statistical analysis. In all figures, IAPP-LI and IRI secretion are presented as means ± SD of replicate (3–5) measurements from a single representative experiment. Such experiments were repeated on at least 2 additional occasions with identical results. In Table 1, because of the greater variation among individual adult rat islet experiments, data are expressed as IAPP-LI and IRI secretion as a percent of control (mean ± SE) from 3–6 individual experiments, with each condition measured in triplicate. Statistical differences were assessed by unpaired Student's *t* test, and *P* < 0.05 was considered statistically significant.

RESULTS

Neonatal rat islet cell monolayers
BFA and CHX inhibit secretion of IAPP but not insulin in the absence of calcium. As expected, in the presence of calcium (to allow regulated secretion to occur), 16.7 mmol/l glucose stimulated the secretion of both IAPP-LI (80.7 ± 10.3 vs. 16.1 ± 2.3 pmol/l; *n* = 5; *P* < 0.0001) and IRI (3.16 ± 0.29 vs. 0.44 ± 0.03 nmol/l; *n* = 5; *P* < 0.0001) from neonatal rat islet cells compared with the secretion levels observed in 1.67 mmol/l glucose. As we have observed previously (21), inhibition of regulated secretion by omission of calcium from the incubation media resulted in only partial (63%) inhibition of glucose-stimulated IAPP-LI release (29.3 ± 14.3 vs. 80.7 ±

10.3 pmol/l; n = 5; P < 0.0005; Fig. 1A), whereas it inhibited IRI secretion by >90% (0.31 ± 0.04 vs. 3.16 ± 0.29 nmol/l; n = 5; P < 0.0001; Fig. 1B). As a result, omission of calcium caused a marked increase in the ratio of IAPP-LI/IRI secreted (9.3 ± 3.6 vs. 2.6 ± 0.4%; P < 0.005).

To determine whether this calcium-independent release of IAPP-LI occurred via a constitutive secretory pathway, we tested the effects of 2 substances predicted to selectively block constitutive secretion on glucose-stimulated secretion of IAPP-LI and IRI. Addition of 10 μg/ml BFA—which is known to inhibit the formation of constitutive vesicles in β-cells (22) but has no effect on the secretion of preformed protein in mature β-cell granules (23)—inhibited calcium-independent, glucose-stimulated IAPP-LI release by 70% (8.7 ± 7.8 vs. 29.3 ± 14.3 pmol/l; P < 0.05; Fig. 1A). However, BFA had no additional inhibitory effect on IRI secretion under these conditions (0.26 ± 0.09 vs. 0.31 ± 0.04 nmol/l; NS; Fig. 1B). Cell contents of both IAPP-LI (2.4 ± 0.3 vs. 2.7 ± 0.6 pmol/well) and IRI (254 ± 42 vs. 255 ± 28 pmol/well) were unchanged by addition of BFA in the absence of calcium.

Because the constitutive secretory pathway is a bulk-flow pathway dependent on new protein synthesis, we examined the effects of the protein synthesis inhibitor CHX on islet hormone release. Like BFA, CHX (20 μg/ml) nearly abolished calcium-independent glucose-stimulated IAPP-LI secretion (4.6 ± 2.1 vs. 35.5 ± 4.1 pmol/l; n = 5; P < 0.0001; Fig. 2A) while having no effect on IRI secretion (1.32 ± 0.67 vs. 1.02 ± 0.15 nmol/l; n = 5; NS; Fig. 2B). Cell contents of IAPP-LI (3.4 ± 0.2 vs. 3.5 ± 0.2 pmol/well) and IRI (214 ± 12 vs. 214 ± 30 pmol/well) were not changed by addition of CHX. Thus, the calcium-independent glucose-stimulated secretion of IAPP-LI can be blocked by either BFA or CHX, indicating that secretion of IAPP-LI occurs via a constitutive pathway when regulated secretion is impaired by omission of calcium.

