Studies of Neuropeptides in Pancreatic Beta Cell Function with Special Emphasis on Islet Amyloid Polypeptide (IAPP)

BY

ELLA KARLSSON
ABSTRACT


The presence of protein amyloid in pancreas and its association to diabetes was first described 100 years ago in 1901, but was not identified as Islet Amyloid Polypeptide (IAPP) until 1986. The aim of the present work was to determine the role of the beta cell hormone, IAPP, in normal pancreatic islet physiology and during early disturbances of islet function.

Intra-islet peptides, i.e. chromogranin peptides and an extra-islet peptide, i.e. leptin, were studied to identify possible endogenous regulators of IAPP and insulin secretion. Chromogranin-B, but not chromogranin-A or pancreastatin, had the ability to inhibit islet IAPP and insulin release, suggesting that chromogranin-B may serve as an autocrine regulator of IAPP and insulin secretion.

Leptin had a more potent effect on IAPP secretion than on insulin secretion, which was dissociated from effects on islet glucose metabolism. Glucose oxidation rates were increased at physiological leptin concentrations, whereas higher leptin concentrations showed an inhibitory effect and chronically high leptin concentrations had no effect.

Female NOD mice were studied to investigate the release of IAPP in the progression to type 1 diabetes. The release of IAPP was lower than that of insulin from immune cell infiltrated islets, indicating preferential insulin release during the early course of the disease.

IAPP is expressed at an early embryonic stage. The effect of IAPP on cell proliferation in neonatal rat islets was studied in the search for a physiological role of IAPP. IAPP concentrations of 1-1000 nM stimulated neonatal islet cell proliferation mostly in beta cells and to a lesser extent in alpha cells. IAPP did not have any marked effect on the islet cell death frequency. These data indicate a role for IAPP as a potential regulator of beta cell proliferation in neonatal pancreatic islet.

It is concluded that IAPP may be involved in regulation of pancreatic beta cell function both in fetal and adult life.

Key words: Alpha cell, amylin, beta cell, chromogranin, growth, IAPP, insulin, islet amyloid polypeptide, leptin, NOD mice, pancreastatin, pancreatic islet, proliferation.

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“Perfekt!” sa jag. “Jag visste att du kunde!”

“Har vi kommit till slutet?” frågade Nasse.

“Ja”, svarade jag, “jag antar det.”

“Det verkar vara slutet”, sa Puh.

“Det gör det. Och ändå-”

“Ja, vadå, Nasse?”

“På sätt och vis verkar det också som en början.”

Benjamin Hoff, Te enligt Nasse, 1992
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<td>Bio-Breeding</td>
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<tr>
<td>BrdU</td>
<td>5-bromo-2’ deoxyuridine</td>
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<td>Cg</td>
<td>Chromogranin</td>
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<td>CGRP</td>
<td>Calcitonin gene related peptide</td>
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<tr>
<td>CIS</td>
<td>Cytokine-inducible sequence</td>
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<tr>
<td>CRLR</td>
<td>Calcitonin receptor-like receptor</td>
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<tr>
<td>DAP</td>
<td>Diabetes associated peptide</td>
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<tr>
<td>Cdk4</td>
<td>Cyclin dependent kinase 4</td>
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<tr>
<td>db</td>
<td>Diabetes gene - mutated leptin receptor gene</td>
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<tr>
<td>E</td>
<td>Embryonic day</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>Erk</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<td>FCS</td>
<td>Fetal calf serum</td>
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<td>GH</td>
<td>Growth hormone</td>
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<td>GIP</td>
<td>Gastric inhibitory polypeptide</td>
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<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte/monocyte colony stimulating factor</td>
</tr>
<tr>
<td>hGH-V</td>
<td>Human placental growth hormone</td>
</tr>
<tr>
<td>IAP</td>
<td>Insulinoma associated peptide</td>
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<tr>
<td>IAPP</td>
<td>Islet amyloid polypeptide</td>
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<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
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<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>Jak</td>
<td>Janus activated kinase</td>
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<tr>
<td>KDP</td>
<td>Komedia diabetes-prone rat</td>
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<tr>
<td>KRBH</td>
<td>Krebs Ringer bicarbonate buffer</td>
</tr>
<tr>
<td>LI</td>
<td>Labelling index</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukocyte inhibitory factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<tr>
<td>NMRI</td>
<td>Naval medical research institute</td>
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<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
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<tr>
<td>Ob</td>
<td>Obesity gene coding for leptin</td>
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<tr>
<td>Ob-R</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>OM</td>
<td>Oncostatin M</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PC</td>
<td>Prohormone convertase</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDX-1</td>
<td>Pancreatic duodenal homeobox gene 1</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PL</td>
<td>Placental lactogen</td>
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<tr>
<td>PMSF</td>
<td>Phenylmethysulphonyl fluoride</td>
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<td>PRL</td>
<td>Prolactin</td>
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<tr>
<td>PST</td>
<td>Pancreastatin</td>
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<tr>
<td>RAMPs</td>
<td>Receptor-activity modifying proteins</td>
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<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell park memorial institute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecylphosphate</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TUNEL</td>
<td>Terminal transferase-mediated deoxynucleotidyl nick end labelling</td>
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<tr>
<td>Wr</td>
<td>Wistar rats</td>
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Background

General introduction

Diabetes mellitus is a disease of increasing incidence worldwide (1). Type 2 diabetes is the most common form of diabetes, corresponding to around 80% of all diabetic cases and it is estimated that the number of type 2 diabetic patients will have exceeded 200 million by the year 2010 (1). When considering these numbers, it is easy to understand that diabetes will have a tremendous impact on future health care.

In the past few years, research has provided growing evidence for a remarkable heterogeneity among patients with diabetes mellitus and particularly so in type 2 diabetes. However, even though several subgroups have been described, diabetes mellitus can still, in large, be divided into two main groups.

Type 1 diabetes is considered to develop as a consequence of an autoimmune destruction of pancreatic beta cells, preferentially in young people (2, 3). The cause of type 2 diabetes appears more complex, involving disturbed action and secretion of insulin as well as inadequate hepatic regulation of nutrients. An important feature in common for type 1 and type 2 diabetes, is the functional loss of insulin. In type 1 diabetes, this is due to an attenuated insulin production, whereas type 2 diabetes partly could be regarded as a situation of a relative lack of insulin due to decreased insulin sensitivity. The lack of insulin in type 1 diabetes gives rise to a number of metabolic changes, e.g. inhibition of glycogen breakdown (4), decreased glucose uptake (5), increased protein breakdown (6) and increased lipolysis, (7) followed by formation of ketone bodies (8). There is increasing evidence for severe metabolic disturbances also in type 2 diabetes. Recent studies have been devoted to clarify a relationship between nutritional intake on one hand and insulin sensitivity, leptin synthesis, leptin’s effect on the endocrine pancreas and hepatic glucose handling, on the other.
Deeper knowledge regarding pancreatic islet physiology is necessary for the understanding of the pathogenesis underlying type 1 and type 2 diabetes, respectively. The present thesis will focus on the role of the beta cell hormone, Islet amyloid polypeptide (IAPP), in normal islet physiology and early in the progression to a disturbed islet function.

**Aspects of Islet amyloid polypeptide (IAPP) in the pancreas**

Although the presence of amyloid deposits in pancreas was first described 100 years ago (9), it was not until 1986 that the protein composition of these deposits was identified. The key component, amylin, (Islet Amyloid Polypeptide (IAPP), Diabetes Associated Peptide (DAP) or Insulinoma Associated Peptide (IAP)), was first isolated from amyloid deposits in a human insulinoma (10). Shortly thereafter, IAPP was also described in amyloid deposits in pancreata of diabetic cats and humans (11, 12). The peptide is most often referred to as IAPP in Europe, whereas the name amylin is more often used in the US.

**IAPP structure and tissue localization**

IAPP is a 37 amino acid peptide that is synthesized in the form of a preproamylin in pancreatic beta cells (Fig. 1). Prohormone convertase 2 (PC2), the enzyme known to convert proinsulin into insulin, is involved in the processing of the 89 amino acid preproamylin (13). Prohormone convertase 1/3 (PC1/PC3) might also be of importance in IAPP formation (13).

