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Molecular Physiology of the Islet Amyloid Polypeptide (IAPP)/Amylin Gene in Man, Rat, and Transgenic Mice

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Abstract Islet amyloid polypeptide ("amylin") is the major protein component of amyloid deposits in pancreatic islets of type 2 (non-insulin-dependent) diabetic patients. Islet amyloid polypeptide consists of 37 amino acids, is co-produced and co-secreted with insulin from islet β-cells, can act as a hormone in regulation of carbohydrate metabolism, and is implicated in the pathogenesis of islet amyloid formation and of type 2 diabetes mellitus. Rat islet amyloid polypeptide differs from human islet amyloid polypeptide particularly in the region of amino acids 25–28, which is important for amyloid fibril formation. In rat and mouse, diabetes-associated islet amyloid does not develop. To study the genetic organization and biosynthesis of islet amyloid polypeptide, we have isolated and analyzed the human and rat islet amyloid polypeptide gene and corresponding cDNAs. Both genes contain 3 exons, encoding precursor proteins of 89 amino acids and 93 amino acids, respectively. Apart from a putative signal sequence, these precursors contain amino- and carboxy-terminal flanking peptides in addition to the mature islet amyloid polypeptide. To understand regulation of islet amyloid polypeptide gene expression, we have identified several potential *cis*-acting transcriptional control elements that influence β-cell-specific islet amyloid polypeptide gene expression. Using antisera raised against synthetic human islet amyloid polypeptide we developed a specific and sensitive radioimmunoassay to measure levels of islet amyloid polypeptide in plasma and tissue extracts. Also antisera raised against the flanking peptides will be used in studying human islet amyloid polypeptide biosynthesis. Elevated plasma islet amyloid polypeptide levels have been demonstrated in some diabetic, glucose-intolerant, and obese individuals, as well as in rodent models of diabetes and obesity. To examine the potential role of islet amyloid polypeptide overproduction in the pathogenesis of islet amyloid formation and type 2 diabetes, we generated transgenic mice that overproduce either the amyloidogenic human islet amyloid polypeptide or the nonamyloidogenic rat islet amyloid polypeptide in their islet β-cells. Despite moderately to highly (up to 15-fold) elevated plasma islet amyloid polypeptide levels, no marked hyperglycemia, hyperinsulinemia or obesity was observed. This suggests that chronic overproduction of islet amyloid polypeptide "per se" does not cause insulin resistance. No islet amyloid deposits were detected in mice up to 63 weeks of age, but in every mouse producing human islet amyloid polypeptide (as in man), accumulation of islet amyloid polypeptide was observed in β-cell lysosomal bodies. This may represent an initial phase in intracellular amyloid fibril formation. The human islet amyloid polypeptide overproducing transgenic mice model offers a unique opportunity to study the biosynthesis, intracellular handling, secretion, and extracellular handling of human islet amyloid polypeptide in vivo.

Key words: CALC gene family, genomic organization, transcription regulation, biosynthesis, islet β-cell, insulin resistance, islet amyloid, type 2 diabetes mellitus, animal model

INTRODUCTION

Islet amyloid polypeptide (IAPP) ("amylin") was discovered in 1986/1987 as a novel polypeptide and putative hormone of 37 amino acids, which turned out to be 43% and 46% homologous to calcitonin gene-related peptide-I (CGRP-I) and CGRP-II, respectively [Westermark et al., 1987a; Cooper et al., 1987, 1988]. In addition, like the CGRPs IAPP has cysteine residues at positions 2 and 7, which enable the formation of an amino-terminal ring structure by means of a disulfide bond (Fig. 1). The CGRPs are produced from the CALC-I and -II genes

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[Steenbergh et al., 1984, 1985a], which in man are located on the short arm of chromosome 11 [Höppener et al., 1984, 1985; Kittur et al., 1985].

The CALC-I gene can produce two mRNAs, due to tissue-specific alternative RNA processing events [Amara et al., 1984; Steenbergh et al., 1985b; Höppener et al., 1986]. In the thyroid C-cell, mRNA encoding the precursor of the 32-amino acid hypocalcemic hormone calcitonin (CT) is produced from exons 1–4. CT itself is encoded by exon 4. Particularly in certain neural cells, the CALC-I gene produces mRNA encoding the precursor of the 37 amino acid neuropeptide CGRP-I. This mRNA contains exons 1–3 plus 5 and 6 and CGRP-I is encoded by exon 5. A highly homologous CALC-II gene only produces CGRP-II mRNA, encoding the precursor of the 37-amino acid neuropeptide CGRP-II, which differs from CGRP-I at only 3 positions [Steenbergh et al., 1986; Höppener et al., 1987]. A third CALC locus on chromosome 11 only contains exon 2 and exon 3 homologous nucleotide sequences and is considered a pseudogene for which no mRNA has been described [Höppener et al., 1988]. Also in the rat two CT/CGRP genes have been identified. As in man, one gene produces both CT and CGRP (-α) [Amara et al., 1982], whereas the other gene produces only a second CGRP (-β) [Amara et al., 1985]. In the salmon, which is known to express several different CTs [Potts and Aurbach, 1977], at least one CT gene (I) was shown also to encode a CGRP molecule [Jansz and Zandberg, 1992]. Whether other salmon genes, encoding CT II or CT III, also encode CGRPs remains to be investigated.

The significant homology between IAPP and the CGRPs indicated that the gene encoding this novel polypeptide might be a fourth member of the CALC gene family. This gene might produce a second (CT-like?) mRNA in addition to the (CGRP-like?) mRNA encoding IAPP. Such a second mRNA potentially might encode additional novel and biologically active polypeptides in man. Apart from this "evolutionary" interest in the IAPP gene, we became interested in the physiological function of this novel CGRP-like peptide as well as in its putative role in the pathogenesis of islet amyloid formation and type 2 diabetes mellitus. IAPP was termed as such because it was isolated, purified, and characterized from amyloid which develops in pancreatic islets in diabetic cats and in human insulinomas [Westermark et al., 1987a]. The major protein component isolated from pancreatic islet amyloid from a type 2 diabetic patient was found to have the same amino acid sequence as IAPP, but was termed "amylin" [Cooper et al., 1987, 1988].

