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Regulation of Insulin Secretion and Blood Glucose Metabolism by Adrenomedullin

ALFREDO MARTÍNEZ, CYPRIAN WEAVER, JOSÉ LÓPEZ, SAM J. BHATHENA, THEODORE H. ELSASSER, MAE-JEAN MILLER, TERRY W. MOODY, EDWARD J. UNSWORTH, AND FRANK CUTTITTA

Biomarkers and Prevention Research Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health (A.M., M.J.M., T.W.M., E.J.U., F.C.), Rockville, Maryland 20850; College of Medicine, Fu Jen Catholic University (C.W.), Hsinchuang, Taipei Hsien, Taiwan, Republic of China; Department of Biology, Universidad Autónoma de Madrid (J.L.), Cantoblanco, Madrid, Spain; and U.S. Department of Agriculture, Agricultural Research Service (S.B., T.H.E.), Beltsville, Maryland 20705

ABSTRACT

Adrenomedullin (AM), a recently discovered hypotensive peptide, is expressed in the endocrine pancreas of different species, as demonstrated by immunocytochemistry. Electron microscopic studies with double immunogold showed colocalization of AM and pancreatic polypeptide. A homogeneous expression of AM receptor was found throughout the islet using *in situ* hybridization. Six different insulin-producing cell lines have been analyzed by reverse transcription-PCR and showed expression of both AM and its receptor. Two experimental models have been used to study the effects of AM in pancreatic physiology. 1) Analysis of isolated rat islets shows that AM inhibits insulin secretion in a dose-dependent manner. The monoclonal antibody MoAb-G6, which neutralizes AM bioactivity, was able to increase insulin release 5-fold; this effect was reversed by the addition of synthetic AM. 2) Oral glucose tolerance tests showed that iv injection of AM reduces the levels of insulin in the bloodstream with a concomitant increase in circulating glucose. These studies implicate AM as a newly defined factor of the insulin regulatory system that could be involved in disorders such as diabetes and obesity. (*Endocrinology* **137:** 2626–2632, 1996)

ADRENOMEDULLIN (AM), a recently characterized regulatory peptide (1–3), is generated from a larger 185-amino acid preprohormone through consecutive enzymatic cleavage and amidation. This process culminates in the liberation of a 52-amino acid bioactive peptide (4). AM has been found in numerous organs, including adrenal gland, heart atrium, kidney, and lung (2, 5). AM's role as a vasodilatory agent has been extensively studied (6–9). It acts through specific receptors (10) in the plasma membrane to activate adenylate cyclase activity and modulate Ca^{2+} flux in the target cells (11, 12). These signal transduction pathways are involved in numerous physiological processes, including the regulation of hormone secretion (13). It is well established that regulation of intracellular cAMP modulates hormone release in the pancreas (14, 15). As AM and its gene related peptide have been reported to influence the secretion rate of several hormones, including catecholamine (16), ACTH (17), and aldosterone (18), we investigated the potential role of AM in regulating the endocrine physiology of the pancreas.

Materials and Methods

Tissues

Human pancreas sections were obtained from Dako Corp. (Carpinteria, CA). Pancreata from rats, hamsters, guinea pigs, cats, and dogs were fixed in Bouin's fluid (Sigma Chemical Co., St. Louis, MO) and embedded in paraffin. Animal tissues were obtained through a contract with Science Application International Corp., located at the Frederick Cancer Research and Development Center (Frederick, MD).

Antibodies

A previously characterized antibody against P072, a fragment of AM (5), was used for immunocytochemistry together with commercially available antisera against insulin, glucagon, somatostatin, and pancreatic polypeptide (Accurate Chemical and Scientific Corp., Westbury, NY).

A monoclonal antibody, designated MoAb-G6, was developed against the P072 peptide of AM following a procedure modified from a previously described methodology (19). In brief, BALB/c mice were hyperimmunized with P072 peptide conjugated to keyhole limpet hemocyanin via glutaraldehyde cross-linkage (1 mg/1 mg coupling ratio). Splenic lymphocytes were fused to mouse myeloma cell line RNS1 following standard protocols. Resulting hybrids were screened for anti-P072 MoAb production using a solid phase enzyme-linked immunosorbent assay technique. Responding hybridomas were subcloned twice, expanded in mass culture, and used as the seed stock for ascites generation. MoAb-G6 (IgAκ isotype) represented the highest titered antibody and was purified from ascites fluid by affinity chromatography on solid phase P072-coupled resin (Affi-Prep 10, Bio-Rad Laboratories, Richmond, CA; coupling efficiency, 10 μmol peptide/ml resin). The antibody was characterized for binding specificity using a solid phase RIA with [^{125}I]protein A as the detector (20) (Fig. 1). In brief, test peptides were passively absorbed to individual wells (50 ng/well; overnight at 4 C) of a 96-well polyvinylchloride microtiter plate (Dynatech Laboratories, Chantilly, VA), after which the plate was coated with 1% BSA in PBS to minimize nonspecific binding. Test peptides (Peninsula Laboratories, Belmont, CA) included AM, P072, calcitonin gene-related peptide (CGRP), gastrin-releasing peptide, glucagon-like peptide 1, vasoactive intestinal peptide, arginine vasopressin, GRF, cholecystokinin, amylin, gastrin, oxytocin, calcitonin, αMSH, pancreatic polypeptide, peptide tyrosine-tyrosine, *Tabanus atratus* hypotrehalosemic hormone,