BFA and CHX inhibit secretion of IAPP but not insulin in the presence of calcium. We next sought to determine whether constitutive secretion of IAPP-LI occurs only when regulated secretion is impaired (e.g., in the absence of calcium) or whether it occurs even when regulated secretion is stimulated, thus making a contribution to total IAPP-LI secretion under normal conditions. Therefore, we examined the effects of BFA or CHX on glucose-stimulated IAPP-LI and IRI secretion in the presence of 2 mmol/l calcium to allow regulated secretion of these peptides to occur. Addition of 10 μg/ml BFA to media containing 16.7 mmol/l glucose and 2 mmol/l calcium resulted in a 41% inhibition of IAPP-LI release (47.7 ± 8.7 vs. 80.7 ± 10.3 pmol/l; n = 5; P < 0.001; Fig. 3A) but had no
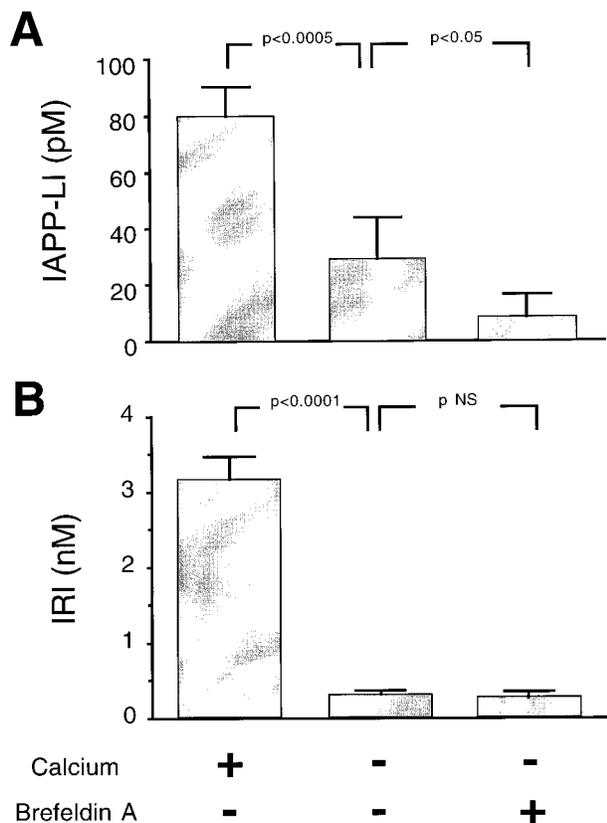
FIG. 1. Effect of BFA on calcium-independent glucose-stimulated secretion of IAPP-LI (*A*) and IRI (*B*) from neonatal rat islet cell monolayer cultures. Cultured cells were incubated for 2 h in 16.7 mmol/l glucose plus 2 mmol/l calcium, no calcium, or no calcium plus 10 μg/ml BFA, as indicated. IAPP-LI and IRI were measured by RIA of the incubation media.
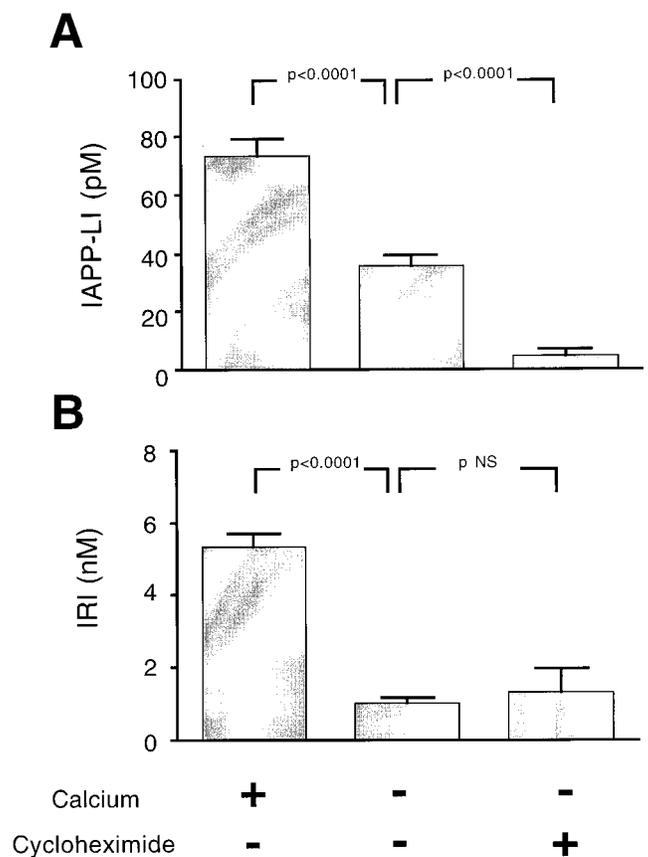
FIG. 2. Effect of CHX on calcium-independent glucose-stimulated secretion of IAPP-LI (*A*) and IRI (*B*) from neonatal rat islet cell monolayer cultures. Cultured cells were incubated for 2 h in 16.7 mmol/l glucose plus 2 mmol/l calcium, no calcium, or no calcium plus 20 μg/ml CHX, as indicated. IAPP-LI and IRI were measured by RIA of the incubation media.