The active form of IAPP contains two posttranslational modifications, an intramolecular disulfide bridge between cysteine residues at position 2 and 7 and a C-terminal amide group (14). A limited region of the human and cat IAPP, amino acid position 20-29, is linked to fibril formation (15-17).
The sequence Ala-Ile-Leu-Ser-Ser at positions 25-29 of human IAPP has been suggested to be particularly amyloidogenic. A proline-for-serine substitution at position 28, as seen in most rodents, abolishes the capacity to form fibrils. In line with this, substitution with three proline residues at positions 25, 28 and 29 as in the human analogue, pramlintide, results in a peptide without any tendency to self-aggregate (15, 18, 19). However, the picture regarding amyloidogenic sections of the IAPP molecule might be modified in the near future since it was recently proposed that several domains of hIAPP as well as one part of the ratIAPP, have the ability to form fibrils (20). However, this has not been shown in vivo.

IAPP is mainly expressed in pancreatic beta cells where it is stored together with insulin in secretory granulas. Immunoreactivity against IAPP can also be found in lysosomes and lipofuscin bodies of the beta cell. The fact that not all IAPP is secreted might be one prerequisite for intracellular accumulation of the peptide (21). At lower concentrations, IAPP has also been described to be present in islet somatostatin producing delta cells (22, 23).
IAPP appears early during embryogenesis, however, there are some species differences. In the mouse fetal pancreas, IAPP and insulin are both detected on the 12th gestational day (24). In contrast, insulin precedes IAPP in the human embryonic pancreas, i.e. IAPP is expressed from 13 weeks of gestation onwards while insulin is present already at 9 weeks (25).

Furthermore, IAPP is distributed in neuroendocrine cells of the gut from the stomach to the rectum (26, 27). IAPP mRNA has been detected in sensory ganglia (28, 29). Moreover, subtypes of sensory neurons expressing IAPP have been further characterized (30). IAPP has been described to alter neurotransmitter metabolism in hypothalamic tissue extracts (31) and there is one report of IAPP expression in brain tissue in chickens (32). However, it can be assumed that IAPP does not have a widespread distribution in the brain since Northern blot analysis for IAPP mRNA has generally been negative in brain tissue. Small amounts of IAPP mRNA have also been detected in the lung (28).

The expression of IAPP in islets is higher than in any other tissue studied. This is consistent with the fact that amyloid formation by IAPP has not been described outside islets.

**IAPP signalling**

A specific IAPP receptor has so far not been possible to identify. However, the peptide family consisting of IAPP, adrenomedullin and CGRPs, have recently been suggested to signal via a group of receptors that involves a calcitonin receptor-like receptor (CRLR) in combination with different receptor-activity-modifying proteins (RAMPs) (33). The specificity of the receptor for either IAPP, adrenomedullin or CGRP is thought to be dependent on the combination of the different RAMP-proteins in the receptor. The calcitonin receptor-like receptor (CRLR) has been demonstrated to be specifically expressed in beta cells within the islet which is of relevance to study IV in this thesis (33).
In the same study, PCR-analysis showed specific expression of CRLR, RAMP1 and RAMP3 in a mouse beta cell line. Interestingly, aortic endothelial cells and COS-7 cells transfected with certain heterodimers of CRLR and RAMP1 or RAMP3, have been demonstrated to have higher affinity for IAPP than CGRP (34, 35).

**Effects of IAPP on islet hormone secretion**

Both CGRP and IAPP are expressed in pancreatic islets although the localization differs. CGRP is mainly expressed in delta cells and sensory nerve fibres, whereas IAPP is predominantly found in the beta cells (36). As already mentioned, there is evidence for the presence of a receptor with high affinity for IAPP on beta cells (33, 37, 38). Several studies have therefore been undertaken to investigate an effect of IAPP on insulin release. At present, there is no consensus regarding a regulatory role for IAPP in insulin secretion. It should also be noted that the role of CGRP in insulin release remains controversial, although most studies have shown an inhibitory effect of CGRP on insulin release (39, 40).

Both in *in vivo* and *in vitro* experiments, an inhibitory effect of IAPP and its closely related peptides have been reported on insulin secretion stimulated by glucose (41-52), arginine (51, 53-55), carbachol (56), glucagon (57) as well as other beta cell secretagogues of potential clinical relevance such as a cholecystokinin fragment, sulfonylurea and KCl (58). Recently, it was demonstrated that IAPP-deficient mice have an accelerated rate of glucose elimination at high glucose due to an increased insulin response (59). Some authors claim a lack of effect of IAPP on insulin release (56, 60-65) whereas others suggest a stimulatory effect (66, 67). However, IAPP of 1000 pmol/l was needed in order to stimulate insulin secretion in the perfused rat pancreas (67). In the same study, there was a tendency towards decreased insulin secretion at lower concentrations of IAPP.

In humans, a physiological role for IAPP in islet insulin regulation has been questioned. IAPP concentrations 90 times higher than the described plasma concentrations of IAPP (2-15 pmol/l) (68), were needed to obtain an inhibitory effect on insulin secretion in one study (40). Furthermore, a reduced
insulin secretion and hyperglycemia has been described in one patient with high circulating IAPP concentrations due to an IAPP-like peptide secreting tumor (69). Two independent experiments with transgenic mice overexpressing human IAPP in beta cells have reported reduced glucose-stimulated insulin release (70, 71). In the study by Ahrén et al (71), IAPP was only effective after an oral glucose tolerance test, giving rise to theories that the insulin release was affected secondary to changes in gastric emptying and release of gut hormones and not as a direct effect in pancreas. However, it was recently presented that hIAPP mice may show inadequate insulin secretion also after an i.p. glucose tolerance test (72).

IAPP has been proposed to inhibit pancreatic glucagon secretion. Thus, at physiological concentrations, IAPP has been shown to dose-dependently inhibit arginine-induced glucagon release (55, 73). In a study by Wang et al, IAPP and somatostatin were suggested to additionally inhibit islet insulin and glucagon secretion (55). IAPP decreases basal circulating glucagon levels in the mouse (74). However, insulin could act as a confounding factor in this context. In a study by Fehmann et al. (67), a decrease in arginine-stimulated glucagon release occurred together with an increased insulin release after IAPP exposure, which makes it impossible to exclude that the effects on glucagon release were secondary to changes in insulin release. For putative treatment with IAPP analogs like pramlintide, it is interesting to note that IAPP’s inhibitory effect on glucagon secretion was recently reported to be specific to arginine- and meal-stimulated glucagon secretion without the ability to affect glucagon secretion caused by hypoglycemia (75).

At the time of its discovery, IAPP was hypothesized to possess favorable effects on glucose homeostasis and thus seen as a strong candidate for the improvement of insulin effects in diabetes treatment. However, it has turned out to be difficult to predict the net effect of IAPP on the glucose concentration and to establish under which circumstances the effect is of physiological relevance. A summary of potential effects of IAPP in glucose homeostasis, i.e. how IAPP affects the prevailing glucose concentration, can be seen in Fig. 2. Some of the IAPP effects
seem contradictory, e.g. the effects on glucagon secretion and gluconeogenesis. The direct effect on glucagon secretion is inhibitory, whereas an inhibition of insulin release, also described for IAPP, results in a stimulation of glucagon secretion. However, the net effect of IAPP is an inhibitory effect on glucagon secretion at the same time as a stimulatory effect is seen on hepatic gluconeogenesis. This indicates that IAPP, most likely, has a direct effect on hepatic gluconeogenesis, which is stronger than that mediated via glucagon.

Figure 2. Potential effects of IAPP on extracellular glucose concentration (adapted from (210) with permission). The counteracting effects of IAPP on the prevailing glucose concentration, makes it difficult to predict the net effect of the peptide.
Regulation of IAPP secretion

IAPP circulates in the range of 2-15 pmol/l in plasma (68) and the secreted and circulating IAPP/insulin ratio is in the range 1-5% (76). Due to technical problems reported for IAPP measurements there is a considerable variation between assays and laboratories (68). Regarding circulating IAPP/insulin ratios, one has to be aware of the differences in clearance for IAPP and insulin. The clearance of IAPP appears to occur via the kidneys, whereas insulin is eliminated both by the liver and the kidneys (77, 78). IAPP has been proposed to have a clearance three to four times slower than that of insulin (77). So, plasma concentration ratios do not necessarily reflect the relation between IAPP and insulin upon secretion.