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Address all correspondence and requests for reprints to: Dr. Alfredo Martínez, Biomarkers and Prevention Research Branch, Division of Clinical Sciences, National Cancer Institute, National Institutes of Health, 9610 Medical Center Drive, Room 300, Rockville, Maryland 20850. E-mail: martineza@bprb.nci.nih.gov.

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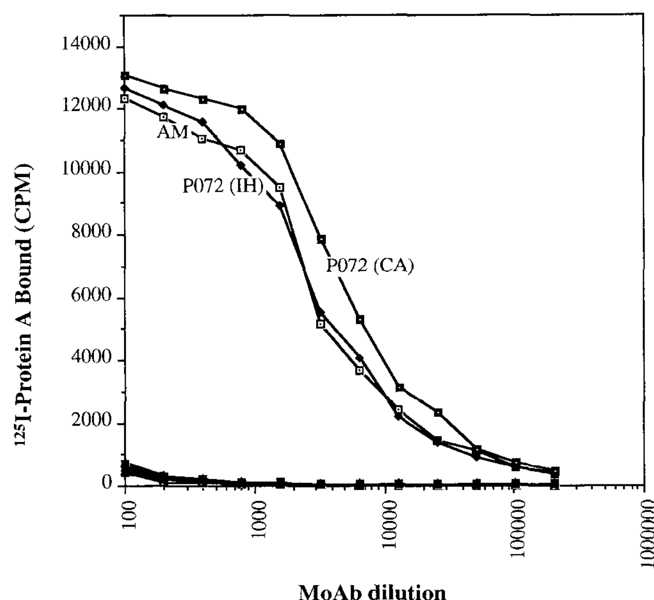

FIG. 1. Titration curve for monoclonal antibody MoAb-G6 binding to solid phase test peptides. The antibody recognized AM and the immunogen P072; both our in-house peptide (IH) and a commercially available product (CA, Peninsula Laboratories). All other target peptides [P070, CGRP, gastrin-releasing peptide (GRP), glucagon-like peptide 1 (GLP 1), vasoactive intestinal peptide (VIP), arginine vasopressin (AVP), GRF, cholecystokinin (CCK), amylin, gastrin, oxytocin, calcitonin, αMSH, pancreatic polypeptide, peptide tyrosine-tyrosine (PYY), *Tabanus atratus* hypotrehalosemic hormone, and BSA] showed negligible binding.

and BSA (Sigma Chemical Co., St. Louis, MO) as a negative control. The titration screen covered a range from 1:100 to 1:204,800 (2-fold dilutions).

Immunocytochemistry (light and electron microscopies)

The avidin-biotin-peroxidase complex method (21) was used for paraffin sections. Negative controls included substitution of the primary antibody with preimmune serum from the same rabbit and preabsorption of the antibody with 10 nmol/ml of the synthetic peptide (AM or CGRP).

For immunoelectron microscopy, three rats were perfused with a mixture of 2.5% paraformaldehyde and 2.0% glutaraldehyde in cacodylate buffer. Small pieces of pancreas were dehydrated and embedded in resin. Ultrathin sections were mounted in nickel grids, and the double immunogold method was used as previously described (22). In brief, sections were incubated in 1% BSA in TBS for 30 min, followed by an overnight incubation at 4 C with a mixture of the antibodies at the optimal dilution. Subsequent steps, all at room temperature, included rinses in 1% BSA-TBS, incubation with the gold-labeled secondary antibodies, rinses with BSA-TBS and distilled water, and double staining in 5% aqueous uranyl acetate and lead hydroxide. In addition to the controls used in light microscopy, one of the primary antisera was omitted in serial sections to exclude possible interactions.

