

**PANCREATIC ISLET AMYLOID POLYPEPTIDE MEMBRANE
BINDING AND PERMEABILIZATION**

by

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LIST OF ABBREVIATIONS

CF	5(6)-carboxyfluorescein
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
EM	electron microscopy
HFIP	1,1,1,3,3,3-hexafluoro-2-propanol
IAPP	islet amyloid polypeptide or amylin
INS	insulin
LDL	low-density lipoprotein
LUV	large unilamellar vesicle
MLV	multilamellar vesicle
NaF	sodium fluoride
NaP _i	sodium phosphate
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
POPG/POPC	equimolar POPG and POPC
POPG	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3- [phosphor- <i>rac</i> -(1-glycerol)]
RFU	relative fluorescence unit
RT	room temperature
TEM	transmission electron microscopy
ThT	Thioflavin T

ABSTRACT

Type II diabetes is often associated with the presence of islet amyloid. Islet amyloid is aggregated fibrils composed primarily of the hormone peptide, islet amyloid polypeptide (IAPP), which is cosecreted with insulin from the secretory granules of the β -cells in the islets of Langerhans of the pancreas. Despite the commonality of IAPP amyloid fibrils in the disease state, recent evidence suggests that the small oligomeric forms of IAPP consisting of a few monomers may be the cause of β -cell toxicity rather than fibrillar amyloid species. In this thesis we explore the relationship of small oligomeric forms of IAPP with their ability to bind and permeabilize the membranes of our model lipid system. We extend previous research by identifying the region of the peptide responsible for membrane binding, which is required for further membrane permeabilization. Insulin inhibits IAPP fibril formation and because of this inhibitory nature it has been suggested that it may also inhibit IAPP membrane binding and permeabilization. We show that insulin, which is co-secreted with IAPP from the secretory granules, does not inhibit the ability of IAPP to permeabilize membranes. Insulin in the act of inhibiting IAPP fibril formation maintains IAPP in a toxic small oligomeric form that more readily permeabilizes membranes than is observed with IAPP fibrils. Type II diabetes is an aging associated disease; one common change that occurs in the aging process is a decrease in membrane-localized cholesterol. Because of the stabilizing effect of cholesterol on the membrane we looked at the effect of IAPP permeabilization of large unilamellar vesicles (LUVs) with incorporated cholesterol and

found that cholesterol dramatically reduces the ability of IAPP to permeabilize lipid membranes. In our dye leakage experiments, cholesterol plays a protective role against membrane permeabilization. In light of the complex environment of the secretory granule and the cell we conclude that insulin in its soluble form does not inhibit the ability of IAPP to permeabilize membranes, and cholesterol has a protective effect against permeabilization by IAPP. Other components of the secretory granule may have a cooperative effect in sequestering the toxic IAPP peptides and protecting the hormone secreting β -cells of the pancreas.

CHAPTER I

Introduction to Islet Amyloid Polypeptide and Its Involvement in Type II Diabetes

1.1 Islet Amyloid Is Present in the Disease State

Diabetes is a commonly used term that is applied to both Type I and Type II diabetes and although this work involves only Type II diabetes, the term diabetes will be used to refer specifically to this form of the disease. Type I and II diabetes occur from fundamentally different processes. Type I diabetes is an autoimmune disease that leads to a T-cell mediated immune system attack and destruction of the insulin secreting β -cells in the islets of Langerhans of the pancreas. The development of Type I diabetes occurs suddenly and almost invariably results in complete insulin dependence, requiring insulin monitoring and injections. Type II diabetes gradually develops and is characterized by defective insulin secretion, insulin resistance, a reduction in β -cell mass, increased β -cell apoptosis, and often the presence of islet amyloid. Several of these conditions alone do not constitute diabetes. Insulin resistance alone does not qualify as diabetes and is only one of the necessary steps that leads to diabetes. Insulin resistance is a precursor to the development of Type II diabetes and is often termed pre-diabetes. Insulin resistance leads to the development of Type II diabetes by requiring greater insulin, IAPP, and lipid synthesis from the β -cells, placing them under a constant state of stress (*1*). Unlike Type I diabetes that occurs suddenly and has little warning, Type II diabetes develops over a number of months to years and can be prevented by changing dietary habits and activity

levels. In the overweight or obese state the tissues become insensitive to insulin. Maintaining a healthy weight encourages insulin sensitivity at the target tissues requiring less insulin secretion. Exercise resensitizes the tissues to insulin by increasing their glucose requirements. With inactivity the cells downregulate the expression of their insulin receptors due to the decreased need for glucose. When activity levels rise the cells begin expressing insulin receptors to meet their increased energy demands. Diet and exercise can prevent Type II diabetes in nine out of ten cases of the disease from progressing from insulin resistance to diabetes (2).

Type II diabetes is a serious health problem that has been increasing in incidence (3). At least 180 million people in world currently have diabetes (3). Type II diabetes accounts for more than 90% of all diabetes cases (3). If left untreated, Type II diabetes progresses to an insulin-dependent state and can be complicated with neuropathy, kidney failure, cardiovascular disease, blindness, and other conditions (4). As the disease progresses from insulin resistance to overt Type II diabetes, a loss in β -cell mass occurs that makes it difficult for the patient to respond to fluctuations in blood glucose levels. To compensate for insulin resistance and decreased β -cell mass, the remaining β -cells have to synthesize and secrete greater amounts of insulin to regulate blood glucose levels. This compounding stress may lead to further β -cell death. Post-mortem, Type II diabetic islets of Langerhans often contain amyloid deposits composed of islet amyloid polypeptide (IAPP). Originally the association of Type II diabetes with the presence of islet amyloid led to the application of the amyloid hypothesis to Type II diabetes. The amyloid hypothesis states that amyloid fibrils and plaques are cytotoxic and responsible for cell death. The hypothesis was originally applied to the amyloid plaques and fibrils composed

of Amyloid- β that are associated with neuronal cell death in Alzheimer's disease. In recent years, a new hypothesis has been suggested, that small oligomeric species of IAPP form ion-channels in the membranes of β -cells leading to a decline in cellular homeostasis and ultimately cell death. Accordingly, recent research has shown that small oligomers and protofibrils are much more toxic than islet amyloid (5-9). Interestingly, the appearance of amyloid fibrils actually lags behind disease progression and is not present in every case of Type II diabetes. The appearance of small oligomeric species in the disease state more closely correlates with the onset of disease symptoms and is a more likely cause of β -cell toxicity, because oligomeric species have been shown to be highly cytotoxic in cell culture experiments (7-9).

1.2 Overview of the Physiological Role of IAPP

Islet amyloid polypeptide (IAPP or amylin) is a 37 amino acid peptide that is amidated at its C-terminus and contains a disulfide bridge between cysteine residues 2 and 7 (figure 1.1). IAPP is co-secreted with insulin from the β -cells in the islets of Langerhans in the pancreas. The islets of Langerhans are clusters of cells that are involved in coordinated hormone secretion. Several cell types including α , β , δ , ϵ , and PP-cells are found in the islets. α -Cells secrete somatostatin, β -cells secrete insulin and IAPP, δ -cells secrete glucagon, ϵ -cells secrete ghrelin, and PP-cells secrete pancreatic polypeptide (10). Insulin and IAPP are costored and secreted from within the same secretory granules. Secretion of these two hormone peptides occurs in a coordinated manner throughout the islet in response to a rise in blood glucose levels. The role of IAPP in humans is currently unknown, but based on murine models IAPP acts to slow

gastric emptying, binds to receptors in the brain to promote satiety, and slows digestion to help down-regulate blood glucose levels post-prandially (11).



Figure 1.1 IAPP sequence. The physiologically active form of the peptide is IAPP₍₁₋₃₇₎ with a cysteine bridge between residues 2 and 7 and the C-terminus is amidated.

1.3 IAPP Oligomers Are Suggested to be Responsible for β -cell Death

The formation of extra- and intra-cellular fibrils is characteristic of amyloid diseases including type II diabetes, Alzheimer's disease, Parkinson's disease, etc. Despite the commonality of amyloid fibrils in these disease states, recent evidence suggests that oligomeric forms of the amyloidogenic proteins, consisting of a few monomers, may be the cause of cellular toxicity rather than fibrillar amyloid species (figure 1.2). Human islets exposed to early oligomeric species of IAPP led to the apoptosis of the outer cells of the islet within 24-48 hours after exposure (12). Similarly, dispersed mouse islet cells demonstrated cell apoptosis and necrosis in response to the addition of oligomeric hIAPP (6, 12).

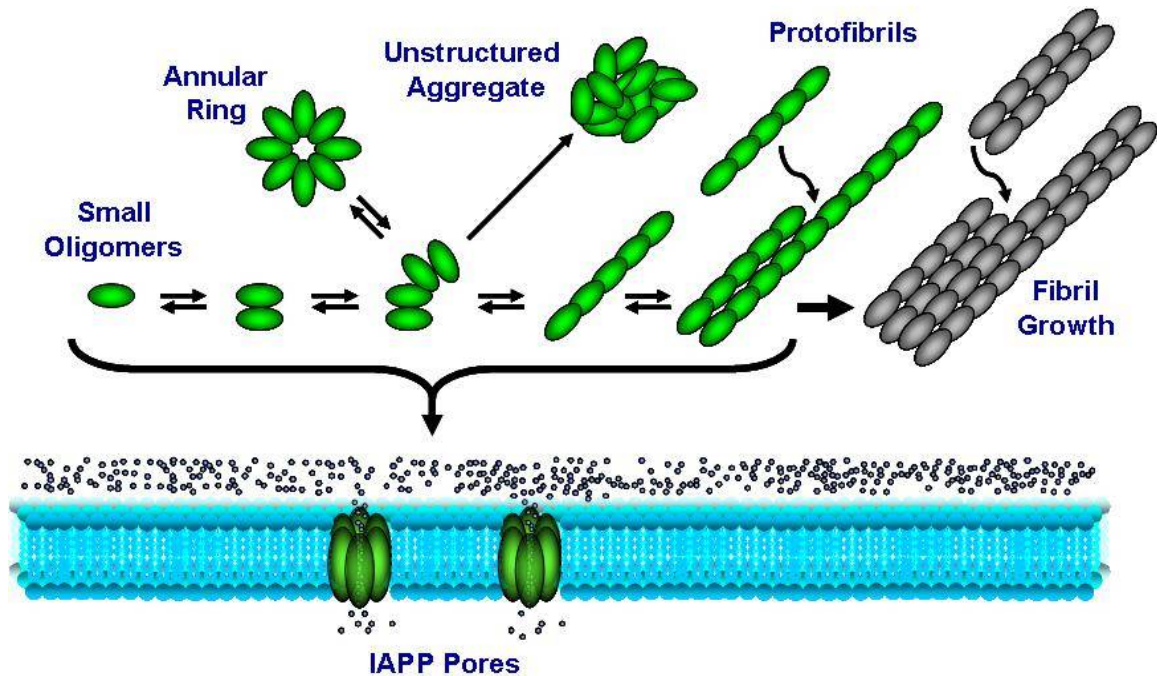


Figure 1.2 Possible pathway of oligomerization and permeabilization. The early oligomeric species permeabilize the membrane, possibly as a barrel stave as shown here, although the toroidal pore mechanism is currently more supported in the literature.

Three main mechanisms of membrane permeabilization are discussed in context with IAPP: the carpet mechanism, the barrel-stave ion-channel, and the toroidal pore (figure 1.3) (13). Membrane permeabilization by any of these three mechanisms could be significant enough to lead to β -cell death, although the exact mechanism for IAPP induced β -cell death is currently unknown. The carpet model suggests that IAPP peptides bind to the lipid head groups based on electrostatic interactions and disrupt the lipids by imposing strain on the membrane. The interaction of the peptide opens up spaces of permeation between lipids at the site of binding. The barrel-stave model suggests that IAPP peptides oligomerize and then insert into the hydrophobic core of the membrane, creating a discrete ion-channel in the membrane that is ion selective. The toroidal model involves IAPP binding first to the membrane and then interacting with the head groups of

the lipids to induce curvature of the membrane where the lipid thins and bends to form a pore with lipid headgroups lining the interior of the pore.

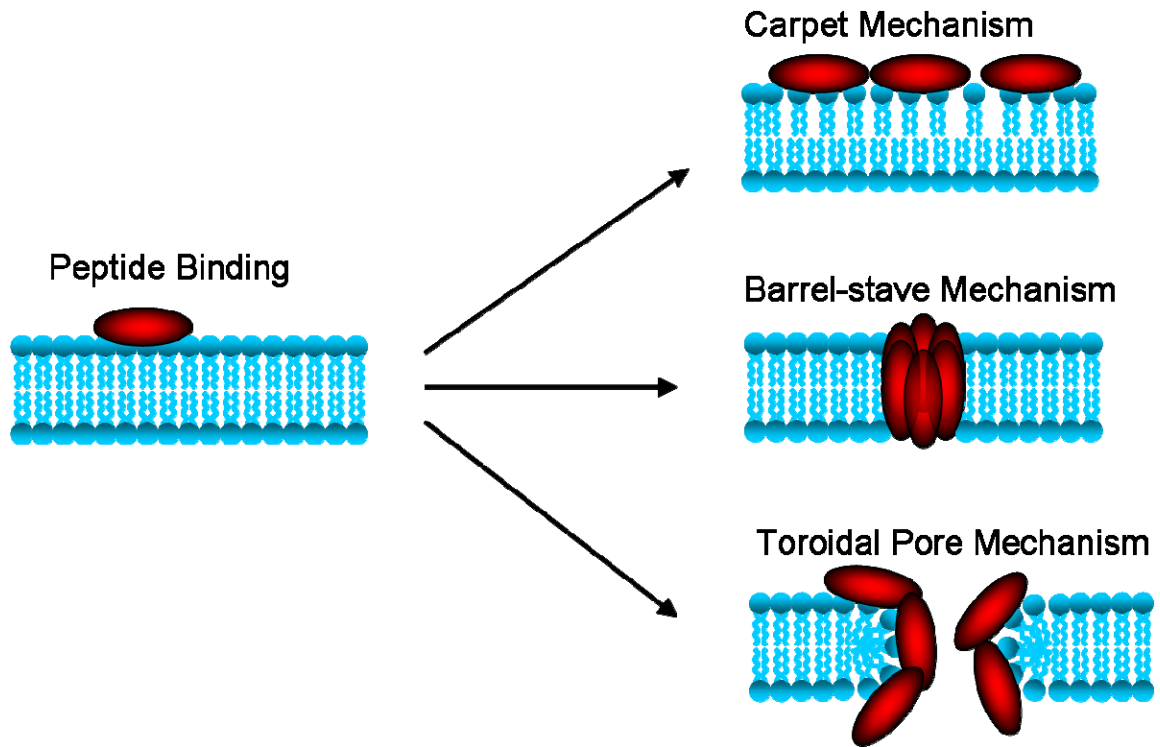


Figure 1.3 Mechanisms of membrane disruption. The mechanism of IAPP membrane permeabilization is currently unknown. Three main models have been proposed in the literature.

1.4 IAPP, Insulin, and the Secretory Granule

Interestingly, the peptide involved in type II diabetes, islet amyloid polypeptide (IAPP), is present at milli-molar concentrations in secretory granules prior to secretion. At these concentrations, IAPP rapidly fibrillizes *in vitro*, yet within secretory granules of healthy individuals IAPP does not fibrillize. In addition to IAPP, insulin is also present within the secretory granules prior to secretion and has previously been shown to inhibit IAPP fibril formation in the absence of lipids *in vitro*.

1.5 Thesis Summary

Previous studies have shown that the N-terminal 1–19 region, rather than the amyloidogenic 20–29 region, is primarily responsible for the interaction of the IAPP peptide with membranes. Liposome leakage experiments presented in this study confirm that the pathological membrane disrupting activity of the full-length hIAPP is also shared by hIAPP_(1–19). The hIAPP_(1–19) fragment at a low concentration of peptide induces membrane disruption to a near identical extent as the full-length peptide. At higher peptide concentrations, the hIAPP_(1–19) fragment induces a greater extent of membrane disruption than the full-length peptide. Similar to the full-length peptide, hIAPP_(1–19) exhibits a random coil conformation in solution and adopts an α -helical conformation upon binding to lipid membranes. However, unlike the full-length peptide, the hIAPP_(1–19) fragment did not form amyloid fibrils when incubated with POPG vesicles. These results indicate that membrane disruption can occur independently from amyloid formation in IAPP, and the sequences responsible for amyloid formation and membrane disruption are located in different regions of the peptide.

In addition to the region responsible for membrane permeabilization, we sought to identify if the cosecreted peptide, insulin, is involved in inhibiting IAPP from permeabilizing the β -cell plasma membrane. Insulin readily binds and inhibits IAPP fibrillization, yet we found that insulin had negligible effects on IAPP oligomerization and toxicity. Contrary to the expected protective effects, insulin maintains the oligomeric species of IAPP in a potentially toxic state that permeabilizes liposomes. Other characteristics and components of the plasma membrane may play a role in IAPP's toxicity. A liposome composition of 30 mol % anionic lipids or more is necessary for

IAPP to permeabilize membranes *in vitro* (1, 14). The anionic charge of the lipid surface may be the key determinant of IAPP binding.

Large unilamellar vesicles (LUVs) are frequently used as a simplified model of the cellular plasma membrane. LUVs are liposomes of approximately 100 nm in diameter with a bilayer encapsulating an internal aqueous cavity. They can be composed of any number of lipids or lipid mixtures depending on the desired conditions. The mol % of cholesterol incorporated into LUV membranes determines the fluidity of the membrane which affects the ability of IAPP to permeabilization the LUV membranes. As cholesterol is increased, membrane permeabilization decreases. Cholesterol appears *in vitro* to inhibit IAPP permeabilization, possibly by filling the interstitial spaces between the lipid acyl tails and eliminate space for peptide insertion.

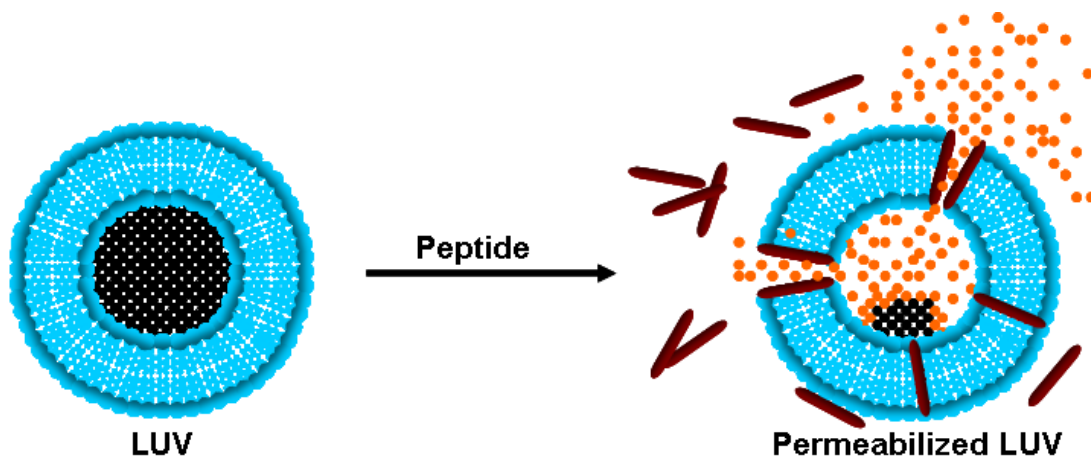


Figure 1.4 IAPP permeabilizes LUVs as measured by dye leakage. Dye leakage experiment with quenched carboxyfluorescein encapsulated inside the LUV. Upon addition of the peptide, the peptide binds and permeabilizes the LUV releasing carboxyfluorescein, which allows it to fluoresce.