The release of IAPP and insulin from pancreatic beta cells seems, at least to some extent, to be independently regulated. The release of IAPP has been suggested to partly take place via constitutive secretion since epinephrine as well as removal of extracellular Ca²⁺ completely inhibited insulin release, whereas IAPP release was unaltered (79). In accordance, it was recently shown that approximately 40% of the IAPP release from neonatal islets occurs constitutively (80). However, no such pathway for IAPP release from adult rat islets was seen in the study (80).

Studies regarding embryonic expression of IAPP and insulin further strengthen the opinion that IAPP and insulin can be separately expressed and thus separately regulated. A null mutation of the pancreatic transcription factor Nkx 2.2 in mice, showed preserved expression of IAPP and low levels of transcription factor pancreatic duodenal homeobox gene 1 (PDX-1) in cells positive for the beta cell marker PC1/PC3, whereas these cells failed to express insulin (81). This is in line with the fact that insulin is more specific to beta cells than IAPP is, and therefore can be seen after further differentiation. IAPP transcription has been suggested to be, at least partly, under the control of PDX-1. The transcription factor PDX-1 is expressed to a high extent in the beta cells and to a lower extent in the delta cells. Indeed, IAPP can be released from delta cells, although the predominant IAPP release most probably occurs from the beta cells.
Some secretagogues have been described to augment islet IAPP and insulin release in parallel, e. g. glucose (76, 82), arginine (83), theophylline (84) and insulinotropic peptides (84). The fact that IAPP release increases together with insulin when islets are exposed to sulphonylurea might be of clinical relevance for the treatment of type 2 diabetes (84).

**The role of IAPP in type 2 diabetes, obesity, insulin resistance, gestational diabetes and type 1 diabetes**

The fact that amyloid was originally described in the pancreas of a type 2 diabetic patient has generated hypotheses about a role for IAPP in diabetes pathogenesis (9). Indeed, an association between the presence of islet amyloid and type 2 diabetes is well established. Humans with type 2 diabetes, as well as some animal models for type 2 diabetes, show amyloid in an increased number of islets in addition to larger amounts of amyloid per islet than age-matched controls (85-91). Moreover, islet amyloid has been demonstrated in humans with type 2 diabetes with a prevalence exceeding 80% (90, 92, 93), which could be compared to the 12% seen in non-diabetic subjects over the age of 40 years (88). In addition, autopsy of individuals suffering from severe type 2 diabetes has shown a close to 100% prevalence of amyloid in the islets of Langerhans (88).

The exact localization of islet amyloid has still not been convincingly clarified. Some data suggest that IAPP aggregation is initiated intracellularly and becomes extracellular at beta cell death (94), whereas other studies support the view of extracellular fibril formation at the site of IAPP release (95). However, as recently proposed, separate mechanisms may underlie amyloid formation in different models thereby favouring either intracellular or extracellular deposition (96).

It has been speculated that amyloid deposits in the vicinity of the islet might contribute to a disturbed microenvironment of the islet cells and thereby lead to an impaired beta cell function. Theoretically, local amyloid deposits might physically disturb the transport of glucose into, and the passage of insulin from the cells, though this has not been possible to establish (97). Moreover, IAPP
might have a harmful effect via direct physical/chemical damage. IAPP has been shown to disrupt phospholipid bilayer membranes and might therefore act as an inducer of apoptosis in islets (98). In line with this, both IAPP and amyloid deposits have been reported to have a directly toxic effect on beta cells (99, 100). Recently, the cytotoxic effect by amyloid was indirectly shown to involve free radicals since antioxidants had a protective effect on amyloid-induced beta cell death (101).

There is no consensus about plasma levels of IAPP in type 2 diabetes. Thus, it is generally believed that plasma IAPP is increased throughout the development of type 2 diabetes, whereas it is decreased at the stage of fully developed disease (68). Insulin resistance is a hallmark in diabetes pathogenesis (102). Interestingly, in obesity, insulin resistance (103, 104) and gestational diabetes (105), there is an increased IAPP concentration in plasma. Dexamethasone-treated rats as a model for insulin resistance and rats made hyperglycemic by glucose injections both show a greater increase in the release of IAPP than insulin at high glucose (106, 107). This increase in IAPP/insulin ratio in insulin resistant rats has also been measured at the mRNA level (108). An increased ratio in circulating IAPP/insulin has also been described in the spontaneously diabetic Goto-Kakizaki rat (109). In humans, dexamethasone treatment increases the secretion ratio IAPP/insulin after an oral glucose tolerance test (110). In addition, it was recently shown that long term exposure of human beta cells to high glucose concentrations results in a reduced islet insulin content, while the IAPP content remains constant but shifts towards a higher proportion of precursor and intermediate forms (111).

A role for IAPP has been less studied in type 1 diabetes. However, some studies have been done, indicating a decreased IAPP production in type 1 diabetic patients, (103, 112) and in some animal models for type 1 diabetes, e.g. BB/Wr rats, STZ-induced rats (113-116) and alloxan-induced diabetic mice. Study III was undertaken to investigate whether changes in IAPP secretion are a cause or a consequence of the development of type 1 diabetes.
Chromogranins present in the pancreas

Chromogranins are acidic secretory glycoproteins with a widespread but specific distribution in neuroendocrine tissues (117). Clinically, measurement of circulating chromogranins has therefore often been used as a tool in the diagnosis of neuroendocrine neoplasia. Under physiological conditions, the chromogranins are stored and secreted together with resident hormones and transmitters and their synthesis can be regulated separately from that of the co-secreted peptide (118-120).

The chromogranin family is heterogenous, consisting of propeptides such as chromogranin-A, chromogranin-B and chromogranin-C, which can either elicit an effect themselves, or serve as precursors to a large number of biologically more active peptides (Fig. 3). The processing of chromogranins is tissue specific (121) and seems to occur intracellularly as well as extracellularly. In the endocrine pancreas, the cleavage is thought to be mediated by PC1/PC3 and PC2 (122, 123), whereas the extracellular processing is considered to be under the control of proteolytic enzymes released together with the chromogranins. Interestingly, the most extensive processing of chromogranins is described in the islet of Langerhans and in endocrine cells of the gut (124).

There is limited amino acid sequence homology between chromogranin-A and chromogranin-B, as also indicated by an obvious lack of immunological cross-reaction (125, 126). Chromogranin-C differs even more in its structure and has thus been suggested to be re-named secretogranin II, in an attempt to limit the nomenclature confusion.

Chromogranin peptides have been suggested to have multiple roles in secretion, intracellularly by targeting hormones and neurotransmitters to granules in the Golgi apparatus and extracellularly by fine tuning of hormone secretion, often proposed to be of inhibitory nature.
In the perfused rat pancreas, physiological concentrations of chromogranin-A have been shown to inhibit glucose-stimulated insulin release (127). Such an inhibitory effect was found to be even more pronounced by pancreastatin (128-130), which is derived from chromogranin-A and by far the most extensively studied chromogranin peptide. The effect of pancreastatin can be modulated by the ambient glucose concentration (131). Pancreastatin has also been demonstrated to inhibit arginine stimulated insulin release in the perfused rat pancreas (127). However, the role of pancreastatin as an inhibitor of insulin release has been questioned (132, 133).
Ultrastructural studies have revealed that chromogranin-A and chromogranin-B appear to be present in all islet cells (134-136), with predominant expression of chromogranin-A in the alpha cells (134). The location of chromogranins within the granules differs between endocrine cell types. Chromogranins are stored together with insulin and IAPP in pancreatic beta cells and study I was initiated to investigate whether chromogranin-B, chromogranin-A or pancreastatin produced within the islet have any regulatory role in the islet IAPP and insulin secretion. In order to study the effect of chromogranins released from the islet, antibodies were used towards epitopes of the human pancreastatin peptide, the human chromogranin-A peptide and the human chromogranin-B peptide.

