Published in final edited form as:

J Mol Biol. 2012 August 10; 421(0): 348–363. doi:10.1016/j.jmb.2012.01.048.

The effect of A β on IAPP aggregation in the presence of an isolated β -cell membrane

Janine Seeliger^a, Katrin Weise^a, Norbert Opitz^b, and Roland Winter^{a,*}

^aPhysical Chemistry I - Biophysical Chemistry, Faculty of Chemistry, TU Dortmund University, Otto-Hahn-Str. 6, 44227 Dortmund, Germany

^bMax Planck Institute of Molecular Physiology, Otto-Hahn-Str. 11, 44227 Dortmund, Germany

Abstract

Fibrillar aggregates of the islet amyloid polypeptide (IAPP) and amyloid- β (A β) are known to deposit at pancreatic β -cells and neuronal cells, and are associated with the cell degenerative diseases type-2 diabetes mellitus (T2DM) and Alzheimer's disease (AD), respectively. Since IAPP is secreted by β -cells and a membrane damaging effect of IAPP has been discussed as a reason for β -cell dysfunction and the development of T2DM, studies of the interaction of IAPP with the β -cell membrane are of high relevance for gaining a molecular level understanding of the underlying mechanism. Recently, it has also been shown that patients suffering from T2DM exhibit an increased risk to develop Alzheimer's disease and *vice versa*, and a molecular link between AD and T2DM has been suggested. In this study, membrane lipids from the rat insulinoma-derived INS-1E β -cell line were isolated and their interaction with the amyloidogenic peptides IAPP, A β , and a mixture of both peptides has been studied.

To yield insight into the associated peptides' conformational changes as well as into their effect on the membrane integrity during aggregation, ATR-FTIR spectroscopy, fluorescence microscopy, and AFM experiments have been carried out. The IAPP-A β heterocomplexes formed were shown to adsorb, aggregate, and permeabilize the isolated β -cell membrane significantly slower than pure IAPP, however, at a rate that is much faster than that of pure A β . In addition, it could be shown that isolated β -cell membranes cause similar effects on the kinetics of IAPP and IAPP-A β fibril formation as anionic heterogeneous model membranes.

Keywords

Amyloid; Peptide-membrane interaction; ATR-FTIR spectroscopy; Fluorescence microscopy; AFM

© 2012 Elsevier Ltd. All rights reserved.

*Corresponding author. roland.winter@tu-dortmund.de, Telephone: +49-231-755-3900, Fax: +49-231-755-3901.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Two of the most prominent diseases affecting the elderly are type-2 diabetes mellitus (T2DM) and Alzheimer's disease (AD). Both diseases are accompanied by the deposition of amyloid fibrils, which are formed upon misfolding and self-association of amyloidogenic peptides and consist of an extended cross- β -sheet structure.¹⁻⁵ These amyloid fibrils are associated with degeneration of β -cells and neuronal cells in T2DM and AD, respectively.^{4, 5} Islet amyloid polypeptide (IAPP) deposits are found in the pancreatic Islets of Langerhans of a substantial proportion of all the individuals suffering from T2DM.^{6, 7} The peptide, also known as human amylin, consists of 37 amino acids and is produced, stored and secreted together with insulin by pancreatic β -cells.⁸ Amyloid- β (A β) is the major component of aggregates located in the brain of AD patients. The 40- (A β 40) or 42-residue (A β 42) containing peptide is a fragment of the membrane associated amyloid precursor protein.^{9, 10}

It has been suggested that β -cell-secreted IAPP disrupts the membranes of β -cells, leading to β -cell dysfunction and the development of T2DM.^{11, 12} In the present study, the first step focussed on the isolation and characterization of membrane lipids from the rat insulinoma-derived INS-1E β -cell line, which were then used to investigate the interaction of A β 40 and IAPP as well as the cross-interaction of both peptides on the isolated β -cell membrane. Utilization of this biological membrane model in biophysical studies is of particular significance as it bridges the gap between *in vivo* studies and *in vitro* work using more simple artificial membrane systems consisting of one or a few lipid components, only. Here, the effect of A β on the interaction of IAPP with the isolated β -cell membrane as well as the pure IAPP- and pure A β -membrane interactions were studied since an increased risk for AD patients to develop T2DM and *vice versa* was reported in clinical studies, which might be based on a molecular interaction between the two peptides.^{13, 14} Moreover, a high sequence similarity of the diseases triggering peptides A β and IAPP together with first investigations of cross-seeded fibrillation in bulk solution was described.^{15, 16} Therefore, an *in vivo* interaction might be possible and, if existent, could indicate a molecular link between T2DM and AD.

In order to gain a detailed picture of the peptide-membrane interaction processes, surface-sensitive attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy and fluorescence microscopy were applied. ATR-FTIR spectroscopy was used to determine time-dependent changes in the peptides' secondary structure upon interaction with a solid-supported bilayer membrane prepared from the isolated β -cell lipids. Localization studies of fluorescent-labeled giant unilamellar vesicles (GUVs) prepared from isolated β -cell lipids and labeled peptides were carried out, together with leakage assays to investigate accompanying peptide-induced membrane permeabilization effects, using confocal fluorescence microscopy. To interpret the effect of β -cell lipids on the aggregation process of the amyloidogenic peptides studied, complementary experiments have been carried out with homogeneous and heterogeneous anionic model membrane systems. Finally, to verify and characterize the species – if oligomers, protofibrils or mature fibrils – that are formed during peptide aggregation on a nanometer-scale, atomic force microscopy (AFM) was applied.

Results

Characterization of the membrane isolated from pancreatic β -cells

An extraction protocol using chloroform and methanol has been applied to isolate membrane lipids from the rat insulinoma-derived INS-1E β -cell line. These isolated β -cell lipids were used for subsequent lipid-peptide interaction studies. Mass spectrometry analysis of the extracted lipid mixture revealed a lipid content of $68.9 \pm 3.5\%$ phosphatidylcholine (PC), $24.0 \pm 3.4\%$ phosphatidylethanolamine (PE), $4.6 \pm 0.2\%$ sphingomyelin (SM), $1.6 \pm 0.4\%$ phosphatidylinositol (PI), $0.6 \pm 0.1\%$ phosphatidylserine (PS), $0.1 \pm 0.03\%$ phosphatidic acid (PA), and $0.2 \pm 0.02\%$ phosphatidylglycerol (PG) (Figure 1). For the zwitterionic phospholipids PC and PE, these data correspond well with the phospholipid contents observed in islets isolated from the pancreas of adult rat and mice.^{17,18} With 2.5 mol%, a lower content of lipids carrying a negatively charged headgroup (PI, PS, PA, and PG) was found in INS-1E cells compared to 13.2 mol% in isolated islets of rat.¹⁷ This may be attributed to the use of pure β -cells in this study whereas whole pancreatic islets including β -cells together with α -, δ - and PP cells were utilized for the lipid analysis of islets directly isolated from the pancreas of the adult rat.