Most studies in the literature to date have focused on IAPP peptide binding at the lipid interface without identifying if the binding also destabilizes or permeabilizes the lipid membrane. We used a dye leakage experiment as a measure of membrane permeabilization (figure 1.4). Peptide binding to the lipid membrane may or may not

induce membrane permeabilization, but it is expected that the toxic oligomeric species permeabilizes the β -cell plasma membrane. We wanted to observe permeabilization, which is more representative of toxicity than peptide binding, with our model lipid membranes *in vitro*. We used permeabilization as a model measure of toxicity. IAPP lipid binding has been well characterized in the literature, but has not previously been connected with permeabilization. We sought to determine the region of IAPP necessary for membrane binding and permeabilization, whether IAPP permeabilizes in the presence of inhibitory insulin and the effect that cholesterol has on the ability of IAPP to permeabilize LUVs.

CHAPTER II

The Toxic Oligomer Hypothesis Is Supported by a Membrane Permeabilizing Nonamyloidogenic Fragment of IAPP

2.1 Introduction

Islet amyloid is a deposit composed of islet amyloid polypeptide (IAPP) in the form of proteinaceous plaques and fibrils deposited in the islets of Langerhans of the pancreas and is a hallmark of type II diabetes in humans. The islets of Langerhans are clusters of approximately 100,000 cells that primarily function to secrete peptide and protein non-steroid hormones like insulin, IAPP, and glucagon. The prevalence of islet amyloid in the disease state and the increase in the presence of amyloid as the disease progresses has led to the assumption that the islet amyloid is in some way responsible for the progression of Type II diabetes (15). Prior to extensive examination, research initially appeared to support the hypothesis that fibrils and plaques are responsible for Type II diabetes. In vitro experiments of rat IAPP (rIAPP), which is nonamyloidogenic due to a difference in the amino acid sequence from that of human IAPP, have shown that it is non-toxic, which is consistent with the finding that rats do not spontaneously develop Type II diabetes (16). This putative link between β -cell death and amyloid formation has in turn led to numerous mechanistic fibril formation studies (17-20). However, fully formed IAPP fibrils of the toxic human form have since been shown to be relatively inert and have little cytotoxic effect on β -cells (5, 6). This finding is in common with other

amyloidogenic peptides; the fibrils exhibit relatively little cytotoxicity in comparison with earlier species of aggregation (7-9).

Also present early in the aggregation of IAPP are small oligomeric species. Instead of mature fibrils and plaques being responsible for cytotoxicity, these small oligomeric species of IAPP may be the culprits. Small oligomeric species of other amyloid peptides have been implicated in membrane permeabilization, through either ion channel formation or a nonspecific disruption of the membrane (7, 12, 21-23). Whether these oligomers form directly on-pathway (24) as precursors to fibril formation or are off-pathway is unknown (5, 8, 9, 25). The nature of the cytotoxic oligomer is transitory, which could fit either pathway of formation. Part of the difficulty in answering this question is due to the fact that fibrils rapidly form in the sample to create a heterogeneous mixture of species that obscures the identity of any single species.

Fragments of IAPP that consist of separate functional regions have been used frequently to simplify the analysis of the function of amyloid peptides and to identify regions that are necessary for cytotoxicity and amyloidogenesis. Two main regions have been identified as responsible for the aggregation of IAPP: IAPP₂₀₋₂₉ and IAPP₃₀₋₃₇ (26). Variations in these sequences have been shown to inhibit amyloid formation analogous to that seen in rIAPP (16, 25, 27, 28). Unfortunately, even though IAPP₂₀₋₂₉ and IAPP₃₀₋₃₇ have a strong tendency to form amyloid, the toxicity of these peptides appears to be less than the full-length peptide (6, 29). Likewise, we wanted to study the ability of the peptide to bind and permeabilize our model membrane system. Understanding the interaction of amyloid peptides with lipid membranes is crucial to understanding how IAPP leads to β -cell death. The interaction, as well as the catalysis of toxic oligomers at the membrane, is

critical to understanding amyloid diseases (30). The IAPP₂₀₋₂₉ fragment is amyloidogenic, but has a much lower affinity for phospholipid membranes in comparison to the full-length peptide (27, 28). This may be due partially to the charge of the peptide at the N-terminus.

Previously, it has been shown through lipid monolayer expansion experiments, infrared reflection-absorption spectroscopy, and fluorescence anisotropy that the N-terminus of IAPP is primarily responsible for membrane binding (1, 27, 28, 31). The IAPP₁₋₁₉ fragment does in fact insert more readily than the full-length peptide (27, 28). Here we show that the N-terminus of IAPP₁₋₁₉ can disrupt synthetic lipid vesicles to a similar extent as the full-length IAPP peptide. In addition, IAPP₁₋₁₉ accomplishes this without the ability to form amyloid, making it a prime model to study the process of IAPP membrane disruption and permeabilization.

2.2 Material and Methods

2.2.1 IAPP Preparation

Thioflavin T, DMSO, HFIP, and carboxyfluorescein were obtained from Sigma-Aldrich; POPG and POPC were purchased from Avanti (Alabaster, AL); Full-length human IAPP with the C-terminus amidated (>95% purity) was obtained from Anaspec (San Jose, CA). The IAPP₁₋₁₉ and IAPP₂₀₋₂₉ peptides were synthesized using standard Fmoc (9-fluorenylmethoxycarbonyl) methods. A PAL-PEG resin was used to provide an amidated C-terminus, and standard Fmoc reaction cycles were used. The first residue attached to the resin, every β -branched residue, and every residue immediately following

a β -branched residue were double coupled. Peptides were cleaved from the resin using standard trifluoroacetic acid protocols and purified using a reversed-phase C18 column with an acetonitrile/water gradient and trifluoroacetic acid as an ion-pairing agent. The hIAPP₂₀₋₂₉ peptide was synthesized as described above but with both an amidated C-terminus and an acetylated N-terminus. After synthesis, the crude hIAPP₁₋₁₉ peptide was subjected to air oxidation to form the disulfide bond. The identity of the hIAPP₁₋₁₉ and hIAPP₂₀₋₂₉ sequences and formation of the disulfide bond for hIAPP₁₋₁₉ was verified by electrospray mass spectroscopy. Peptide purity was measured as greater than 95% by analytical HPLC.

2.2.2 LUV Preparation

POPG LUVs were prepared for dye leakage and circular dichroism (CD) experiments by first dissolving the powdered POPG lipids to 20 mg/ml concentration in chloroform. The chloroform solvent was then removed from the lipids by first evaporating it under a stream of N₂ gas, which deposits a thin lipid film on the walls of a glass test tube. The sample was then dried under a vacuum overnight to remove residual solvent. Then the appropriate buffer was used to rehydrate the lipid film creating multilamellar vesicles (MLVs) with a 40 mg/ml concentration. Ten freeze-thaw cycles were used to equilibrate the buffer and vesicles. LUVs were made from the MLVs by extruding the sample through a 100 nm polycarbonate filter in an extruder 21 times (figure 2.1). To prepare the LUVs for dye leakage experiments, the lipid film was rehydrated with 50 mM NaP_i buffer containing 40 mM carboxyfluorescein at pH 7.5. Carboxyfluorescein not encapsulated in the LUVs was removed via size exclusion

chromatography with a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The concentration of the phospholipids were determined according to the Stewart Method (32). Rehydrated LUV solutions were prepared and used prior to each experiment to avoid lipid degradation.

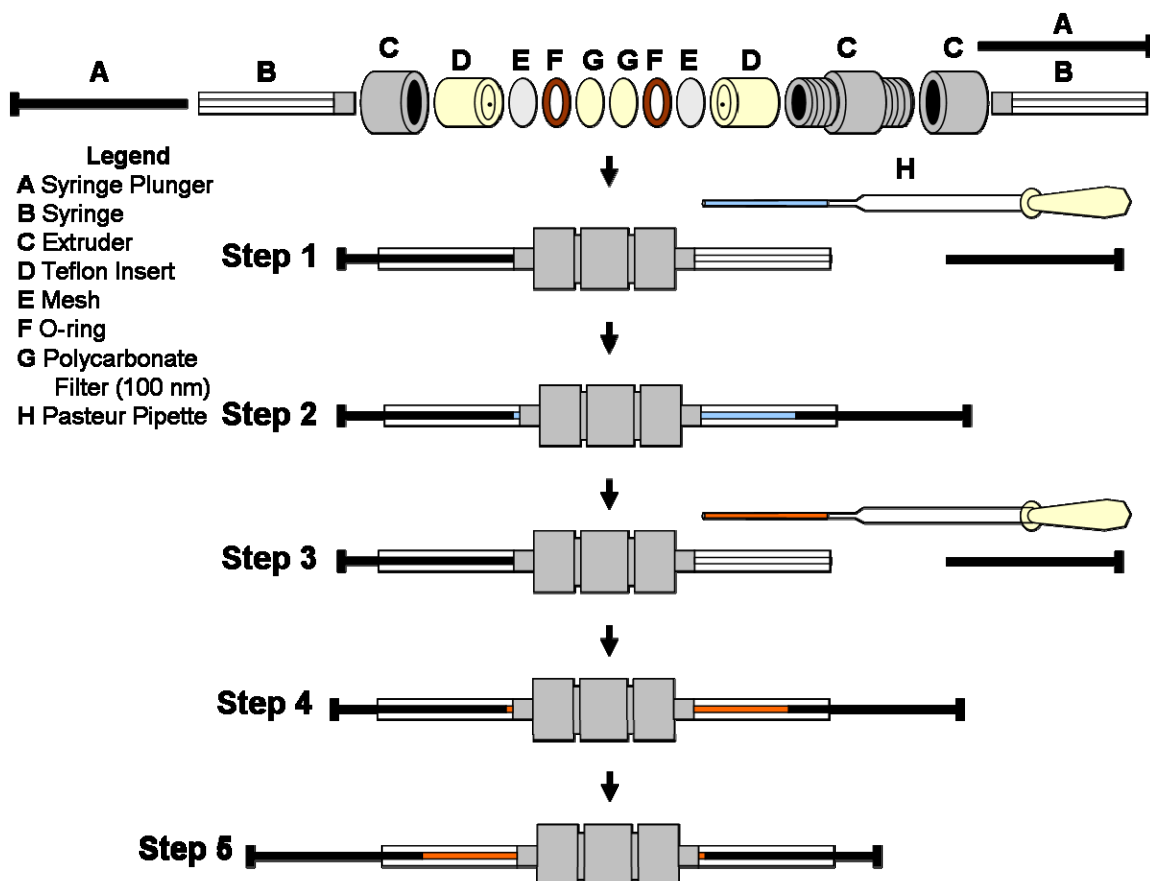


Figure 2.1. Extruder assembly. The extruder is assembled with all of the parts A through G in the order shown above. (Step 1) After assembly, the polycarbonate filter is hydrated with the addition of the sample buffer and (Step 2) extruded through the membrane from side to side a few times and then discarded. (Step 3) LUVs are added to the syringe and (Step 4) extruded from side to side. (Step 5) Extrusion is complete upon the 21st extrusion, which ends the extrusion on the opposite side to the starting position. Ending on the opposite side prevents contamination of multilamellar LUVs that failed to pass through the polycarbonate filter from entering the final sample.

2.2.3 CD Spectroscopy

IAPP₍₁₋₃₇₎ and IAPP₍₁₋₁₉₎ were lyophilized and dissolved for 1 h in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to prepare a 4 mg/ml peptide solution. Aliquots were re-lyophilized with HFIP and then solubilized for the experiment in either 10 mM NaP_i buffer, pH 7.3, or 10 mM NaP_i buffer, 150 mM NaF, pH 7.3. After resolubilization, the peptide solutions were briefly vortexed, sonicated for ~15 s, and transferred to a 1 mm path-length cuvette. An initial CD spectral scan of the hIAPP solution was made prior to the addition of POPG vesicles. POPG vesicles were prepared as a 40 mg/ml stock solution and then were added to the IAPP solution to a final concentration of 400 μ M LUV concentration. The spectra were then measured from 190-260 nm at 1nm intervals at a 50 nm/min scanning speed, and with a 5 nm bandwidth. Each spectrum was an average of four accumulations after subtracting the baseline spectrum of buffer, vesicles, and cuvette without the presence of the peptides. The experiments were conducted at RT. The CONTINLL option in CDPRO was used to estimate the secondary structure of the peptides with SMP56 (13 membrane proteins and 43 soluble proteins) used as the basis set (33). Peptides like IAPP exist in a number of conformational states and have a tendency to adsorb to quartz and other surfaces. The results of this analysis of the secondary structure of IAPP by CD must be treated cautiously as the basis sets were developed for large proteins and the applicability of the analysis, for short peptides that oligomerize and aggregate is undetermined. Because of these complicating factors in the qualitative analysis we listed the approximate secondary structure value instead of the exact secondary structure of the peptide. To support our conclusions about amyloid formation we used two additional techniques (thioflavin T binding and electron

microscopy) to verify of the formation amyloid fibrils. The conformational change of the IAPP₍₁₋₃₇₎ peptide observed with ThT and CD spectroscopy occurred on a similar timescale and in agreement with previous values reported in the literature (31).

2.2.4 Dye Leakage Assay

IAPP₍₁₋₃₇₎ and IAPP₍₁₋₁₉₎ stock solutions were dissolved in HFIP and lyophilized as described under the CD spectroscopy methods. A concentrated peptide stock solution was prepared at 10 mg/ml in pure DMSO. At each data point, the baseline fluorescence of the POPG LUV mixture (containing unlabeled LUVs and LUVs with carboxyfluorescein encapsulated inside) was measured for 15 s. The fluorescence of the baseline is relatively stable with only slight changes due to the slow process of dye leakage. After recording the baseline, an aliquot of the DMSO-prepared peptides were added to the lipid mixture at 250 nM or 1 μ M and measured at 100 s, which allowed sufficient time for the IAPP to bind and permeabilize the LUVs. The dye leakage plateaued at approximately 100 s, which was thus determined to be an acceptable stopping point for each data point. Triton X-100 detergent was then added to the sample to 0.2% as a measure of the total fluorescence of the caboxyfluorescein within the sample. The % dye leakage was calculated according to eq. 2.1:

$$\% \text{ dye leakage} = \frac{F_{\text{sample}} - F_{\text{baseline}}}{F_{\text{detergent}} - F_{\text{baseline}}} \times 100 \quad (\text{eq. 2.1})$$

The dye leakage was performed at RT with 50 mM NaP_i buffer, pH 7.5, and was performed in triplicate from separate stock solutions of peptide.

2.2.5 Thioflavin T Fluorescence

Peptide and LUVs (27.5 μ M peptide, 440 μ M lipid) were prepared as described for the CD experiments. Thioflavin T (ThT) stock was prepared to 250 μ M and added to the sample at a final concentration of 25 μ M. The ThT was excited at 450 nm and the fluorescence emission was measured at 486 nm. The relative fluorescence intensity was plotted over the timecourse of the experiment. The ThT $t_{1/2}$ value for the measure of fibril growth was calculated as the time point at which the fluorescent intensity was one-half of the maximum intensity.

2.2.6 Electron Microscopy

IAPP₍₁₋₃₇₎ was incubated for 7 days, IAPP₍₁₋₁₉₎ was incubated for 15 days, and the control vesicles without peptide were incubated for 15 days prior to preparing the EM grids. Formvar-coated copper grids (Ernest F. Fullam, Inc., Latham, NY) were incubated with 10 μ l aliquots of peptide sample for 2 min, washed with 10 μ l of deionized water twice, and then negatively stained with 2% uranyl acetate for 90 s. The samples were imaged with a Philips CM10 Transmission Electron Microscope.

2.3 Results and Discussion

2.3.1 IAPP₍₁₋₁₉₎ Binds and Permeabilizes LUVs, yet Does Not Form Amyloid.

To measure the extent of membrane permeabilization induced by IAPP₍₁₋₁₉₎ and IAPP₍₁₋₃₇₎, large unilamellar vesicles (LUV) of POPG (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-(1-glycerol)) were made to measure the leakage of carboxyfluorescein

dye as a measure of permeabilization (i.e. toxicity) (Figure 2.2). At 250 nM peptide concentrations, IAPP₍₁₋₁₉₎ rapidly permeabilized the LUVs almost identically to the full-length peptide, IAPP₍₁₋₃₇₎. At a higher concentration of peptide (1 μ M), IAPP₍₁₋₁₉₎ is more effective than IAPP₍₁₋₃₇₎ at inducing membrane disruption, most likely due to the full-length peptide quickly aggregating to inert fibrils above the critical concentration for fibril formation. The critical concentration varies based on buffer conditions but is approximately 1 μ M in our experiments. IAPP₍₂₀₋₂₉₎ is an amyloidogenic fragment that quickly forms amyloid fibrils, yet did not induce membrane permeabilization (Figure 2.2).

To investigate the relationship between amyloid fibril formation and membrane disruption by IAPP₍₁₋₃₇₎ and the IAPP₍₁₋₁₉₎ fragment, conformational changes of the peptides associated with membrane binding and fibril formation were measured by CD in both low (10 mM phosphate buffer) and high (10 mM phosphate buffer and 150 mM NaF) ionic strength solutions. The secondary structures of both peptides in solution are similar initially (figure 2.3), as well as in the presence of POPG LUVs (figure 2.4). IAPP₍₁₋₁₉₎ appears completely unstructured in solution (Figure 2.3), and IAPP₍₁₋₃₇₎ appears primarily unstructured in conformation (Figure 2.3A). However, at a high ionic strength IAPP₍₁₋₃₇₎ contains a small amount of β -sheet structure shown by a greater minimum at 218 nm (Figure 2.3B).