Islet amyloid is an amorphous mass of fibrils, formed from IAPP molecules and located primarily between islet β-cells and capillaries [Westermark, 1973]. The histopathological finding of such extracellular protein deposits as a characteristic feature of pancreatic islets in type 2 diabetic patients was recognized already at the beginning of this century [Opie, 1901; Weichselbaum and Stangl, 1901; Cecil, 1909]. The recent discovery of IAPP/amylin as principal component of this islet amyloid and as a putative novel islet hormone implicated in carbohydrate metabolism (see section on Metabolic Actions of IAPP/Amylin) raised the opportunity to investigate novel mechanisms which might be involved in the pathogenesis of the common age-related hereditary disease type 2 diabetes mellitus.

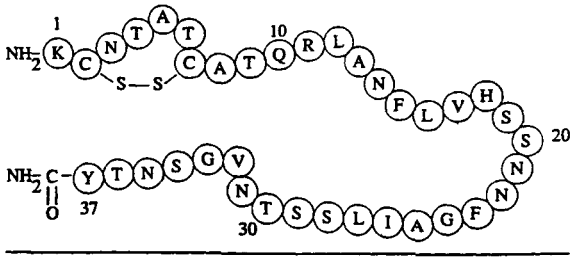

Fig. 1. Primary and predicted secondary structure of human IAPP/amylin. Amino acids are represented in single letter code. The cysteine residues at positions 2 and 7 can form a disulfide bond, creating an amino-terminal ring structure. The tyrosine residue at position 37 can be amidated, with a glycine residue (flanking IAPP in its precursor) as donor of the amide group. (From Lips et al., 1993.)

ISOLATION AND CHARACTERIZATION OF THE HUMAN IAPP/AMYLIN GENE

In order to isolate the human gene encoding IAPP, we screened a genomic DNA library with two oligonucleotide probes whose sequence was based on different parts of the published amino acid sequence of IAPP (Fig. 2). Because of the degeneracy of the genetic code, we made use of the preferential codon usage in higher eukaryotes [Lathe, 1985] when developing these probes. In addition, we took in consideration the DNA sequence encoding the evolutionary related CGRP molecules. Each of the two IAPP oligonucleotides recognized about 100 clones in the DNA library. However, only 4 clones hybridized to both IAPP oligonucleotides. These clones were

negative with a CGRP-specific oligonucleotide probe and all 4 clones turned out to contain nucleotide sequences encoding human IAPP [Mosselman et al., 1988]. Using (part of) the human IAPP gene as a probe for Southern blot analysis of a panel of human-rodent somatic cell hybrids [Mosselman et al., 1988] and for in situ hybridization to human chromosome spreads [Buckle et al., 1989; Hoovers et al., 1993], we demonstrated that the human IAPP gene is a single copy gene located in the region p12.1–12.3 on the short arm of chromosome 12. The fact that the short arm of chromosome 12 is evolutionary related to the short arm of chromosome 11 [Craig et al., 1986], which carries the CALC genes, lent further support to the notion that the IAPP gene is a fourth member of the CALC gene family.

Using the IAPP gene probe for Northern blot analysis, we detected human IAPP RNAs of 1.6 kilobases (kb) and 2.1 kb in insulinoma tissue [Mosselman et al., 1988]. cDNA sequences obtained from a human insulinoma cDNA library corresponded to 3 exons in the human IAPP gene and predicted the complete amino acid sequence of an IAPP precursor of 89 amino acids [Mosselman et al., 1989]. Additional analyses of human IAPP RNAs by means of 5′ RACE (rapid amplification of cDNA ends) reactions, Northern blot analyses and nucleotide sequence analyses of the 3′ end of the gene revealed the exon composition and complete nucleotide sequence of the two major IAPP mRNAs (Fig. 3) [Höppener et al., 1992b]. Both mRNAs start at the same position, 25 nucleotides downstream from a consensus TATA box (TATAA), thus defining the beginning of exon 1 (cap site), which is 103 base pairs (bp) long. Both mRNAs contain exon 2 (95 bp) and exon 3 sequences. The 1.6 kb RNA is generated using polyadenylation signal 2, which is located at a position 1224 bp downstream from the beginning of exon 3. The 2.1 kb RNA extends further downstream and uses one or both of the polyadenylation signals 3 and 4, which are located 1686 and 1716 bp downstream from the beginning of exon 3, respectively. Polyadenylation signal 1, which was used for the generation of mRNA from which one of

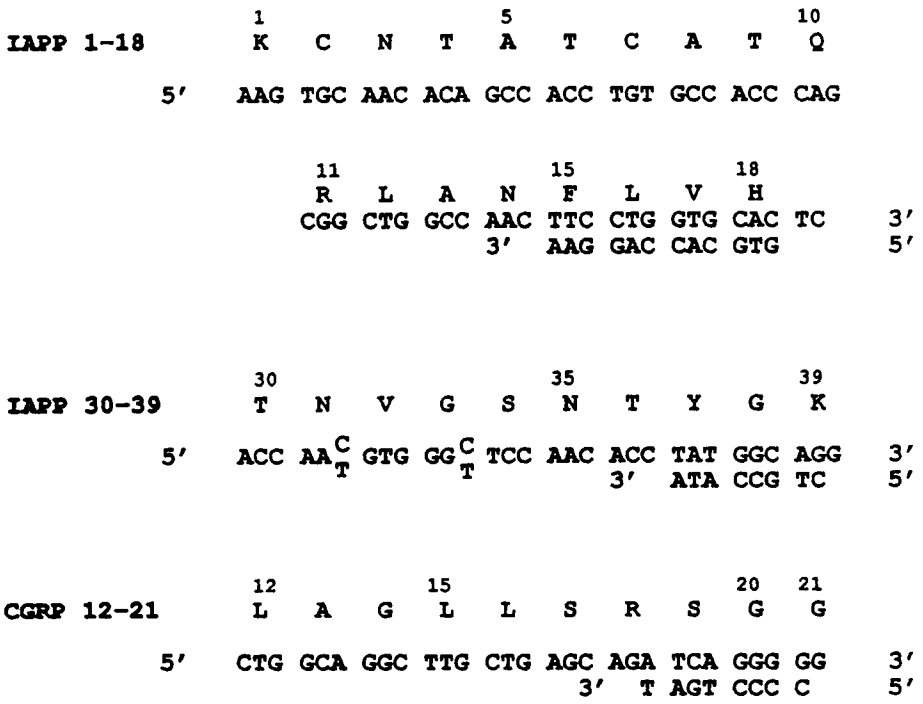

Fig. 2. Nucleotide sequence of template oligonucleotides IAPP 1–18, IAPP 30–39, CGRP 12–21 (middle lines) and their respective oligonucleotide primers (bottom lines), designed on the basis of the corresponding amino acid sequences (top lines). The IAPP 1–18 and IAPP 30–39 oligonucleotides were about 65% and about 90% homologous to the DNA sequences encoding the corresponding amino acids of both CGRP-I and -II. The CGRP 12–21 oligonucleotide was 100% homologous to the DNA sequences encoding amino acids 12–21 of both CGRP-I and -II. Primers were elongated using Klenow polymerase. Incorporation of labeled dNTPs resulted in labeled strands, which could be separated from the template oligonucleotides due to the difference in length. The labeled strands were used as probes to screen a human genomic DNA library.

our IAPP cDNAs was derived [Mosselman et al., 1989], is apparently used very infrequently in insulinoma or pancreas tissue [Höppener et al., 1992b]. Human IAPP cDNA and genomic clones have also been reported from other laboratories and they all predict the same 89 amino acid IAPP precursor [Sanke et al., 1988; Roberts et al., 1989; Nishi et al., 1989a; Christmanson et al., 1990].