inhibitory effect on glucose-stimulated IRI secretion (3.34 ± 0.64 vs. 3.17 ± 0.29 nmol/l; $n = 5$; NS; Fig. 3B). Cell contents of IAPP-LI (2.2 ± 0.2 vs. 2.4 ± 0.2 pmol/well; NS) and IRI (197 ± 18 vs. 238 ± 50 pmol/well; NS) were unchanged by the addition of BFA in the presence of calcium. Similarly, 20 µg/ml CHX inhibited glucose-stimulated IAPP-LI secretion in 2 mmol/l calcium by 43% (37.3 ± 5.8 vs. 73.3 ± 6.2 pmol/l; $n = 5$; $P < 0.0001$; Fig. 4A) but had no significant effect on IRI secretion (5.08 ± 0.53 vs. 5.33 ± 0.39 nmol/l; NS; Fig. 4B). As with BFA, CHX did not change the cell content of either IAPP-LI (3.2 ± 0.4 vs. 3.4 ± 0.2 pmol/well; NS) or IRI (188 ± 12 vs. 198 ± 26 pmol/well; NS) under these conditions. Thus, even during stimulation of regulated secretion of IAPP-LI from neonatal rat β-cells, ~40% of total IAPP-LI secretion occurs via a constitutive secretory pathway.

IAPP-LI/IRI ratio. If IAPP-LI secretion occurs via both the regulated and constitutive secretory pathways whereas IRI secretion occurs predominantly via the regulated pathway, the secreted ratio of IAPP-LI/IRI should increase when regulated β-cell secretion is inhibited, because under such conditions the relative contribution of constitutively released IAPP-LI to this ratio would increase and the contribution of constitutively

secreted insulin would remain negligible (<2%) (32; C.B.V. S.E.K, and P.A. Halban, unpublished data). To test this hypothesis, the data from 203 incubations of neonatal rat islet cells in 16.7 mmol/l glucose plus stimulators or inhibitors of regulated secretion were plotted (Fig. 5) as the IAPP-LI/IRI ratio against the degree of regulated β-cell secretion (estimated as IRI secretion because constitutive IRI release is negligible). These data are also considered as IAPP-LI versus IRI as a linear regression, and the resulting parameters are then used to define a hyperbolic curve as detailed in RESEARCH DESIGN AND METHODS (Fig. 5). This analysis shows that as regulated secretion from the β-cell increases, the IAPP-LI/IRI ratio approaches an asymptote (Fig. 5) of 0.635%, a value that closely approximates that of extracts (and thus presumably secretory granules) of neonatal rat islet cells (6). Furthermore, as regulated secretion decreases, the IAPP-LI/IRI ratio increases in magnitude, consistent with the hypothesis that IAPP-LI is secreted by both the constitutive and regulated secretory pathways.