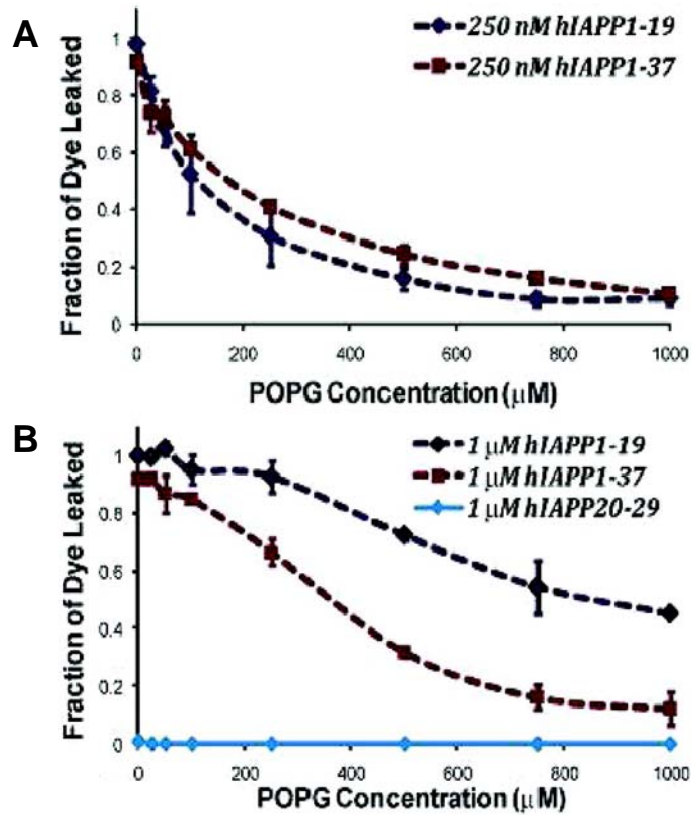


Figure 2.2. Peptide induced dye leakage of carboxyfluorescein from POPG LUVs. A constant concentration of 1.5 μM of carboxyfluorescein containing POPG LUVs along with the listed concentration of dye-free POPG LUVs were added to each peptide sample. (A) The LUVs were added to 250 nM IAPP₍₁₋₁₉₎ or IAPP₍₁₋₃₇₎. (B) The LUVs were added to 1 mM IAPP₍₁₋₁₉₎, IAPP₍₁₋₃₇₎, or IAPP₍₂₀₋₂₉₎. The fraction of dye leaked was recorded at 100 s. The buffer was 50 mM NaP_i, pH 7.5. The experiments were conducted in triplicate (error bars = 1 standard deviation from the average).

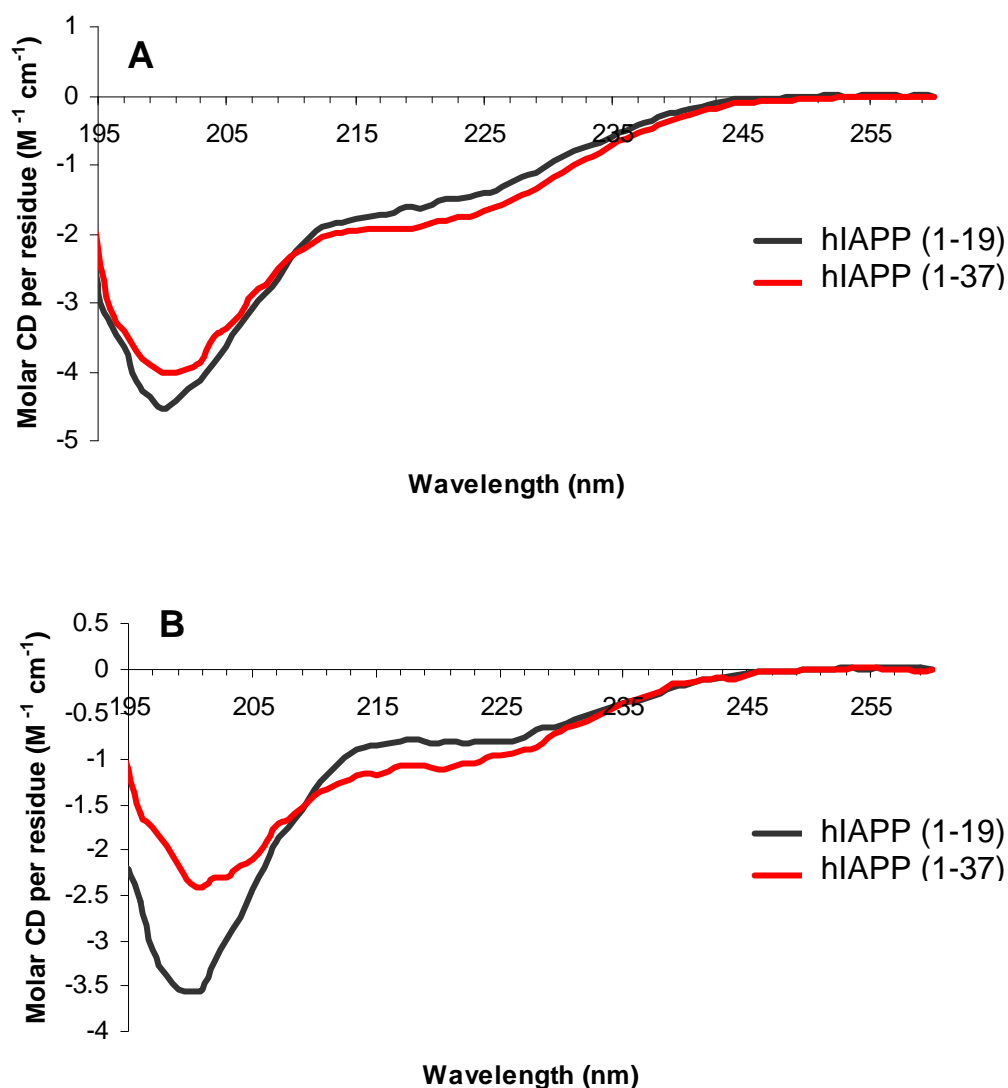


Figure 2.3. CD secondary structures of IAPP₍₁₋₁₉₎ and IAPP₍₁₋₃₇₎ at high and low ionic conditions without LUVs. (A) Spectra of IAPP₍₁₋₁₉₎ (black line) and the IAPP₍₁₋₃₇₎ (red line) peptides at low ionic strength buffer (no salt, 25 μ M NaP_i buffer, pH 7.3) and (B) peptides at a high ionic strength buffer (150 mM NaF, 25 μ M NaP_i buffer, pH 7.3). IAPP₍₁₋₃₇₎ exhibits lower solubility at a higher ionic strength.

With the addition of POPG LUVs, IAPP₍₁₋₃₇₎ and IAPP₍₁₋₁₉₎ both adopt a primarily α -helical structure with the characteristic minima at 208 and 222 nm (34). IAPP₍₁₋₁₉₎ remained stable in this conformation for at least 17 h (Figure 2.4A). After 17 h, aggregation of the lipid vesicles was visible to the naked eye and the CD signal

diminished in intensity without altering shape. IAPP₍₁₋₃₇₎ previously has been shown to facilitate the aggregation of liposomes at a low ionic strength (35). The CD signal after 56 min showed no evidence of the β -sheet structure that is indicative of amyloid fibril formation. On the other hand, the IAPP₍₁₋₃₇₎ peptide showed a conformational change from a predominantly α -helical structure to a β -sheet structure indicative of amyloid fibril formation within 40 min after the addition of POPG liposomes (Figure 2.4B). With high ionic strength conditions more similar to the physiological range (150 mM NaF), IAPP₍₁₋₁₉₎ remained stable in an α -helical conformation for at least 13 days without a decrease in the CD signal intensity or the aggregation of the LUVs (Figure 2.5A). IAPP₍₁₋₃₇₎ by contrast, showed a conversion of the initial α -helical structure to a β -sheet conformation indicative of amyloid fibril formation (Figure 2.5B). The Thioflavin T (ThT) fluorescence measured in the presence of POPG LUVs revealed a $t_{1/2}$ for IAPP₍₁₋₃₇₎ amyloid fibril formation as 64 min (3840 s). This value of 64 min is similar to other reports in the literature (Figure 2.6) (36). IAPP₍₁₋₁₉₎ did not induce an increase in the ThT fluorescence over the duration of the experiment, indicating a lack of amyloid fibril formation for this peptide. ThT itself is known to not inhibit amyloid fibril formation of IAPP (8, 9).

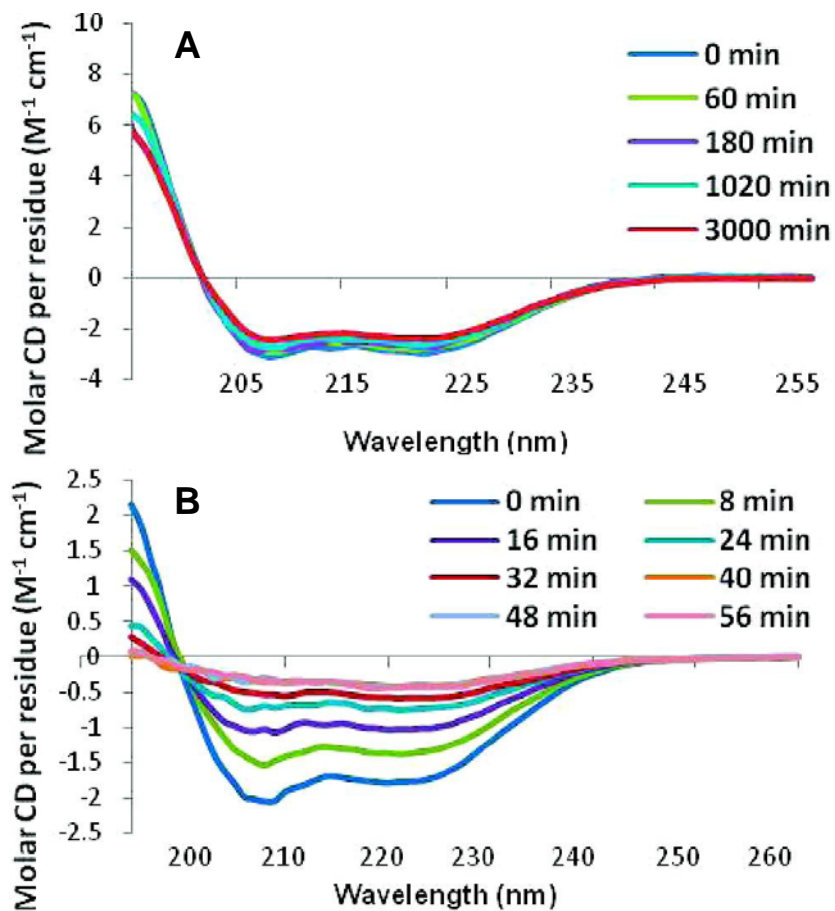


Figure 2.4. CD spectra of IAPP₍₁₋₁₉₎ and IAPP₍₁₋₃₇₎ at low ionic strength in the presence of POPG LUVs. (A) IAPP₍₁₋₁₉₎, 400 μM POPG LUVs (B) IAPP₍₁₋₃₇₎, 400 μM POPG LUVs. The buffer solution (no salt, 25 μM NaP_i buffer, pH 7.3) was measured as a function of time after the addition of 400 μM POPG to each sample.

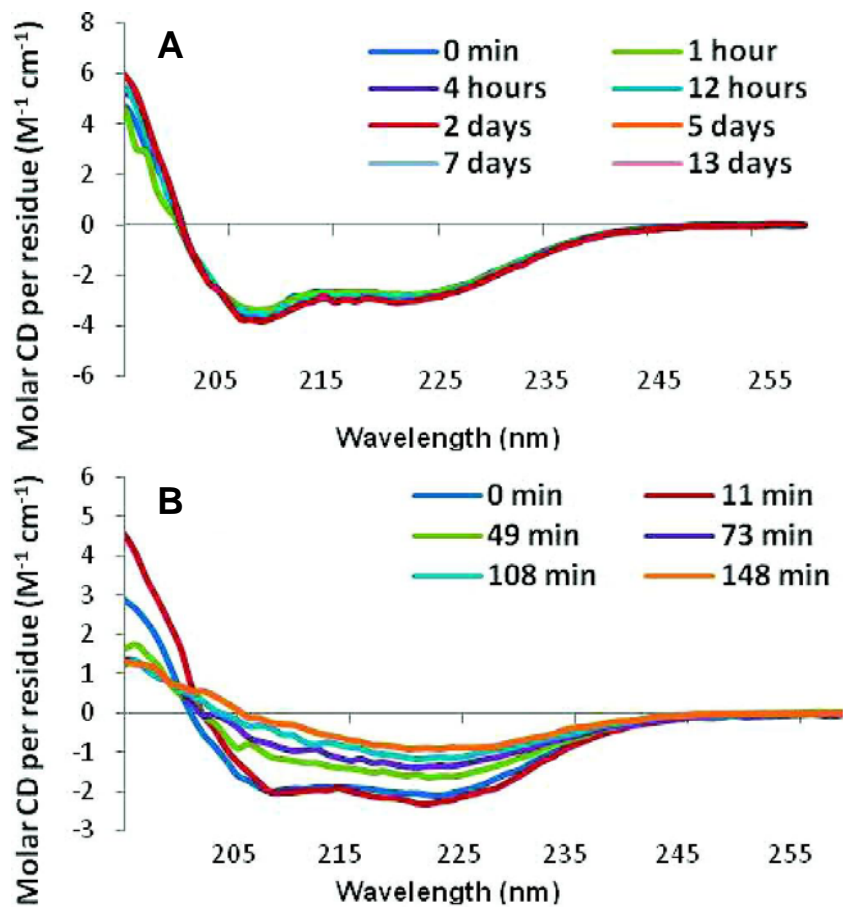


Figure 2.5. IAPP₍₁₋₃₇₎ undergoes a structural change to β -sheet structure, while IAPP₍₁₋₁₉₎ does not. CD spectra of IAPP₍₁₋₁₉₎ and IAPP₍₁₋₃₇₎ at high ionic strength (150 mM NaF) and in the presence of POPG LUVs. (A) IAPP₍₁₋₁₉₎ (B) IAPP₍₁₋₃₇₎. The buffer solution (150 mM NaF, 25 μ M NaP_i buffer, pH 7.3) was measured as a function of time after the addition of POPG LUVs to 400 μ M POPG to each sample.

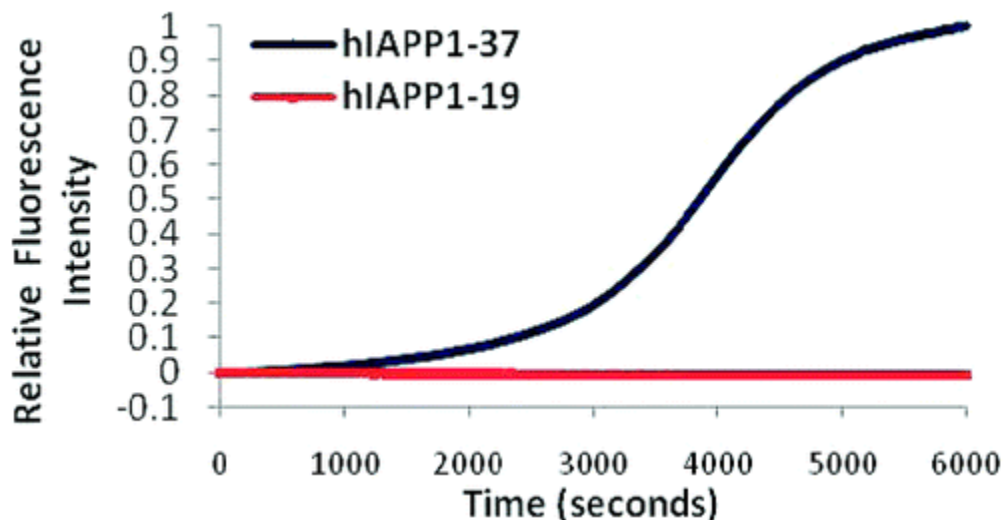


Figure 2.6. IAPP₍₁₋₁₉₎ does not form amyloid. Thioflavin T (ThT), an amyloid specific dye, binds and fluoresces in the presence of hIAPP₍₁₋₁₉₎ and full-length hIAPP measured by fluorescence. IAPP₍₁₋₃₇₎ or IAPP₍₁₋₁₉₎ were incubated at 25 μ M with 400 μ M of POPG LUVs and 25 μ M ThT.

To confirm the absence of IAPP₍₁₋₁₉₎ amyloid fibrils in the CD experiments, aliquots at the final timepoint were taken and negatively stained for transmission electron microscopy (TEM). The electron micrographs of the IAPP₍₁₋₃₇₎ sample containing POPG LUVs showed amyloid fibrils branching out from the LUVs (Figure 2.7A). Remarkably, amyloid fibrils were not detected with IAPP₍₁₋₁₉₎, which confirms the absence of amyloid fibril formation for IAPP₍₁₋₁₉₎ (Figure 2. 7B).

Related N-terminal IAPP fragments, like IAPP₍₁₋₁₈₎ at 2.14 mM, an extremely high peptide concentration, have shown a weak ability to form amyloid (26, 36). However, FTIR spectroscopy showed that the amyloid fibrils that were detected by TEM were only a minor fraction of the IAPP₍₁₋₁₉₎ sample. The self-association of the amyloid fibrils formed from IAPP₍₁₋₁₉₎ appeared to be much less than the self-association of amyloid fibrils formed from the IAPP₍₁₋₃₇₎ peptide. Additionally, the IAPP₍₈₋₂₀₎ amino acid sequence of IAPP has also been shown to be weakly amyloidogenic (26).

Nevertheless, IAPP₈₋₂₀ and IAPP₁₋₁₉ fragments have several important differences between them. The disulfide bridge between cysteine residues 2 and 7 conformationally restrict this region of IAPP from adopting the β -sheet conformation and may alter the amyloidogenicity of the entire peptide (37, 38). The studies on both IAPP₍₁₋₁₈₎ and IAPP₍₈₋₂₀₎ were done in solution, without the presence of lipid vesicles. Lipid vesicles may bury hydrophobic residues that are exposed in solution within the membrane and would be expected to alter the energetics of amyloidogenesis.

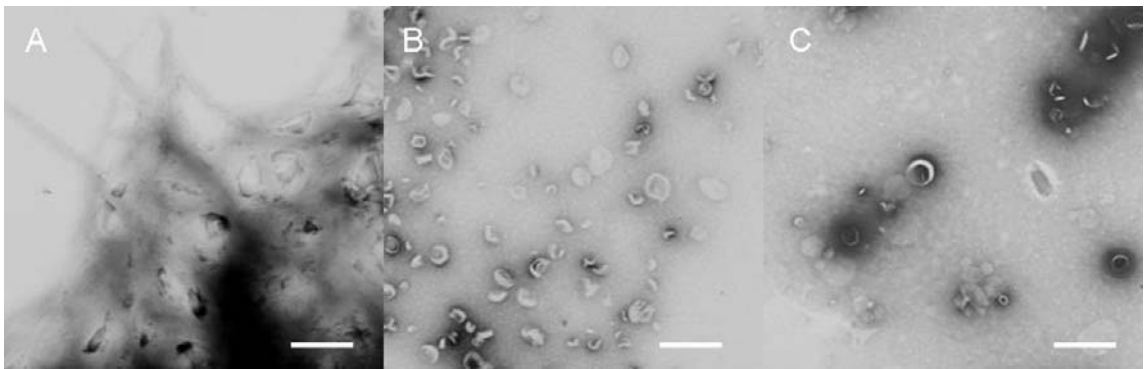


Figure 2.7. IAPP₍₁₋₁₉₎ does not form visible amyloid fibrils by TEM. Electron Micrographs of IAPP peptides incubated with POPG LUVs (A) IAPP₍₁₋₃₇₎ incubated with POPG LUVs for 7 days. Amyloid fibrils can be seen emanating from the peptide engulfed POPG LUVs. (B) IAPP₍₁₋₁₉₎ incubated with POPG LUVs for 15 days. No amyloid fibrils were visibly detected. (C) POPG LUVs incubated without peptide for 15 days (scale = 500 nm).