The amino terminal 22 amino acids of the human *IAPP* precursor exhibit features of a signal peptide, indicating secretion into the bloodstream. Within the precursor, IAPP is separated from amino-terminal (9 amino acids) and carboxy-terminal (16 amino acids) flanking peptides by dibasic proteolytic cleavage sites lys-arg [Sanke et al., 1988; Mosselman et al., 1989]. A glycine residue flanking IAPP after the tyrosine residue at position 37 indicates amidation of IAPP at its carboxy terminus (Fig. 1), as is the case with e.g., CT and CGRP. IAPP was co-localized with insulin in islet β-cell secretory granules [Westermark et al., 1987b; Lukinius et al., 1989]. The proteolytic enzymes which generate insulin from proinsulin probably also liberate IAPP from its precursor (preproIAPP).

Comparison of the structural organization and nucleotide sequence of the human IAPP gene with those of the CALC-I and -II genes showed that exon 1 of the IAPP gene is analogous to exons 1 of the CALC genes (Fig. 4). IAPP gene exon 2 and the IAPP-encoding exon 3 are both analogous and homologous to exons 2 and the CGRP-encoding exons 5 of the CALC genes. Thus, we wondered whether the region of approximately 5 kb in between exons 2 and 3 in the IAPP gene might contain nucleotide sequences homologous to exons 3 and/or 4 of the CALC genes. Such sequences might be contained within a mRNA encoding a CT-like protein. However, nucleotide sequence analysis of the entire IAPP gene intron 2 region did not reveal sequences with significant homology to either exons 3 or exon 4 of the CALC genes. Thus, exons 1, 2, and 3 are the only exons within the human IAPP gene and only preproIAPP-encoding mRNAs are produced from this gene.

ISOLATION AND CHARACTERIZATION OF THE RAT IAPP/AMYLIN GENE

Islet amyloid associated with diabetes is found in man, cats, and monkeys, but not in rats or mice [Clark, 1992]. To investigate whether IAPP is produced at all in rat, we hybridized a rat pancreas cDNA library with a human IAPP gene probe and isolated a clone containing a nucleotide sequence encoding rat IAPP [van Mansfeld et al., 1990]. Additional analyses of rat IAPP

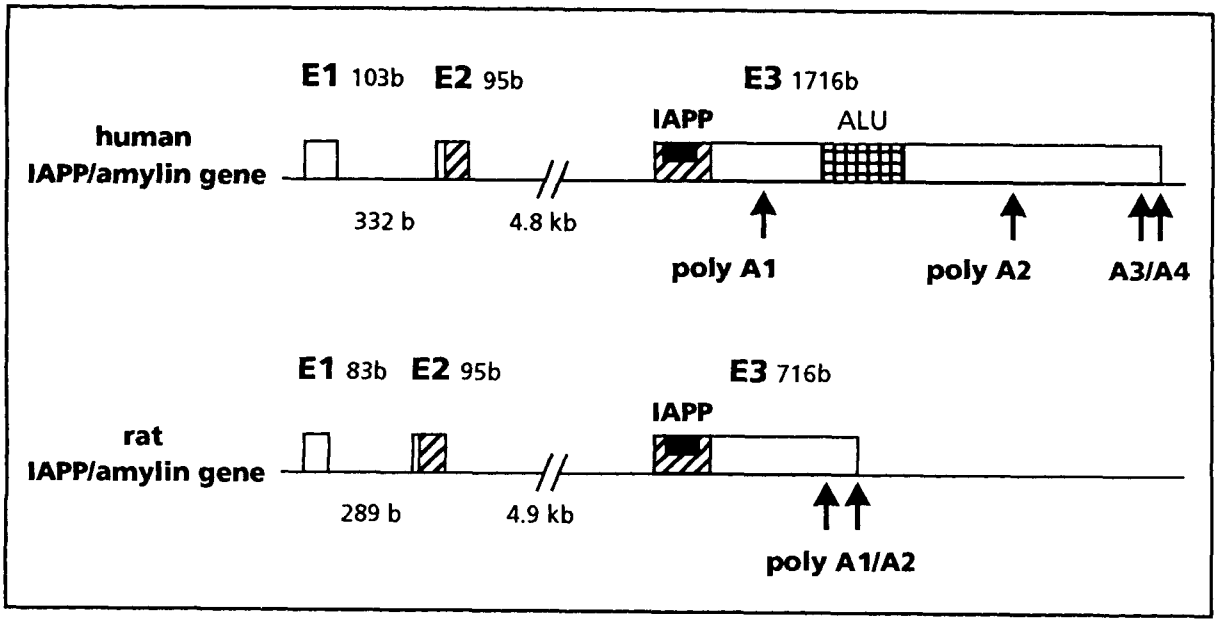

Fig. 3. Comparison of the structural organization of the human and rat IAPP/amylin gene. Exons are represented by numbered boxes, the hatched parts indicate protein-encoding regions, and in black the parts encoding the mature IAPP peptides are shown. Also indicated are the positions of an *Alu* repetitive sequence, characterized polyadenylation signals, and sizes of the exons and introns. b, bases; kb, kilobases.