Immunohistochemistry. To determine whether non–β-cells could be a significant source of constitutively released IAPP-LI, we immunostained neonatal rat islet cells for colo-
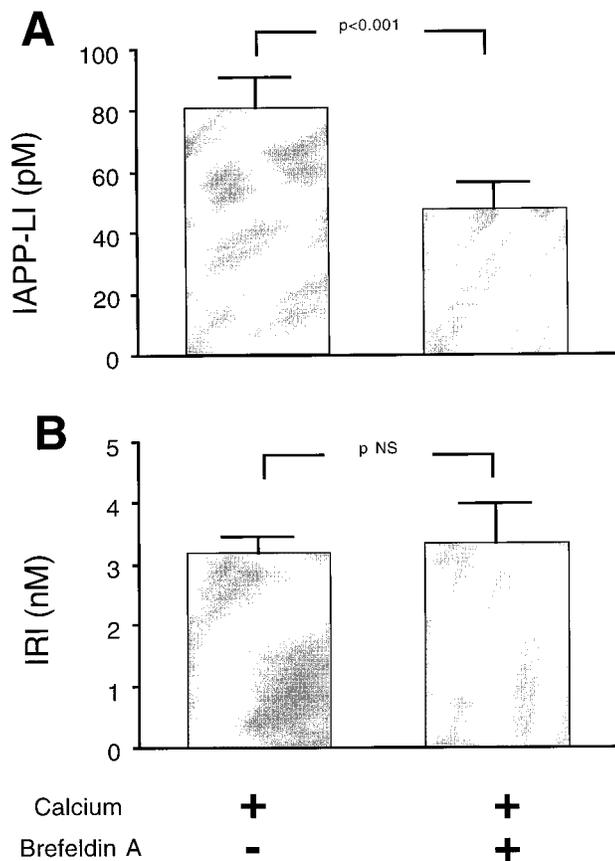
FIG. 3. Effect of BFA on glucose-stimulated secretion of IAPP-LI (*A*) and IRI (*B*) from neonatal rat islet cell monolayer cultures in the presence of calcium. Cultured cells were incubated for 2 h in 16.7 mmol/l glucose plus 2 mmol/l calcium in the presence or absence of 10 µg/ml BFA, as indicated. IAPP-LI and IRI were measured by RIA of the incubation media.
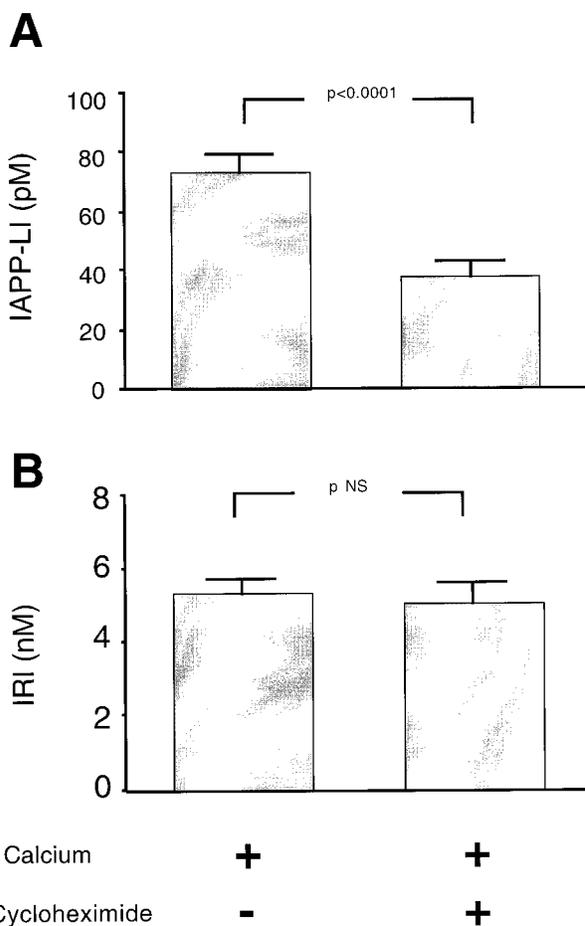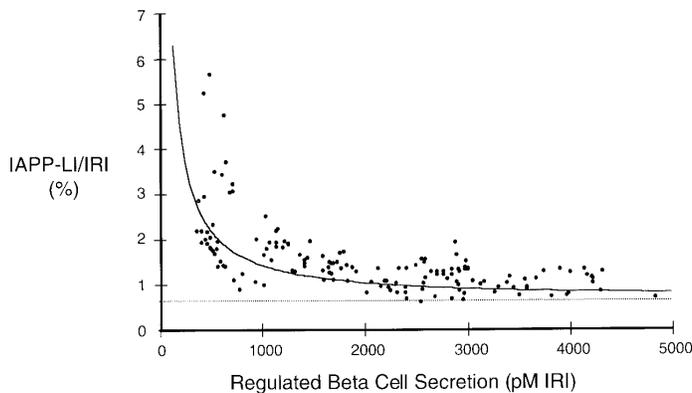
FIG. 4. Effect of CHX on glucose-stimulated secretion of IAPP-LI (*A*) and IRI (*B*) from neonatal rat islet cell monolayer cultures in the presence of calcium. Cultured cells were incubated for 2 h in 16.7 mmol/l glucose plus 2 mmol/l calcium in the presence or absence of 20 µg/ml CHX, as indicated. IAPP-LI and IRI were measured by RIA of incubation media.