2.3.2 Amyloid Fibril Formation and Membrane Disruption Are Separate Processes

Localized in Two Distinct Regions of IAPP

Our results indicate that IAPP amyloid fibril formation is not necessary for the peptide to permeabilize membranes. As established in the literature, amyloid fibrils themselves are not especially toxic (5, 6), but it is debatable whether amyloid formation is necessary for the generation of small toxic oligomers and the membrane

permeabilizing activity of amyloid peptides. Our results suggest that the membrane disrupting activity of IAPP can be achieved irrespective of amyloid formation and occurs primarily as a result of other factors not necessarily related to amyloidogenesis (39). One of the strongest pieces of evidence supporting the amyloid hypothesis instead of the oligomer hypothesis is the lack of a cytotoxic response from the nonamyloidogenic rat IAPP. However, Knight et al. have recently shown that the nonamyloidogenic rIAPP does have the ability to disrupt membranes under certain conditions (14). The detection of a small degree of β -cell apoptosis in diabetic rats also supports the conclusion that rat IAPP can be toxic, albeit to a lesser degree than the human IAPP (40). The binding of IAPP to the membrane is cooperative and dependent on the formation of small oligomers on the surface of the membrane. In addition to being unable to form amyloid fibrils, rat IAPP is less effective at forming the small oligomers necessary for membrane binding than is human IAPP (14). The difference in toxicity between the rat and human versions of IAPP therefore may be largely due to a difference in the formation of these small oligomers rather than the inability of rat IAPP to form amyloid fibrils.

It is likely that several processes that lead to cell death are simultaneously operational *in vivo*, some of which are dependent on the formation of amyloid fibrils and others that are not. The instability of the proposed toxic intermediates, a particularly acute problem for the IAPP peptide due to its extremely fast rate of aggregation, considerably complicates analysis. The IAPP₍₁₋₁₉₎ fragment, which does not form amyloid fibrils but disrupts membranes to a similar extent as that for the IAPP₍₁₋₃₇₎ peptide, will be a useful tool to disentangle this complex process.

Previous studies of the secondary structure of IAPP have shown that residues 1–7 and 19–37 are likely to be disordered after the peptide initially binds to the membrane (1). Packing density arguments suggest that the N-terminal segment of the IAPP peptide (residues 1–18) adopts a transmembrane orientation, while the C-terminal region is believed to lie outside the membrane in a disordered state (figure 2.8) (1, 14, 30). The formation of secondary structure in the C-terminal region is expected to drive the process of amyloidogenesis in the full-length IAPP peptide (14, 41). The IAPP_(1–19) peptide shows a similar amount of helical content to that of IAPP_(1–37) (Figure 2.3A). However, the N-terminal segment (residues 1–7) is conformationally constrained from adopting a secondary structure by the presence of a disulfide bond between residues 2 and 7 (37, 38). Without the formation of an additional secondary structure as a driving force, the amyloidogenic propensity of IAPP_(1–19) is expected to be far less than that of IAPP_(1–37). Nevertheless, the amphipathic helical structure of the full-length peptide implicated in membrane-disrupting properties is retained in IAPP_(1–19) (14).

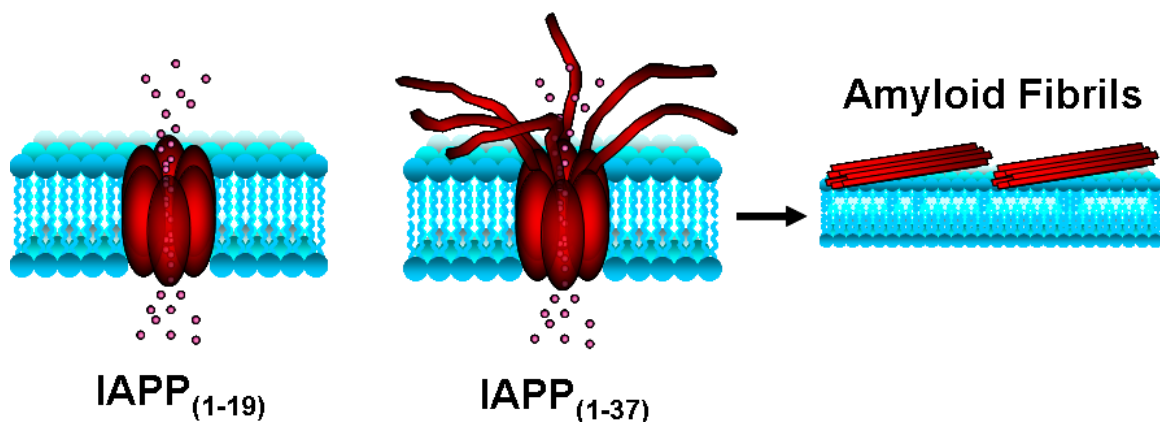


Figure 2.8 Cartoon of IAPP₍₁₋₁₉₎ and IAPP₍₁₋₃₇₎ showing a possible mechanism of peptide action, with only IAPP₍₁₋₃₇₎ forming fibrils. IAPP₍₁₋₁₉₎ binds and permeabilizes anionic LUVs without forming amyloid fibrils. IAPP₍₁₋₃₇₎ binds, permeabilizes, and forms amyloid fibrils in the presence of LUVs.

Because of the stability of the IAPP₍₁₋₁₉₎ fragment in membranes, we expect that it will be useful as a model system to study the membrane disrupting effects of IAPP and other amyloid peptides, using high-resolution techniques such as solid-state NMR spectroscopy, without the complications introduced by time-dependent aggregation.

CHAPTER III

Insulin Stabilizes the Membrane Permeabilizing Species of Islet Amyloid Polypeptide

3.1 Introduction

While the exact causes of β -cell death in overt Type II diabetes are largely unknown, there is strong evidence that the human Islet Amyloid Polypeptide (IAPP) plays an important role (42-44). It is well known through *in vitro*, tissue culture, and transgenic animal studies, as well as postmortem clinical examinations, that large β -sheet aggregates of IAPP, known as islet amyloid, are often associated with the development of Type II diabetes (42-44). Similar extracellular proteinaceous deposits composed of large aggregates of proteins in a characteristic cross β -sheet conformation have been found in an increasing number of pathologies, including Alzheimer's, Huntington's, and Parkinson's disease, among many others (42, 43). An aggregated (but not necessarily fibrillar) form of the peptides and proteins implicated in these amyloid associated diseases have been found to disrupt the integrity of cellular membranes to varying degrees: allowing the uncontrolled influx of calcium into the cell, subjecting the cell to membrane-associated oxidative stress, and triggering the initial stages of apoptosis.

The relevant factors that trigger IAPP aggregation and pancreatic β -cell membrane disruption in some individuals but not others are elusive, as the pathology of IAPP aggregation occurs most often with the wild-type peptide and is not associated with mutations of the IAPP sequence, except for the rare S20G polymorphism (45-48). Since

the wild-type IAPP is both aggressively amyloidogenic and toxic at low (<5 μM) concentrations *in vitro*, yet is stored in the secretory granule of β -cells at concentrations orders of magnitude higher (0.8-4 mM) without apparent dysfunction in non-diabetic individuals, it is unknown how IAPP is regulated to control both its aggregation and its inherent cytotoxicity. In fact, considering the high toxicity of IAPP *in vitro*, it may be more relevant to question what stops IAPP from attacking β -cells in healthy individuals, rather than what triggers IAPP aggregation and toxicity in diabetics.

Insulin, which is copackaged and cosecreted with IAPP from within the secretory granule, has emerged as a possible regulator of IAPP fibrillogenesis. Several studies have reported that insulin can inhibit the formation of amyloid by IAPP in solution (49-53), although this effect was not consistent in all studies, and some reported either little change or a slight increase in the rate of fibril formation at lower ratios of IAPP to insulin (54). Moreover, the relationship between amyloid fibril formation and β -cell toxicity is not entirely obvious. Mature amyloid fibrils composed of IAPP have little toxicity to cells in culture (5, 6, 12, 21, 55). Furthermore, amyloid formation is not necessarily correlated with the loss of β -cell mass in Type II diabetes in all cases. Deposits of islet amyloid have been found in both non-diabetics and Type II diabetics, yet some diabetics present no amyloid deposits in the advanced stages of the disease (56, 57). In addition, the appearance of islet amyloid is often spatially separated from the site of β -cell apoptosis and the onset of symptoms correlates poorly with the initial appearance of islet amyloid (58-60). Instead of amyloid fibrils, the formation of small oligomeric species that form either in association with the plasma membrane or in solution are a critical step in amyloid-induced apoptosis (61-68). Although the kinetics of these species have been

intensely studied, relatively little is known about the equilibrium between oligomer states in this process of forming the small oligomeric species of IAPP.

In contrast to the effect of insulin on IAPP amyloid formation, the effect of insulin on IAPP-induced membrane disruption and cell toxicity has been relatively unexplored. The use of compounds that bind to the end-stage of aggregation, that consists mostly of amyloid fibrils are largely unsuccessful in reducing the toxicity of these amyloidogenic proteins, most likely due to the low toxicity of the end-stage amyloid fibrils (5, 69). Other compounds have been shown to actually increase the toxicity of amyloid proteins by inhibiting the formation of the relatively inert amyloid fibrils, while increasing the amount of peptide in the early oligomeric and more toxic forms. An example of this is clusterin (apolipoprotein J), a protein found in Alzheimer's disease plaques, that binds and inhibits amyloid- β_{1-42} fibrillization, yet at the same time increases the cytotoxic amyloid- β_{1-42} species (61, 70, 71). Conversely, other inhibitors can block amyloid induced toxicity without affecting or even increasing amyloidogenesis.

The purpose of this research was twofold: (1) to further understand the separate functions of IAPP amyloid formation and membrane binding and (2) to determine if insulin inhibits IAPP not only from fibrillization but also from forming the toxic oligomeric species. We show that the binding of IAPP to the membrane and IAPP-induced membrane disruption are present even when amyloid formation is blocked by coincubation with insulin, which lends support to the oligomeric hypothesis of β -cell death. Thus, IAPP has the ability to permeabilize membranes without forming amyloid, and insulin actually maintains IAPP in a potentially toxic state by inhibiting IAPP fibrillization.

3.2 Material and Methods

3.2.1 IAPP and Insulin Preparation

Human IAPP was purchased from Anaspec Inc., recombinant human insulin expressed in yeast was purchased from Sigma-Aldrich. The IAPP peptide used in this study was amidated at the C-terminus like the natural peptide. Preformed aggregates in both peptides were solubilized by first dissolving the peptide in a 75% acetonitrile/water solution at a concentration of 1 mg/ml. The peptides were then lyophilized, and the lyophilized powder was redissolved in hexafluoroisopropanol (HFIP) at a concentration of 2-4 mg/ml. HFIP was removed by lyophilization overnight at high vacuum (72). The peptide was first dissolved in pure water to a concentration of 25 μ M in siliconized eppendorf tubes and then diluted with 2x buffer to create the final working solution (final buffer composition: 100 mM NaCl, 10 mM sodium phosphate, pH 7.4) (73).

3.2.2 Liposome Preparation

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (POPG) and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) dissolved in chloroform were purchased from Avanti Inc. (Alabaster, AL). To make liposomes, chloroform lipid stocks were dried with a stream of nitrogen and then placed under vacuum overnight to remove residual solvent. The lipid films were then rehydrated for 1-2 hours with 10 mM NaP_i buffer at pH 7.4 containing 100 mM NaCl. The rehydrated lipids were vortexed and then subjected to 8 freeze-thaw cycles. The resulting multilamellar vesicles were extruded 21

times through two 100 nm polycarbonate Nucleopore membrane filters (Whatman) to create unilamellar vesicles.

For liposomes with dye encapsulated inside, the vesicles were created as above except the buffer also contained 50 mM 5(6)-carboxyfluorescein. Free dye was separated from the lipid encapsulated dye by running the samples through a 10 mL Sephadex G-50 gel filtration column (Sigma-Aldrich). Lipid concentrations were determined using the Stewart method (32).

3.2.3 Circular Dichroism

Circular dichroism was performed using a J-715 spectropolarimeter (Jasco, Easton, MD) at RT with a 1 mm pathlength cuvette. IAPP, IAPP/Insulin mixtures and liposomes were prepared as above except NaF was used in place of NaCl. Each spectrum was obtained from the average of 10 accumulations between 190-260 nm. Spectra were collected at 50 nm/min, with a response time of 2 s and a bandwidth of 5 nm. Spectra of the buffer, liposomes, and cuvette were subtracted from the IAPP spectra. To prevent masking of the IAPP signal by insulin, the insulin signal was also subtracted from the spectra of IAPP and insulin mixtures

3.2.4 Dye Leakage

IAPP or IAPP/insulin (25 μ M) was preincubated for the indicated amount of time and then diluted to 1 μ M immediately before addition to a 200 μ M mixture of LUVs (195 μ M empty POPG vesicles, 5 μ M dye-encapsulated POPG vesicles). The fluorescence was recorded for the LUV mixture prior to the addition of the peptide and subtracted

from the sample. At the completion of dye leakage, the total signal was normalized by adding Triton-X detergent to 0.2% v/v to permeabilize any remaining intact LUVs with encapsulated dye. Dye leakage was plotted according to equation 2.1.

3.2.5 Glutaraldehyde Crosslinking and Western Blotting.

IAPP (25 μM) and IAPP/insulin (25 μM : 25 μM) were incubated at room temperature with POPG/POPC LUVs (200 μM) in 100 mM NaF, 10 mM NaP_i , pH 7.4 for the indicated times. After incubation, 0.01% (v/v) glutaraldehyde was added to initiate cross-linking at each time-point. The cross-linking reaction was allowed to continue for 30 min and was then quenched by the addition of 100 mM lysine. 3x SDS-PAGE loading buffer was then added and the samples were placed on a heat block at 90°C for 2 min. The samples were electrophoresed on 10-20% (w/v) polyacrylamide SDS Tris-Tricine Ready Gel (Bio-Rad, Hercules, CA), transferred with CAPs transfer buffer pH 11.0, immunoblotted on nitrocellulose with rabbit polyclonal α -IAPP antibody (Anaspec Inc., San Jose, CA). The primary antibody was visualized with a horseradish peroxidase conjugated α -rabbit antibody and ECL (Bio-Rad, Hercules, CA).

3.2.6 Transmission Electron Microscopy

Samples (10 μL of 25 μM) were incubated for 2 min on Formvar-coated copper grids (Ernest F. Fullam, Inc., Latham, NY). The grids were then washed twice with 10 μL deionized water and then negatively stained with 10 μL of 2% uranyl acetate for 1.5 min. The prepared grids were imaged using a Philips CM10 or CM100 Transmission Electron Microscope (TEM).

3.2.7 Thioflavin T Assay

Peptides were initially solubilized with deionized water, 2x buffer was added, and then Thioflavin T was added to final concentrations of 25 μ M for each peptide with 5 μ M Thioflavin T. The fluorescence intensity of the ThT mixture was measured with a BioTek Synergy 2 plate reader (BioTek, Winooski, VT).

3.3 Results

3.3.1 Insulin in the Monomeric Form Inhibits IAPP Amyloid Fibril Formation Both in Solution and in the Presence of Membranes

Human IAPP in solution normally exists in a predominantly random coil conformation, but converts into a β -sheet conformation indicative of amyloid fibrils in a nucleation-dependent process. Several studies have reported that insulin can inhibit this process (49-53), although this effect was not consistent in all studies, and some reported either little change or a slight increase in the rate of fibril formation at lower ratios of IAPP to insulin(54). Since the effect of insulin on IAPP has been reported to be dependent on the oligomeric state of insulin, we have accordingly first tested the effect of insulin on the formation of amyloid by IAPP at near neutral pH and at an ionic strength approximating the conditions found in the extracellular space.

Amyloid fibril formation was measured using the amyloid-specific dye, thioflavin T (ThT), which has a large increase in quantum yield upon binding to the cross-beta sheet structure characteristic of amyloid fibrils. The growth of fibrils is measured by a relative increase in ThT fluorescence intensity often called a relative fluorescence unit (rfu),

which is the relative change in fluorescence intensity from the baseline fluorescence. The presence of ThT in the reaction mixture does not significantly affect the kinetics of fibril formation by IAPP (8, 74). The kinetics of amyloid formation occurs by a nucleation-dependent process that is characterized by an initial lag phase, where amyloid formation is very slow, and rapid formation of amyloid in the later stages after the formation of nucleating particles. Fibril formation for IAPP in the absence of insulin occurred after a lag time of approximately 1000 minutes, similar to previous reports under similar conditions (Fig. 3.1A). In the presence of an equimolar amount of insulin the lag time for fibril formation increased dramatically, and significant fibril formation was not observed after 7000 minutes, indicating insulin inhibits the formation of amyloid fibrils by IAPP in solution under our conditions.

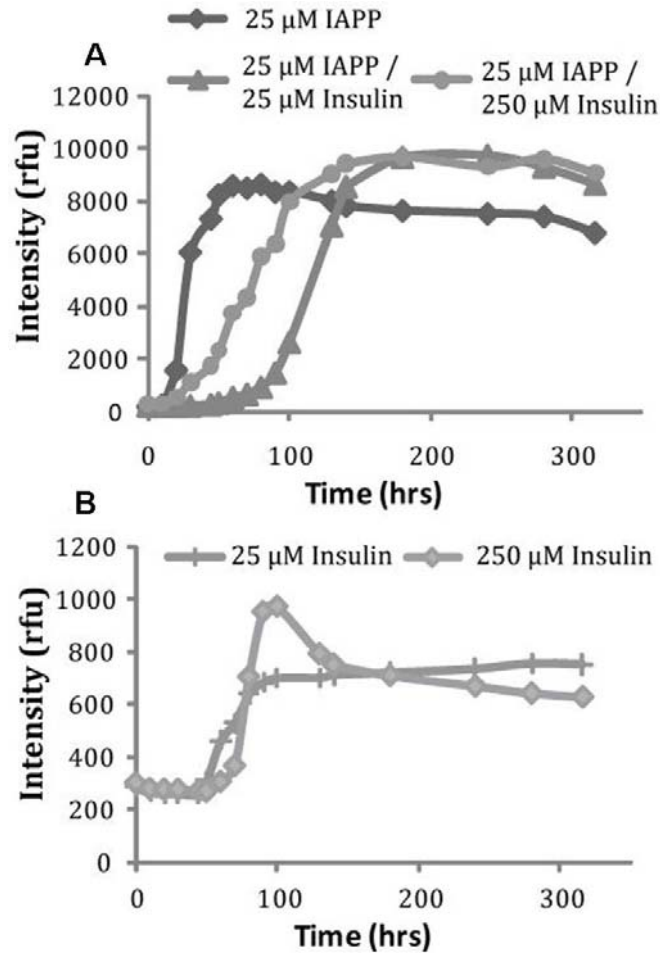


Figure 3.1. Insulin inhibits IAPP fibril formation. Enhancement of thioflavin T fluorescence showing fibril formation by IAPP and by (A) IAPP and insulin mixtures and by (B) insulin alone. Mixtures of IAPP and insulin form fibrils at a reduced rate. Insulin also has the ability to form oligomers and fibrils.