In situ hybridization

Detection of the AM receptor (AM-R) messenger RNA (mRNA) was performed using *in situ* hybridization, as previously described (23). The full-length complementary DNA was ligated into the expression vector pcDNA1 (10) and used to generate riboprobes. The plasmid was linearized with *Eco*RV and *Bam*HI and was used as a template to synthesize digoxigenin-labeled sense and antisense RNA transcripts. Hybridization was performed in a moist chamber at 46 C for 20 h in a 20-μl volume containing 2.5 ng probe/μl. After stringency washes, visualization of digoxigenin was performed using the Digoxigenin detection kit (Boehringer Mannheim, Indianapolis, IN). Controls included the use of the sense probe and digestion with ribonuclease before hybridization.

Cell culture

Six well characterized, insulin-producing cell lines (RINm, N289, TR4, CRL 2057, CRL 1777, and CRL 2055) were obtained from the American Type Culture Collection (Rockville, MD). A small cell lung carcinoma, H187, was obtained from the NCI-Navy Medical Oncology Branch and was used as a negative control for AM-R expression.

Reverse transcription-PCR (RT-PCR) and Southern blot

Polyadenylated mRNA from human normal tissue (adrenals, pancreas, and thymus) was purchased from Clontech (Palo Alto, CA). The Micro-Fast Track kit (Invitrogen, San Diego, CA) was used to extract mRNA from cell lines. RT-PCR and Southern blot were carried out as previously described (5). A set of primers that recognizes the most conserved regions of the AM gene were designed and are shown in Table 1, which also contains the sequences for the oligonucleotides used to amplify the AM-R mRNA. Base sequence analysis of isolated bands confirmed identity of the RT-PCR products.

Islet isolation, insulin secretion, and cAMP assays

Islets from six Sprague-Dawley rats were isolated following well established protocols (24). Assays were performed in 24-well plates (90 islets/well). After a 45-min incubation in RPMI-1690 medium containing 5.6 mM glucose, a second incubation was performed in RPMI containing 20.6 mM glucose and various concentration of AM in the presence or absence of MoAb-G6 (2.5 μg/ml). Supernatants from both incubations were tested by RIA for insulin (Amersham, Arlington Heights, IL). After collecting the medium, the islets from the same experiments were saved for analysis of cAMP contents (25). Islets were extracted in 50% ethanol and centrifuged, and the supernatants were tested for cAMP using a RIA kit (New England Nuclear Corp., Boston, MA) following manufacturer's instructions. The experiments were repeated three times.

Insulin secretion and cAMP assays in cultured β-cells

Insulin-producing cell lines were seeded in 24-well plates at 2×10^5 cells/well, and analyses of insulin secretion and cAMP were performed as described above.

In vivo studies

Glucose tolerance methods have been previously described (26). Six Sprague-Dawley rats were administered a glucose solution (400

TABLE 1. Sequences of the oligonucleotides synthesized for this study

Adrenomedullin	
Sense (AM 250–270)	5′-AAG-AAG-TGG-AAT-AAG-TGG-GCT-3′
Antisense (AM 523–542)	5′-TGT-GAA-CTG-GTA-GAT-CTG-GT-3′
Probe (AM 430–450)	5′-TCT-GGC-GGT-AGC-GCT-TGA-CTC-3′
Adrenomedullin receptor	
Sense (AM-R 476–497)	5′-AGC-GCC-ACC-AGC-ACC-GAA-TAC-G-3′
Antisense (AM-R 923–946)	5′-AGA-GGA-TGG-GGT-TGG-CGA-CAC-AGT-3′
Probe (AM-R 788–811)	5′-GGT-AGG-GCA-GCC-AGC-AGA-TGA-CAA-3′