FIG. 5. Effect of stimulation of β-cell regulated secretion on the IAPP-LI/IRI ratio secreted. Neonatal rat islet cell monolayer cultures were incubated in 16.7 mmol/l glucose with or without a variety of stimulators (10 μmol/l to 10 mmol/l carbachol, 10 nmol/l to 1 μmol/l isoproterenol, 10 mmol/l arginine, 0.1 mmol/l IBMX) or inhibitors (0.1–100 μmol/l epinephrine, 1–100 nmol/l somatostatin analogs, no calcium) of regulated β-cell secretion. The IAPP-LI/IRI ratio decreases as regulated β-cell secretion increases, approaching an asymptote of 0.635% (dotted line).
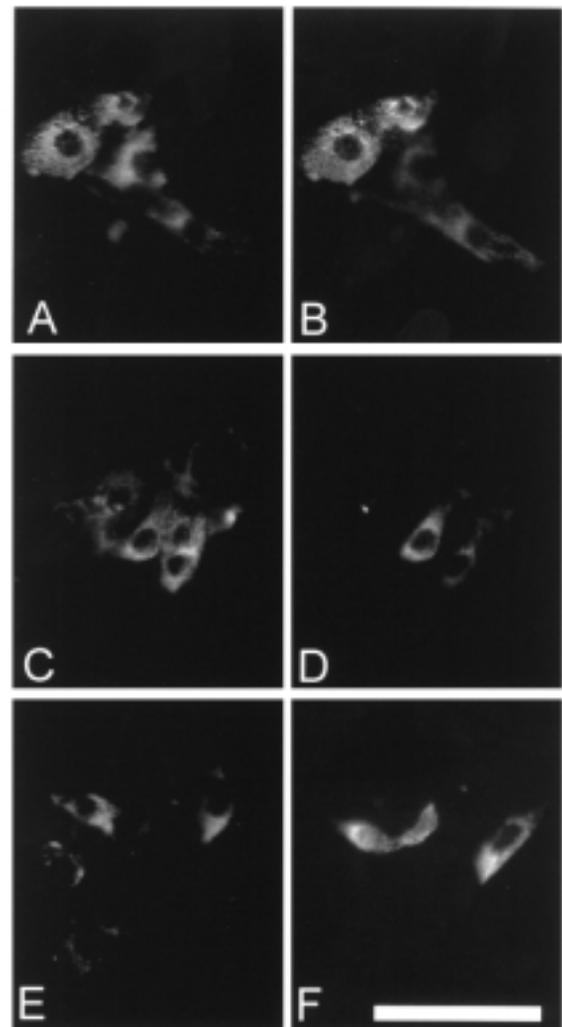
calization of IAPP-LI with other islet hormones. As expected, there was extensive colocalization of IAPP and insulin immunofluorescence in neonatal islet cells (Fig. 6*A* and *B*): 86% of IAPP-immunopositive cells also contained immunoreactivity for insulin. Almost all (97%) of somatostatin-positive cells were also IAPP-positive, indicating that IAPP is present in neonatal rat δ-cells; however, these cells represent only a small proportion of all IAPP-containing cells in this preparation, since only 8% of IAPP-positive cells also contained somatostatin immunoreactivity (Fig. 6*C* and *D*). Colocalization of IAPP and glucagon immunofluorescence was observed in only 2% of glucagon-positive cells (Fig. 6*E* and *F*).
Adult rat isolated islets. To determine whether constitutive secretion of IAPP also occurs in adult rat β-cells or whether it is a particular characteristic of neonatal rat β-cells, we examined the effects of calcium omission, BFA, or CHX on glucose-stimulated IAPP-LI and IRI release from isolated adult rat islets. As expected, incubation of adult rat islets ($n = 3$) in 16.7 mmol/l glucose produced a marked stimulation of both IAPP-LI (108.0 ± 41.9 vs. 2.0 ± 1.9 pmol/l; $n = 3$; $P < 0.05$) and IRI (20.8 ± 4.3 vs. 1.2 ± 0.3 nmol/l; $n = 3$; $P < 0.005$) secretion over that observed in 1.67 mmol/l glucose (Fig. 7). Blockade of regulated secretion by omission of calcium from the incubation medium nearly abolished not only glucose-stimulated IRI release (% inhibition = $80 \pm 5\%$; $P < 0.005$) but also glucose-