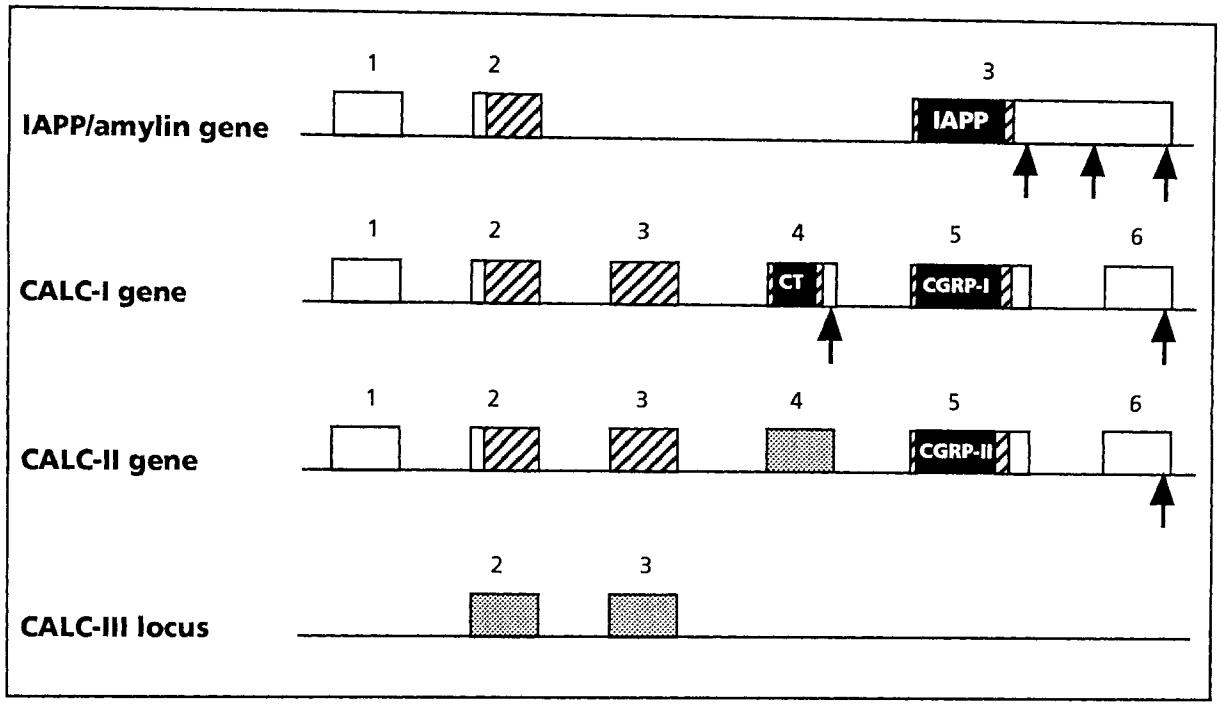

Fig. 4. Schematic representation illustrating a structural comparison between the IAPP/amylin gene, the CALC-I and -II genes and the CALC-III locus. Exons are represented by numbered boxes, the hatched parts indicate protein-encoding regions, and in black the parts encoding the mature IAPP, CT, and CGRP peptides are shown. Spotted boxes indicate 'pseudo-exons' with homology to either exon 2, 3, or 4 of the CALC-I gene. No significant nucleotide sequence homology is found between exons 1 of the IAPP, CALC-I, and -II genes. Exon 2 of the IAPP gene is 42% and 50% homologous to exon 2 of the CALC-I and -II gene, respectively. Highest homology between the IAPP gene and the CALC genes is found between the 111 nucleotides encoding the 37 amino acids of IAPP or the CGRPs (55% with CALC-I, 59% with CALC-II).

mRNAs [Nishi et al., 1989b; Leffert et al., 1989; Ferrier et al., 1989] and of the rat IAPP gene [van Mansfeld et al., 1990] revealed that the structural organization of the rat IAPP gene is very similar to that of the human IAPP gene, also comprising three exons (Fig. 3). Exon 1 is 83 bp long and noncoding. Exon 2 is 95 bp long and contains the ATG start codon for translation. Within exon 3 two polyadenylation signals can be used, which are 90 bp apart and generate mRNAs of approximately 1 kb in pancreas tissue [van Mansfeld et al., 1990]. Rat IAPP mRNAs encode a preproIAPP of 93 amino acids. Like human IAPP, rat IAPP is 37 amino acids long and is separated from amino- and carboxy-terminal flanking peptides by lys-arg and gly-lys-arg sequences, respectively, indicating carboxy-terminal amidation. Also like human IAPP and the CGRPs, rat IAPP contains cysteine residues at positions 2 and 7, predicting an amino-terminal ring structure by disulfide bond formation. The predicted amino acid sequence of rat IAPP differs at 6 positions from that of human IAPP. All these differences are located in the region of amino acids 18-29 (Table I).

Using a combination of immunohistochemistry and in situ RNA hybridization, we demonstrated that rat pancreas β-cells contain both IAPP protein and IAPP RNA [Denijn et al., 1992]. Thus we could actually prove that islet β-cells not only contain IAPP protein but also express the IAPP gene. IAPP is co-released with insulin in response to β-cell secretagogues [Kanatsuka et al., 1989; Kahn et al., 1990; van Jaarsveld et al., 1990].

IAPP/AMYLIN AND ISLET AMYLOID

Amyloid fibrils are formed from polypeptide molecules which contain a region of β-pleated sheet conformation and are held together by hydrogen bonding. Amyloidogenic peptides, like the Alzheimer's A4 or β-peptide [Kirschner et al., 1987] and human IAPP [Westermark et al., 1990] can form amyloid-like fibrils in vitro. Amyloid fibril formation from rat or mouse IAPP (which are identical) has not been described in vitro or in vivo. It was shown that the amino acid sequence in the region 25–28 of human and cat IAPP is very important for amyloid fibril formation and mutation of one or two amino

acids within this region can abolish the capacity to form such fibrils in vitro [Westermark et al., 1990]. The amino acid sequences of IAPP from several species which do or do not form islet amyloid in vivo (Table I), support the notion that the region 25–28 of IAPP is critically important for islet amyloid fibril formation in vivo as well. However, if it were to be the one and only determining factor for amyloidogenesis, then why do only some people (particularly those with type 2 diabetes) develop islet amyloid and why then is islet amyloid not seen in the pancreas of the dog, since the amino acid sequence of dog IAPP 25–28 is identical to cat IAPP 25–28? The causative factors for amyloidogenesis of IAPP are unknown. Type 2 diabetes is not related to a mutation in or around the IAPP gene [Cook et al., 1991]. The amino acid sequence of IAPP as determined from amyloid deposits is identical to the amino acid sequence as predicted by the genomic DNA sequence in both nondiabetic [Mosselman et al., 1988] and diabetic [Nishi et al., 1990] individuals. Overproduction, decreased breakdown or abnormal cellular handling of IAPP could be involved in amyloid formation. The fact that IAPP-immunoreactive amyloid is present in insulinomas in man [Westermark et al., 1987] and in the dog [O'Brien et al., 1990b], indicates that apart from its amino acid sequence, the rate of production/secretion of IAPP might be an additional important factor in islet amyloid formation.