To further characterize the extracted β -cell membrane system, particularly with regard to potential phase transitions and the conformational order of the lipid acyl chains, large unilamellar vesicles (LUVs) were prepared from the β -cell lipid mixture and temperature-dependent FTIR spectroscopic measurements were carried out. The strongest lipid bands observed in the IR spectra are the symmetric ($2849\text{--}2855\text{ cm}^{-1}$) and antisymmetric ($2916\text{--}2925\text{ cm}^{-1}$) CH_2 -stretching vibration modes, which are highly sensitive to changes in the *trans/gauche* ratio and the number of kinks in the acyl chains.^{19,20} The symmetric CH_2 -stretching mode wavenumbers, of the isolated β -cell membrane lipids are shown in Figure 2a as a function of temperature. The band maximum of the shifts linearly to higher wavenumbers in the temperature range from 5 to 14°C. This region exhibits the lowest wavenumbers within the temperature range covered. The magnitude of these wavenumbers is indicative for a lipid phase with high conformational order and can be assigned to a liquid-ordered phase. At higher temperatures, the sigmoidal change in slope indicates the formation of a new phase that extends up to about 22 °C. This phase region may be attributed to a coexistence region of liquid-ordered and liquid-disordered lipid phases. At higher temperatures, the slope decreases again, pointing towards an essentially monophasic liquid-disordered region, which is confirmed by corresponding AFM measurements displaying one homogeneous lipid phase of $\sim 4\text{ nm}$ in height (Supporting Information, Figure S1). From these lipid preparations, giant unilamellar vesicles can be formed that also exhibit one homogeneous phase (on the micrometer scale) and that are used for leakage and peptide localization studies (Figure 2b). Furthermore, LUVs (large unilamellar vesicles) can be spread onto germanium crystals for ATR-FTIR spectroscopic measurements.

ATR-FTIR studies of the IAPP and A β interaction with the isolated β -cell membrane

The surface-sensitive ATR-FTIR spectroscopy was applied to reveal time-dependent secondary structural changes of 3 μM IAPP, 3 μM A β or an equimolar mixture of both peptides ($c = 3\text{ }\mu\text{M}$ each) upon interaction with the isolated β -cell membrane system. As

shown in Figure 3a, the maximum of the amide-I' band of pure IAPP undergoes a pronounced shift from 1644 cm^{-1} towards 1625 cm^{-1} . This shift points towards a secondary structure change from a disordered/ α -helical conformation at $t = 5\text{ min}$ to a structure mainly consisting of intermolecular β -sheets ($\sim 40\%$) after $\sim 3\text{ h}$, which indicates aggregation of IAPP (cf Supporting Information, Figure S2a for details). The normalized FTIR-spectra of IAPP seem to cross in an isosbestic kind of point (Figure 3a, right), indicating a highly cooperative conversion of IAPP's disordered conformation into the aggregated β -sheet structure without populating other secondary structure motifs significantly. Furthermore, an increasing adsorption of IAPP to the isolated β -cell membrane was observed within the first two hours of incubation as the amide-I' band signal intensity considerably increases within this time period (Figure 3a and Supporting Information, Figure S3a).

For the interaction of pure A β with the isolated β -cell membrane, a different behavior has been observed (Figure 3b). A reasonable amide-I' band signal could first be detected after 20 h of incubation of A β with the β -cell membrane, which can be interpreted in terms of a poor adsorption of A β to the membrane system. Even after 40 h, the A β signal is eight times lower than the amide-I' band signal observed for IAPP at the same time point (cf. Figure S3a, Supporting Information). These differences of IAPP and A β in their adsorption propensities to the 2.5 mol% negatively charged β -cell membrane can be attributed to their different net charge, which is positive for IAPP and negative for A β at neutral pH. Therefore, electrostatic interactions with the membrane would be favorable for IAPP, only. The delayed interaction of the amphiphilic A β with the β -cell membrane could be initialized by its hydrophobic C-terminus and the presentation of its positively charged residues. Owing to the low adsorption of A β to the membrane at the beginning of the measurement, analysis of secondary structural changes is difficult. However, in the time period from 20 to 40 h an increased band intensity at $\sim 1625\text{ cm}^{-1}$ was observed, indicating an increase in intermolecular β -sheet content and hence aggregation of A β (Figure 3b, right).

Figure 3c displays the corresponding results for the incubation of the isolated β -cell membrane with an equimolar mixture of IAPP and A β . A slow shift of the amide-I' band maximum from 1644 cm^{-1} to 1634 cm^{-1} was observed during 40 h of measurement. This indicates a secondary structural change from a mainly disordered/ α -helical structure to a more β -sheet containing conformation (β -sheet content of $\sim 33\%$, Supporting Information, Figure S2b), which is retarded compared to the spontaneous conformational change observed for pure IAPP. Consequently, a heterocomplex formation between IAPP and A β that slows down the aggregation process seems to take place. Moreover, the aggregation process of this heterocomplex seems to follow a similar mechanism as that found for pure IAPP since all normalized spectra of the IAPP + A β aggregation cross in an isosbestic kind of point at a similar wavenumber. Adsorption of the IAPP-A β mixture to the isolated β -cell membrane is completed after 5 h, which is only slightly slower than the adsorption of pure IAPP to the membrane, however significantly faster than the adsorption of pure A β (Supporting Information, Figure S3a).

Influence of IAPP and A β on the morphology of GUVs composed of isolated β -cell lipids

Fluorescence microscopy leakage assays have been performed to reveal the membrane permeabilizing effect of IAPP, A β , and IAPP + A β on the isolated β -cell membrane. To this end, GUVs of the extracted β -cell lipids were generated by electroformation. To detect permeabilization of the vesicles, which is supposed to occur during aggregation and fibril formation, the interior of the GUVs was filled with buffer containing the fluorophore ATTO647. Furthermore, the lipids were labeled by addition of *N*-Rh-DHPE to the mixture before electroformation. For peptide visualization, IAPP was C-terminally labeled with Bodipy-FL by an additional lysine residue and A β was either N-terminally labeled with the fluorophore fluorescein or with diethylaminocoumarin (DAC). Fluorescence microscopy images of the interaction of 5 μ M IAPP-K-Bodipy-FL, 5 μ M Fluorescein-A β (DAC-A β), and a mixture of IAPP-K-Bodipy-FL and Fluorescein-A β (DAC-A β , $c = 5 \mu$ M each) are depicted in Figures 4–7. The first row (at $t = 0$ min) in each figure shows the GUVs before peptide was added to visualize the ATTO647 containing buffer solution (blue) inside the *N*-Rh-DHPE labeled GUVs (red).