FIG. 6. Colocalization of immunoreactivity for IAPP with other islet hormones. Neonatal rat islet cell cultures were double immunostained for the presence of IAPP immunoreactivity (*A, C, E*) plus either insulin (*B*), somatostatin (*D*), or glucagon (*F*). Note the extensive colocalization of IAPP with insulin (compare *A* and *B*), and the presence of IAPP immunoreactivity in somatostatin-positive cells (*C* and *D*). Glucagon immunoreactive cells that also contain IAPP immunoreactivity are rare (*E* and *F*). Size bar = 20 μm.

stimulated IAPP-LI release (% inhibition = $86 \pm 3\%$; $P < 0.05$; Fig. 7). Thus, in adult rat islets, unlike those of neonatal rats, the ratio of IAPP-LI/IRI secreted was not affected by omission of calcium (0.51 ± 0.11 vs. $0.37 \pm 0.15\%$; NS). In addition, when regulated secretion was stimulated by the presence of 16.7 mmol/l glucose plus 2 mmol/l calcium, neither BFA nor CHX had any significant effect on either IAPP-LI or IRI secretion (Table 1). Thus, in contrast to our observations in neonatal rat islet cells, the secretion of IAPP-LI and IRI from adult rat islets could not be dissociated by selective inhibition of either regulated or constitutive secretion.

DISCUSSION
Endocrine cells such as the pancreatic β-cell possess an efficient mechanism for sorting specific proteins into secretory granules, where they are stored until their release. This pathway for hormone secretion, termed the regulated secretory

TABLE 1
Lack of effect of BFA or CHX on glucose-stimulated secretion of IRI and IAPP-LI from adult rat islets

	IRI (% control)	IAPP-LI (% control)
BFA (10 μg/ml)	96 ± 15	111 ± 11
CHX (20 μg/ml)	85 ± 18	87 ± 10

Data are expressed as secretion (mean ± SE) of IRI or IAPP-LI as a percent of that observed in 16.7 mmol/l glucose. $n = 3$–6 individual experiments with triplicate determinations in each experiment.
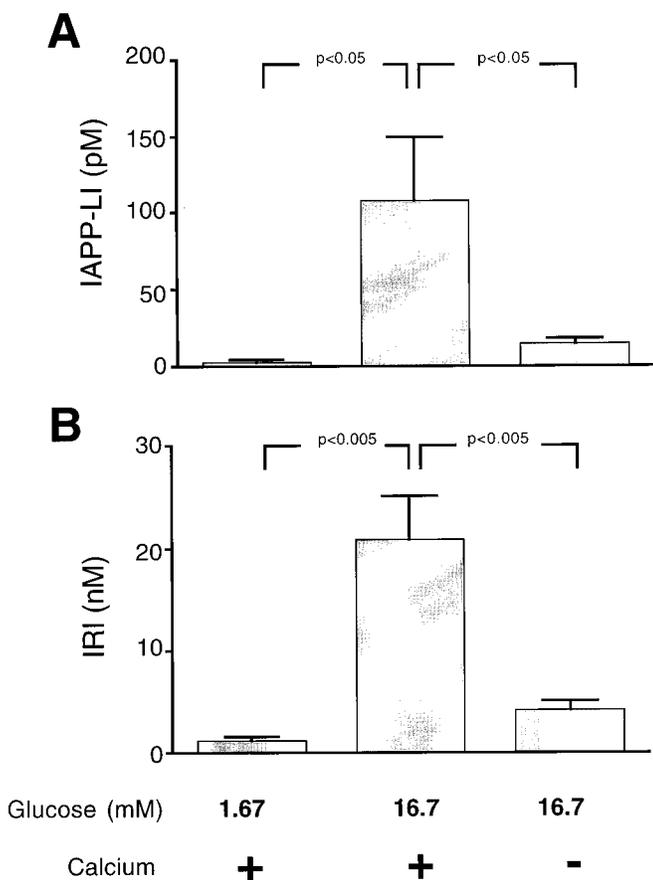
A