TABLE I. Amino Acid Sequence (in Single-Letter Code) of the IAPP/Amylin Precursor in Man, Rat, and Mouse, as Well as of IAPP in Monkey, Cat, and Dog*

	Signal peptide	Amino-terminal flanking peptide	KR	IAPP/Amylin 1 KCNTATCATQRLANFLVHSSNNFG AILS STNVGSNTY 25 28 37	GKR	Carboxy-terminal flanking peptide NAVEVLKREPLNYLPL
Human	MGILKLQVFLIVLSVALNHLKA	TPIES---HQVE	KR	KCNTATCATQRLANFLVHSSNNFG AILS STNVGSNTY	GKR	NAVEVLKREPLNYLPL
Rat	MRC·SR·PAV·LI·····G··R·	··VG·GTNP···D	··	·····R···L· PV·P P·····	···	·VA·DPN··S·DF·L·
Mouse	M·C·S··PAV·LI····S···R·	··VR·GSNP·MD	··	·····R···L· PV·P P·····	···	··AGDPN··S·DF·KV
Monkey				·····R····· T····· ····D··		
Cat				·····IR···L· ···· P·····		
Dog				·····RT··L· ···· P·····		

*The region of the IAPP molecule that is very important for amyloid fibril formation (amino acids 25–28) is well conserved in man, monkey, cat, and dog, which develop amyloid in pancreatic islets or insulinomas. For references, see Clark [1992].
–, no amino acid at that position in the human sequence; ·, identical amino acid as in the human sequence at that position.

METABOLIC ACTIONS OF IAPP/AMYLIN

In vitro and in vivo experiments have revealed several potential physiological functions of IAPP. In particular, effects on carbohydrate metabolism have been described. In addition, IAPP mimics certain of the known actions of CGRP and CT, like vasodilation and hypocalcemia [Datta et al., 1989; Brain et al., 1990; Gardiner et al., 1991; Kassir et al., 1991]. However, IAPP is much less potent than CGRP and CT in performing these CGRP- and CT-like activities.

The first metabolic effect to be described for IAPP was inhibition of insulin-stimulated glycogen synthesis in rat skeletal muscle in vitro [Leighton and Cooper, 1988]. Subsequently it was shown that in rat skeletal muscle, IAPP can inhibit the enzyme glycogen synthase [Deems et al., 1991], can stimulate activity of the enzyme glycogen phosphorylase [Deems et al., 1991; Young et al., 1991a], and can increase lactate release [Young et al., 1991b, 1992a]. IAPP thus

may act as a catabolic antagonist of insulin in skeletal muscle [Young et al., 1992b]. In liver, IAPP may act as an anabolic partner to insulin by promoting the conversion of muscle lactate into liver glucose and glycogen (Cori cycle) [Roden et al., 1992]. Also in vivo it was shown that administration of IAPP can reduce peripheral glucose disposal and counteract insulin-mediated inhibition of hepatic glucose production [Johnson et al., 1990; Molina et al., 1990; Frontoni et al., 1991; Koopmans et al., 1991].

With respect to a possible inhibitory effect of IAPP on insulin secretion, conflicting results have been published. In some studies, using perfused rat pancreas or isolated islets, an inhibition of insulin secretion was demonstrated [Ohsawa et al., 1989; Silvestre et al., 1990; Kogire et al., 1991] and administration of an IAPP antagonist in vivo enhanced the insulin response to glucose [Wang et al., 1993].

Initially, the reported effects of IAPP were seen with pharmacological rather than physiological concentrations of the peptide. Basal circulating levels of IAPP in man are 2–6 pMol as measured by radioimmunoassay (RIA) [Butler et al., 1990; Hartter et al., 1991; Sanke et al., 1991] (van Hulst et al., in press), whereas the effects described above were usually seen with concentrations of >1 nM. More recently however, inhibition of insulin-stimulated glucose uptake in cultured myocytes was seen with 10-100 pMol [Sheriff et al., 1992] and the EC50 for inhibition of insulin-stimulated glycogen synthesis in skeletal muscle by human IAPP was 220–440 pMol [Rink et al., 1993]. Inhibition of glucose-stimulated insulin secretion in perfused rat pancreas was seen with 75 pM [Dégano et al., 1993]. These are (patho)physiological concentrations in rodents [Bretherton-Watt et al., 1989; Gedulin et al., 1991; Gill and Yen, 1991; Kreutter et al., 1991].

Apart from the studies referred to above, several studies failed to detect effects of IAPP on either insulin action on peripheral tissues [Ghatei et al., 1990; Tedstone et al., 1990; Kassir et al., 1991] or on insulin secretion [Bretherton-Watt et al., 1990; Nagamatsu et al., 1990; O'Brien et al., 1990a; Broderick et al., 1991]. Part of the discrepancies might be explained by reported differences (upto 100-fold) in biological potency of commercially available synthetic IAPP preparations [Young et al., 1991a]. As mentioned before, native IAPP is modified post-translationally in two ways: amidation of the carboxy-terminal tyrosine residue 37 with an amino group from the adjacent glycine residue and disulfide bridge formation between the two cysteine residues at position 2 and 7 to form an amino-terminal ring structure. Both modifications are essential for full biological activity [Cooper et al., 1988].

IAPP/AMYLIN PRODUCTION IN RELATION TO TYPE 2 DIABETES MELLITUS

Type 2 diabetes mellitus is characterized by hyperglycemia, impaired insulin secretion, peripheral insulin resistance and pancreatic islet amyloid formation. High doses of synthetic IAPP have been shown in several studies, although not all, to inhibit insulin secretion as well as insulin action on peripheral tissues (insulin resistance). Islet amyloid associated with diabetes is found in man, cats, and monkeys. This is related to the species-specific amino acid sequence-dependent ability of IAPP molecules to form amyloid fibrils. A second prerequisite for islet amyloid formation might well be overproduction of IAPP, as indicated by fibril formation in vitro [Westermark et al., 1990] and amyloid formation in insulinomas [Westermark et al., 1987a; O'Brien et al., 1990b]. The data with respect to reported metabolic actions of IAPP and to its amyloidogenic properties have led to the hypothesis that an increased production/secretion of IAPP might be involved in the pathogenesis of both islet amyloid formation and type 2 diabetes mellitus. However, a clear picture has not yet emerged. This is probably due to difficulties with the development of a reliable RIA for IAPP, progressive loss of islet β-cell mass as a consequence of progressive amyloid formation, and possible disease stage-specific changes in the pathogenesis of type 2 diabetes. Several studies report elevated basal levels or increased glucose-stimulated levels of IAPP in type 2 diabetic, glucose intolerant or obese individuals [Ludvik et al., 1990; Hartter et al., 1991; Sanke et al., 1991; Eriksson et al., 1992; Koda et al., 1992]. In addition, plasma IAPP levels are markedly elevated (upto 600 pMol) in all rodent models of diabetes/obesity [Bretherton-Watt et al., 1989; Gedulin et al., 1991; Gill and Yen, 1991; Kreutter et al., 1991]. An additional finding *pointing to* the potential importance of IAPP overproduction is the reported increase in IAPP/insulin ratio during periods of prolonged stimulation by elevated glucose levels [Gedulin et al., 1991; O'Brien et al., 1991]. More recently, it was also