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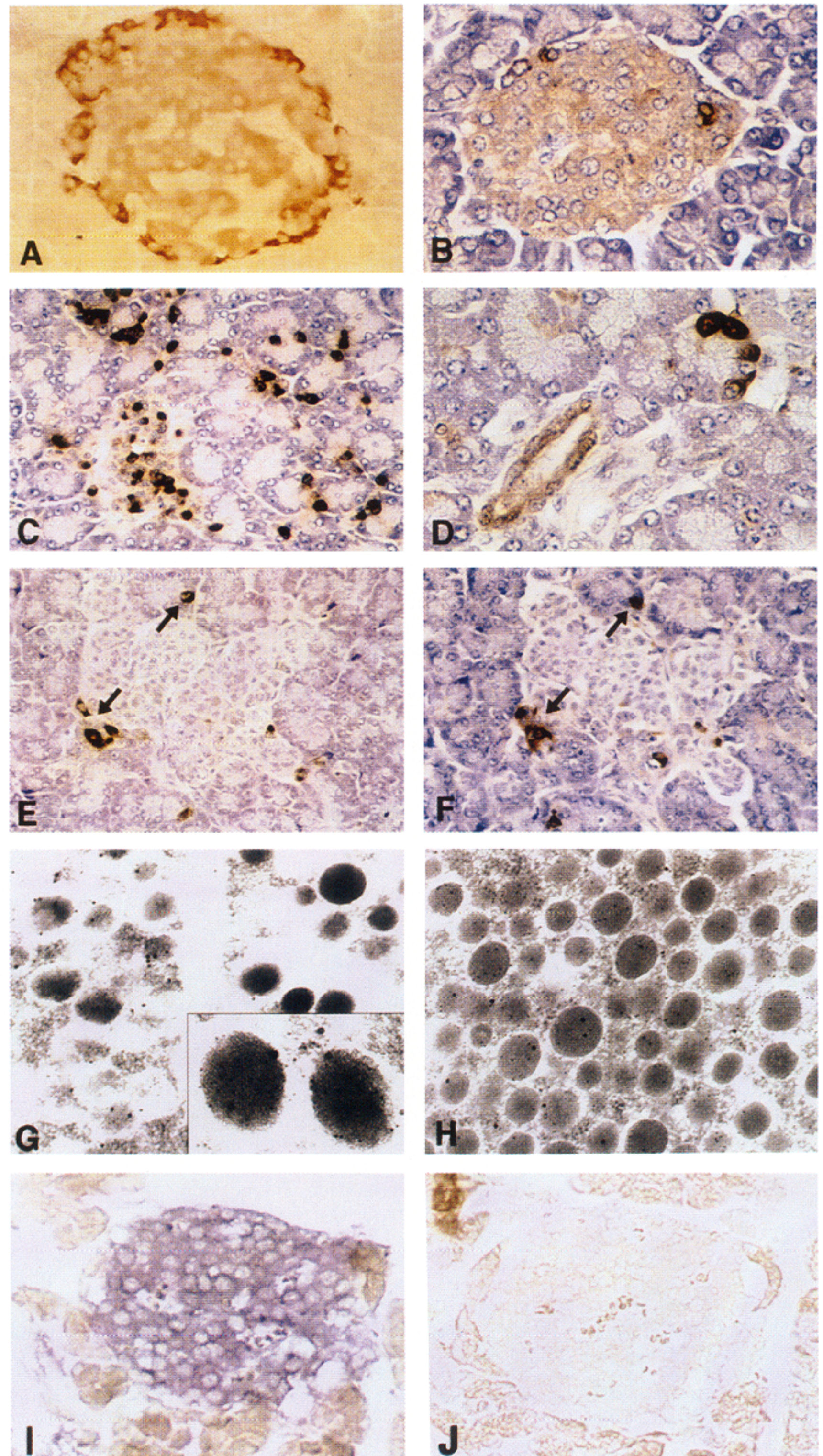

FIG. 2. Distribution of AM in the pancreas, as shown by immunocytochemistry. A, Rat pancreas shows mild immunoreactivity throughout the entire islet of Langerhans and strongly stained cells in the periphery. No counterstaining was applied to this section. B, Hamster pancreas displays a similar pattern. Magnification, ×370. C, Dog pancreas contains numerous immunoreactive cells scattered throughout the parenchyma. Magnification, ×180. D, Immunoreactivity in ductal system of guinea pig pancreas. Note that endocrine cells are more intensely stained than duct cells. Magnification, ×370. Serial sections of hamster pancreas immunostained for AM (E) and pancreatic polypeptide (F) show colocalization of both immunoreactivities (*arrows*). Magnification, ×180. G, Double immunogold staining by electron microscopy in rat pancreas shows colocalization of AM (small gold particles, 10 nm) and pancreatic polypeptide (large gold particles, 20 nm) in the cell situated to the left. A small fragment of a negative α-cell can be observed to the right. Magnification, ×27,000. The *inset* shows two secretory granules containing both immunoreactivities at higher magnification. Magnification, ×62,000. H, Detail of a D cell showing some immunoreactivity for AM (large particles) in the somatostatin-containing (small particles) secretory granules. Magnification, ×27,000. I, *In situ* hybridization for AM-R in rat pancreas with the antisense probe; J, negative control using the sense probe. Magnification, ×180.

mg/100 g BW) via gastric intubation (protocol 95–062, NCI). Ocular blood samples were collected at intervals after glucose loading, and the glucose concentration was determined by a colorimetric assay (Sigma). Blood insulin levels were determined on the same samples by RIA (Amersham). Three days later, the same rats received 1 μl AM (60 μM)/g BW through iv injection immediately after glucose administration, and glucose tolerance tests were repeated. The overall effects of AM on plasma glucose and insulin responses to oral glucose gavage were initially compared by ANOVA (Proc Anova, 1986, SAS Institute, Cary, NC). To satisfy the basic statistical assumptions for the proper use of ANOVA, animals with missing data cells were dropped from the computations. Because of significant treatment by time interactions in the ANOVA, data were further analyzed by regression analysis using a general linear model (Proc GLM, 1986, SAS) to compare differences in treatment means at specific time points using all animals. Furthermore, in this analysis, differences by

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treatment in the time to peak response after glucose gavage and the areas under the insulin and glucose response curves were compared.