B

| Glucose (mM) | **1.67** | **16.7** | **16.7** |
| Calcium | **+** | **+** | **–** |

FIG. 7. Effect of omission of calcium on glucose-stimulated secretion of IAPP-LI (*A*) and IRI (*B*) from adult rat isolated islets. Islets were incubated for 2 h in the presence of 1.67 mmol/l glucose or 16.7 mmol/l glucose in the presence or absence of 2 mmol/l calcium. IAPP-LI and IRI were measured by RIA of the incubation media.

pathway, is a distinguishing characteristic of neuroendocrine secretory cells and allows for a rapid response of the cell to stimuli. In the β-cell, for example, insulin is very efficiently (>98%) sorted to the regulated secretory pathway, where it is stored in granules until secretion is acutely stimulated by secretagogues such as glucose (32). Most nonendocrine cells also secrete proteins but do so by the constitutive secretory pathway, a nonstorage, bulk-flow pathway driven by new protein synthesis. In β-cells and other secretory cells, proteins that are not sorted to granules (e.g., receptors) can be delivered to the plasma membrane and/or secreted via the constitutive pathway. Although the β-cell peptide IAPP is colocalized with insulin in secretory granules (4,5) and can be released from β-cells by the regulated secretory pathway, our findings demonstrate that a significant proportion (~40%) of glucose-stimulated IAPP release occurs via the constitutive secretory pathway in neonatal rat β-cells.

The present study provides several pieces of evidence to support the idea that IAPP is released constitutively from neonatal rat β-cells. First, 2 different compounds (BFA and CHX), each predicted to block constitutive secretion by distinct mechanisms without having any effect on the secretion of preformed granules, were found to partially inhibit glucose-

stimulated IAPP release. Neither BFA nor CHX had any effect on glucose-stimulated insulin release in the presence of calcium, demonstrating that these 2 compounds have negligible effects on regulated secretion. Moreover, neither agent had any effect on cellular content of IAPP or insulin, arguing against the possibility that the 2-h exposure to BFA or CHX might impact routing of IAPP to granules or to degradative pathways. Second, the calcium-independent component of glucose-stimulated IAPP release, which we previously postulated to occur via a constitutive pathway (21), was nearly abolished by these agents. The small but measurable release of insulin observed in the absence of calcium (Fig. 2) is likely regulated secretion due to the presence of residual intra- or extracellular calcium in these experiments or due to physical handling of the cells during media changes. It is unlikely to be constitutive release because, in separate experiments (C.B.V., S.E.K., P.A. Halban, unpublished data), we have shown that constitutive secretion of (pro)insulin from this preparation is <2%. Finally, we found that when regulated secretion from the β-cell was stimulated by nonglucose secretagogues, the ratio of IAPP/insulin secreted decreased such that at maximally stimulated levels it approximated the cellular IAPP/insulin ratio, which probably reflects the ratio in secretory granules. Conversely, when regulated secretion was inhibited, the IAPP/insulin ratio increased as the contribution of constitutively released IAPP became unmasked. Taken together, these data indicate that a large proportion of glucose-stimulated IAPP, but not insulin, release occurs via the constitutive secretory pathway in neonatal rat β-cells.