shown that in rat islets IAPP gene expression increases with age, and it increases more than insulin gene expression [Giddings et al., 1993]. In man, IAPP secretion was reported to be greater in young and elderly than in middle-aged persons [Edwards et al., 1992]. Thus, age-related changes in IAPP gene expression/secretion might (also) contribute to the pathogenesis of the age-related disease type 2 diabetes.

In view of the potential importance of altered IAPP gene expression for the pathogenesis of islet amyloid formation and type 2 diabetes, as well as for its potential therapeutic prevention/intervention, we decided to study β-cell-specific regulation of IAPP gene expression.

REGULATION OF IAPP/AMYLIN GENE EXPRESSION

The major site of IAPP gene expression is the pancreatic β-cell [Ferrier et al., 1989; Leffert et al., 1989]. Minor sites of expression are the stomach, duodenum and dorsal root ganglia [Ferrier et al., 1989; Asai et al., 1990]. In pancreatic β-cells, IAPP is co-produced and co-secreted with insulin in response to glucose and other β-cell secretagogues, with insulin levels generally being at least 10-fold higher than IAPP levels [Kanatsuka et al., 1989; Fehmann et al., 1990; Kahn et al., 1990; Ogawa et al., 1990]. However, regulation of expression of these two "β-cell-specific" genes is not necessarily coupled. As previously mentioned, prolonged hyperglycemia causes a relative upregulation of IAPP gene expression [Gedulin et al., 1991; O'Brien et al., 1991], as does administration of dexamethasone [Bretherton-Watt et al., 1989]. Also in transformed murine islet cell cultures, uncoupling of IAPP and insulin gene expression has been observed [Madsen et al., 1991] and in human islet β-cell tumors very different ratios of IAPP/insulin mRNA concentrations have been demonstrated [Nieuwenhuis et al., 1992; Stridsberg et al., 1992].

Regulation of gene expression is mostly performed at the level of gene transcription. It is generally accepted that this is established through binding of nuclear proteins (transcription factors/*trans*-acting factors) to particular DNA sequences (*cis*-acting sequences) within, or mostly upstream, of the gene of interest. Tissue-specific gene expression is mediated by binding of tissue-specific transcription factors, or a combination of tissue-specific and ubiquitous transcription factors, to such *cis*-acting elements. To study β-cell-specific regulation of IAPP gene expression, and possibly differential regulation from insulin gene expression, we screened different regions of the human and rat IAPP gene for transcriptional activity. The studied IAPP gene fragments all contained exon 1, intron 1, and exon 2 up to the ATG start codon, which was combined with the ATG of a luciferase reporter gene. The IAPP gene fragments differed with respect to the size of the region upstream of exon 1 (up to −2,800 in the human IAPP gene and up to −3,000 in the rat IAPP gene, respectively). Transcriptional activity of these constructs was tested in a transient expression system in β-cells and non-β-cells. Since human islet β-cell lines are not available, we used rat (RIN) and mouse (βTC3) insulinoma cells for these studies. Both the human and the rat IAPP gene constructs revealed higher transcriptional activity in β-cells than in the non-β-cell lines Hep3B or BRL3A. In both IAPP genes, potential *cis*-acting transcription elements (with homology to insulin gene transcription elements) were identified [Mosselman et al., 1990; de Wit et al., 1993]. In particular in the rat IAPP gene, inclusion of the region −152 to −249 caused a 20-fold increase in gene expression in β-cells. Exact determination of the nucleotide sequences within these constructs which bind β-cell nuclear proteins and identification of these *trans*-acting factors will contribute to elucidation of the molecular mechanisms which regulate β-cell-specific IAPP gene expression [German et al., 1992]. In addition, nucleotide sequences important for glucose- or dexamethasone-induced stimulated IAPP gene expression may be identified by similar analyses.

HUMAN IAPP/AMYLIN TRANSGENIC MICE AS A MODEL FOR TYPE 2 DIABETES MELLITUS

To examine in an in vivo model the hypothesis that overproduction of IAPP is a primary event in the pathogenesis of islet amyloid formation and type 2 diabetes, we have generated transgenic mice that overproduce either the amyloidogenic human IAPP or the nonamyloidogenic rat IAPP (which is identical to mouse IAPP). To target transgene expression to islet β-cells and to obtain overexpression, we placed the IAPP transgenes under transcriptional control of a rat insulin 2 gene promoter (RIP2) fragment. Thus, effects of chronically elevated plasma IAPP concentrations on glucose metabolism and islet β-cell function could be studied. In addition,

transgenic mice producing human IAPP are suitable for examination of factors involved in islet amyloid formation. Such transgenic mice represent a more "natural" and powerful model to study biological actions of native IAPP, produced in vivo in (supra)physiological amounts from pancreatic islet β-cells.