In case of pure IAPP, already 5 min after peptide addition IAPP-K-Bodipy-FL (Figure 4, green) can be detected mainly at the interface of the lipid vesicles. Within the next minutes, the amount of IAPP at the membrane increases further and leads to permeabilization and leakage of the membrane. The ATTO647 fluorophore is no longer visible in the vesicles after 10 min of incubation. Nevertheless, IAPP aggregates of vesicle-like shape are still detectable even after $t = 40$ min and permeabilization of the vesicles, indicating that some of the lipids are incorporated into the growing peptide aggregates. However, another part of the β -cell lipids and the IAPP aggregates are separated from each other and randomly distributed in the bulk solution.

Conversely, by following the interaction of pure A β with GUVs consisting of isolated β -cell lipids, a homogeneous distribution of Fluorescein-A β (Figure 5, green) was initially detected in the buffer solution around the vesicle, with only a small amount of accumulation at the lipid membrane being observable ($t = 10$ min and $t = 24$ h). Even after 40 h of incubation, A β fluorescence intensity is still mainly found in the buffer solution. However, membrane permeabilization was detected at this time point. This can be explained by the dominating membrane activity of A β oligomers whose fluorescence intensity might be too faint to be observed and discriminated from the intensity in the surrounding solution. Several previous studies have pointed out A β oligomers to be the essential membrane active species.^{21,22} These data are in agreement with the results of the ATR-FTIR spectroscopy measurements in showing a strong adsorption of IAPP to the membrane system, whereas a poor membrane interaction was detected for A β .

While incubating the mixture of IAPP + A β with the GUVs prepared from β -cell lipids, a homogeneous distribution of both peptides (Figure 6, green) in the buffer solution was observed at the beginning of the measurement ($t = 7$ min). After 30 min of incubation, an increased peptide concentration at the lipid membrane is visible. The membrane-associated peptide concentration increases further with time until the membrane is permeabilized after 6 h. To confirm that both peptides – IAPP and A β – are localized at the membrane system,

additional measurements using IAPP-K-Bodipy-FL and DAC-A β , whose fluorescence emissions can be detected separately, were performed. The fluorescence microscopy images (Figure 7) display colocalization of both IAPP and A β at the lipid vesicle. It can be assumed from these data that the IAPP-A β interaction is initialized in solution where heterocomplexes may be formed. These IAPP-A β heterocomplexes still tend to adsorb to the isolated β -cell membrane surface and permeabilize the GUVs with time. However, accumulation of heteroaggregates is found in bulk solution as well (Figure 6, white arrows).

Effect of isolated β -cell lipids compared to anionic model membrane systems

The influence of the isolated β -cell lipids on the kinetics of fibril formation in comparison to other well established model membrane systems was investigated using Thioflavin T (ThT), which displays an enhanced fluorescence upon non-covalent binding to amyloid fibrils. As shown in Table 1, β -cell lipid vesicles reduce the lag time of pure IAPP as well as IAPP-A β aggregation significantly by a factor of 12 and 17 and enhance the apparent growth rate, k_{app} , 4- and 5-fold, respectively. A similar behavior was observed by the use of an anionic heterogeneous (raft) model membrane consisting of 15% DOPC, 10% DOPG, 40% DPPC, 10% DPPG, and 25% cholesterol (Figure 8), which exhibits lateral heterogeneity (i.e., coexisting liquid-ordered and liquid-disordered domains) as well as negatively charged lipid headgroups.²³ To further confirm the observed similarity of the two membrane systems regarding their influence on the aggregation kinetics, the interaction of IAPP, A β , and the peptide mixture with the anionic raft membrane system was studied in more detail by ATR-FTIR spectroscopy (cf. Supporting Information, Figure S4).²⁴ The ATR-FTIR spectra display the same time-dependent shift and shape of the amide-I' band as the spectra that were recorded in the presence of the isolated β -cell membrane, besides a slightly different kinetics. However, the adsorption of IAPP and IAPP + A β to the anionic raft membrane is less than half that strong as to the isolated β -cell membrane (Supporting Information, Figure S3b). In contrast, the influence of a homogeneous model membrane that consists of DOPC/DOPG in a molar ratio of 7:3 on the aggregation kinetics is significantly smaller. Only a slight decrease of k_{app} was determined for the IAPP-A β mixture and a 3-fold reduced lag phase as well as a triplication of k_{app} in the case of pure IAPP (Figure 8 and Table 1).

Kinetics of IAPP-A β heteroaggregation compared to the pure peptides' fibrillation as monitored by AFM

In the ATR-FTIR measurements, an about 7% lower β -sheet content was found for the IAPP-A β heteroaggregates compared to the $\sim 40\%$ β -sheet content detected for pure IAPP aggregates after 40 h of incubation. To identify which species – if oligomers, protofibrils or mature fibrils – are formed during incubation on a negatively charged surface, the aggregation process of IAPP + A β ($c = 3 \mu\text{M}$, each) was followed on mica by AFM measurements in phosphate buffer (Figure 9), since previous experiments showed that IAPP oligomers spontaneously disrupt solid-supported lipid membranes and fibrillation occurs mainly at the exposed mica surface.³¹ Due to the muscovite mica composition, the negatively charged aluminosilicate layers constitute a negatively charged surface in aqueous solution owing to the dissociation of the potassium ions that were electrostatically bonded to the aluminosilicate layers before. In addition, the aggregation kinetics of IAPP, A β , and the equimolar mixture on mica were compared (Figure 10). The time-lapse AFM experiments

on IAPP + A β revealed the formation of first short fibrils after ~ 3 h of incubation, but they are clearly outnumbered by oligomers and small protofibrils at this stage of aggregation. Early oligomers detected exhibit a mean height of 1.05 ± 0.40 nm (Supporting Information, Figure S5). With time, the amount of protofibrils and fibrils increases and fibril elongation can be observed (Figure 9). After 24 h, a sufficiently large amount of fibrils was detected and the samples were dried to further characterize the IAPP-A β heteroaggregates (fibrils). For comparison, the aggregation of 3 μ M IAPP and 3 μ M A β on mica was followed under the same conditions. In Figures 10a and b, representative AFM images with the corresponding section profiles are shown for IAPP and IAPP + A β at selected time points (4 and 24 h of incubation, respectively). In case of pure IAPP, mainly protofibrils and mature fibrils could be detected already at 4 h of incubation, indicating a fast and almost completed fibrillation of IAPP at this time point. Even if the fibril formation is retarded for the IAPP-A β mixture, the early protofibrils of pure IAPP and IAPP-A β exhibit nearly the same mean height of 0.67 ± 0.15 nm and 0.81 ± 0.14 nm, respectively. Furthermore, a mean height of about 2.9 nm and ~ 5 nm was detected for later protofibrils and mature fibrils of both IAPP and IAPP + A β , respectively. Therefore, the different stages of aggregation could be distinguished for both amyloidogenic systems, with IAPP mature fibrils at the final stage of aggregation already after 4 h (5.21 ± 1.62 nm, Figure 10a) and slightly thinner IAPP + A β fibrils (3.52 ± 0.68 nm) as the main species even after 24 h of incubation being indicative for an earlier stage of aggregation, i.e., the late exponential fibril growth phase (Figure 10b). Thus, while the kinetics of IAPP and IAPP + A β aggregation are clearly different (in agreement with the ATR-FTIR experiments), both IAPP and IAPP + A β show the same characteristics in shape, branching and size of the aggregate species within the accuracy of the analysis.