Results

Distribution of AM in the pancreas

In humans, rats, and hamsters, the islets of Langerhans were immunoreactive for AM in all cells. However, specific cells in the periphery presented a stronger positivity than others (Fig. 2, A and B). In addition, a few strongly stained cells were found scattered through the pancreatic parenchyma or among the ductal epithelial cells. In the guinea pig, cat, and dog pancreata, the staining pattern was different. Most of the AM-like cells were scattered in the parenchyma, and only occasional immunoreactive cells were found in the periphery of the islets. In these cases, no immunoreactivity was evident in the β-cells (Fig. 2C). In addition, low intensity staining was consistently found in the ductal epithelia of the guinea pig pancreas (Fig. 2D).

To further characterize the nature of the cells containing the AM-like material, serial sections were stained with antibodies against AM and the major pancreatic hormones. Where weak immunoreactivity for AM was noted throughout the islet, colocalization with all other hormones was evident, but the cells strongly positive for AM colocalized only with pancreatic polypeptide (Fig. 2, E and F). Double immunogold staining at the electron microscopic level confirmed the colocalization of AM with pancreatic polypeptide in peripheral cells of rat pancreas (Fig. 2G). Consistent with the light immunohistochemical data, few immunogold particles detecting AM were found in other endocrine cell types by electron microscopy (Fig. 2H).

Distribution of the AM-R was determined by *in situ* hybridization in paraffin sections of rat pancreas. A homogeneous distribution of the mRNA was observed throughout the islets of Langerhans (Fig. 2, I and J).

We were able to confirm these morphological results with molecular techniques. Six insulin-producing cell lines showed expression of mRNA for both the ligand and the receptor (Fig. 3).

Physiological effects of AM in endocrine pancreas regulation

The addition of AM to freshly isolated islets resulted in a dose-dependent reduction of insulin secretion (Fig. 4A). This inhibition reached 78% for an AM concentration of 1 μM. Using the neutralizing monoclonal antibody MoAb-G6, we observed a dramatic 5-fold increase in insulin secretion in the absence of extrinsic AM (Fig. 4A). The addition of extrinsic AM again resulted in a dose-dependent competitive inhibition (Fig. 4A). Consistent with this observation, cAMP levels increased in the islets when AM was added (Fig. 4B). Similar studies were performed in β-cell lines in culture, and no variation was observed in either insulin secretion or cAMP content after the addition of AM (results not shown).

In another experiment we measured the influence of AM on oral glucose tolerance testing of nonanesthetized rats (Fig. 5). In control animals, the plasma insulin concentration increased rapidly to a peak 20 min after glucose administration and remained elevated until 60 min (Fig. 5A). In contrast, rats

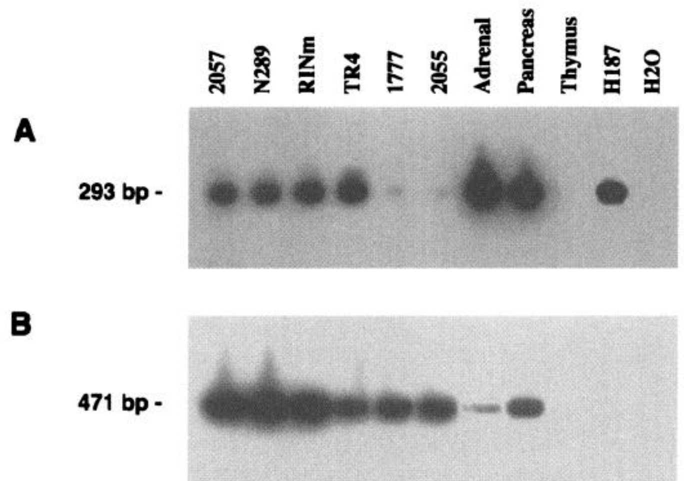

FIG. 3. Southern blot for AM (A) and AM-R (B) in six cell lines expressing insulin and in human adrenal and pancreas mRNA. Human thymus and the cell line H187 (small cell lung carcinoma) were included as negative controls for AM and AM-R, respectively.

treated with AM did not begin to respond to glucose until 40 min, with a peak response 60 min after glucose treatment. In addition, plasma insulin concentrations decreased 20 min ($P < 0.06$) after glucose challenge and tended to be greater than control values at 60 min ($P < 0.06$). Twenty minutes after glucose administration, plasma concentrations of insulin were approximately 2-fold greater in control rats *vs.* AM-treated rats ($P < 0.009$). Plasma glucose concentrations in control rats increases uniformly over time, peaking 4–6 h after feeding (Fig. 5B). In association with the depressed insulin response, plasma glucose levels in AM-treated rats increased rapidly to peak 1 h postoral glucose and decreased in association with a progressive change in plasma glucose. The time to the glucose peak was significantly different between control and AM-treated rats ($P < 0.0025$). The difference between treatments at 1 h was also highly significant ($P < 0.005$).