The inhibitory effects of insulin on IAPP fibrillization in solution was confirmed by transmission electron microscopy (TEM). IAPP was incubated alone and with insulin at an equimolar concentration (25 μM) for seven days and then negatively stained and imaged with transmission electron microscopy (TEM) (Fig. 3.2). In the absence of insulin, IAPP fibrillizes extensively to form a meshwork of amyloid fibrils (Fig. 3.2). In agreement with the ThT results, TEM indicates IAPP fibrillization is greatly inhibited in

a concentration-dependant manner by the presence of insulin (Fig. 3.2B). The few fibrils that formed appeared short and fragmented, consistent with a previous report that the primary effect of insulin is to suppress fibril elongation.(49).

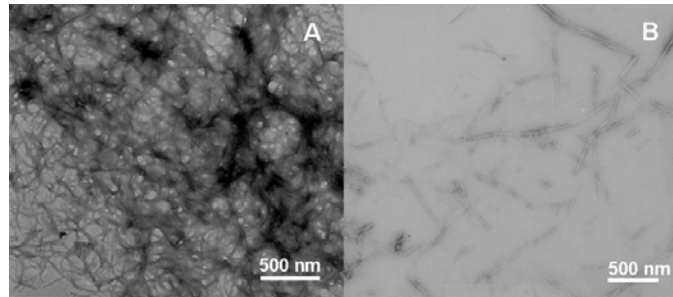


Figure 3.2. Insulin reduces, but does not fully prevent fibril formation in the absence of liposomes. Transmission electron microscopy images of (A) IAPP and a (B) 1:1 mixture of IAPP/Insulin incubated without liposomes at room temperature and pH 7.4 for seven days. Fibrils formed in all cases, but the incidence was reduced with increasing insulin concentration.

The interaction of IAPP with insulin in the presence of membranes is less well characterized. Binding of IAPP to the membrane converts the peptide to an α -helical form at a rate that is rate-limiting for IAPP fibrillogenesis (1, 75). A previous study showed an inhibitory effect of insulin on IAPP fibrillogenesis in the presence of LUVs, although considerably less than in the absence of membranes (51). However, this study used the hexameric, low pH, form of insulin (T6). Accordingly, a similar experiment was performed with the addition of LUVs. To differentiate the ability of IAPP to permeabilize liposomes on the basis of the LUV charge ratio, IAPP was added to dye-labeled LUVs prepared at several different ratios of anionic POPG to zwitterionic POPC (Fig. 3.3). IAPP permeabilized the LUVs mixtures until the anionic lipid composition of the LUVs was reduced to POPG/POPC molar ratios of 1:3 and 0:4. The effect of monomeric insulin

on the ability of IAPP to form fibrils in the presence of the POPG/PC LUVs at neutral pH was even more distinct than in solution (Fig. 3.4). IAPP fibrillizes extensively to form a meshwork of IAPP fibrils in the presence of LUVs seven days after mixing (Fig. 3.4A), yet with an equimolar concentration (25 μM) of insulin coincubated with IAPP we see that insulin again strongly inhibits IAPP fibrillization (Fig. 3.4B). Only a few fibrils were found in the sample showing that when IAPP is incubated with insulin and LUVs there is a greater reduction in fibril growth than without LUVs.

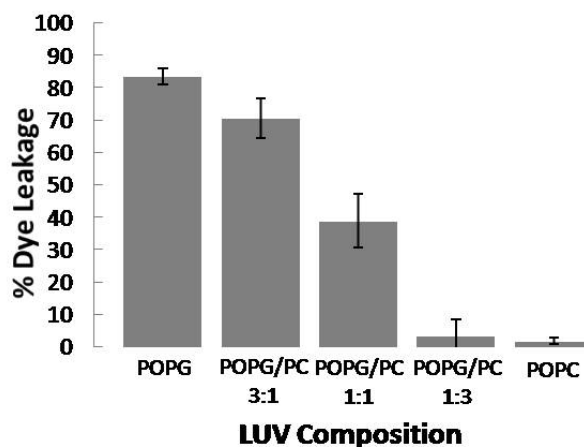


Figure 3.3. IAPP permeabilizes liposomes based on the anionic charge of the lipids. The ability of IAPP to permeabilize liposomes decreases as the ratio of zwitterionic POPC lipid increases, decreasing the anionic effect of POPG. IAPP was 1 μM and each liposome composition totaled 200 μM . The fluorescent reading of the sample was recorded at 100 s minus the baseline (without peptide) at 100 s. This value was divided by the total fluorescence at 100 s after adding a Triton-x detergent minus the baseline at 100 s (five measurements per condition, mean \pm SEM).

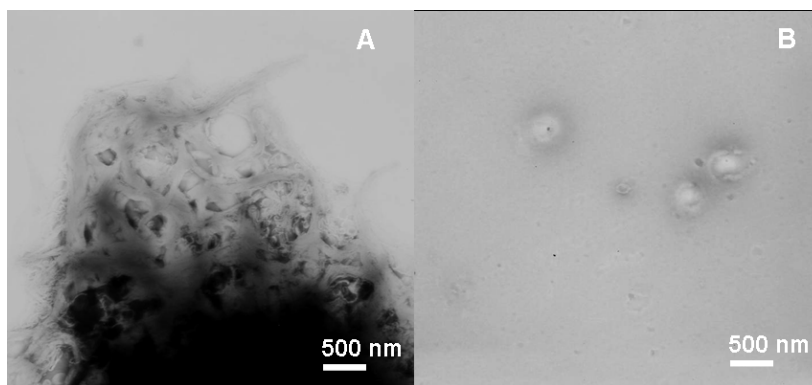


Figure 3.4. Insulin strongly inhibits fibril formation in the presence of POPG/PC liposomes. Transmission electron microscopy images of (A) IAPP and (B) IAPP/insulin in a 1:1 incubated at 25 μ M with 200 μ M POPG/PC liposomes at room temperature and pH 7.4 for seven days.

The structural change of IAPP in the presence of POPG/PC liposomes was also monitored by circular dichroism (Fig. 3.5). For this experiment, the insulin signal was subtracted from that of the IAPP/insulin sample. This procedure allowed a comparison of the pure IAPP signals in both the IAPP and IAPP/insulin samples, without the contribution of the strong insulin signal (53). A very clear structural change occurred in the IAPP sample indicative of β -sheet fibril formation, which was prevented in the presence of insulin. As a control, IAPP and IAPP/insulin samples were observed without the presence of liposomes (figure 3.6). IAPP in the presence of insulin appears α -helical and then precipitates out of solution by 50 h without forming β -sheet structure, which is on the same timescale that IAPP sample without insulin and LUVs forms β -sheet structure. The effects of insulin on the structural properties of IAPP, without liposomes to accelerate the structural transition of IAPP to β -sheet structure and without liposomes to create a high local IAPP concentration at the membrane surface, appears to inhibit β -sheet structure, but the resulting data is less clearly discernable than the IAPP samples with LUVs (figure 3.6B).

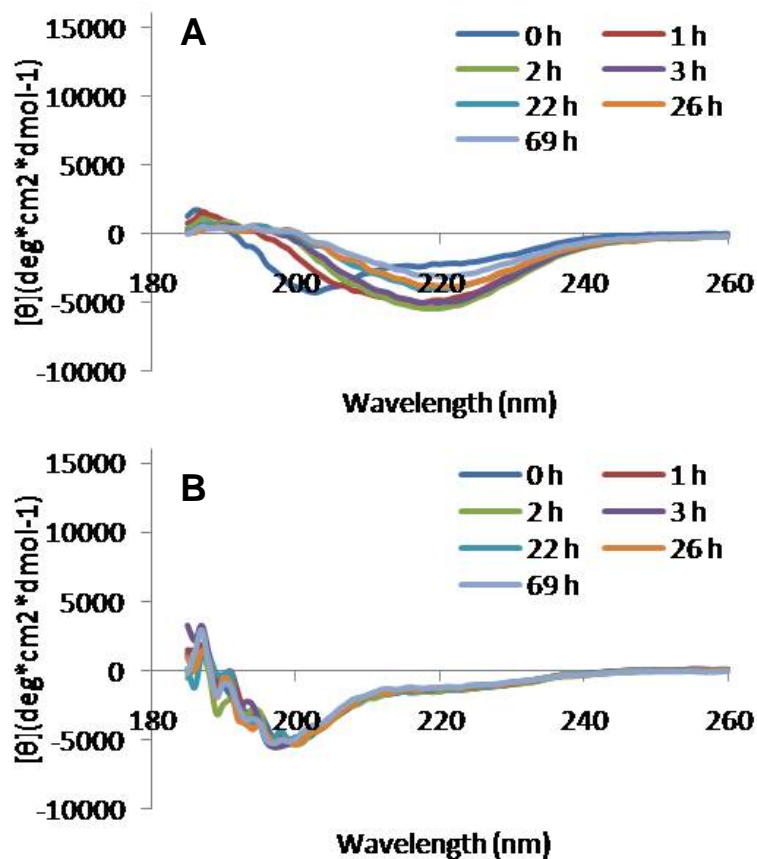


Figure 3.5. Insulin slows IAPP from forming β -sheet secondary structure in the presence of POPG/PC liposomes. (A) In the presence of POPG/PC LUVs, IAPP undergoes a structural shift from a predominantly random coil structure to a β -sheet indicative of around 1 h. (B) IAPP in the presence of insulin and POPG/PC LUVs does not undergo a structural transition to β -sheet within 69 h. The components of each signal were subtracted, except for the IAPP signal, to compare the effects insulin had on the time to β -sheet formation of IAPP. The samples contained 25 μM IAPP (A) or 25 μM IAPP/25 μM insulin (B) and 200 μM POPG/PC, 100 mM NaF, 10 mM NaP_i , pH 7.4.

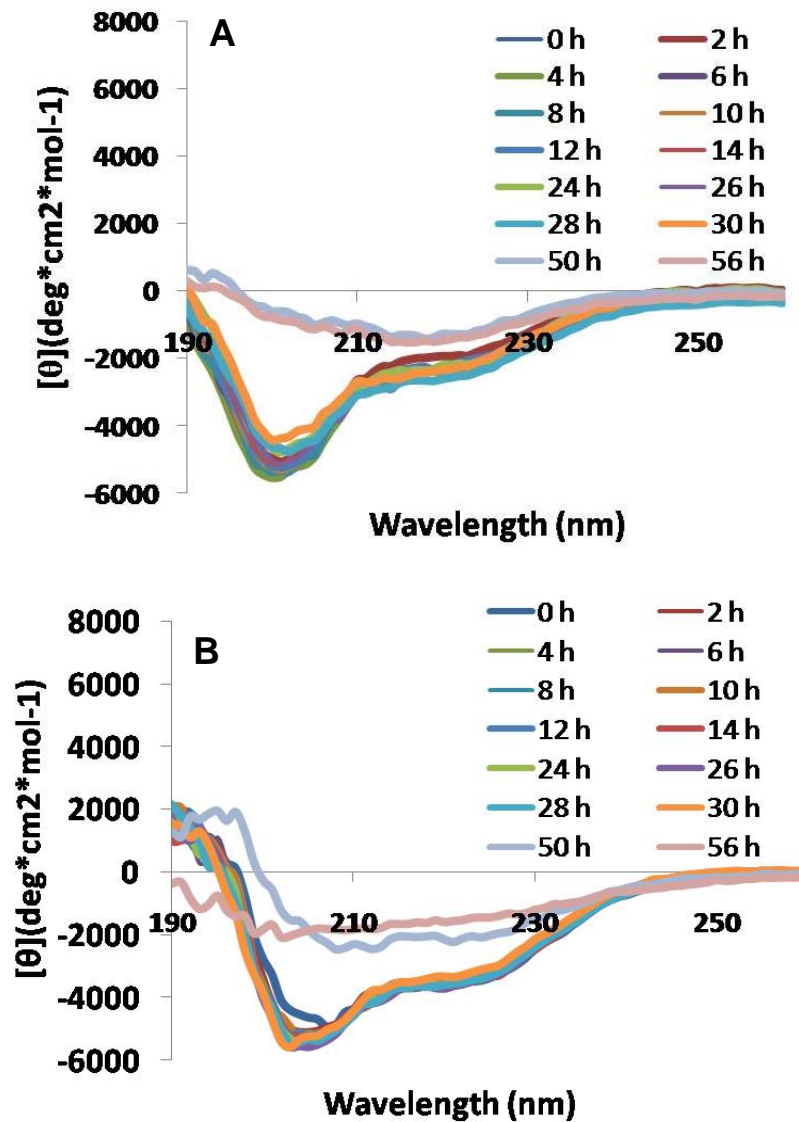


Figure 3.6. Insulin has little effect on the secondary structural change of IAPP without liposomes. (A) IAPP undergoes a structural shift from a predominantly random coil structure to a β -sheet structure around 50 h. (B) IAPP in the presence of insulin precipitates out of solution by 50 h without forming β -sheet structure. The components of each signal were subtracted, except for the IAPP signal, to compare the effects insulin had on the time to β -sheet formation of IAPP. The samples contained 25 μM IAPP (A) or 25 μM IAPP/25 μM insulin (B) and 100 mM NaF, 10 mM NaP_i , pH 7.4.

3.3.2 IAPP Has a Stimulatory Effect on Insulin Amyloidogenesis at Neutral pH

Monomeric insulin forms amyloid fibrils through the formation of a partially unfolded intermediate. Insulin in the absence of IAPP also undergoes an amyloidogenic transition with a lag time approximately three times longer than that of IAPP (approximately 50 h) (Fig 3.1B). Interestingly, increasing the concentration of insulin to a 10:1 molar excess over IAPP decreased the lag time relative to the 1:1 mixture of IAPP and insulin, indicating the overall rate of amyloid fibril formation increased as the concentration of insulin was increased (Fig 3.1B). This effect was not due simply to the increase in insulin concentration, as the kinetics of 25 μ M and 250 μ M insulin solutions without IAPP are similar. Although the data is not conclusive, the decrease in lag time suggests that IAPP may act to catalyze the amyloidogenesis of insulin.

3.3.3 Insulin Does Not Prevent IAPP-induced Membrane Disruption

One of the primary mechanisms of IAPP-induced toxicity is disruption of the cellular membrane.^(14, 21, 23, 24, 76-80) Our results indicate that insulin strongly inhibits fibril formation both in solution and in a membrane environment, in agreement with previous reports. However, recent research indicates membrane disruption by amyloid proteins and amyloid formation are separate, although related processes, and the effect of insulin on membrane disruption, as opposed to amyloid fibril formation, is unknown. Accordingly, the ability of IAPP to disrupt model membranes composed of either POPG or a 1:1 mixture of POPG/POPC in the presence of insulin was tested using a dye leakage assay. In the absence of insulin, IAPP strongly disrupts both POPG (Fig.

3.7A) and, to a somewhat lesser degree, POPG/POPC (Fig. 3.7B) vesicles as has been previously reported for this peptide (1).

Insulin did not block IAPP from permeabilizing the liposomes, despite its inhibitory effect on amyloid formation (Fig. 3.7). Insulin also binds membranes and has been shown to cause membrane disruption by a similar mechanism under certain conditions.(81) As shown in Fig. 3.7, insulin alone had a slight disruptive effect on both types of vesicles that was not dependent on the charge of the lipid headgroup.

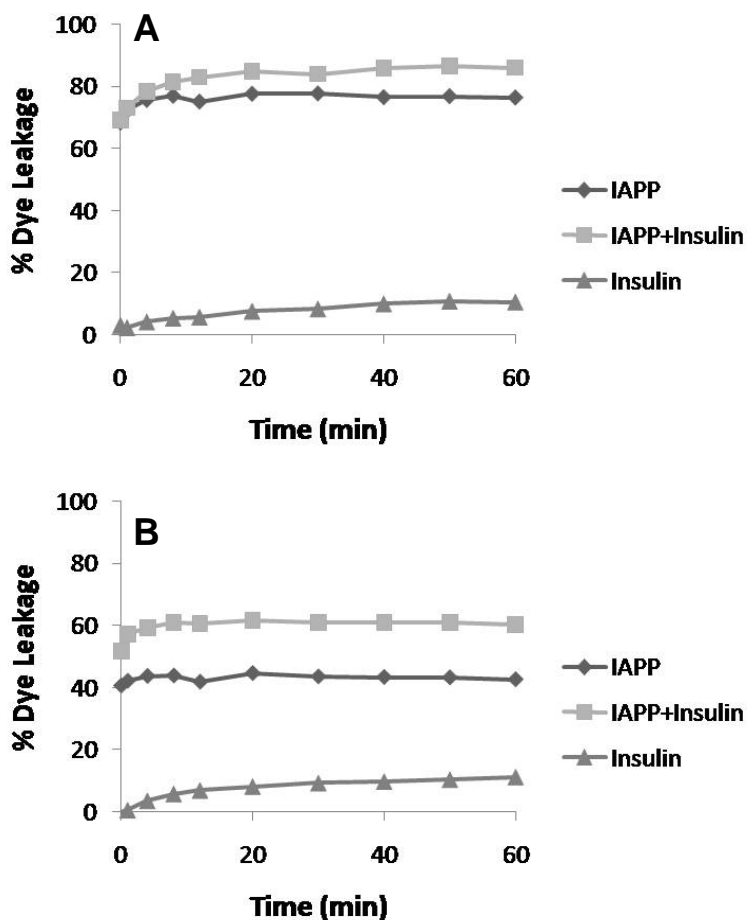


Figure 3.7. Permeabilization of liposomes by IAPP in the presence of insulin. (A) Samples of 1 μ M IAPP, 1 μ M IAPP + 1 μ M insulin mixture, and 1 μ M insulin were added to 200 μ M of POPG liposomes and monitored for dye leakage over 60 min. (B) The same experiment repeated with POPG/POPC dye leakage lipids. Rather than adding the peptide at distinct time points of aggregation to new liposomes with encapsulated dye (as shown in fig. 3.8), the peptides were added at zero time and monitored for the duration of the timecourse.