In contrast to the IAPP-A β mixture and pure IAPP, only a few fibrils could be detected for the aggregation of A β on mica, with the fibrils exhibiting a height of ~ 3 nm after 72 h (Figure 10c). The predominance of A β oligomers and low amount of fibrils even after prolonged incubation times argues for an extremely slow kinetics of aggregation for A β , which is in accordance with the FTIR-spectroscopic experiments.

Discussion and Conclusions

In this study, a natural lipid mixture, which exists in a fluid-like state at room temperature, was extracted from INS-1E β -cells and utilized for the preparation of large and giant unilamellar vesicles for further investigations. This isolated β -cell membrane was used to study the interaction of the amyloidogenic IAPP, A β , and a mixture of both peptides with the membrane system. By ATR-FTIR spectroscopy and fluorescence microscopy, an immediate adsorption of IAPP to the β -cell membrane was detected, which is apparently fostered by electrostatic attraction between the two positive charges in the N-terminal region of IAPP and the negatively charged membrane lipid headgroups. Immediately after adsorption, a conformational transition from an unordered/ α -helical to a mainly β -sheet containing conformation was observed in the ATR-FTIR spectra, indicating rapid IAPP fibril formation. Immediate fibril formation of IAPP at negatively charged surfaces has been confirmed by AFM studies where mature branched fibrils (~ 5 nm in height) could be detected already after 4 h. Applying confocal fluorescence microscopy, membrane

permeabilization and disintegration of GUVs could be visualized after IAPP localization at the isolated β -cell membrane. Similar effects have been observed in previous studies on the interaction of IAPP with anionic model membranes.²⁵⁻³⁰ The results led to the postulation of a multiple step mechanism where an insertion of the positively charged N-terminus of IAPP into the headgroup and upper chain region of the anionic membrane is followed by a rapid conformational transition from an unordered via an α -helical to a β -sheet conformation.²⁷⁻³⁰ Furthermore, viability studies of the INS-1E cell line interacting with IAPP showed significant cytotoxicity of IAPP oligomers,³¹ which may permeabilize the β -cell membrane as shown by a fluorescence microscopy leakage assay on isolated β -cells in the present study.

In a second step, the interaction of A β with the isolated β -cell membrane system was investigated. In contrast to IAPP, only a poor adsorption of A β to solid-supported membranes and GUVs could be observed in ATR-FTIR spectroscopy and fluorescence microscopy experiments, respectively. Furthermore, A β was shown to aggregate extremely slowly at a negatively charged mica surface in corresponding AFM measurements. This may on the one hand be attributed to the negative net charge of A β at neutral pH, which repels the peptide from the 2.5 mol% negatively charged β -cell membrane and the mica surface. On the other hand, previous studies on the A β -lipid interaction have shown a slow adsorption and aggregation of A β also in the presence of zwitterionic lipid bilayers.^{32,33} Nevertheless, with time an interaction of A β with the isolated β -cell membrane occurred as spectra of β -sheet-rich A β appeared in the ATR-FTIR measurements after 20 h and permeabilization of GUVs took place. This delayed adsorption may be driven by a conformational change in the secondary structure of amphiphilic A β (e.g., to β -sheet-rich oligomers or protofibrils) in such a way that the peptide domain rich in positive amino acid residues is able to interact with the negatively charged membrane surface together with the interaction of A β 's hydrophobic C-terminus with the lipid system. These results are in line with recent reports stating that the A β -membrane interaction is driven electrostatically but also contains a hydrophobic component.³³⁻³⁵

To reveal the cross-amyloid interaction of IAPP and A β in the presence of the β -cell membrane, comparative experiments were carried out on an equimolar mixture of IAPP + A β . The results show that the peptide aggregation and adsorption to the slightly negatively charged membrane or mica surface is considerably retarded compared to pure IAPP. Heterocomplex formation of IAPP and A β may be inferred from these data, which is probably initiated by hydrophobic interactions as well as electrostatic interactions between the basic amino acid residues of IAPP and acidic amino acid residues of A β . Direct evidence for IAPP-A β heterocomplex formation in solution in the absence of membranes was already obtained by pull-down assays in previous studies.¹⁶ Based on the recent identification of the IAPP-A β cross-interaction interface, a 1:1 binding model was taken as basis for our studies.⁴⁶ Significant interaction of the heterocomplex with the isolated β -cell membrane takes place, as an accumulation and colocalization at the membrane interface could be observed for both amyloidogenic peptides. An IAPP-like aggregation behavior, i.e., a conformational transition from an initially unordered/ α -helical to a β -sheet conformation and subsequent fibril formation, was detected for the IAPP-A β heterocomplexes in ATR-FTIR and has been confirmed by the observation of oligomers, protofibrils and fibrils with

similar characteristics in AFM measurements. It may be concluded that the IAPP-A β heterocomplexes initially formed in solution adsorb to the isolated β -cell membrane, most likely due to electrostatic and hydrophobic interactions, where they start to fibrillate, which leads, although retarded, to a permeabilization of the membrane. These data could also explain the attenuated but cytotoxic effect of IAPP-A β heterocomplexes found recently.¹⁶

Finally, the effect of the isolated β -cell membrane on the peptide aggregation has been compared with that of other anionic model membrane systems. The data show that the previously established, heterogeneous anionic model membrane system²³ closely resembles the impact of the natural β -cell membrane in terms of aggregation kinetics and membrane permeabilization, whereas a homogeneous anionic membrane system does not. This indicates that besides the negative charge of the β -cell membrane, its heterogeneous composition - with the possibility for extensive lipid sorting - affects peptide aggregation as well. In addition, initial peptide insertion at the liquid ordered/disordered domain boundaries of the heterogeneous model membrane - thus decreasing the unfavorable line energy - could provide an additional initiation mechanism of membrane associated aggregation and fibril formation.