The transcription rate of the insulin genes in man and rat is probably at least 10-fold higher than that of the IAPP gene [Bretherton-Watt et al., 1989; Leffert et al., 1989] and the RIP2 fragment we employed (−695/+8) was known from previous studies to direct β-cell-specific expression of a coupled gene in transgenic mice [Hanahan, 1985]. Several founders with the human or rat IAPP gene construct were obtained and transgenic IAPP mRNA of the correct size was detected in the pancreas of every transgenic animal investigated [Höppener et al., 1992a]. Human IAPP mRNAs were 4–22 times more abundant in human IAPP transgenic mouse pancreas, whereas rat IAPP mRNAs were 2–3 times more abundant in rat IAPP transgenic mouse pancreas as compared to normal human or rat pancreas, respectively. The levels of endogenous mouse pancreas IAPP and insulin mRNA were apparently not influenced by expression of the transgenes. To investigate the actual production of protein from the human transgene, we performed immunohistochemistry using an antiserum raised against the carboxy-terminal flanking peptide in the human IAPP precursor. The homology between this peptide and the corresponding peptide in the mouse IAPP precursor is only 38% [Nishi et al., 1989b], whereas human and mouse IAPP are 84% homologous. The antiserum immunostained β-cells in human pancreas and in human IAPP transgenic mouse pancreas, but not in rat IAPP transgenic or control mouse pancreas, thus confirming human protein production (Fig. 5). Using a RIA for IAPP, which was developed in our laboratory (van Hulst et al., in press), significantly elevated (up to 15-fold) plasma IAPP levels were demonstrated in most transgenic lines. In the transgenic mice, plasma glucose and insulin levels were not elevated (Fig. 6) and transgenic animals did not develop obesity. This indicates that in mice chronically elevated plasma IAPP levels "per se" do not inhibit insulin biosynthesis and suggests that they do not induce insulin resistance [Höppener et al., 1993]. In some short-term studies IAPP-induced inhibition of insulin secretion has been demonstrated. However, changes of insulin secretion at long-term elevated IAPP concentrations have not yet been explored. In theory, both a direct inhibitory effect of IAPP on β-cell insulin secretion and a stimulatory influence due to IAPP-induced peripheral insulin resistance might counterbalance each other, without an obvious effect on plasma insulin levels. However, in that case the resulting insulin level would probably be insufficient to prevent hyperglycemia as a consequence of the insulin resistance. Additional in vitro and

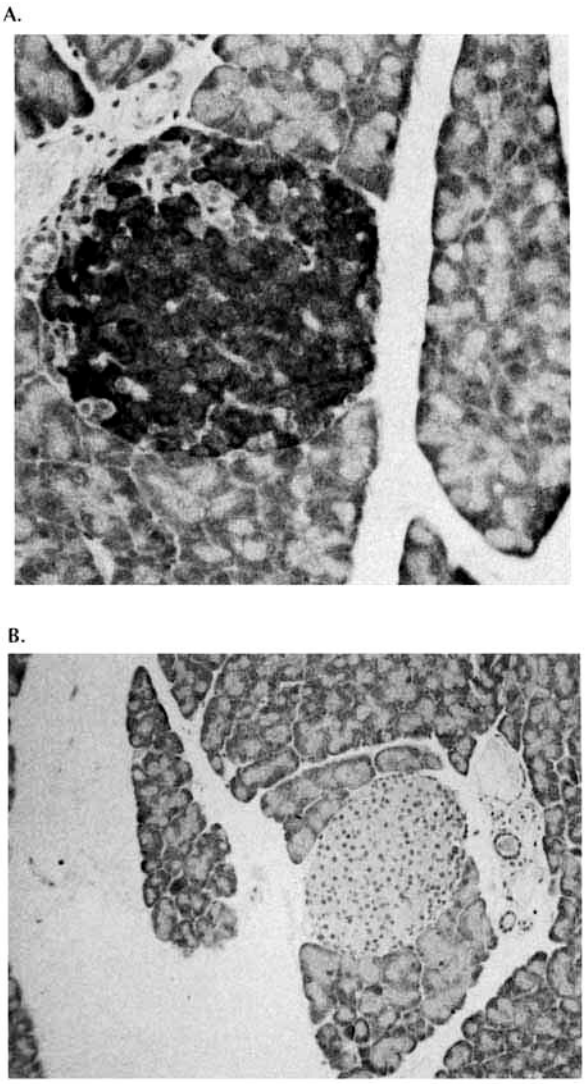

Fig. 5. Detection of human IAPP/amylin gene encoded protein in pancreas tissue using immunoperoxidase staining with a rabbit antiserum raised against the carboxy-terminal flanking peptide in the human IAPP precursor. The antiserum immunostained islet β-cells in human IAPP transgenic mouse pancreas (**A**), but not in nontransgenic control mouse pancreas (**B**), ×200.

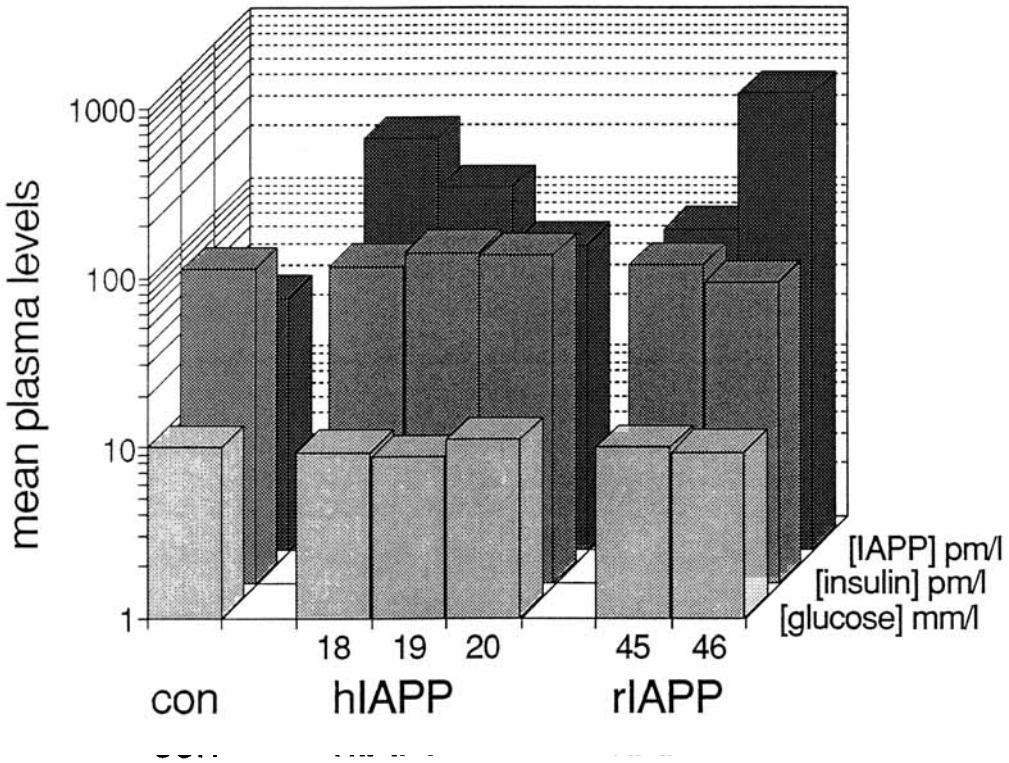

Fig. 6. Plasma IAPP, insulin and glucose levels of nontransgenic control mice, human IAPP transgenic mice (lines 18, 19, and 20) and rat IAPP transgenic mice (lines 45 and 46). The mean value of 4–10 nonfasted animals (both males and females) is indicated. The IAPP levels in lines 18, 19, and 46 were significantly higher, as compared to the controls. Plasma glucose and insulin levels were not statistically significant between transgenic mice and controls.