Discussion

Our morphological data show colocalization of AM with pancreatic polypeptide in the islets of Langerhans. This result differs from the findings of a previous report (27), which suggested a colocalization with somatostatin based only on the peripheral distribution of AM immunoreactivity.

AM has some structural similarities to CGRP and amylin (28), both of which are involved in pancreatic physiology. Nevertheless, the distribution of these three peptides in the pancreas varies. Amylin is mainly located in the β-cells, colocalizing with insulin (29), and CGRP is present in pancreatic nerves as well as in D cells of the islets (30, 31). The differential distribution of the immunoreactivities together with the absorption controls excludes a possible cross-reactivity of the AM antibody with amylin or CGRP.

We have shown, by *in situ* hybridization analysis, that the AM-R mRNA is homogeneously distributed throughout the islet. These data suggest that all endocrine cell types of the pancreas have an inherent potential for expressing re-

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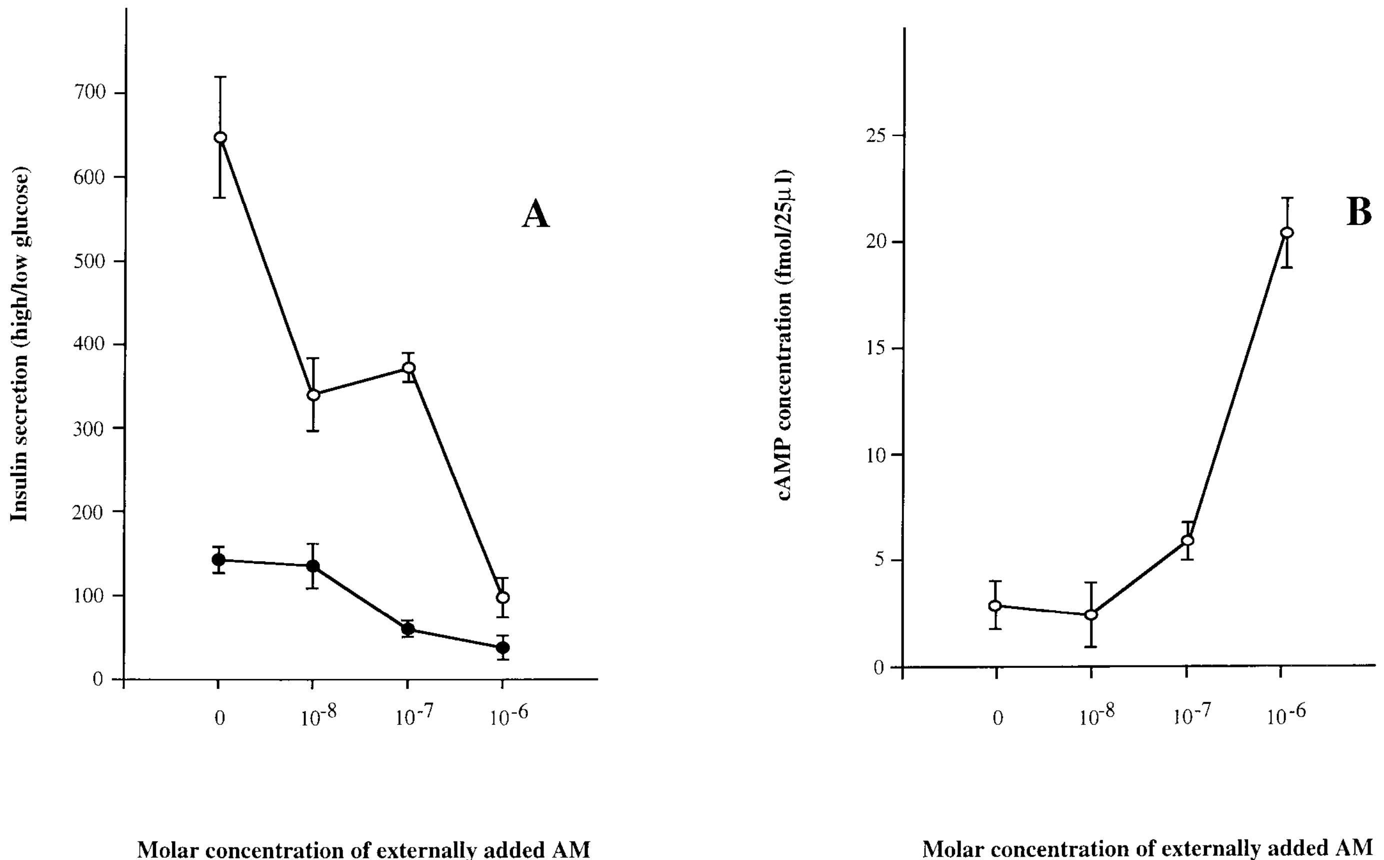