In summary, our results provide novel mechanistic evidence on the interaction of the two key amyloidogenic peptides in T2DM and AD as well as their heterocomplexes with the β -cell membrane. This cross-interaction is thought to be a potential molecular link between the two diseases and their cell toxicity. Significant differences were found for the IAPP-A β heterocomplex interaction compared to the individual membrane-associated aggregation propensities of IAPP and A β . The IAPP-A β complexes formed were shown to adsorb, aggregate and permeabilize the isolated β -cell membrane system much slower than IAPP alone, however, at a rate that is faster than that of pure A β .

Materials and methods

Materials

Sodium hydrogen phosphate (NaH₂PO₄ and Na₂HPO₄), sodium chloride (NaCl), sodium pyruvate, potassium chloride (KCl), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris-HCl), deuterium oxid (D₂O), 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), 2-mercaptoethanol, penicillin, streptomycin, chloroform, methanol, perchloric acid, (NH₄)₆Mo₇O₂₄·4 H₂O, the Fiske-Subbarow-reducer, and the standard phosphate solution were purchased from Sigma-Aldrich (Steinheim, Germany). Human IAPP (Amylin, human) was obtained from Calbiochem (Darmstadt, Germany). A β (Amyloid β (1-40)) as well as the fluorescent-labeled (7-diethylaminocoumarin-3-yl)carbonyl-A β (DAC-A β) were purchased from Bachem (Bubendorf, BL, Switzerland). The fluorescent-labeled IAPP-K-Bodipy-FL and Fluorescein-A β were obtained from Peptide Specialty Laboratories (Heidelberg, Germany). *N*-(Lissamine-rhodamine-B-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (*N*-Rh-DHPE) and ATTO647 were purchased from Molecular Probes (Karlsruhe, Germany) and ATTO-TEC (Siegen, Germany), respectively. RPMI 1640 medium as well as trypsin-EDTA solution were purchased from Invitrogen (Darmstadt, Germany) and fetal bovine serum from PAA (Cölbe, Germany). The INS-1E cell line was a

gift from the group of Dr. Pierre Maechler (Geneva University Hospital, Switzerland).³⁶ All chemicals used were of the highest analytical grade available and used without further purification.

INS-1E cell culture

INS-1E cells were cultured at 37 °C under 5% CO₂ in complete medium composed of RPMI 1640 medium (with 2 mM glutamine) supplemented with 5% fetal bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100 U mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin, pH 7.4. Once a week, cultured cells were passaged by gentle trypsinization and seeded at a density of 4·10⁴ cells/cm² into fresh 75-cm² Falcon bottles (BD Biosciences, Franklin Lakes, NJ) containing 25 mL complete medium.

Lipid extraction, quantification and analysis

For lipid extraction from INS-1E cells, a modified procedure primarily described by Bligh and Dyer³⁷ was used. INS-1E cells were used after one week of growth, with one medium change at day 3. Cells were washed twice with 10 mL of TBS buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4), before they were scraped off the plate with 3 mL of TBS. The maintained cell suspension of two plates was centrifuged to collect the cell pellet, which was then resuspended in 0.8 mL ddH₂O and homogenized in a 2 mL Dounce homogenizer. Immediately, 1 mL of chloroform and 2 mL of methanol were added to the cell suspension and mixed well, before additional 1 mL chloroform and 1 mL ddH₂O were added. After shaking, the suspension was centrifuged (at 450 g) for 5 min and the lower chloroform/lipid-layer was collected. The extraction was repeated two times more by addition of 1 mL chloroform to the upper aqueous layer, shaking, centrifugation (450 g, 5 min), and collection of the chloroform/lipid-layer. The three chloroform/lipid extracts were combined and washed by addition of 400 µL KCl, shaking, and removing of the upper layer, as well as by adding 400 µL ddH₂O, shaking, centrifugation and collection of the lower chloroform/lipid-layer. The chloroform was removed using a rotary evaporator followed by lyophilization over night.

A modified Bartlett assay³⁸ was used to quantify the extracted phospholipids. The lipids were dissolved in 200 µL chloroform, thereof 10 µL aliquots were dried in glass tubes and used for quantification. First, 75 µL ddH₂O and 400 µL perchloric acid were added and incubated for 1.5 h at 200 °C. The solution maintained was cooled down before addition of 2.4 mL of a 0.44% (w/v) (NH₄)₆Mo₇O₂₄·4 H₂O-solution and 2.4 mL of 2.5% (w/v) Fiske-Subbarow-reducer, and heated in boiling water for 10 min. Subsequently, the solution was cooled on ice and the absorbance at 820 nm was measured. To calculate the amount of phospholipids, the quantification process was performed with aliquots of a standard-phosphate solution (0-100 nmol) as well. All measurements were performed in triplicate.

The mass spectrometry analysis of the extracted lipids was performed at the Kansas Lipidomics Research Center Analytical Laboratory.

Temperature-dependent Fourier-transform infrared (FTIR) spectroscopy

For preparation of large unilamellar vesicle (LUV) solutions at a concentration of 15 mg/mL, the appropriate amount of dried extracted lipids was dissolved in 10 mM phosphate buffer pH 7.4 and sonicated for 15 min. After five freeze-thaw cycles (thawing at 70 °C), a homogeneous solution of multilamellar vesicles (MLV) was obtained. To prepare LUVs, the solution was passed through a preheated extruder (60 °C) with a membrane filter made of polycarbonate (pore diameter: 0.1 µm).

Temperature-dependent FTIR spectra were recorded with a Nicolet 5700 FT-IR spectrometer equipped with a liquid nitrogen cooled MCT (HgCdTe) detector (Thermo Scientific, Waltham, MA, USA). Measurements were carried out using a cell with CaF₂ windows, which were separated by a 50 µm thick mylar spacer. Spectra of 128 scans were taken with a spectral resolution of 2 cm⁻¹. To control the temperature, an external circulating water thermostat was used. Details on the assignment and interpretation of lipid bands can be found in references 19, 20.

Peptide preparation

Peptide solutions were prepared by dissolving IAPP (IAPP-K-Bodipy-FL) and Aβ (Fluorescein-Aβ, DAC-Aβ) in hexafluoroisopropanol (HFIP) yielding a concentration of 0.5 mg/mL for each peptide. Thus, any preformed fibrils of IAPP and Aβ were dissolved. The required amount of IAPP, Aβ or a mixture of both peptides was dried using a gentle stream of nitrogen and subsequent lyophilization. For the ATR-FTIR and AFM measurements, a phosphate buffer solution (10 mM NaH₂PO₄, pH 7.4, in D₂O for ATR-FTIR) was added to obtain three different peptide sample solutions with final concentrations of 3 µM for pure IAPP as well as for pure Aβ, and 3 µM each in the solution containing both peptides. In case of fluorescence microscopy measurements, the peptides were dissolved in Tris buffer (5 mM Tris-HCl, pH 7.4) to yield a final concentration of 5 µM for pure IAPP as well as for pure Aβ, and 5 µM each in the solution containing both peptides.