**Leptin and pancreas**

Leptin, the product of the ob/ob gene, is a peptide of 16 kDa, mainly expressed in white adipose tissue. Leptin deficiency is present in a “naturally developed knock-out mouse” (spontaneous mutation of the ob/ob gene), the ob/ob mouse, which has been extensively studied as a model for type 2 diabetes. The genetic background of the ob/ob mouse seems to be of considerable importance for the symptoms of the leptin deficiency. A null mutation of the ob gene on the C57BL/Ks background results in more severe diabetes than if the ob gene is absent in C57BL/6 mice (137). The ob/ob mouse was initially characterized in 1950 (138), however, it was not until 1994 that the ob/ob gene was finally described (139). The ob/ob mouse was then demonstrated to have a mutated ob/ob gene, resulting in either a truncated form of leptin or a total lack of leptin mRNA (139). Linking leptin to the symptoms shown by the ob/ob mouse, was a starting point for the search for leptin’s role in obesity and weight related diseases, e.g. type 2 diabetes.

Whether leptin has an effect on the pancreas is controversial. Leptin receptors have been demonstrated in pancreatic beta cells (140) and with the disturbed glucose homeostasis and hyperinsulinemia in the ob/ob mouse in mind, leptin was initially suggested to have a direct inhibitory effect on insulin secretion.
Indeed, there is evidence for such an inhibitory action of leptin on insulin release (141, 142), although there are also studies indicating that leptin does not affect insulin secretion (143). Studies with human islets have shown an inhibition of insulin release by leptin in long term experiments but a lack of effect in short term experiments (144). One of the aims of study II, was therefore to clarify leptin’s role in islet insulin secretion. Moreover, obesity and thus type 2 diabetes appears associated with increased levels of insulin, IAPP and leptin. Hence, a further aim of study II was to study leptin’s effects on islet IAPP secretion.

**Leptin signalling**

Leptin signalling is mediated via the leptin receptor (Ob-R), which exists in three main isoforms; a long receptor, a short receptor and a soluble, circulating receptor. The three different forms of the leptin receptor are generated by differential splicing of the Ob-R gene (145). The membrane bound receptors have identical extracellular domains but differ intracellularly. The long, functional form of the receptor has a long intracellular domain which is considered to be a prerequisite for further signal transduction (145). In contrast, the intracellular part of the short receptor is truncated and was therefore initially described as non-functional (145). Lately, this has been somewhat questioned and there are data suggesting that the short isoform may take part in leptin transport and clearance (146, 147). Also, from a teleologic point of view, it would seem strange if a receptor, expressed to such an extent as the short leptin receptor, would lack function. The truncated form of the leptin receptor has been shown to be dominantly expressed in an animal model for type 2 diabetes, i.e. the db/db-mouse (148, 149). As in the case with the ob/ob mouse, a more severe form of diabetes is seen on a C57BL/Ks background compared to C57BL/6 (137). Less is known about the soluble isoform of the leptin receptor, but it has been suggested to function as an inhibitory leptin-binding protein, reducing the free leptin concentration (145).
The leptin receptor is a member of the cytokine receptor superfamily that acts by associating to tyrosine kinases of the janus activated kinase (Jak) family. The leptin receptor is structurally similar to some receptors, e.g. the receptors for granulocyte/monocyte colony stimulating factor (GM-CSF), leukocyte inhibitory factor (LIF), oncostatin M (OM), and in particular, to the interleukin-6 (IL-6) receptor (150). Binding of leptin to the receptor causes receptor homo-dimerization, activation of Jak kinases and further phosphorylation of cytoplasmic transcription factors such as members of the signal transduction and activator of transcription (STAT) family (151). It appears as if leptin mostly signals via homo- or heterodimers of STAT 3, STAT 5 and STAT 6, which enter the nucleus and activate genes of importance for leptin’s effects.

The response to leptin can be regulated at different levels. The total amount of leptin receptors, and the ratio between long and short forms of the receptor, vary between different tissues. Receptors for leptin appear widespread but tissues clearly involved in energy metabolism, e.g. hypothalamus and pancreas, have been shown to have a higher expression of the long form of the receptor (145). Variation of the amount and efficacy of different intracellular Jaks and STATS, might be of importance for a regulated leptin response.

**Beta cell proliferation in the context of diabetes**

The beta cell mass can be increased by neogenesis via differentiation from precursor cells, or by proliferation of differentiated endocrine cells. However, both human and rodent adult islet cells, show a limited capacity to proliferate. The majority of the beta cells are considered to be in the resting G0-phase of the cell cycle and most of these cells will eventually be senescent and die. However, a minor fraction of the beta cells in G0-phase may retain their ability to undergo mitosis and may therefore be recruited to the active G1-phase upon stimulation. It has been estimated that this fraction corresponds to less than 3% of the rodent adult islet cells and to approximately 10% in rodent neonatal islet cells (152). It is still not clarified how the cell cycle is regulated in beta cells but
cyclin dependent kinase 4 (Cdk4) is proposed to be one important regulator of the proliferative capacity by stimulating the re-entry from \( G_0 \) to \( G_1 \) (153).

The low proliferation rate of beta cells is a negative factor in diabetes development since it decreases the repair potential of the beta cell mass and reduces its ability to recover after damage. Nevertheless, the proliferation rate should not be underestimated since it can be stimulated to meet increased needs. Most often, an adjustment of the beta cell mass occurs during pregnancy, mild obesity and insulin resistance. Even people suffering from severe forms of these conditions may be able to adjust their beta cell mass to such an extent that they avoid diabetes onset. It has been shown that 15-20\% of individuals suffering from obesity or insulin resistance do not develop diabetes (154). Thus, the beta cell mass is, to some extent, dynamic. By mathematic calculations, Finegood et al. have estimated that a complete renewal of the beta cell mass would take approximately a month in rats (155). However, this is controversial, and some authors argue that stimulated beta cell proliferation reaches a plateau with time probably reflecting that the cells only can undergo a limited number of divisions (156).

For islet transplantation and thus diabetes therapy, the need for pancreatic islets at present highly exceeds the organ access. This makes beta cell growth and differentiation an important research field. Expanding the beta cell mass, either \textit{in vivo} in the diabetic patient, or \textit{in vitro} prior to transplantation, could be one way to limit the amount of organs required in the future for beta cell transplantation.

\textbf{Regulation of beta cell proliferation \textit{in vitro}}

A number of candidates have been studied in the context of islet cell proliferation throughout the years. Glucose has been convincingly proposed as a stimulator of beta cell proliferation (157-161), an effect which most likely is dependent on glucose phosphorylation (160). Glucose has even been suggested to be the main driving force behind most beta cell growth.
The beta cell mass expands during pregnancy to meet an increased insulin demand, and some factors that circulate at elevated concentrations during pregnancy, e.g. prolactin (PRL), placenta lactogen (PL) and placental growth hormone (hGH-V), have been shown to be some of the more potent stimulators of beta cell growth identified to date (162). Moreover, in pregnancy, this appears correlated to an up-regulated expression of receptors for growth factors such as PRL and GH (163). PRL and GH are more potent stimulators of beta cell growth in rat islets compared to human islets (164). However, the mechanisms of beta cell growth in human pregnancy has not been fully elucidated.

Among intra-islet hormones, insulin has been discussed in the context of islet cell proliferation. Insulin was initially shown to stimulate beta cell proliferation in monolayer cultures of neonatal rat islets (165). In contrast, mice with a target disruption of the genes for insulin I and II were demonstrated to possess enlarged islets giving rise to theories of a role for insulin as an inhibitor of islet growth (166). However, a more recent study of mice lacking the insulin receptor showed a mild form of glucose intolerance, thus the beta cell mass was almost sufficient to meet the insulin requirements (167). The conclusion from these studies could be that insulin plays a minor role for pancreatic beta cell growth.

Another intra-islet peptide of potential interest for islet cell growth would be IAPP. This peptide has been shown to stimulate proliferation in some tissues, e.g. osteoblasts (168), the endothelial cells of the umbilical vein (169) and proximal kidney tubules (170, 171). Study IV was undertaken to investigate whether IAPP has any effect on islet cell proliferation with special reference to the beta cells.
**Aims of the thesis**

The overall aim of this thesis was to determine the role of the beta cell hormone, IAPP, in pancreatic islet physiology and during early disturbed islet function. The more specific aims were as follows:

To study possible endogenous regulators of islet IAPP and insulin secretion by intra- and extra-islet peptides, i.e. chromogranin peptides (I) and leptin (II)

To study IAPP’s role during the development of type 1 diabetes (III)

To elucidate IAPP’s role as a putative growth factor in the endocrine pancreas (IV)

**Materials and methods**

**Animals**

Female NOD mice, aged 6-9 weeks or 12-15 weeks (III) were obtained either from a local breeding colony (Biomedical Center, Uppsala, Sweden) or from Bommice (Bomholtgård, Denmark). The cumulative diabetes incidence in the former colony was about 62% at the time of study (III) and in the latter colony 85%, in the females at 30 weeks of age (172). In both colonies, pancreatic insulitis was observed in 85-100% of female mice from 10-12 weeks of age. The cumulative diabetes incidence in male NOD mice was estimated to ≈10% in both colonies. The reasons behind the increased diabetes susceptibility in female NOD mice compared to males are still unknown.