FIG. 4. Effects of AM and MoAb-G6 (α-AM) on the release of insulin from rat isolated islets. A, Increasing concentrations of AM reduce insulin secretion in the presence (○) or absence (●) of MoAb-G6 antibody. Note the dramatic increase in insulin secretion mediated by the antibody. The ratio between the insulin concentration found in the high glucose supernatant and that measured in the low glucose solution is represented on the *ordinates*. As a reference, absolute insulin concentrations in the wells without AM or MoAb-G6 were 0.08 ± 0.01 ng/ml (low glucose) and 10.5 ± 0.6 ng/ml (high glucose). With the addition of the antibody, these values were 0.10 ± 0.02 ng/ml (low glucose) and 62.5 ± 3.2 ng/ml (high glucose). B, Dose-dependent increase in intracellular cAMP in the islets after the addition of AM. Data are the mean ± SD of two wells.

ceptor protein, and hence, certain aspects of their functional role may be regulated by AM. It should be noted, however, that although β-cell lines expressed AM-R, as analyzed by RT-PCR, none responded to exogenous AM treatment by insulin release or cAMP production. Thus, translational regulation of receptor protein expression may play a critical role in mediating the biological effects of AM. On the other hand, we had previously demonstrated expression of AM in lung tumors (5), and the presence of this peptide-receptor system in insulinomas could be more related to the neoplastic phenotype than to the original source of the tumor.

Our experimental data on isolated islets clearly demonstrate the inhibitory role of AM on insulin secretion. AM has been previously described as an antisecretagogue in different systems (16–18), and our study with isolated islets is but another example of this ability. These results are further strengthened by the results observed with the neutralizing monoclonal antibody MoAb-G6, which blocked both endogenous and exogenous AM effects on insulin secretion. Such studies implicate the existence of a continuous inhibitory tone in the islet that may contribute to pancreatic homeostasis.

It has been previously shown that AM triggers increases in both cAMP and Ca^{2+} flux in the target cell (11, 12), and we have been able to demonstrate an increase in cAMP in the islets. However, no response to AM was observed in cultured β-cells even though expression of AM-R was detected by RT-PCR. These results, in agreement with previous studies showing that substances able to increase cAMP and Ca^{2+} in β-cells induce insulin secretion rather than inhibit it (32), indicate that the regulation of insulin secretion through AM is not directly processed in the β-cell; rather, more complex interactions, involving other endocrine cell types, take place at the islet level. A similar mechanism has been described for amylin (33), and further investigation is needed to determine which cell type is responsible for these actions. The apparent contradiction in β-cells between expression of AM-R and lack of cAMP response could be explained by the presence of phosphodiesterases; these enzymes regulate levels of intracellular cAMP by catalyzing its degradation and have been found in the islets of Langerhans (34). Other possibilities consist of AM acting through a different intracellular pathway or through other receptors. There is evidence that AM binds to CGRP receptors with lower affinity (11, 35, 36) and could bind amylin receptors similarly, because the three peptides are structurally related (28).

The *in vivo* experiments fully supported the observations made in isolated islets; AM was able to attenuate and delay