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy

Large unilamellar vesicle solutions of the dried extracted cellular lipid mixture or the model membrane system at a concentration of 0.5 mg/mL in phosphate buffer (10 mM NaH₂PO₄, pH 7.4, in D₂O) were prepared as mentioned above. ATR-FTIR spectra were recorded using a Nicolet 6700 infrared spectrometer equipped with a liquid nitrogen cooled MCT (HgCdTe) detector (Thermo Scientific, Waltham, MA, USA). Spectra of 128 scans were taken with a spectral resolution of 2 cm⁻¹. The ATR out-of compartment accessory consists of a liquid jacketed PikeTech ATR flow-through cell with a trapezoidal Ge-crystal (PikeTech, Madison, WI, 80×10×4 mm³, angle of incidence: 45°). The ATR flow-cell was tempered to 25 °C and a buffer spectrum was taken before each measurement. The freshly prepared solution of large unilamellar vesicles was injected into the ATR flow-cell, which was heated to 60 °C. This led to the spontaneous formation of supported lipid bilayers on the surface of the Ge-crystal, which was controlled by following the increase of the CH₂ lipid IR band intensities over time. After adsorption overnight and cooling to 25 °C, the membrane was washed with buffer for 2 h. Then, 1 mL of peptide solution was injected into the ATR cell and spectra were collected every 5 min. Data analysis was performed using the GRAMS

software (Thermo Electron). After subtraction of buffer together with the membrane, the spectra were baseline corrected between 1710 and 1585 cm^{-1} and normalized to the amide-I' band area. Initial peak positions for curve fitting were determined from the second-derivative and FSD of the area normalized spectra. These peaks were then fitted to the normalized raw spectra according to the least squares condition, using a Levenberg–Marquardt curve-fitting routine with bands of Voigt line shape, and the relative peak areas (integral intensities) of all secondary structure elements revealed were determined. The starting width at the half height of each peak was 8 cm^{-1} . Each peak position was restricted to move not farther than 1-3 cm^{-1} (as determined by shifts in the second derivative and FSD) from their centers during the fitting routine. The output relative peak areas of the amide-I' band have an approximate error of $\pm 3\%$. Typical R^2 -values of the peak fitting statistics were in the range of 99.3–99.9%. We note that the results of this method need to be treated with caution for the determination of absolute values of the secondary structure elements because their transition dipole moments may be different and because theoretical predictions of the absorbance frequencies of model polypeptide secondary structures showed that they may be influenced by structural distortions, variable (hydrogen/deuterium) H/D exchange, etc. No problems arise, however, from the application of the fitting method to the study of relative changes in conformations of the protein backbone, which was the primary goal of this study.³⁹⁻⁴¹

Fluorescence microscopy

Giant unilamellar vesicles (GUVs) were prepared by electroformation⁴²⁻⁴⁴ on optically transparent and electrically conductive indium tin oxide (ITO) coated glass slides (SPI Supplies, Unterföhring, Germany) in a home-made preparation chamber consisting of a closed bath imaging chamber RC-21B mounted on a P-2 platform (both from Warner Instruments Co., MA, USA) topped with a flow-through temperature block. *N*-Rh-DHPE (0.2 mol%) was added to a solution of the extracted β -cell lipids in chloroform, which was then spread onto an ITO-coated cover slip (20 μL , $c = 2 \text{ mg/mL}$), spin-coated at 800 rpm for 1 min and subsequently dried under vacuum for at least 2 h. Afterwards the lipids were hydrated in buffer containing the water-soluble fluorophore ATTO647 (5 mM Tris-HCl, 5 μM ATTO647, pH 7.4) within the preparation chamber and heated to 42 $^{\circ}\text{C}$, before a low frequency alternating current (AC) field (10 Hz, 1.7 V) was applied to the ITO electrodes by using a function generator (Thurlby Thandar Instruments TG315). After two hours of electroformation the temperature was slowly reduced (1 $^{\circ}\text{C}/\text{min}$) to room temperature. To remove the water soluble ATTO647 that was not enclosed in the interior of the vesicles, the preparation chamber was carefully rinsed with 2 mL of Tris buffer (5 mM Tris-HCl, pH 7.4). Thereafter, a region of interest for imaging of the GUVs was chosen under the microscope before $\sim 300 \mu\text{L}$ of peptide solution was added.

Images were recorded by a confocal laser scanning microscope (Biorad MRC 1024, extended for multiphoton excitation, now Zeiss, Germany) coupled via a side-port to an inverted microscope (Nikon, Eclipse TE-300DV, infinity corrected optics), enabling fluorescence excitation in the focal plane of an objective lens (Nikon Plan Apo 60 $\times$ WI, NA = 1.2, collar rim corr). Fluorescence of Bodipy-FL, *N*-rhodamine, and ATTO647 was sequentially acquired by alternating the excitation with the 488, 568, and 647 nm lines of a

KrAr-laser (Dynamic Laser, Salt Lake City, UT, USA). Signals were detected in three different PMT-channels (emission band pass filters 522 nm/FWHM 35 nm, 580 nm/FWHM 32 nm, and 680 nm/FWHM 32 nm). Emission of the fluorophore DAC was measured using a PMT-channel with an emission band pass filter of 455 nm/FWHM 30 nm, after two-photon excitation at 820 nm with a TiSa laser (Coherent Mira 900, Santa Clara, CA, USA). Image acquisition was controlled by the software LaserSharp2000 (formerly Biorad, now Zeiss). Analysis of the data was performed using the software Fiji (Max Planck Society for the Advancement of Science e.V., Munich, Germany). Images were background corrected and the contrast was enhanced.

Thioflavin T (ThT) fluorescence spectroscopy

The benzothiazole dye Thioflavin T (ThT) displays an enhanced fluorescence upon non-covalent binding to amyloid fibrils and has been used to monitor the kinetics of fibril growth. Vesicles were prepared by dissolving the corresponding lipid composition in phosphate buffer (10 mM NaH₂PO₄, 10 μM ThT, pH 7.4) to a concentration of 0.5 mg/mL. After sonication for 15 min, five freeze-thaw cycles were performed (thawing at 70 °C), followed by additional 5 min of sonication. The obtained vesicle solution was directly added to the lyophilized peptides. ThT fluorescence intensity was measured as a function of time at 482 nm after excitation at 440 nm in a 96-well plate at a stable temperature of 25 °C (96 Well Black Flat Bottom Polystyrene NBS™ Microplate, Corning, Wiesbaden) using a plate reader (Infinite M200, Tecan). The data were background corrected and normalized by dividing the intensity at every point by the intensity recorded for the final aggregates. Curves were fitted by a sigmoidal curve as described previously⁴⁵ using Origin 7 (OriginLab, Northampton, MA, USA).