Male NMRI mice, age 10-12 weeks (I, II, III), were purchased from B&K Universal (Sollentuna, Sweden).

Male Sprague-Dawley rats (IV) of approximately 300 g were obtained from a local colony, (Biomedical Center, Uppsala, Sweden). Pregnant Sprague-Dawley rats (IV) were killed on day 21 of gestation for isolation of fetal islets.
The animal room had 12-h light/dark cycles, the temperature was 21°C, and the atmosphere was 30-35% humidified air. The animals had free access to pelleted food and water. The NOD mice were housed behind a barrier separated from other animals and had free access to autoclaved pelleted food and water.

Islet isolation and culture

After removal, the pancreata were digested by collagenase at 37°C in a shaking water bath (173), rinsed with Hanks’ solution and handpicked using a braking pipette. Islets were subsequently cultured free-floating in medium RPMI 1640 at 11.1 mM glucose supplemented with 10% (v/v) fetal calf serum (FCS), benzylpenicillin (100 U/ml) and streptomycin (0.1 mg/ml) at 37°C in an atmosphere of air/5% CO2 for approximately 6 days unless otherwise stated (174). Medium was changed every second day.

For isolation of fetal islets (IV), pancreata from the litter were pooled, digested by collagenase and thereafter cultured attached to the bottom of Petri dishes in RPMI 1640 at 11.1 mM glucose supplemented with 10% (v/v) fetal calf serum (FCS), benzylpenicillin (100 U/ml) and streptomycin (0.1 mg/ml), at 37°C in an atmosphere of air/5% CO2 for 48 h (175). The islets were then gently detached from the bottom of the culture dishes by blowing culture media through a sterile Pasteur pipette. The islets were thereafter handpicked with a braking pipette and subsequently cultured at a decreased glucose and serum concentration in RPMI 1640 at 5.6 mM glucose and 1% FCS to obtain suitable experimental conditions for the study of growth factors (176).

In vitro exposure to leptin (II)

For long-term exposure experiments, adult NMRI mouse islets were exposed to leptin (recombinant murine leptin, PeproTech, London, UK) in the range of 1-100 nM for 48 h in medium RPMI 1640 (11.1 mM glucose +10% FCS) (II). The acute effect of leptin (1-100 nM), on pre-cultured islets was examined by measuring glucose oxidation rates and islet IAPP and insulin release at different glucose concentrations (1.7-16.7 mM), in the presence of leptin (1-100 nM).
The biological activity of the leptin preparation used in paper (II) was studied after intraperitoneal (i.p.) injection once daily for 5 days (2.5 µg/g body weight) in obese (ob/ob) and lean (+/+ ) C57BL/6 mice. This resulted in a significant reduction in body weight and food intake in both types of mice (177).

**In vitro exposure to IL-6 (II)**

Experiments were undertaken to study similarities between leptin and IL-6 (II). For this purpose, pre-cultured islets were exposed to murine IL-6 (PeproTech); 1 ng/ml or 5 ng/ml for 48 h in medium RPMI 1640 (11.1 mM glucose + 10% FCS).

**In vitro exposure to antibodies against chromogranin-B, chromogranin-A and pancreastatin (I)**

For long-term experiments, antibodies towards specific epitopes on the chromogranin-B peptide, the chromogranin-A peptide and on pancreastatin, were present during 48 h culture (I).

The anti-chromogranin antibodies used in this study were directed against the human amino acid sequences chromogranin-A 17-38 (A17-38), chromogranin-A 284-301 (C-terminal end of pancreastatin) and chromogranin-B 312-321 (B312-321). Production and testing of the two latter antibodies have been described earlier (178). The A17-37 - antibody was raised against a Fmoc-synthesized peptide, where the amino acid in position 36 was replaced by a tyrosine residue. Before injection into rabbits, the peptide was coupled to Imject malemid activated keyhole limpet cyanogen (KLH) according to the manufacturer’s instructions (Pierce, Rockford, IL, USA). The antibodies were made by Dr. Mats Stridsberg, Dept. of Medical Sciences, Uppsala University. Control islets were exposed to normal rabbit serum from a local animal colony.
**In vitro exposure to IAPP (IV)**

Neonatal islets were cultured free-floating at 5.6 mM glucose and 1% FCS during the 24 h exposure period to rat IAPP concentrations of 1-1000 nM (Peninsula Laboratories, England). The IAPP exposure was preceded by a 24 h pre-treatment period at 5.6 mM glucose and 1% FCS.

Additional experiments with IAPP exposure were undertaken at concentrations of 1 nM or 10 nM. This was due to the fact that 10 nM IAPP seemed to be the most potent concentration in initial experiments. However, as the study progressed, 1 nM IAPP appeared to be even more effective and was therefore investigated further in some respects.

**Islet IAPP release, insulin release, islet hormone and DNA content (I, II, III, IV)**

Groups of 10 islets were transferred in triplicate to glass vials containing 0.25 ml Krebs-Ringer bicarbonate buffer supplemented with 10 mM HEPES (Sigma) and 2 mg/ml bovine serum albumin (fraction V; Miles, Slough, UK), hereafter designated as KRBH buffer. For long-term experiments, the KRBH buffer contained 1.7 mM glucose during the first hour of incubation at 37°C (O₂/CO₂, 95:5). The medium was then carefully removed and replaced by 0.25 ml KRBH supplemented with 16.7 mM glucose and incubated for an additional hour.

The insulin and IAPP concentrations in the incubation media were determined by radioimmunoassay (I, II, III, IV) (179). After the incubations, the islets were harvested, pooled in groups of 30, and homogenized by sonication in 0.2 ml redistilled water. Two 50 µl aliquots of the aqueous homogenate were then used for DNA measurement by fluorophotometry (180). A fraction of the homogenate was mixed with acid-ethanol (0.18 M HCl in 96% (v/v) ethanol) from which insulin and IAPP were extracted overnight at 4°C to analyze islet hormone content.
Insulin and IAPP were measured by radioimmunoassay (179). After culture, the culture medium was removed and analyzed for insulin accumulation (I, II, IV) by radioimmunoassay (179).

Human IAPP was used for standard and tracer preparations (Peninsula, Belmont, CA, USA) and polyclonal rabbit antiserum raised against human IAPP was used as antibody. The antibody cross-reacts 100% with both rat and mouse IAPP. The sensitivity of the IAPP assay was 0.125 fmol/tube and there is no cross-reactivity with calcitonin-gene related peptide. The interassay variation was 9.9% and the intra-assay variation was 8.2%. The procedure for determination of IAPP and insulin has been described in detail elsewhere (179, 181).

**(Pro)insulin and total protein biosynthesis (I, II, IV)**

Duplicate groups of 10 islets were incubated at 37°C in 100 µl of KRBH containing 16.7 mM glucose and 50 µCi/ml of L-[4.5-3H]leucine (AmershamPharmacia Biotech, Uppsala, Sweden) in an atmosphere of humidified air plus 5% CO₂. After 2 h, the islets were washed in buffer containing non-radioactive leucine (10 mM) and sonicated in 200 µl of redistilled water. The amount of labelled (pro)insulin was determined by an immunoabsorption technique (182), and the total protein biosynthesis was measured in trichloroacetic acid precipitates of the islet homogenate.

**DNA synthesis (II, IV)**

For estimation of islet cell DNA synthesis, groups of 50 islets were exposed during the last 6 h of culture to 1 µCi/ml [methyl-3H]thymidine (AmershamPharmacia Biotech). The islets were washed in Hanks' solution containing 10 mM thymidine and disrupted in 0.2 ml redistilled water by ultrasonication.
Duplicate aliquots of the homogenates were precipitated with 10% (v/v) TCA and the labeled DNA was separated from unbound $^3$H-thymidine by filtering through glass microfiber filters (GF/A 2.5 cm; Whatman, Maidstone, UK). After drying, the radioactivity on the filters was determined by liquid scintillation.