Since the amyloid form of the peptide is less effective at disrupting cellular and artificial membranes than the freshly dissolved peptide, the ability of IAPP to permeabilize membranes declines over time as the percentage of amyloid in the sample increases. This effect was monitored by adding IAPP (with or without an equimolar amount of insulin) to fresh lipid preparations at each timepoint to measure how toxicity changes over the course of the experiments (Fig. 3.8). IAPP's ability to permeabilize liposomes in the absence of insulin declines rapidly after 5 hours of preincubation in solution before being added to the liposomes, which corresponds with the start of exponential fibril growth in solution measured by ThT fluorescence at ~15 hours (Fig. 3.1A). This result is in agreement with numerous reports that have shown mature amyloid fibrils to be non-disruptive to membranes and non-toxic to cells, in contrast to earlier forms of the peptide.

The presence of insulin changes the influence of IAPP pre-incubation time on membrane disruption dramatically. In contrast to the rapid decrease with preincubation time seen in the absence of insulin, the ability of IAPP/insulin mixtures to permeabilize liposomes declines only slowly over the course of the experiment. The toxicity of a 1:1 IAPP/insulin mixture does not fully disappear until approximately 300 h when it starts to slowly polymerize (Fig. 3.8A), matching the slow decline in permeability over time. Insulin at this concentration alone had no ability to permeabilize liposomes or form fibrils. At a higher concentration (250 μ M or 10x the concentration of IAPP) insulin eventually oligomerizes and permeabilizes liposomes after 100 h. (Fig. 3.8B) Insulin blocks fibril formation, but is ineffective at blocking oligomer formation and actually

maintains the toxicity of the IAPP long past the normal life of the toxic IAPP oligomer that forms without insulin.

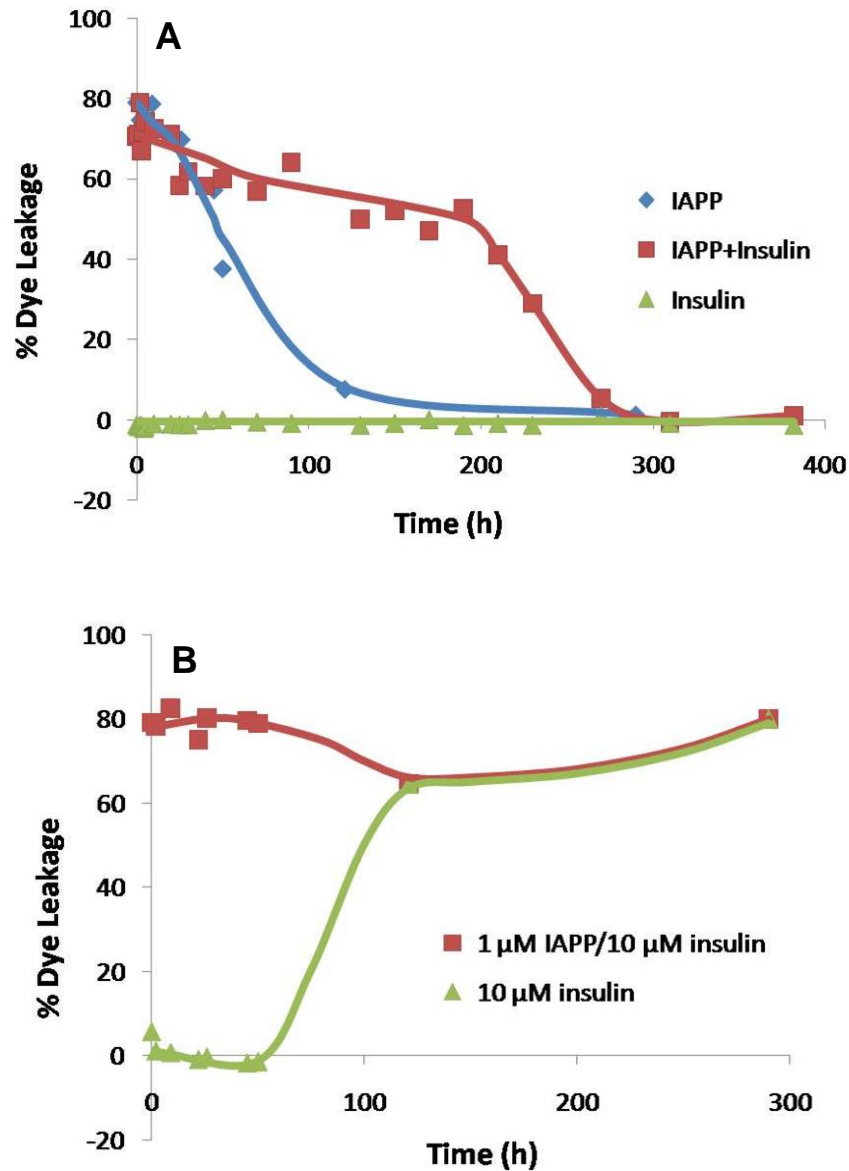


Figure 3.8. Insulin does not protect against membrane damage and maintains IAPP in an active permeabilizing form for a lengthened period of time. Peptides were incubated and added to fresh 200 μM POPG liposomes (5 μM POPG liposomes with encapsulated dye and 195 μM POPG liposomes) at each timepoint. (A) Dye leakage conducted with equimolar concentrations of 1 μM IAPP and 1 μM insulin and (B) 10 \times insulin concentration showing that when insulin is incubated at high concentrations it is capable of forming a toxic amyloid species. To prevent the interference of insulin toxicity, insulin was used at low equimolar concentrations to IAPP.

3.3.4 Insulin Does Not Prevent the Membrane-catalyzed Formation of Small Oligomeric Species

Insulin is able to effectively inhibit the fibrillization of IAPP, but has a stimulatory effect on vesicle permeabilization by IAPP. A current hypothesis holds that membrane disruption by amyloid proteins is not due to the action of amyloid fibrils but rather to the action of smaller oligomeric species. The processes for the formation of small oligomeric species and amyloid fibrils have been shown to be distinct, and in some cases can be uncoupled from each other so that small, toxic oligomers form but not amyloid fibrils or vice versa. Glutaraldehyde crosslinking was used to detect the populations of small membrane bound IAPP oligomers at a number of time points during the membrane-catalyzed aggregation of IAPP (Fig. 3.9). An IAPP specific antibody was used to distinguish IAPP oligomers from insulin oligomers. In the absence of insulin, bands corresponding to monomers, dimers, trimers and tetramers were detected, along with a relatively strong band corresponding to high molecular weight species that exceed the resolving power of the gel. A decrease in the intensity of the monomer band and a corresponding increase in the oligomer bands can also be detected by comparing the relative intensities of bands from the initial time point (lane 1) and later time points (lanes 2-4).

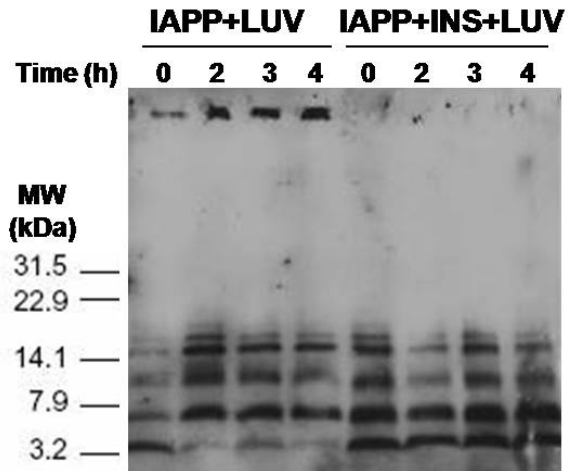


Figure 3.9. Cross-linking of IAPP and insulin bound to POPG/POPC LUVs. IAPP (25 μ M) or IAPP/insulin (25 μ M /25 μ M) were incubated with POPG/POPC LUVs (200 μ M) at room temperature to allow the peptides to oligomerize. At each timepoint aliquots of each sample were removed and crosslinked with 0.01% (v/v) glutaraldehyde. The reactions were quenched with the addition of lysine and loading buffer, boiled, and separated with SDS-PAGE. The gel was transferred and then immunoblotted with a rabbit polyclonal α -IAPP antibody.

In contrast to its effect on amyloidogenesis (Figs. 3.1-3.4) the addition of one molar equivalent of insulin has little effect on the formation of IAPP oligomers (lanes 5-8 in Fig. 3.9). The western blot of the cross-linked IAPP-insulin complex is similar to that of cross-linked IAPP, except the bands corresponding to high molecular weight species are absent. In addition, the decrease in the intensity of the monomer band over time is not seen in the IAPP-insulin sample. Significantly, the bands are in the same positions for samples with and without insulin, indicating that IAPP does not cross-link to insulin under these conditions. The absence of a shift in mass in the Western-blot due to cross-linking to insulin suggests that the membrane-disruptive species are largely composed of IAPP peptides and not an IAPP-insulin complex.

3.4 Discussion

3.4.1 Insulin Stabilizes IAPP Oligomers and Maintains IAPP Toxicity.

Islet amyloid deposits are frequently found in Type II diabetics but only rarely in non-diabetics; as a result, the toxicity of IAPP has been proposed by others to be linked directly to IAPP amyloid formation (82, 83). Since IAPP is stored together with an excess of insulin within the β -cell secretory granules, and several studies have shown the inhibitory effect of insulin on IAPP fibrillogenesis, it has been frequently implicitly assumed that insulin would also inhibit the inherent toxicity of IAPP (49, 53, 84). However, recent experiments on other amyloid proteins have challenged this notion. While the extent of amyloid formation in type II diabetes (as well as other amyloid-associated diseases) is correlated with progression of the disease, amyloid formation by itself does not cause pathological dysfunction. In general, however, inhibition of amyloid fibril formation is correlated with a concomitant reduction in amyloid toxicity. However, notable exceptions to this rule exist. A prime example is the failure of the A β antibody to prevent toxicity. The anti-amyloid- β_{1-11} antibody binds and inhibits amyloid- β_{1-42} fibrillization, yet at the same time it stabilizes the cytotoxic amyloid- β_{1-42} species (85). Although the antibody inhibited fibril formation, toxicity was not completely prevented. The toxicity was somewhat reduced but likely only because the antibody has a partial ability to bind to the early oligomeric species in addition to protofibrils and fibrils. Given that smaller oligomers of the peptide typically have a much greater toxicity than the mature fibrils, inhibitors of amyloid formation may increase the toxicity of the peptides if

they do not inhibit the binding of the peptide to the membrane and the formation of small oligomers on the membrane.

While insulin has been demonstrated to be an *in vitro* inhibitor of IAPP fibrillization, its effects on IAPP membrane disruption and toxicity are less understood. To test the impact of insulin on IAPP toxicity, we have observed the ability of insulin to inhibit IAPP fibril formation and have tested IAPP's ability to disrupt membranes in model membranes under buffer conditions that approximate the pH and ionic strength found after secretion. We found that (a) insulin inhibits only amyloid fibrillization and not the formation of small oligomeric species of IAPP on the membrane, (b) IAPP rapidly permeabilizes lipid membranes even in the presence of soluble insulin, (c) the ability of IAPP to permeabilize membranes decreases rapidly with time as the peptide is incubated in solution in the absence of membranes, and (d) insulin permits IAPP to continue to permeabilize liposomes far longer than in the absence of insulin as long as stoichiometric or excess amounts of insulin are present. Taken together, these results show that insulin alone may not have the ability to protect β -cells from IAPP toxicity at the site of secretion, and alternate hypotheses should be considered for the mechanism by which the cell maintains IAPP in a stable and nontoxic state in non-diabetic individuals.

3.4.2 Insulin Enhances IAPP Membrane Disruption by Inhibiting the Conversion of Membrane-active Protofibrillar Species into the Fibril Form

Membrane disruption is believed to be one of the primary causes of cell death caused by amyloidogenic proteins (77, 80, 86). Among amyloid peptides IAPP is somewhat unusual in that the highest amount of membrane disruption is detected

immediately after solubilization and then decreases monotonically afterwards (fig. 3.8) (22, 87). This is in contrast to most other amyloid proteins in which membrane disruption first increases with time as toxic, membrane-binding oligomers form in solution and then decreases as the protofibrillar species are converted into relatively inert mature amyloid fibrils (62-68, 88-90). The transient nature of the protofibrillar species limits the amount of time the β -cell is exposed to the highly toxic protofibrillar species, as IAPP either diffuses away from the secretion point where it rapidly decreases to non-aggregating concentrations or it is converted to less toxic amyloid fibrils near the secretion point (14, 31, 91, 92).

Our results are consistent with insulin inhibiting IAPP fibril formation, but being ineffective at blocking the formation of smaller IAPP oligomers both in solution and on the membrane. A schematic representation is shown in Fig. 3.10. Insulin inhibits IAPP formation both in the absence and presence of membranes (Figs. 3.1-3.2 and 3.4-3.5). However, insulin, which blocks fibril formation, prolongs the time-period of IAPP toxicity, long past the normal life of the toxic IAPP oligomer in the absence of insulin (Fig. 3.8). In the absence of insulin IAPP forms fibrils in solution and the ability of IAPP to cause membrane disruption is rapidly reduced after 50 h (Fig. 3.1A and 3.8A). In the presence of an equimolar amount of insulin the ability of IAPP to induce membrane disruption was maintained for far longer and only decreases slowly after ~ 275 hours (Fig. 3.8A). A similar result was obtained with a 10-fold excess of insulin, except the toxicity did not decrease after ~ 275 hours, apparently due to the formation of membrane-disruptive species of insulin itself (Fig. 3.8B). The ability of insulin to suppress the formation of large oligomers of IAPP but not small oligomers of IAPP on the membrane

was confirmed by time-lapse cross-linking of IAPP and IAPP-Insulin solutions added to POPG/POPC liposomes, Small oligomers could be detected in both samples whether insulin is present with IAPP or not, but the monomer in the sample with IAPP alone quickly disappeared and large oligomeric species at the top of the gel were not seen when IAPP was co-incubated with insulin (Fig. 3.9). From this we surmise that insulin by itself does not have the ability to protect β -cells from IAPP toxicity and only inhibits fibrillization under these conditions.

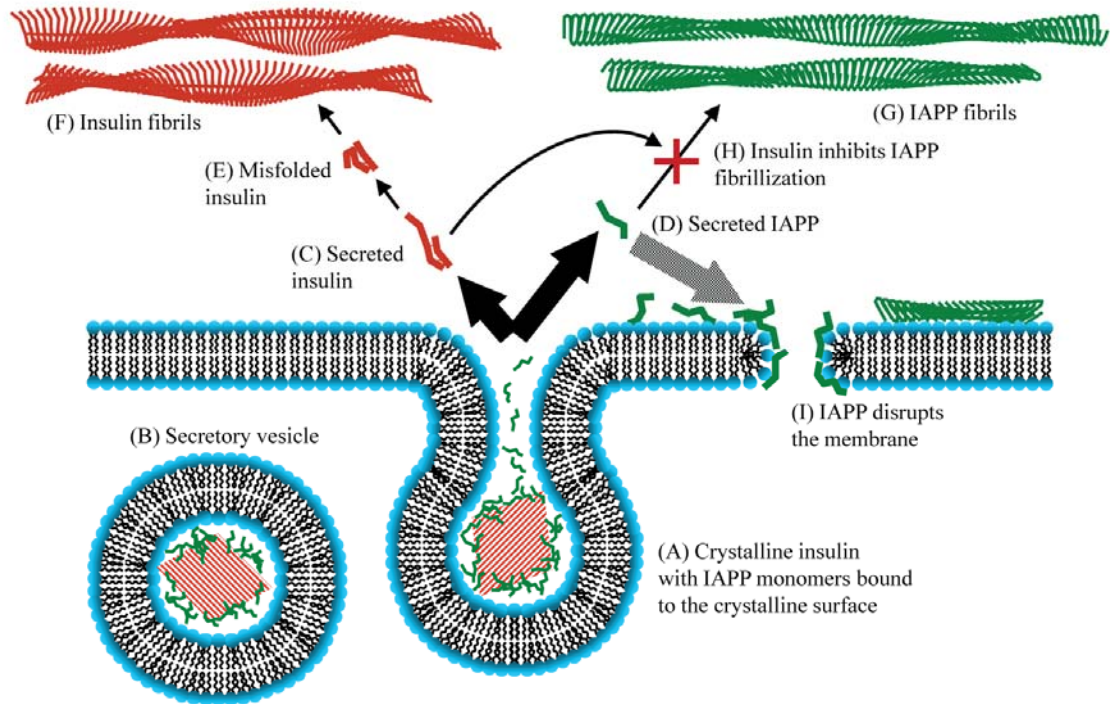


Figure 3.10. Cartoon of IAPP and insulin secretion and interactions. The secretory vesicles fuse with the plasma membrane releasing their contents into the extracellular space. (A) IAPP is costored with crystalline insulin prior to secretion in response to a rise in blood glucose within a (B) secretory vesicle. Upon secretion IAPP dissociates from crystalline insulin and insulin dissolves to its active form. Secreted (C) insulin and (D) IAPP can both stay in their unaggregated forms and fulfill their biological functions as peptide hormones. Alternatively, (E) insulin can misfold under certain largely non-physiological conditions and initiate insulin aggregation into (F) insulin fibrils. IAPP forms (G) islet amyloid readily at the high concentrations like those envisioned at the site of peptide secretion. Interestingly, (H) islet amyloid would be greatly inhibited by the high soluble insulin concentration also experienced at the secretion site. Finally, (I) soluble IAPP may bind to the plasma membrane at or near the site of secretion under certain conditions: high anionic lipid content of the plasma membrane or the lack of other secretory components that would inhibit IAPP membrane binding.

3.4.3 Other Conditions in the Secretory Granule Besides Insulin May Act to Retard its Aggregation and Toxicity

If insulin is not solely responsible for inhibiting the membrane disruption by IAPP, what factors may be responsible for maintaining IAPP in a non-toxic state in the secretory granule and immediately after secretion? First, although insulin appears to have

little effect on reducing membrane disruption by IAPP in model membranes, it may reduce the toxicity of IAPP through other, currently undiscovered mechanisms in the complex physiological environment at the protein-lipid interface of the β -cell. It is known that the fibrillar form of IAPP can react with copper and zinc to generate reactive oxygen species and can also apparently mediate the redox-sensitive JNK apoptotic cascade (93, 94). In contrast to IAPP membrane disruption, which occurs immediately when IAPP is added to cells or membranes, ROS formation by IAPP occurs slowly and is associated with IAPP amyloid formation. Insulin may therefore suppress IAPP toxicity by these redox-sensitive mechanisms through the suppression of IAPP amyloid formation.