Atomic force microscopy (AFM)

For the time-dependent AFM studies of peptide aggregation on muscovite mica, initially phosphate buffer (10 mM NaH₂PO₄, pH 7.4) was injected into the AFM fluid cell at room temperature to image the mica surface before peptide addition (AFM image shown in Figure S6 of the Supporting Information). Afterwards, 200 μL of peptide solution were slowly injected into the fluid cell and imaged until a high amount of fibrils was detected. This sample – exhibiting the aggregated peptide adsorbed on mica – was removed from the AFM setup, rinsed with ~ 6 mL of deionized water and dried using a gentle stream of nitrogen; all solvent was subsequently removed in the vacuum over night.

All AFM measurements were performed in the tapping mode on a MultiMode scanning probe microscope equipped with a NanoScope IIIa controller (Digital Instruments, Santa Barbara, CA, USA). The time-dependent measurements in buffer were carried out by using a J-Scanner (scan size 125 μm) and sharp nitride lever (SNL) probes mounted in a fluid cell (MTFML, both Veeco, Mannheim, Germany). Tips with nominal force constants of 0.32 N/m were used at driving frequencies around 9 kHz and drive amplitudes around 400 mV. AFM images of the dried samples were achieved by using an E-Scanner (scan size 15 μm) and a MMMC cantilever holder (both Veeco, Mannheim, Germany) equipped with a silicon SPM sensor (PPP-NCHR, NanoandMore, Wetzlar, Germany). The tips used exhibit a force constant of 42 N/m and dried samples were scanned in air with drive frequencies around 320

kHz and drive amplitudes between 147 and 522 mV. All AFM measurements were performed at room temperature and scan frequencies between 0.75 and 1.5 Hz. Height and amplitude images of samples regions were acquired with resolutions of 512×512 pixels. For image analysis and processing, the software Nanoscope 5 (Veeco Instruments, Mannheim, Germany) and Origin 7 (OriginLab, Northampton, MA, USA) was used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the DFG and the Max Planck Society (IMPRS of Chemical Biology, Dortmund). The lipid analyses described were performed at the Kansas Lipidomics Research Center Analytical Laboratory (Manhattan, KS, USA). The Kansas Lipidomics Research Center is supported by the National Science Foundation (EPS 0236913, MCB 0455318, DBI 0521587), Kansas Technology Enterprise Corporation, K-IDEA Networks of Biomedical Research Excellence (INBRE) of National Institute of Health (P20RR16475), and Kansas State University. The INS-1E cell line was a gift from the group of Dr. Pierre Maechler (Geneva University Hospital, Switzerland). We are grateful to Simone Möbitz for technical assistance.

References

1. Dobson CM. Protein folding and misfolding. *Nature*. 2003; 426:884–890. [PubMed: 14685248]
2. Morgado I, Fändrich M. Assembly of Alzheimer's A β peptide into nanostructured amyloid fibrils. *Curr Opin Colloid Interface Sci*. 2011 in press.
3. Eichner T, Radford SE. A diversity of assembly mechanisms of a generic amyloid fold. *Mol Cell*. 2011; 43:8–18. [PubMed: 21726806]
4. Westermarck P. Aspects on human amyloid forms and their fibril polypeptides. *FEBS J*. 2005; 272:5942–5949. [PubMed: 16302959]
5. DeToma AS, Salamekh S, Ramamoorthy A, Lim MH. Misfolded proteins in Alzheimer's disease and type II diabetes. *Chem Soc Rev*. 2011; 40:1039/c1cs15112f
6. Westermarck P, Wernstedt C, Wilander E, Hayden DW, O'Brien TD, Johnson KH. Amyloid fibrils in human insulinoma and islets of Langerhans of the diabetic cat are derived from a neuropeptide-like protein also present in normal islet cells. *Proc Natl Acad Sci U S A*. 1987; 84:3881–3885. [PubMed: 3035556]
7. Cooper GJS, Willis AC, Clark A, Turner RC, Sim RB, Reid KBM. Purification and characterization of a peptide from amyloid-rich pancreases of type 2 diabetic patients. *Proc Natl Acad Sci U S A*. 1987; 84:8628–8632. [PubMed: 3317417]
8. Nishi M, Sanke T, Nagamatsu S, Bell GI, Steiner DF. Islet Amyloid Polypeptide – A new β cell secretory product related to islet amyloid deposits. *J Biol Chem*. 1990; 265:4173–4176. [PubMed: 2407732]
9. Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun*. 1984; 120:885–890. [PubMed: 6375662]
10. Selkoe DJ, Abraham CR, Podlisny MB, Duffy LK. Isolation of lowmolecular-weight proteins from amyloid plaque fibers in Alzheimer's disease. *J Neurochem*. 1986; 146:1820–1834. [PubMed: 3517233]
11. Khemtémourian L, Killian JA, Höppener JWM, Engel MFM. Recent Insights in Islet Amyloid Polypeptide-Induced Membrane Disruption and Its Role in β -Cell Death in Type 2 Diabetes Mellitus. *Exp Diabetes Res*. 2008; 10:1155/2008/421287
12. Höppener JW, Lips CJ. Role of islet amyloid in type 2 diabetes mellitus. *Int J Biochem Cell Biol*. 2006; 38:726–736. [PubMed: 16459127]