**Glucose oxidation rate (II, IV)**

Triplicate groups of ten islets were exposed to 1-100 nM leptin either acutely (during the glucose oxidation experiment) or 48 h prior to the glucose oxidation in study II and to 10 nM IAPP during 24 h in study IV. The islets were then transferred to glass vials containing 100 µl of KRBH supplemented with D-[U-$^{14}$C]glucose (AmershamPharmacia Biotech) and non-radioactive glucose to a final glucose concentration of 1.7 mM glucose or 16.7 mM glucose (spec. act. 0.5 mCi/mM). The islet glucose oxidation rates during a 90 min incubation at 37°C ($O_2/CO_2$, 95:5), were measured as described elsewhere (183).

**Immunohistochemistry and autoradiography (IV)**

Groups of 100 islets were exposed during the last 6 h of culture to 1 µCi/ml [methyl-$^3$H]thymidine (IV). The islets were washed in Hanks’ solution containing 10 mM thymidine and then further washed in phosphate-buffered saline (PBS). Islets were fixed in formalin before paraffin embedding. Sections (5 µm) were stained for insulin with an anti-bovine insulin antibody (BioMakor, Rehovot, Israel) and for glucagon using an anti-porcine antibody (Novo, Copenhagen, Denmark). The slides were rinsed in tap water for at least 15 min to remove diaminobenzidine tetrahydrochloride (Kem-En-Tec A/S, Copenhagen, Denmark). Wet slides were dipped in 50% film-emulsion (autoradiography emulsion, Kodak, NY, USA), in 0.75 mM ammonium-acetate, and kept in a dark chamber to dry overnight. The films were then exposed for 3 days before being developed, fixed, and counter-stained with Mayer’s hematoxylin.
Microscopic evaluation (IV)

Insulin positive and glucagon positive cells were counted using a light microscope (400x) and cells with \( \geq 10 \) black silver grains covering the nuclei were considered to be in the S-phase of the cell cycle (IV) (184). During the examination, the observer was unaware of the sample identity. The fraction of labelled cells was determined and expressed as labelling index (LI) (number of labelled cells \( \times 100 \)/total number of cells). To obtain accurate measurements of LI, at least 500 neonatal cells and 2000 adult cells, were counted in each preparation. A number of the samples were counted by two independent observers, generating similar results.

Measurement of insulin stained area (IV)

The mean value of the insulin positive area of three sectioned neonatal islets was estimated by measuring the insulin stained area in relation to the total islet area, from five different islet isolations, using a computerized system for morphometry (IV) (MOP-Videoplan, Carl Zeiss, Stockholm, Sweden). The analysis was made on the same slides that were autoradiographically studied.

Terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) (IV)

Islets were fixed in 10\% formalin overnight before paraffin embedding. The TUNEL labelling was carried out essentially according to Gavrieli et al. (IV) (185). TUNEL labelling of sectioned ileum was used as a positive control.

Analysis of propidium iodide positive nuclei (IV)

Neonatal islet cells were analyzed by fluorescence activated cell sorting (FACS) for propidium iodide (PI) staining as a measure of dead cells after exposure to 1 or 10 nM IAPP. Groups of 20-30 islets were incubated with IAPP for 24 h, then exposed to PI (10 \( \mu l/ml \)) in the culture dish and trypsinized for 5 min at 37\(^\circ\)C.
Groups of 10000 purified islet cells were thereafter analyzed for changes in light scattering and fluorescence emission by exciting the cells at 488 nm and examining fluorescence at 650 nm. PI is a polar dye that stains apoptotic and necrotic cells, the latter with high intensity (186). Cell shrinkage and loss of cell volume, decreases the forward-scattered light. Untreated cells were gated on a forward scatter versus PI fluorescence dot plot, and the same gates were then applied to the IAPP exposed cells for calculation of the fraction of dead cells. The FACS analysis was performed in a FACSCalibur™ (Becton Dickinson, San José, USA, CA).

**PDX-1 expression in islets (IV)**

Islets in groups of 200 were exposed to IAPP of 1 nM for 24 h, rinsed twice in PBS, sonicated and lysed by boiling in 40 µl SDS-gel sample buffer (2% SDS, 100 mM Tris pH 6.8, 100 mM β-mercaptoethanol, 0.01% bromophenol blue, 10% glycerol) supplemented with phenylmethylsulphonyl fluoride (PMSF) (20 µl/ml). Equal amounts of the sonicates were separated on sodium dodecylphosphate (SDS)-polyacrylamide gels. A purified polyclonal anti ratgst-PDX-1 antibody (a kind gift from Dr. O.D. Madsen, Gentofte, Denmark) and a horseradish-peroxidase conjugated anti-rabbit antibody was used for incubations. An enhanced chemiluminescence (ECL)-kit was used for detection (AmershamPharmacia Biotech). The expression of PDX-1 was related to that of Extracellular signal-regulated kinase-1 (Erk-1) to ascertain that equal amounts of protein had been applied to the gel.

**Islet cAMP content (IV)**

Islets in groups of 50 were exposed to 10 nM IAPP for either 30 min or 24 h. The medium was rapidly withdrawn and 0.15 ml 6% (v/v) TCA was added and the samples instantly freeze-dried. The cAMP content was measured using an assay kit (RPA 509; AmershamPharmacia Biotech) as described previously (187). The samples were acetylated to increase the assay sensitivity.
Statistical Analysis

Every experiment represented different animal islet donors. For neonatal islets, one observation corresponded to islets from one litter. Means ± SEM were subsequently calculated and groups of data were compared. When only two groups were compared, probabilities (P) of chance differences were calculated using Student's paired or unpaired t-test. ANOVA was used when multiple comparisons were made (III), including Fisher’s protected least statistical difference test, using StatView (Abacus Concepts, Berkeley, CA, USA).
Results and discussion

1a. The effect of islet chromogranins for a regulated IAPP and insulin release

Study I was undertaken to study the role of pancreatic chromogranin peptides in the regulation of islet IAPP and insulin release. The aim of the study was to focus on endogenous effects of chromogranins. Hence, the study is a contribution to knowledge of regulation of IAPP and insulin release. Moreover, it provides new information about a local role for chromogranins within the pancreas. An autocrine role for chromogranin-B as a regulator for islet IAPP and insulin release is proposed.

The hypothesis behind study I was that chromogranins synthesized in the pancreas have a feedback inhibition on islet hormone secretion. For the study, antibodies were designed towards epitopes on chromogranin-B, chromogranin-A and pancreastatin. It is assumed that these antibodies act by blocking chromogranin-B, chromogranin-A and pancreastatin, synthesized in, and secreted from, the islets. It is reasonable to believe that the antibodies do not penetrate the center of the islets. Therefore, it could be assumed that some of the effects of chromogranin antibodies will not be detected due to counteraction by chromogranins released in more central parts of the islet. Therefore putative changes must be of a certain magnitude to be detected by the method.

Antisera against chromogranin-B potentiated glucose-stimulated IAPP and insulin release both acutely and after a 48 h exposure. Acutely, the stimulatory effect was most obvious for IAPP release as further indicated by increased IAPP/insulin release ratios. No effect was seen by antisera against chromogranin-A or pancreastatin on basal or glucose-stimulated islet IAPP or insulin release. This may be a reflection of the chromogranin distribution within the pancreas.
Chromogranin-A is predominantly expressed in alpha cells and the release of the peptide therefore does not increase at high glucose. This can also be the case for pancreastatin since it is a product of chromogranin-A cleavage.

In the beta cells, chromogranin-B is stored together with insulin which may indicate, at least to some extent, associated release of insulin and chromogranin-B. Furthermore, rat islet IAPP and insulin release has been demonstrated to parallel the release of chromogranin-B, but not chromogranin-A in preliminary experiments (Dr. Mats Stridsberg, Dept. of Medical Sciences, Uppsala University, personal communication). This is in line with the lack of response seen by chromogranin-B antibodies at 1.7 mM glucose when it can be assumed that low concentrations of chromogranin-B are released from the islets. High glucose is probably required to observe an effect of the chromogranin-B antibodies since sufficient quantities of islet chromogranins have to be released in order to block a potential chromogranin effect. Thus, islet exposure to antisera against chromogranin-B significantly increased both the 48 h medium insulin accumulation and the islet IAPP and insulin release, indicating an inhibitory effect of chromogranin-B on islet hormone release.