Other modulators of IAPP pathology besides insulin should also be taken into account when considering the non-toxicity of IAPP in non-diabetic subjects. Lipid binding by IAPP is known to be strongly dependent on the anionic charge of the lipids (95, 96). Similarly, we found that the ability of IAPP to permeabilize LUVs is dependent on the anionic charge of the LUVs. Previously, 30 mol % anionic lipid content has been shown to be the minimum for IAPP binding (1). To avoid working at the breaking point, 25 mol % anionic content was chosen instead of 30 mol %. We found that at 25%:75% POPG/POPC not much permeabilization occurs, yet at 50%:50% POPG/POPC the permeabilization increases to \sim 40%. (Fig. 3.3) At 100% POPG concentration the permeabilization of LUVs reaches above 80% permeabilization. This confirms the importance of anionic lipid content in not only the binding of IAPP, but also the disruption and permeabilization of the LUVs. This supports the hypothesis that increased anionic lipid content may be a key factor in the development of Type II diabetes arising from the increased lipid required for the secretory granules to hypersecrete insulin in

response to elevated blood glucose levels in the insulin resistant state. When the anionic lipid content is reduced to 25 mol % or 0 mol % in our lipid permeabilization experiment, the permeabilization is minimal. The anionic charge ratio of a healthy individual is below 20 mol % anionic content, well below this critical level for peptide binding and permeabilization (1). What would cause a breakdown in the lipid synthesis that leads to increased anionic lipid content? Type II diabetes often involves an overall state of dyslipidemia as well as a specific increase in anionic phospholipid production in the β -cells (1, 97). An anionic lipid content above 30 mol % may allow IAPP to bind and permeabilize these cells, which would further weaken β -cells and lead them to apoptosis. Interestingly, the islets have a limited ability to regenerate β -cell mass. As insulin resistance continues to require elevated levels of insulin secretion coordinated with a disproportionate increase in anionic lipid synthesis, eventually a significant population of β -cells would become overwhelmed and die. The continually stressed islets, after a certain tipping point, would be incapable of regenerating the lost β -cell mass faster than their destruction.

Other peptides exist within the secretory granule that may also play a role in sequestering IAPP to prevent self-toxicity of the β -cell. Insulin in the crystalline and soluble forms may only play the role of inhibiting fibril growth within the secretory granule. Upon secretion, other components may bind to IAPP monomers to inhibit the formation of oligomers and to inhibit the peptides from binding to the cellular membranes. Preproptin, C-peptide I and II, and other protein components found within secretory granules (98) may be involved in this cooperative inhibition of membrane binding and toxicity. Further work needs to be done to determine the components in

combination with the lipid charge that prevents IAPP from forming toxic oligomers at the site of secretion. Our work shows that insulin does inhibit the formation of IAPP fibrils, as previously seen, but does not potentially inhibit the toxic effects of IAPP. Low anionic charge in the plasma membrane may be the most important factor preventing IAPP from binding and permeabilizing β -cells.

CHAPTER IV

Cholesterol Protects LUVs Against IAPP Permeabilization

4.1 Introduction

Type II diabetes is an amyloid disease much like Alzheimer's and Parkinson's disease. It involves a peptide hormone, IAPP, which has been implicated in β -cell apoptosis, a reduction in β -cell mass, and the progression of insulin resistance to overt Type II diabetes. IAPP in the early stages of aggregation correlates well with β -cell toxicity, unlike the later forming islet amyloid, which has been suggested to form as an end product of aggregation and act as a sink for toxic oligomeric species. Within the context of β -cell toxicity, certain requirements are necessary for IAPP to bind and permeabilize the plasma membrane.

The likelihood of developing Type II diabetes increases with age. Previously it was termed adult onset diabetes until an increase in childhood cases of Type II diabetes emerged in recent years. Two aging associated changes in the plasma membrane are discussed in this chapter that could lead to an increase in IAPP binding. These changes include (1) an increase in anionic phospholipids, explained previously as possibly deriving from a breakdown in lipid regulation, and (2) a decrease in the level of membrane incorporated cholesterol.

4.1.1 An Increase in Anionic Phospholipids

The plasma membrane is a bilayer composed of asymmetrically distributed lipids and proteins. The lipids and proteins form functional groups or domains within the membrane, called lipid rafts. Secreted IAPP, in order to permeabilize the β -cell plasma membrane, has to first bind at the protein-lipid interface. A number of characteristics of the outside leaflet of the plasma membrane impart an interface that may promote or inhibit IAPP binding and subsequent permeabilization. Some of these characteristics are membrane surface charge, membrane curvature, phase of the lipid membrane, membrane elasticity, chemical nature of the lipids, amount of hydration, lipid acyl chain type (saturated or unsaturated), hydrophobic matching of the protein-lipid interface with the protein that binds, and the dynamics and conformation of the lipid headgroups (99). The anionic phospholipid composition of the plasma membrane has been suggested to be the most critical factor for IAPP oligomerization and toxicity (1, 24).

In healthy individuals the mol % anionic charge of the plasma membrane is relatively low—below 20% (1). While it is currently unknown what changes occur to the β -cell plasma membrane in the diabetic state, it has been suggested that elevated levels of anionic lipids are present in the Type II diabetic state (1). Dyslipidemia and the hypersecretion of IAPP and insulin from secretory granules may lead to a breakdown in the cell machinery involved in lipid synthesis and turnover. To secrete the high volumes of IAPP and insulin, vast amounts of secretory granule lipids have to be synthesized and recycled. A breakdown in lipid regulation in Type II diabetes has been suggested to lead to an increase in phosphatidic acid (PA), which is negatively charged and would lead to

an increase in the overall negative charge of the membrane. An increase to about 30 mol % anionic lipid content is known to be needed for peptide binding *in vitro* (1, 14).

Zhu et al. showed that when the overall membrane surface charge is opposite to the overall charge of the amyloid peptide, the electrostatic charge attracts the peptides creating a local amyloid peptide concentration roughly two orders of magnitude greater than that observed in solution (100, 101). The electrostatic charge interaction occurs when positively charged IAPP is incubated with lipids that impart an anionic charge to the membrane. Using lipids from Type II diabetic pancreatic tissue, Knight and Miranker found that lipids from diabetic tissue accelerate aggregation more than ten-fold over the absence of lipid (1). Type II diabetics' pancreatic lipid composition may be highly prone to IAPP binding and permeabilization.

4.1.2 A Decrease in the Level of Membrane Incorporated Cholesterol

Cholesterol (figure 4.1) is an unusual lipid molecule made of four hydrocarbon rings, making it extremely hydrophobic, and it has a very small hydroxyl moiety as its head group (102). The hydrophobic region of cholesterol preferentially associates with the hydrophobic tail regions of the lipid molecule of the plasma membrane. The hydroxyl group of cholesterol associates with the phospholipid head groups of the lipids. Because of the small size of cholesterol and its lack of a large head group, it fits within the interstitial spaces between neighboring lipid tails and functions to restrict and order the acyl chains of neighboring lipids, thickening the bilayer in the process (102). The ring system in effect rigidifies the membrane making it more impervious to permeabilization

(102, 103). This lipid-ordered state restricts permeability, yet still allows fluidity of the membrane (102).

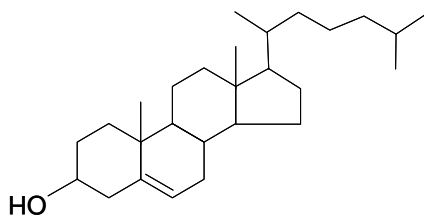


Figure 4.1. Cholesterol structure. Cholesterol is amphiphathic with a hydrophobic ring structure and a small hydroxyl head group

As an individual ages, membrane localized cholesterol is known to decrease in some tissues like the brain, while circulating levels typically rise. Thus, aging-associated changes to the plasma membrane may play a role in the development of Type II diabetes. Overall, no significant changes in total incorporated cholesterol in the pancreas have been observed with aging (104), although this drop in cholesterol could still occur in a subset of pancreatic cells like the β -cells. Several conditions could result in a decrease in incorporated cholesterol within cellular membranes. One possibility is that the β -cells hypersecrete insulin in the insulin-resistant state, creating large quantities of secretory granule lipids. The secretory granule lipids in this stressed state may have a disproportionately low cholesterol content. Other pancreatic cells do not have these secretory requirements, so an unchanged total cholesterol composition of the pancreas may not accurately represent localized changes in cholesterol content. Decreases in cholesterol have been implicated and studied with amyloid- β and other amyloid peptides, but have hardly been explored with IAPP.

The plasma membrane is a highly ordered membrane and has a high concentration of incorporated cholesterol in comparison to organelles within the cell. Cholesterol is the

main sterol found in mammals with the majority (90%) incorporated into the plasma membrane and the rest in other organelle membranes and the circulation (105). In comparison with the plasma membrane, the endoplasmic reticulum (ER) where cholesterol is synthesized is very low in incorporated cholesterol and high in unsaturated lipids (102). The majority of the cholesterol in the cell is synthesized and transported from the ER to the other organelles of the cell, most notably the plasma membrane (105). The secondary or minor sources of cholesterol are from the hydrolysis of cholesterol esters in the lysosomes and late endosomes or from the endocytosis of lipoproteins that contain cholesterol, e.g. low-density lipoprotein (LDL) (106).

Despite the negative publicity, cholesterol is actually required for cell viability and proliferation (105). The plasma membrane typically has a cholesterol concentration of 30 mol % (107). Cholesterol does not uniformly distribute itself within the plasma membrane, but clusters in transitory lipid rafts with a diameter <10 nm (108). These lipid rafts with cholesterol form patches of lipids that are in the liquid ordered phase and surrounded by a liquid disordered phase with lower cholesterol content and more unsaturated lipids (105). Much research on lipid rafts or microdomains has led to the understanding that they are essential for protein recognition and signal transduction pathways (108). It is not known if IAPP preferentially binds to the lipid rafts or to non-raft containing regions.

IAPP in itself is sensitive to the membrane environment, especially the lipid charge and the presence of cholesterol incorporated into the membrane. Cholesterol has a significant impact on reducing permeabilization by IAPP. Previously, Pamela Wong in our laboratory used lauridan to characterize the rigidity of the membrane upon the

incorporation of cholesterol into POPG/POPC membranes (109). Laurdan is a fluorescent molecule with a long hydrophobic tail that intercalates into the membrane situating its naphthalene ring structure in the region of the lipid glycerol backbone near the polar headgroups and hydrophilic protein-lipid interface. The naphthalene ring of laurdan is sensitive to polarity and has two emission peaks, one at 480 and another at 435 nm. Depending on the fluidity of the membrane environment, the emission spectrum changes from red shifted in a fluid membrane to blue shifted in a rigid cholesterol-containing membrane environment. When the membrane is more fluid, water molecules can interact with the fluorescent naphthalene ring. Pamela Wong showed that the membrane becomes more rigid with the incorporation of cholesterol. Less space is available between the phospholipid head groups for water molecules to interact with the naphthalene ring, which changes the polar environment of the naphthalene ring and emission peaks. Pamela Wong found that 25 mol % cholesterol was similar in packing density to a gel phase membrane, showing that cholesterol rigidifies the membrane and could greatly reduce the ability of amyloid peptides to insert into the membrane (109).

The status of cholesterol in terms of amyloid diseases is unclear, as the research literature presents various reports ranging from findings that cholesterol is protective against membrane permeabilization to findings that cholesterol is detrimental to membrane stability. Cholesterol has been identified as a possible risk factor for some amyloid diseases like Alzheimer's disease and Type II diabetes because it is often found in plaques and fibrils, and high serum levels have been reported to be associated with the disease states (103, 110). Yet in an interesting scientific finding, cholesterol-lowering statins have been found to increase amyloid production in humans and rodents (111,

112). Several studies show the possible benefits of cholesterol, especially when it is incorporated into the membrane, rather than the circulation (109, 113, 114). If cholesterol is protective against amyloid peptide membrane permeabilization, then statins and other cholesterol lower drugs may increase cell death and accelerate the progression of Type II diabetes. The exact role cholesterol plays at the interface of the membrane and IAPP at the site of secretion is currently in question. The presence of cholesterol in the membrane should make it more difficult for an amyloid peptide to insert between the headgroups and acyl tails to permeabilize the membrane. It takes several times more work for an amyloid peptide to insert into a cholesterol containing membrane to create a vacancy between the lipids than it does with a membrane without cholesterol (108, 115, 116). The studies presented here show that membrane incorporated cholesterol inhibits IAPP permeabilization of model lipid membranes.

4.2 Material and Methods

4.2.1 IAPP and Insulin Preparation

Human IAPP was purchased from Anaspec Inc., recombinant human insulin expressed in yeast was purchased from Sigma-Aldrich. The IAPP peptide used in this study was amidated at the C-terminus like the natural peptide. Preformed aggregates in both peptides were solubilized by first dissolving the peptide in a 75% acetonitrile/water solution at a concentration of 1 mg/ml. The peptides were then lyophilized and the lyophilized powder was redissolved in hexafluoroisopropanol (HFIP) at a concentration of 2-4 mg/ml. HFIP was removed by lyophilization overnight at high vacuum (72). The

peptide was first dissolved in pure water to a concentration of 25 μ M in siliconized eppendorf tubes and then diluted with 2x buffer to create the final working solution (final buffer composition: 10 mM Sodium Phosphate, pH 7.4 100 mM NaCl) (73).

4.2.2 Liposome Preparation

1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-[Phospho-*rac*-(1-glycerol)] (POPG) and 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) dissolved in chloroform were purchased from Avanti Inc. (Alabaster, AL). To make liposomes, chloroform lipid stocks were dried with a stream of nitrogen and then placed under vacuum overnight to remove residual solvent. The lipid films were then rehydrated for 1-2 hours with 10 mM NaP_i buffer at pH 7.4 buffer containing 100 mM NaCl. The rehydrated lipids were vortexed and then subjected to 8 freeze-thaw cycles. The resulting multilamellar vesicles were extruded 21 times through two 100 nm polycarbonate Nucleopore membrane filters (Whatman) to create unilamellar vesicles.

For liposomes with encapsulated dye, the vesicles were created as above except the buffer also contained 50 mM 5(6)-carboxyfluorescein. Free dye was separated from the vesicles with encapsulated dye by running the sample through a 10 mL Sephadex G-50 gel filtration column (Sigma-Aldrich, St. Louis, MO). Lipid concentrations were determined using the Stewart method (32).

4.2.3 Liposome Preparation with Cholesterol

Cholesterol (Sigma-Aldrich, St Louis, MO) was dissolved in chloroform, mixed with POPG, POPC, or POPG/PC in mol % ratios used in the experiments. The lipid stock

mixtures were aliquoted into test tubes, dried with a stream of nitrogen, and then placed under vacuum overnight to remove residual solvent. Rehydration and extrusion were conducted as described for general liposome preparation.

4.2.4 Dye Leakage

IAPP or IAPP/insulin was solubilized to a 25 μM concentration and then diluted to 1 μM upon addition to a 200 μM mixture of LUVs (195 μM empty POPG vesicles, 5 μM dye-encapsulated POPG vesicles). The fluorescence of leaked dye was recorded for the LUV mixture prior to the addition of the peptide and subtracted from the measurements of the sample. The dye leakage was monitored for an hour or more over the course of the experiment. At the end of the incubation, the total fluorescence was determined and normalized by adding Triton-X detergent to 0.2% v/v to permeabilize any remaining intact dye-encapsulated LUVs. Dye leakage was plotted according to equation 2.1.

4.3 Results

4.3.1 Cholesterol Has an Inhibitory Effect on IAPP Permeabilization of POPG/POPC Membranes

Cholesterol is a predominant cellular component and has been suggested to play a role in amyloid diseases and toxicity, although there have been disagreeing statements about the effects of cholesterol. Using POPG/POPC LUVs with carboxyfluorescein encapsulated inside, varying levels of incorporated cholesterol were monitored for their

effects on the ability of IAPP to permeabilize these liposomes as measured by released dye. At 25 mol % cholesterol a slight reduction in IAPP permeabilization was observed and at 50 mol % cholesterol permeabilization by IAPP was almost completely blocked (figure 4.2).

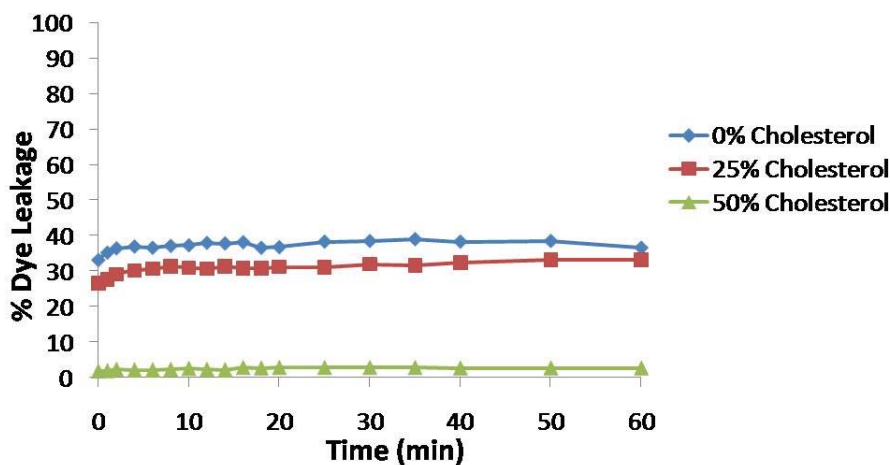


Figure 4.2. Cholesterol inhibits IAPP from permeabilizing POPG/POPC LUVs. IAPP was added to POPG/POPC dye leakage lipids with cholesterol and monitored over a 60 min timecourse. The peptides were added at time zero and the fluorescence was monitored for the duration of the experiment. The cholesterol containing samples were composed of mol % cholesterol and mol % lipid.

4.3.2 Cholesterol Has an Inhibitory Effect on IAPP Permeabilization of POPG Membranes

To better observe the permeabilization of LUVs, POPG LUVs were used in a similar manner to see if cholesterol was capable of inhibiting IAPP permeabilization, even with 100% anionic POPG component. Cholesterol reduced the IAPP induced permeabilization from above 70% to 10% leakage with 50 mol % cholesterol incorporated into the POPG LUVs (figure 4.3). Cholesterol (40 mol %) was nearly as

effective as 50 mol % with roughly only a 15% reduction in dye leakage. The effect of cholesterol was strong enough to inhibit IAPP permeabilization, although it did not prevent permeabilization completely.

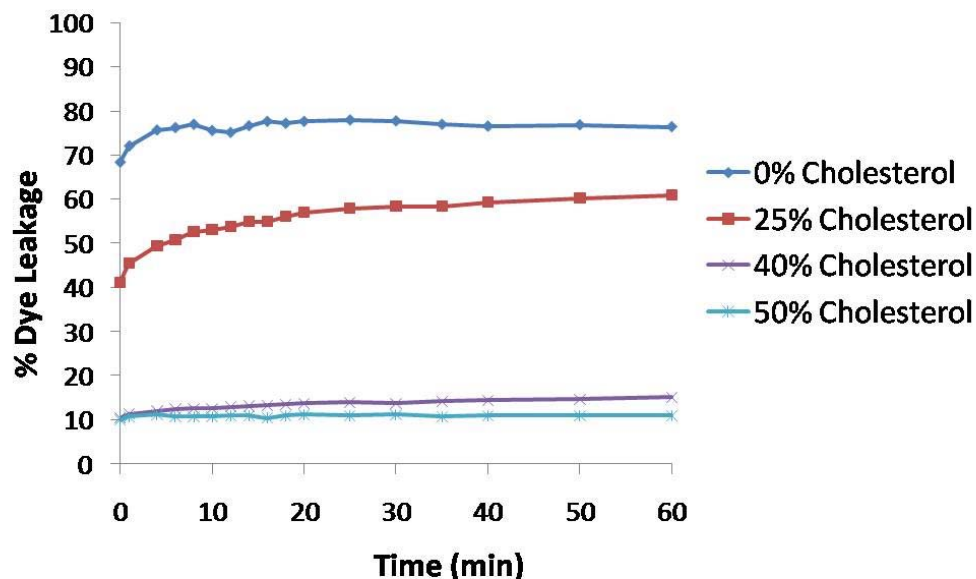


Figure 4.3. Cholesterol inhibits IAPP from permeabilizing POPG LUVs. The same basic experiment as figure 4.1 was repeated with POPG LUVs. IAPP was added to POPG dye leakage liposomes one time and monitored for 60 min for dye leakage in the presence of cholesterol. The experiment used 1 μM IAPP and 200 μM POPG LUVs (5 μM POPG LUVs with encapsulated dye and 195 μM unlabeled POPG LUVs). The cholesterol containing samples were composed of mol % cholesterol and mol % lipid.