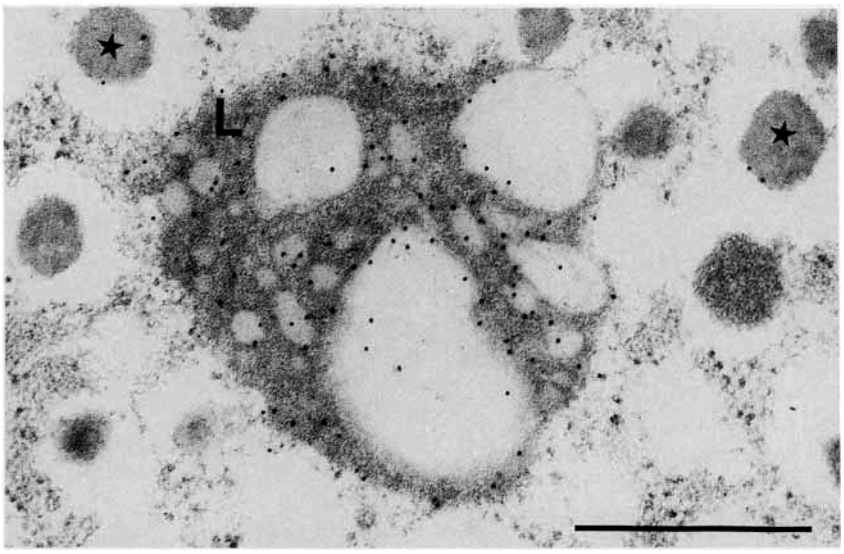

Fig. 7. Subcellular localization of IAPP in islets of Langerhans of human IAPP transgenic mice, using electron microscopy and immunogold labeling. IAPP immunoreactivity was detected in β-cell secretory granules (*) in all transgenic and control mice. In addition, a more dense immunoreactivity was present in β-cell lysosomal bodies (L) of human IAPP transgenic mice only. Scale bar = 0.5 μm.

in vivo experiments are in progress to study this in more detail.

Using light microscopy and amyloid-specific histological staining techniques, no amyloid deposits could be detected in the pancreas of transgenic mice up to 63 weeks of age. Using electron microscopy and immunogold labeling, IAPP was demonstrated in β-cell secretory granules in all mice examined. However, the highest level of IAPP immunoreactivity was observed in β-cell

lysosomal bodies in all human IAPP transgenic mice [de Koning et al., 1992; Höppener et al., 1993] (Fig. 7), as in man [Clark et al., 1989]. IAPP immunoreactivity in lysosomes was not seen in rat IAPP transgenic or control mice. These data suggest that excess β-cell granules are degraded via lysosomes and that breakdown of human IAPP is slower than that of rat or mouse IAPP. In human insulinoma and monkey islet β-cells, intracellular IAPP-immunoreactive amyloid fibrils have been demonstrated [Clark et al., 1991]. If islet amyloid fibril formation occurs as a consequence of accumulation of IAPP, lysosomal bodies represent a potential intracellular site for its initiation, particularly since both lysosomal accumulation of IAPP and amyloid fibril formation are related to the amino acid sequence of the IAPP molecule. Whether such intracellular fibrils contribute to extracellular amyloid deposits remains to be investigated.

Transgenic mice overproducing human IAPP (up to 5-fold elevated plasma IAPP levels) under control of a rat insulin 1 gene promoter, were recently described by Fox et al. [1993]. Also in these mice, hyperglycemia, hyperinsulinemia, or islet amyloid deposits were not observed.

CONCLUSIONS

The IAPP gene is a fourth member of the CALC gene family, producing mRNAs encoding preproIAPP of 89 (man) or 93 (rat) amino acids. Within the precursor IAPP is bordered by amino- and carboxy-terminal flanking peptides of unknown function. Native IAPP contains an amino-terminal ring structure between cysteine residues and is carboxy-terminally amidated. These post-translational modifications are essential for full biological activity. IAPP is the major protein component of amyloid in pancreatic islets of type 2 diabetic patients and in insulinomas. Amyloidogenic properties of IAPP are related to its amino acid sequence in the region 25–28 and possibly to the rate of its synthesis. Both in the human and rat IAPP gene, potential *cis*-acting elements that influence β-cell-specific IAPP gene expression have been identified and corresponding *trans*-acting factors are being discovered. Antisera raised against IAPP and its flanking peptides will aid in elucidating the biosynthetic pathway of IAPP and study possible alterations in its regulation in disease. Metabolic actions of IAPP are mainly related to carbohydrate metabolism in skeletal muscle (catabolic antagonist of insulin), liver (anabolic partner of insulin), and pancreas (inhibition of insulin secretion). These reported actions, as well as elevated IAPP levels in diabetic, glucose-intolerant, and obese individuals and in animal models, have led to the hypothesis that IAPP overproduction may be a primary event in the pathogenesis of islet amyloid formation and insulin resistance in type 2 diabetes. Recent data indicate that chronic overproduction of human IAPP in transgenic mice does not inhibit insulin secretion and no indications for insulin resistance (e.g., hyperglycemia or hyperinsulinemia) were obtained. At the level of skeletal muscle and liver this is being investigated in more detail. Human IAPP is handled similarly by transgenic mouse β-cells as by human β-cells, causing accumulation in lysosomal bodies. Further studies are needed to investigate whether this can lead to fibril formation and amyloidogenesis. Since no amyloid deposits were found in transgenic mice up to 63 weeks of age, other factors apart from IAPP overproduction are probably required for the process of amyloidogenesis (e.g., hyperglycemia and/or hyperinsulinemia?). Alternatively, mice do simply not grow old enough to develop islet amyloid deposits as we know them in man. In the pathogenesis of type 2 diabetes, increased co-production of insulin and IAPP may induce and/or contribute to progressive islet amyloid formation. In the long run this may lead to β-cell dysfunction, insufficient insulin secretory capacity and hyperglycemia. The human IAPP overproducing transgenic mice model offers a unique opportunity to further investigate the biosynthesis, intracellular handling, secretion and extracellular handling of human IAPP in vivo.

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