1b. Validity of the method to use antibodies as a tool for the study of physiological effects

Using antibodies to study effects or rather lack of effects by specific peptides might be the most simple and direct mode of studying physiological effects. With this procedure there are only minor risks of up-regulation of compensatory mechanisms, something which could be a confounding factor with knock-out animal models. The method has been successfully used previously. Potentiating effects on parathyroid secretion was seen by antibodies directed against chromogranin-A, in a study by Fasciotto et. al. (188). Moreover, antiserum against IAPP and somatostatin was recently used in one study to investigate the paracrine effects of IAPP and insulin (55).
One reason for that the method of using antibodies in order to study physiological effects has not been so widely applied, might be that the method demands specifically directed antibodies, which may be difficult to design in many laboratories.

2a. Leptin’s role for islet IAPP and insulin release

The relation of increased circulating levels of leptin and insulin in human obesity is a complex issue (189). Prolonged hyperinsulinemia seems to increase leptin secretion (190). Furthermore, leptin has been suggested to aggravate insulin resistance since it can counteract some effects of insulin on a human hepatoma cell line (190). In this context, it would be of interest to elucidate whether an increased leptin concentration would affect the insulin secretion.

In study II, leptin seemed to have a more potent effect on IAPP secretion than on insulin secretion, i.e. leptin reduced islet IAPP secretion both acutely and in long-term experiments. The most physiological concentration of leptin used in the study (1 nM), had an inhibitory effect on the islet insulin secretion, whereas higher leptin concentrations failed to affect the insulin secretion significantly. Another important finding was that the changes in IAPP secretion did not appear to be correlated to changes in glucose metabolism. An increased glucose oxidation rate was seen at 1 nM leptin, while higher leptin concentrations showed an inhibitory effect. No effect was seen by leptin on the glucose oxidation rate when islets had adapted to leptin concentrations of 1-1000 nM during a 48 h culture.

Possible targets for leptin action in the beta cell have been discussed. The key event in leptin action seems to be to reduce intracellular concentrations of Ca^{2+} (192, 193). However, this could be achieved in various ways. Opening of K_{ATP}-channels (192) as well as disturbed signalling via PKA (194) and PKC (195), within beta cells, have been proposed. This fits well with our data of a glucose-dependent leptin action in study II, suggesting that a raised metabolic activity leads to increases in [Ca^{2+}]_i and inhibition of leptin signalling in the beta cells.
Conflicting results in the literature regarding leptin’s effect on insulin secretion, have given rise to theories that the different results described, may partly be due to the use of different leptin preparations of different biological activity and stability. Moreover, it has been proposed that the different results could vary depending on whether or not islets have been cultured prior to the experiments. Theoretically, the extent of FCS exposure during islet culture might affect the expression of leptin receptors as well as the amount of intracellular targets for leptin signalling. To exclude that the effect of leptin on islet insulin secretion in study II was a result of the islets being cultured for several days prior to the experiments, we also undertook some additional experiments where we studied the acute effect of 10-100 nM leptin on acutely isolated NMRI mouse islets. Leptin was present during the actual IAPP and insulin release incubations with the acutely isolated islets. High glucose significantly stimulated the islet insulin release under all experimental conditions. Leptin concentrations of 10 or 100 nM had no ability to counteract the glucose-stimulated insulin secretion (control: 2.6 ± 0.2 pmol/10 islets vs 10 nM leptin: 3.0 ± 0.6 pmol/10 islets vs 100 nM leptin: 3.3 pmol/10 islets; n=7). Moreover, no effect was seen on the islet insulin content by leptin (control: 49.0 ± 4.5 pmol/10 islets vs 10 nM leptin: 50.2 ± 4.4 pmol/10 islets vs 100 nM leptin: 46.7 ± 5.9 pmol/10 islets; n=7). Thus, the culture process does not seem to be a confounding factor in study II.

Besides activating the Jak/STAT pathway, leptin may signal via the mitogen activated protein kinase (MAPK) pathway. In accordance with this, leptin has been described to increase proliferation of insulinoma cells such as RINm5F cells and MIN6 cells (196, 197). However, in study II, 100 nM leptin failed to affect the cell replication in primary beta cells. It has been stated that proliferation in response to leptin most likely is a non-physiological phenomenon, exclusively seen in transformed cell lines (198).

The concentration dependency of leptin seen in study II most probably reflects the concentration range in which leptin exerts its physiological effect. It has previously been suggested that an attenuated leptin response at a high leptin concentration could be the result of an inhibited receptor oligomerization (199).
However, this could not explain the decreased effect of leptin at higher concentrations as in study II since such a situation would increase the probability of leptin binding to other structurally similar receptors, e.g. the IL-6 receptor on beta cells. Moreover, in the study, IL-6 was shown to inhibit IAPP and insulin secretion (II).

2b. Hyperleptinemia - what are the functional implications? Discussion about high leptin concentrations vs high functional leptin concentrations, in obesity

In the context of leptin effects, it is important to discuss what obesity-associated hyperleptinemia signifies. Linking effects of leptin to obesity and type 2 diabetes, requires a standpoint regarding whether leptin resistance is similar to insulin resistance. If that is so, putative effects of leptin should rather be interpreted inversely, i.e, prolonged high levels of circulating leptin may reflect a functional lack of leptin due to decreased leptin sensitivity.

Human obesity is very rarely associated with mutations in the genes coding for leptin and leptin receptors (200, 201). Thus, in contrast to what has been described for the ob/ob mouse, administration of leptin does not seem to efficiently reduce the body weight in the majority of obese subjects (202). Circulating leptin is directly correlated to the amount of body fat. The paradox that hyperleptinemia in obese persons occurs together with decreased energy metabolism and increased food intake, indicates a decreased leptin sensitivity in these persons.

Possible mechanisms for leptin resistance may include reduced transport of leptin across the blood-brain barrier, disturbed leptin signal transduction in central neurons and peripheral organs or antagonism of leptin effects. There is evidence for leptin affecting its own signalling. A group of inhibitors of cytokine signal transduction, including CIS (cytokine-inducible sequence) and SOCS-1, 2 and 3 (suppressor of cytokine signalling), has been described (203) and these are thought to function as modulators of a cytokine response since their expression is cytokine-induced.
Interestingly, leptin has been shown to increase the expression of SOCS-3 and possess a negative feed-back on leptin-activation of STAT 1 and STAT 3 (204). Whether this has any clinical significance, remains to be shown.

The circulating concentrations of leptin described for humans vary between different studies. It is therefore difficult to establish a physiological range and an obesity range of serum leptin. However, a serum leptin concentration of approximately 2-20 ng/ml has been proposed for healthy subjects by several authors.

3a. IAPP’s role during the progression to type 1 diabetes

IAPP has been proposed to be a pathogenetic factor in the development of type 2 diabetes. However, less is know about IAPP in the context of type 1 diabetes. Therefore, study III was undertaken to investigate the release of IAPP in the progression to type 1 diabetes. The study also provides data of importance for how islet IAPP and insulin release is regulated. The release of the two peptides can be independently regulated, but it is still not fully envisaged under which circumstances and via which mechanisms such a dissociation may occur.

High glucose proved to be a more potent stimulus for acute islet insulin release compared to IAPP release in study III. This may reflect a preferred release of insulin. More extensively immune cell infiltrated islets showed a tendency towards a relatively increased insulin release. Several mechanisms may contribute to that these islets failed to significantly decrease the IAPP/insulin secretion ratio at high glucose. Acutely isolated NOD islets probably reflect their previous in vivo milieu better than cultured NOD islets, and the more the islet hormone secretion is decreased due to islet cell destruction, the more prioritized the insulin release will be. An impairment of insulin release early in type 1 diabetes has been described (205). However, an impairment of IAPP release at this state of diabetes, seems even more pronounced.
3b. Validity of the NOD mouse as a model for type 1 diabetes

Essentially, three animal models for spontaneous diabetes development have been described; the NOD mouse, the lymphopenic BioBreeding (BB) rat and the Komedia diabetes-prone (KDP) rat. Both the NOD mouse and the BB rat develop diabetes in a narrow time span, preceded by insulitis. Immunodeficiency is thought to underlie the vulnerability to diabetes and other autoimmune disturbances, e.g. thyroiditis, described in the two models. The KDP rat was first described in 1998 (206) and will have to await further characterization. Of the animal models, the NOD mouse is the most widely used model for type 1 diabetes and has also the advantage of lower maintenance costs (207).