IAPP was incubated with equimolar insulin to more closely approximate the conditions at the site of secretion, where soluble insulin and IAPP are co-secreted. Even with the presence of insulin, IAPP permeabilization of LUVs was nearly identical to IAPP alone, showing that insulin and cholesterol do not inhibit IAPP binding to anionic lipid membranes (figure 4.4).

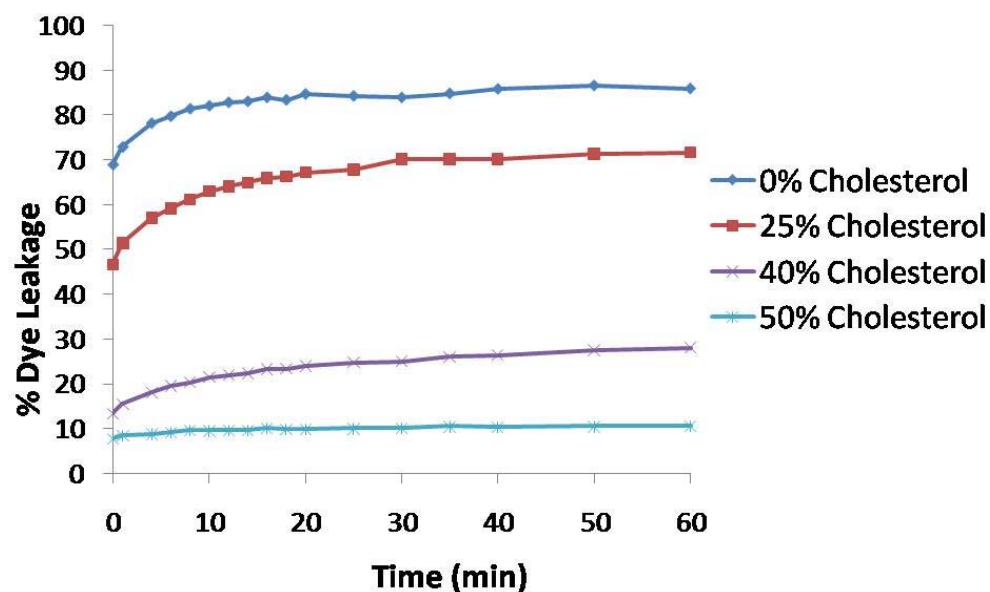


Figure 4.4. Cholesterol inhibits IAPP/insulin from permeabilizing POPG LUVs. 1 μ M IAPP/1 μ M insulin. The cholesterol containing samples were composed of mol % ratios.

Insulin was added to POPG dye labeled LUVs to see if cholesterol altered the affinity of insulin for the membrane. This shows that cholesterol does not have any significant alteration of the interaction of insulin with anionic lipid membranes (figure 4.5).

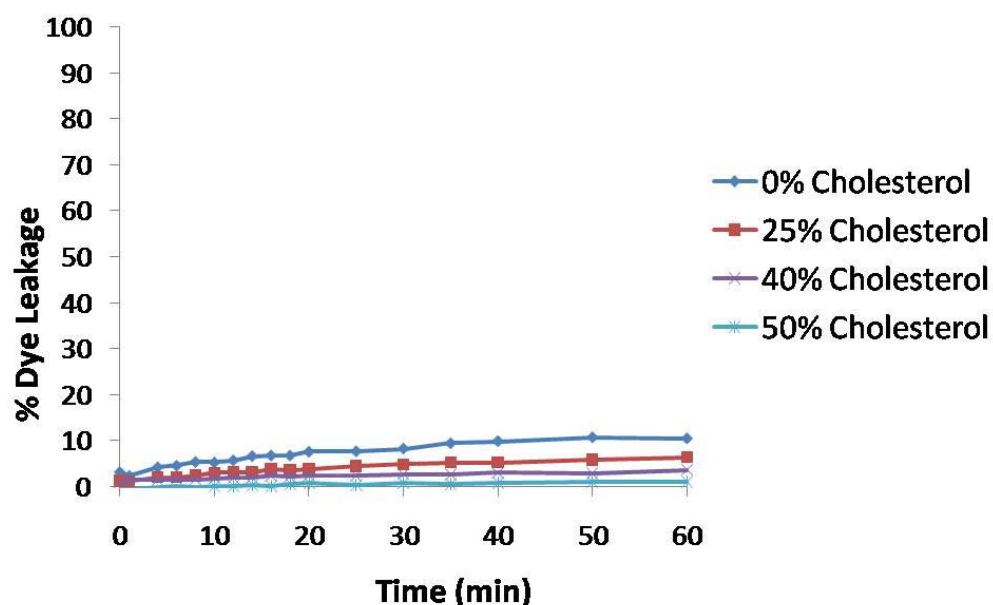


Figure 4.5. Insulin has little effect on POPG LUVs. 1 μ M IAPP/1 μ M insulin. in mol % ratios. The effect of sphingomyelin on dye leakage with 1 μ M insulin. Cholesterol:POPG (C:P) in mol % ratios. The cholesterol containing samples were composed of mol % cholesterol and mol % lipid.

4.4 Discussion

4.4.1 Membrane Charge Is a Key Determinant of IAPP Binding

The IAPP identified in plaques and fibrils in diabetic patients has not been found to be altered from the wild-type amino acid sequence, leading to the idea that changes in the plasma membrane and the surrounding environment increase or decrease the potential toxicity of IAPP (*I*). The most abundant protein in the secretory granule is insulin, which has been suggested to inhibit IAPP toxicity when insulin is in its crystalline form. Shown previously in Chapter III, soluble insulin does not inhibit IAPP from forming oligomers, binding to lipid membranes, or permeabilizing them. Other secretory granule components

or membrane components in the outer leaflet of the plasma membrane, like membrane charge, may play a role in inhibiting IAPP toxicity in the non-diabetic state.

Large unilamellar vesicles (LUVs) containing anionic lipids have been known for some time to accelerate the aggregation of several amyloid peptides, including IAPP and amyloid- β . IAPP rapidly binds and permeabilizes these LUVs, while amyloid- β binding and permeabilization is relatively low early in the incubation. One key difference exists between these two peptides. Amyloid- β is natively negatively charged until it undergoes a conformational change to expose its positively charged residues. IAPP is natively positively charged and is immediately capable of binding to anionic LUVs. The charged plasma membrane acts as a local attractant to IAPP peptides, congregating them in a localized area that creates a high concentration capable of forming the toxic oligomeric species and in later stages or at higher concentration forms IAPP fibrils extending out from the membrane (see Figure 3.4A). This explains how membrane charge can act as an accelerant for IAPP aggregation by creating localized pockets of high peptide concentration. This high local peptide concentration has been proposed to overcome the nucleation energy barrier, skipping the lag phase or nucleation phase of amyloid formation (see IAPP in Figure 3.1A) (*101, 117, 118*).

In the diabetic state, a breakdown in lipid biosynthesis may lead to an imbalance in zwitterionic and anionic lipids with a greater percentage of anionic lipid content. In addition, when phospholipases cleave the choline head group of PC, it results in the negatively charged phosphatidic acid (*119*). If there is breakdown in lipid turnover, anionic PA would temporarily accumulate again, leading to an increase in anionic charge and affinity for IAPP binding (*1*).

4.4.2 Is Cholesterol Protective Against Permeabilization of LUVs?

Why would cholesterol inhibit IAPP permeabilization? Cholesterol has a rigid hydrocarbon ring structure that intercalates between the lipid acyl chains. The hydroxyl head group of cholesterol binds to the phospholipid head group. Cholesterol fills the interstitial space, reducing the ability of the lipid tails to move in response to peptide binding or insertion. Cholesterol adds a steric constraint on the lipid membrane that inhibits IAPP permeabilization. Whether the interaction of cholesterol with the polar lipid headgroup exerts a decrease in the electrostatic charge of the lipid surface that would reduce the affinity of IAPP is unknown, as this work only sought to address the permeabilization at varying levels of cholesterol.

In the absence of cholesterol, IAPP readily binds to membranes that contain a minimum anionic lipid content of 30 mol % (figure 4.6A). As the cholesterol content is increased to 25 mol % the ability of IAPP to permeabilize the membrane decreases (figure 4.6B). At high concentrations of incorporated cholesterol (50 mol %) IAPP is almost entirely inhibited from permeabilizing the membrane (figure 4.6C).

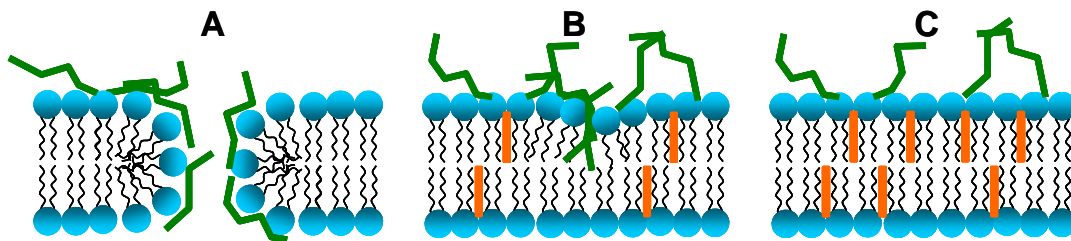


Figure 4.6 Cartoon of membranes with incorporated cholesterol inhibiting IAPP permeabilization of LUVs. The cholesterol (orange rectangles) stabilizes the membrane, preventing IAPP (shown in green) from permeabilizing the membrane. (A) A toroidal pore caused by IAPP permeabilization of the membrane. The toroidal mechanism is one of several pore types hypothesized as the mechanism of IAPP permeabilization of the β -cell membrane. (B) Incorporating a small amount of cholesterol into the membrane reduces membrane permeabilization. (C) Higher concentrations of cholesterol incorporated into the membrane completely exclude IAPP from permeabilizing the membrane similar to what would be expected with gel phase membranes.

Cholesterol in the membrane is a necessity for cellular function, liquid-order phase, and lipid rafts. Cholesterol has been termed the most important regulator of lipid organization, and complex controls are involved to maintain correct levels of incorporated cholesterol (106). In light of this, the notion that cholesterol is always bad and that it needs to be reduced in individuals is potentially inappropriate. Incorporated cholesterol decreases with age, changing liquid-ordered to liquid-disordered regions. Care needs to be applied to separate incorporated cholesterol levels from circulating cholesterol. The effects of statin drugs may also pose a particular problem if they, in the process of reducing circulating cholesterol, reduce the ability of cells to synthesize and incorporate cholesterol into their plasma membranes. A decrease in incorporated cholesterol could foster the progression of Type II diabetes as well as other amyloid diseases. Further work needs to be done to elucidate the role that cholesterol and other cellular components play in protecting cells from toxic amyloid peptides. Regions high in anionic charge and low in cholesterol content may be the at risk regions of the β -cell plasma membrane where IAPP readily binds and permeabilizes the cell if the anionic content is ~ 30 mol % or higher.

CHAPTER V

Conclusions

5.1 Introduction

This body of work has focused on identifying the IAPP peptide region responsible for membrane binding, elucidating the interaction of insulin with IAPP, and the role that cholesterol plays in regulating membrane fluidity and in protecting from IAPP permeabilization of model lipid membranes. This chapter seeks to summarize the conclusions and findings of the previous chapters, Chapters I through IV. The Significance and Future Studies section focuses on future directions and experiments involving IAPP oligomerization and toxicity.

5.2 Thesis Summary

5.2.1 Chapter I.

Typically eukaryotic cells are largely composed of zwitterionic (effectively neutral) lipids with an anionic component of 20 mol % or less. Accordingly, many of the antimicrobial peptides produced by eukaryotes bind specifically to anionic membranes that would be found on microbial membranes (13). This is also the case with IAPP. IAPP is positively charged and binds readily to negatively charged surfaces similarly to antimicrobial peptides. IAPP is also known to interact readily with anionic lipids, such as

the specifically identified lipids, glycerol and serine-containing phospholipids (120). Lys₁, Arg₁₁, and His₁₈ are all located within the N-terminal membrane binding region of IAPP and are positively charged at neutral pH, creating strong electrostatic interactions with negatively charged substances. Insulin is also negatively charged at pH 7.4, leading to its electrostatic interactions with IAPP. IAPP preferentially binds anionic lipids over insulin when both are present. These charge interactions appear to play a large role in the ability of IAPP to bind and subsequently permeabilize LUV membranes. Conditions that favor an anionic lipid membrane would allow IAPP binding if the anionic charge was 30 mol % or greater unless cooperative inhibition from secreted secretory granules could temporarily bind and inhibit IAPP interaction with anionic membranes.

5.2.2 Chapter II. Amyloid Fibril Formation and Membrane Disruption are Separate Processes Localized in Two Distinct Regions of IAPP, the Type-2-Diabetes-Related Peptide

In Chapter II the fragment IAPP₍₁₋₁₉₎ was compared with the full-length amidated IAPP₍₁₋₃₇₎ for their ability to permeabilize LUVs and form amyloid. This study made a key finding by separating the membrane binding region of IAPP located in the (1-19) region from the amyloid forming regions of the peptide (20-29) and (30-37). This showed for the first time that amyloid formation is not a necessary step for permeabilization. IAPP₍₁₋₃₇₎ had a reduced ability to permeabilize LUVs when the concentration was above the critical concentration for aggregation. This supports the idea that amyloid formation may be protective, acting as a sink for toxic peptides, and supports the oligomeric hypothesis of toxicity. IAPP₍₁₋₁₉₎ did not form amyloid as measured by circular dichroism

or thioflavin T assay. No amyloid formation occurred even in the presence of anionic LUVs, unlike the full-length peptide that underwent a rapid conformational change from α -helical to β -sheet conformation with the addition of anionic LUVs. The C-terminus drives amyloid formation, whereas the N-terminus is responsible for membrane binding and toxicity. In addition the disulfide bond blocks the ability of the N-terminal region to form amyloid.

5.2.3 Chapter III. Insulin Stabilizes the Membrane Permeabilizing Species of Islet Amyloid Polypeptide

The interaction between IAPP and insulin has been suggested to be protective because it is well known that insulin inhibits IAPP fibrillization. Unfortunately insulin does not also inhibit IAPP oligomerization and IAPP induced permeabilization of LUVs.

Insulin appears to bind and inhibit fibril formation and does not have much if any effect on IAPP in the early stages of oligomerization. In accordance with this, dye leakage experiments show that insulin, by inhibiting the fibrillization, prolongs the oligomeric state of IAPP. This also prolongs the time period that IAPP can readily bind and permeabilize lipid membranes. Insulin only inhibits amyloid formation, not small oligomers, and most surprisingly IAPP rapidly permeabilizes membranes even in the presence of insulin.

Interestingly IAPP may bind directly to the membrane as a monomer based on the electrostatic interaction between the membrane and peptide, not requiring a structural change and oligomerization prior to membrane binding. Oligomerization of IAPP may

occur on the membrane surface, which would explain the immediate permeabilization after the addition of IAPP to LUVs.

Oligomerization appears to be independent of the effects of insulin as observed in the crosslinking experiment. The same sized oligomeric species were present in both samples, showing that insulin does not readily bind the early states of IAPP aggregation, only later fibrillar forms.

Membrane charge appears to be the main determinant of IAPP binding in our experiments. Whether insulin is present or not, a minimum anionic charge of 30 mol % is necessary for permeabilization. At 25 mol % anionic lipid content, not much permeabilization occurs.

5.2.4 Chapter VI. Cholesterol Protects LUVs Against IAPP Permeabilization

The role of cholesterol in amyloid diseases is unclear at the present time, but this research shows support for cholesterol as playing a protective role inhibiting IAPP permeabilization of model lipid membranes. Cholesterol at 25 mol % reduced IAPP binding by approximately 15%. At 50 mol % the rigidity of the membrane becomes similar in fluidity to a gel phase membrane based on the stabilization of cholesterol. Accordingly, cholesterol at 50 mol % lipid concentration was highly effective at inhibiting IAPP permeabilization.

5.3 Significance and Future Studies

The significance of this work is that it shows that amyloid formation and amyloid peptide toxicity are separate parts of the peptide. This work supports the oligomeric

hypothesis, that smaller oligomeric species are responsible for β -cell death in Type II diabetes rather than the formation of islet amyloid and the growth of the fibrils. This research supports and extends Knight and Miranker's recent work involving IAPP binding and anionic charge (1), but shows that not only binding is occurring but permeabilization is occurring as well. Miranker also showed that crystalline insulin is protective against IAPP oligomerization and toxicity within the secretory granules (51). In addition, the greatest risk of IAPP toxicity may be at the site of secretion from the secretory granules when insulin becomes soluble, as we clearly show that insulin is only effective at inhibiting amyloid fibril formation and not the early oligomeric species or the ability of these species to permeabilize the membrane. In addition, we showed that incorporated cholesterol is protective against IAPP permeabilization.

It would be interesting to look at the interaction of IAPP with other secretory granule components, like Prepeptin, C-peptide I, and C-peptide II, and then observe the effect IAPP has on membrane permeabilization when they are added in conjunction with these other components. Further work needs to be done to determine the components and lipid charge that prevent IAPP from forming toxic oligomers within the secretory granules.

Further investigation needs to be conducted to elucidate the exact changes that occur in the plasma membrane in the diabetic state, both changes in the lipid components and the anionic charge of the membrane. Greater understanding of the conditions and mechanism of IAPP binding and permeabilization could allow the development of better prevention strategies and treatments to prevent further β -cell loss in the insulin resistant and diabetic states.

In all of these experiments we observed membrane permeabilization of our model lipid membranes. It would be interesting to observe toxicity in cell culture experiments with MIN6 cells (immortalized insulin secreting murine pancreatic cell line). However, MIN6 cells express insulin receptors and are sensitive to changes in insulin concentrations. Adjusting the insulin level could lead to apoptosis in MIN6 and other insulin sensitive cell lines. Insulin insensitive cells would need to be used to conduct experiments that use a combination of insulin and IAPP. A possible candidate for these experiments would be Madin-Darby canine kidney (MDCK) cells, which lack insulin receptors and are insulin insensitive (121).

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