Other interpretations of these data must also be considered. First, it should be noted that IAPP has also been found in the somatostatin-secreting δ-cells in rats (33–35), and it is possible that these cells may contribute to the BFA- and CHX-sensitive component of glucose-stimulated IAPP secretion. However, our immunostaining data (Fig. 6) indicate that somatostatin-positive δ-cells compose a very small (~8%) proportion of the total IAPP-containing cells in neonatal rat islet cell cultures. The vast majority of IAPP-positive cells are also insulin positive, indicating that the major source of IAPP in this preparation is likely to be β-cells. It therefore seems quite likely that IAPP derived from δ-cells does not make a significant contribution to the total IAPP immunoreactivity secreted from islets. A second possibility is that IAPP, like the proinsulin connecting peptide (C-peptide), may be secreted in part from β-cells via the "constitutive-like" pathway in which vesicles rich in C-peptide are thought to be released from the halo of immature secretory granules (36,37), where IAPP is also thought to reside (38). We previously observed, however, that this constitutive-like pathway makes a relatively minor contribution to C-peptide secretion in this neonatal rat islet cell preparation (39). Moreover, the constitutive-like pathway is thought to be insensitive to the effects of BFA (23), and BFA inhibited IAPP secretion in the present study. Thus, it seems unlikely that a significant proportion of IAPP release occurred via this constitutive-like pathway. Rather, we believe that the most likely explanation for our findings is that selective sorting of peptides, in this case IAPP, may occur in the β-cell.

In marked contrast to neonatal rat islet cells, glucose-stimulated IAPP release from adult rat isolated islets was abolished by omission of calcium from the medium and unaffected by addition of either BFA or CHX. These data suggest that constitutive secretion of IAPP is a characteristic of β-cells in the

newborn rat but not the normal adult rat. This finding is supported by another study that found no dissociation of IAPP and insulin release from adult rat islets under a variety of conditions, including the absence of calcium (40). Whether constitutive release of IAPP serves a physiological role in the newborn or whether it represents an immaturity of IAPP sorting in neonatal β-cells remains unknown. However, neonatal rat β-cells clearly possess the intracellular machinery required to efficiently sort proinsulin (a molecule present in 100-fold greater amounts than proIAPP) to the regulated secretory pathway, as indicated by the blockade of glucose-stimulated insulin release by omission of calcium. This efficient sorting of insulin relative to IAPP in newborn rat β-cells suggests that the sorting of IAPP and insulin may occur via distinct mechanisms.

If the constitutive release of IAPP that we observed in neonatal rat but not adult rat β-cells represents a deficiency in IAPP sorting to the regulated pathway that is characteristic of immature cells, our findings may have implications for understanding the mechanism of islet amyloid formation in insulinomas and in type 2 diabetes. Immature (fetal and to a lesser extent neonatal) β-cells are characterized by a decreased regulated secretion of peptide in response to glucose stimulation (15). Such impaired sensitivity to glucose is also a characteristic of β-cells in insulinomas, type 2 diabetes, and transformed β-cell lines (15,41). Interestingly, such cell lines are further characterized by impaired sorting and processing of both proinsulin (42) and proIAPP (43), the impaired processing presumably due to the lack of prohormone convertase enzyme activity in the constitutive secretory pathway (44). Although we have not yet characterized the molecular forms of (pro)IAPP immunoreactivity being secreted constitutively from neonatal β-cells, we speculate that unprocessed or partially processed forms of proIAPP may be secreted by this route. Indeed, a high molecular weight form of IAPP immunoreactivity (presumably proIAPP) was found to be constitutively secreted by the insulinoma-derived β-cell line βTC3 (43). Considering that NH_2-terminal proIAPP immunoreactivity has been demonstrated in human islet amyloid deposits by immunostaining (45) and proIAPP has been shown to be present in isolated human islets after prolonged exposure to elevated glucose levels (46), we speculate that constitutive release of proIAPP may represent a mechanism for islet amyloid formation in insulinomas and possibly type 2 diabetes as well. Although it is known that proIAPP can spontaneously form fibrils in vitro (47), it is currently unknown whether proIAPP or other higher molecular weight forms of IAPP may be more amyloidogenic than the fully processed molecule.

In conclusion, we have demonstrated that neonatal rat β-cells secrete IAPP via both constitutive and regulated secretory pathways. Further, the constitutive pathway appears to contribute to IAPP secretion only in newborn and not adult rat β-cells and does not appear to be available to insulin in either newborn or adult rat β-cells. It remains unclear whether constitutive release of IAPP by the neonatal rat β-cell serves a physiological function or represents an immaturity in peptide sorting by the newborn β-cell in the rat.

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