4a. IAPP as a growth factor in the endocrine pancreas

The early embryonic onset of the IAPP expression at gestational day 12 (in mouse), supports the view that an effect of IAPP is of physiological importance. In a discussion about a physiological role of IAPP, the IAPP concentration needs to be taken into account. Indeed, IAPP circulates at much lower concentrations (2-15 pmol/l), than the concentrations used in study IV. However, it may be presumed that IAPP occurs at several-fold higher concentrations within pancreatic islets compared to in plasma, although this has not been possible to demonstrate.

One purpose of study IV was to the search for a physiological role for IAPP. Moreover, the study adds knowledge to the field of beta cell proliferation. In the study, we found that IAPP concentrations of 1-1000 nM stimulated neonatal beta cell proliferation. This was studied by biochemical measurements and by counting the autoradiographic LI of immunocytochemically stained cells. Islet beta cell proliferation was more pronounced than alpha cell proliferation after exposure to IAPP. In contrast, both cell types increased their LI when the serum concentration was raised from 1 to 10%. The cell specific effect raises questions regarding the existence of IAPP receptors on the surface of alpha and beta cells as well as possible differences in the cell cycle regulation of the two cell types. As already mentioned, IAPP appears to signal via a receptor complex involving
CRLR, RAMP1 and RAMP3. Of interest for study IV, CRLR has been demonstrated
to be specifically expressed in beta cells within the islet (33). The recent studies
of the IAPP receptor have been based on adult islets or beta cell lines. Whether
neonatal islets differ regarding the expression of IAPP receptors is unknown.

By studying the proliferative effect using autoradiographs, we were able to
distinguish between effects on insulin positive and glucagon positive cells. This
is a strength of our study regarding beta cell proliferation. Important work was
carried out by Swenne and Hellerström in the early 1980’s about the replicatory
capacity of beta cells. However, it should be kept in mind that these studies
dealt with whole neonatal islets and the results obtained were interpreted as
beta cell specific due to the estimated dominance of beta cells (90%) in the islets.
Thus, much of our assumptions regarding beta cell proliferation may partly
involve other cells than beta cells, i.e. glucagon, somatostatin and pancreatic
polypeptide positive cells, in addition to non-endocrine cells. Furthermore,
many studies in the field today only supply indirect signs of beta cell
proliferation by using cocktails of anti-glucagon, anti-somatostatin and
anti-pancreatic polypeptide to exclude beta cells. This is frequently used when
5-bromo-2’deoxyuridine (BrdU) is administered as an analogue for
$^3$H-thymidine (208).

The lowest and possibly most physiological concentration of IAPP studied by us,
turned out to be the most potent in some respects, i.e. for medium insulin
accumulation and islet insulin content. One possible explanation could be that
the binding of IAPP to a putative receptor has higher affinity in a particular
concentration range, a phenomenon described for several hormone receptors.
If that is the case in our study, one would assume that concentrations of IAPP
below 1 nM, would result in a decreased response to IAPP. However, this was
not tested in the present study.

In a study by Wookey et. al, activation of adenylate cyclase activity and
subsequent cAMP production was described in association with the proliferative
effects of IAPP in the renal cortex (171). This is of interest since cAMP’s
involvement in proliferation has been debated. Furthermore, CGRP and calcitonin have the ability to activate cAMP production (209, 210). This might be the case also for IAPP if IAPP and CGRPs signal via the same group of receptors. We decided to analyze whether IAPP affected the islet cAMP content in study IV. It is difficult to assume at what time-point after the IAPP exposure such an estimation ought to be done. Therefore, we measured the cAMP content at an early and late time-point. The first time-point, 30 min, was chosen according to the work by Wookey et. al and the second time-point, 24 h, was selected at the end of the exposure period (211). We observed no effect on the islet cAMP content either 30 min or 24 h after IAPP was added. From the study it can, however, not be excluded that there had been a transient change in cAMP at any other time-point.

4b. Validity of the cell proliferation methodology

The analysis of incorporated $^3$H-thymidine as a measure of DNA synthesis is based on a number of assumptions, i.e. that thymidine incorporation mainly occurs during the S-phase of the cell cycle, that the duration of the S-phase is constant and that the experimental conditions do not affect the amount of $^3$H-thymidine available for incorporation. Theoretically, $^3$H-thymidine metabolism in islet cells could affect the latter prerequisite. In this context it is of interest that thymidine oxidation has been shown to be low in rodent islets, especially in the presence of glucose (212). Therefore, it is assumed that alterations in the availability of $^3$H-thymidine is not a major source of error in study IV. Whether $^3$H-thymidine uptake into DNA can occur under other circumstances than prior to mitosis, has been debated. In a study by Sandler and Swenne it was shown that $^3$H-thymidine can be incorporated in the DNA even when the cells are not dividing, i.e. due to DNA repair (213). In addition, the rate of DNA repair in non injured islets was demonstrated to be very low (213). It could therefore be excluded that this affected the analysis in study IV.
Concluding remarks

Despite the effects described for chromogranin-A (214, 215) and chromogranin-B (III), no receptors have been identified for these peptides. In fact, the concept of a receptor for such large molecules has been questioned. In line with this, it has been suggested that intracellular effects by chromogranin-A and chromogranin-B may be due to effects that can be attributed to the intact molecules. In contrast, it is reasonable to believe that most extracellular effects described for chromogranins are the result of chromogranin-derived bioactive peptides. Proteases that may contribute to the cleavage of chromogranins are widely distributed, e.g. in plasma, in tissues and in plasma membranes. Indeed, we can not exclude that the effects seen by chromogranin-B in study I, is caused by an active product of the peptide. Interestingly, the sequence of chromogranin-B that was investigated in study I was recently purified from human cerebrospinal fluid, showing its existence in vivo (216). Thus, it will be most interesting to learn the results of functional studies with this chromogranin-B-derived peptide in the future.

From our experiments and others, it can be concluded that leptin does not seem to have a crucial role in the regulation of glucose-stimulated insulin release. In contrast, an effect by leptin on islet glucose-stimulated IAPP release seems more apparent. As has been suggested, and by some referred to as “the Ella-loop”, an inhibition of islet IAPP release by leptin could counteract the known inhibitory effect by IAPP on insulin secretion. A summary of potential effects of leptin on islet IAPP and insulin secretion is seen in Fig. 4. Thus, a lowered IAPP concentration would modulate a putative effect by leptin on the islet insulin secretion.

Leptin has been proposed to function as an inhibitory control signal to prevent overstimulation of insulin release by glucose and gastrointestinal hormones at food intake.
Moreover, leptin has also been described to inhibit neuro-stimulated insulin release at food intake. It is reasonable to believe that IAPP can interfere with these effects and have a modulatory role in leptin’s effect on insulin secretion.

Fig. 4. Potential effects of leptin on islet IAPP and insulin secretion. The remaining uncertainties, make it difficult to predict the net effect of the peptide.
To eventually be able to elucidate whether leptin contributes to the development of type 2 diabetes, leptin resistance has to be studied more thoroughly. As previously noted, for meaningful discussions about putative leptin effects, knowledge regarding the clinical relevance of leptin resistance, its onset and causes, are required.

Study IV provides a new basis for the study of a putative IAPP function. There are indications of IAPP receptors on beta cells and thus it seems logical that IAPP should have a local effect within the pancreas.

The view of IAPP as a growth factor generates a number of questions. To be able to proceed, it is of importance to eventually identify a true IAPP receptor, i.e. to clarify the involvement of RAMP isoforms and CRLR under different circumstances. Furthermore, little is known about the intracellular IAPP signalling. It would be of interest to find the intracellular targets for the IAPP response. G protein-coupled receptors have been proposed to be the most diverse signal transduction system in eukaryotic cells (217). Thus, searching for intracellular pathways involved in IAPP signalling might be like trying to find a needle in a haystack. However, intracellular domains of the G protein-coupled receptors have been shown to activate pathways leading to MAPK activation in other systems (218). The MAPKinease pathway might therefore be one target for IAPP’s proliferative response that could be worthwhile investigating.
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