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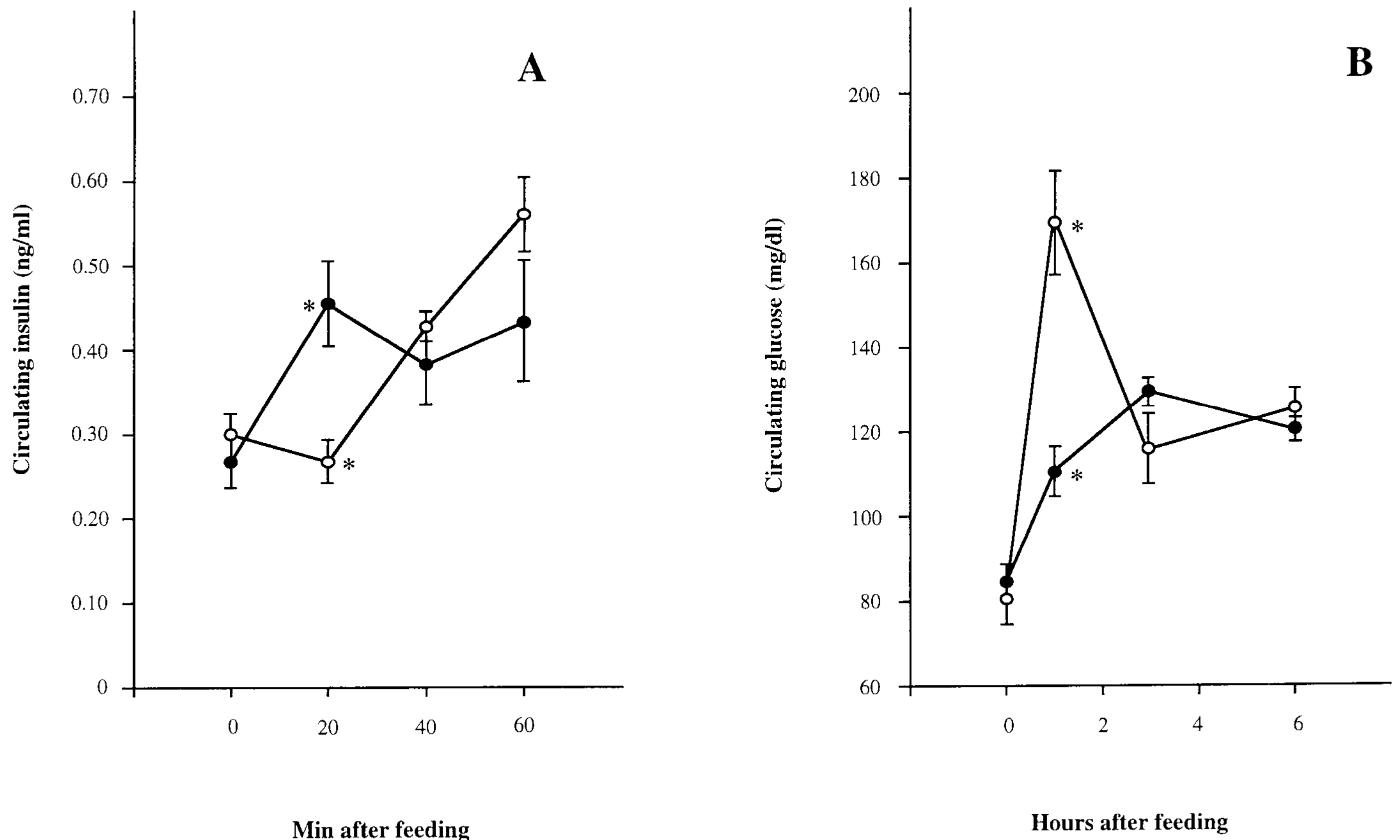

FIG. 5. Glucose tolerance tests were performed on Sprague-Dawley rats (250–300 g) in the presence (○) or absence (●) of AM. A, Significant differences ($P < 0.01$) in insulin levels were observed 20 min after iv injection of AM. B, The difference in levels of circulating glucose was also highly significant ($P < 0.005$) 1 h after injection. Data are the mean ± SD for six animals. *, Statistical significance ($P < 0.01$).

the insulin response to oral glucose challenge, resulting in elevated glucose levels early in the response. The well known vasodilatory effect of AM may also influence the insulin secretion rate by increasing pancreatic perfusion. Although this cannot be the main mechanism, as demonstrated in the islet experiments where blood flux is irrelevant, it merits further evaluation.

As the expression of AM (at least in the cardiovascular system) is affected by levels of tumor necrosis factor-α, interleukin-1, lipopolysaccharide, interferon-γ, endothelin-1, angiotensin II, substance P, bradykinin, thrombin, and vasoactive intestinal peptide (37), any combination of these bioactive substances may be involved with AM in regulating insulin secretion. In fact, some reports link different forms of diabetes with these substances (38, 39).

It has also been observed that AM's physiological effects are somehow connected with those produced by nitric oxide (9). This relationship may be due to the cross-talk in the target cell between the signal transduction pathways for AM, which increases cAMP, and those for nitric oxide, which increases cGMP (40). As nitric oxide synthase is present in the islets of Langerhans, and nitric oxide regulates insulin secretion (41, 42), it would be interesting to investigate the interactions between these two regulatory systems.

A better understanding of the interactions of AM in normal pancreatic physiology and in different pathological states, such as diabetes and obesity, may help define new areas of therapeutic intervention to obliterate these metabolic disorders.

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