13. Janson J, Laedtke T, Parisi JE, O'Brien P, Petersen RC, Butler PC. Increased risk of type 2 diabetes in Alzheimer disease. *Diabetes*. 2004; 53:474–481. [PubMed: 14747300]
14. Sims-Robinson C, Kim B, Rosko A, Feldman EL. How does diabetes accelerate Alzheimer disease pathology? *Nat Rev Neurol*. 2010; 6:551–559. [PubMed: 20842183]
15. O'Nuallain B, Williams AD, Westermarck P, Wetzel R. Seeding Specificity in Amyloid Growth Induced by Heterologous Fibrils. *J Biol Chem*. 2004; 279:17490–17499. [PubMed: 14752113]
16. Yan LM, Velkova A, Tatarek-Nossol M, Andreetto E, Kapurniotu A. IAPP Mimic Blocks A β Cytotoxic Self-Assembly: Cross-Suppression of Amyloid Toxicity of A β and IAPP Suggests a Molecular Link between Alzheimer's Disease and Type II Diabetes. *Angew Chem Int Ed*. 2007; 46:1246–1252.
17. Turk J, Wolf BA, Lefkowitz JB, Stump WT, McDaniel ML. Glucose-induced phospholipid hydrolysis in isolated pancreatic islets: quantitative effects on the phospholipid content of arachidonate and other fatty acids. *Biochim Biophys Acta*. 1986; 879:399–409. [PubMed: 3535899]
18. Hallberg A. Effects of starvation and different culture conditions on the phospholipid content of isolated pancreatic islets. *Biochim Biophys Acta*. 1984; 796:328–335. [PubMed: 6391554]
19. Reis O, Winter R, Zerda TW. The Effect of High External Pressure on DPPC-Cholesterol Multilamellar Vesicles - A Pressure-Tuning Fourier-transform Infrared Spectroscopy Study. *Biochim Biophys Acta, Biomembr*. 1996; 1279:5–16.
20. Reis O, Winter R. Pressure and Temperature Effects on Conformational and Hydrational Properties of Lamellar and Bicontinuous Cubic Phases of the Fully Hydrated Monoacylglyceride Monoelaidin - A Fourier Transform-Infrared Spectroscopy Study Using the Diamond Anvil Technique. *Langmuir*. 1998; 14:2903–2909.
21. Wong PT, Schauerte JA, Wissner KC, Ding H, Lee EL, Steel DG, Gafni A. Amyloid-beta membrane binding and permeabilization are distinct processes influenced separately by membrane charge and fluidity. *J Mol Biol*. 2009; 286:81–96. [PubMed: 19111557]
22. Valincius G, Heinrich F, Budvytyte R, Vanderah DJ, McGillivray DJ, Sokolov Y, Hall JE, Lösche M. Soluble Amyloid β -Oligomers Affect Dielectric Membrane Properties by Bilayer Insertion and Domain Formation: Implications for Cell Toxicity. *Biophys J*. 2008; 95:4845–4861. [PubMed: 18515395]
23. Kapoor S, Werkmüller A, Denter C, Zhai Y, Markgraf J, Weise K, Opitz N, Winter R. Temperature–pressure phase diagram of a heterogeneous anionic model biomembrane system: Results from a combined calorimetry, spectroscopy and microscopy study. *Biochim Biophys Acta, Biomembr*. 2011; 1808:1187–1195.
24. Seeliger J, Evers F, Jeworrek C, Kapoor S, Weise K, Andreetto E, Tolan M, Kapurniotu A, Winter R. Cross-amyloid Interaction of A β and IAPP at Lipid Membranes. *Angew Chem Int Ed*. 2012; 1002:201105877.
25. Knight JD, Miranker AD. Phospholipid catalysis of diabetic amyloid assembly. *J Mol Biol*. 2004; 341:1175–1187. [PubMed: 15321714]
26. Jha S, Sellin D, Seidel R, Winter R. Amyloidogenic Propensities and Conformational Properties of ProIAPP and IAPP in the Presence of Lipid Bilayer Membranes. *J Mol Biol*. 2009; 389:907–920. [PubMed: 19427320]
27. Hebda JA, Miranker AD. The interplay of catalysis and toxicity by amyloid intermediates on lipid bilayers: insights from type II diabetes. *Annu Rev Biophys*. 2009; 38:125–152. [PubMed: 19416063]
28. Evers F, Jeworrek C, Tiemeyer S, Weise K, Sellin D, Paulus M, Struth B, Tolan M, Winter R. Elucidating the mechanism of lipid membrane-induced IAPP fibrillogenesis and its inhibition by the red wine compound resveratrol: a synchrotron X-ray reflectivity study. *J Am Chem Soc*. 2009; 131:9516–9521. [PubMed: 19583433]
29. Lopes DHJ, Meister A, Gohlke A, Hauser A, Blume A, Winter R. Mechanism of Islet Amyloid Polypeptide Fibrillation at Lipid Interfaces Studied by Infrared Reflection Absorption Spectroscopy. *Biophys J*. 2007; 93:3132–3141. [PubMed: 17660321]
30. Jayasinghe SA, Langen R. Lipid membranes modulate the structure of islet amyloid polypeptide. *Biochemistry*. 2005; 44:12113–12119. [PubMed: 16142909]

31. Weise K, Radovan D, Gohlke A, Opitz N, Winter R. Interaction of hIAPP with Model Raft Membranes and Pancreatic β -Cells: Cytotoxicity of hIAPP Oligomers. *ChemBioChem*. 2010; 11:1280–1290. [PubMed: 20440729]
32. McLaurin J, Chakrabartty A. Characterization of the interactions of Alzheimer beta-amyloid peptides with phospholipid membranes. *Eur J Biochem*. 1997; 245:355–363. [PubMed: 9151964]
33. Williams TL, Serpell LC. Membrane and surface interactions of Alzheimer's A β peptide – insights into the mechanism of cytotoxicity. *FEBS J*. 2011; 278:3905–3917. [PubMed: 21722314]
34. Sani MA, Gehman JD, Separovic F. Lipid matrix plays a role in Abeta fibril kinetics and morphology. *FEBS Lett*. 2011; 585:749–754. [PubMed: 21320494]
35. Curtain CC, Ali FE, Smith DG, Bush AI, Masters CL, Barnham KJ. Metal ions, pH, and cholesterol regulate the interactions of Alzheimer's disease amyloid-beta peptide with membrane lipid. *J Biol Chem*. 2003; 278:2977–2982. [PubMed: 12435742]
36. Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P. Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology*. 2004; 145:667–678. [PubMed: 14592952]
37. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol*. 1959; 37:911–917. [PubMed: 13671378]
38. Bartlett GR. Colorimetric assay methods for free and phosphorylated glyceric acids. *J Biol Chem*. 1959; 234:469–471. [PubMed: 13641242]
39. Tatulian SA. Attenuated total reflection Fourier transform infrared spectroscopy: a method of choice for studying membrane proteins and lipids. *Biochemistry*. 2003; 42:11898–11907. [PubMed: 14556620]
40. Cordeiro Y, Kraineva J, Suarez MC, Tempesta AG, Kelly JW, Silva JL, Winter R, Foguel D. Fourier transform infrared spectroscopy provides a fingerprint for the tetramer and for the aggregates of transthyretin. *Biophys J*. 2006; 91:957–967. [PubMed: 16698785]
41. Cordeiro Y, Kraineva J, Gomes MPB, Lopes MH, Martins VR, Lima LMTR, Foguel D, Winter R, Silva JL. The amino-terminal PrP domain is crucial to modulate prion misfolding and aggregation. *Biophys J*. 2005; 89:2667–2676. [PubMed: 16040743]
42. Angelova MJ, Dimitrov DS. Liposome electroformation. *Faraday Disc Chem Soc*. 1987; 81:303–311.
43. Dimitrov DS, Angelova MJ. Lipid swelling and liposome formation mediated by electric fields. *Bioelectrochem Bioenerg*. 1988; 19:323–333.
44. Ambroggio, EE.; Bagatolli, LA. Giant Unilamellar Vesicles, Fluorescence Microscopy and Lipid-Peptide Interactions. In: Castanho, MARB., editor. *Membrane Active Peptides: Methods and Results on Structure and Function*. Intl. Univ. Line; USA: 2009. p. 169
45. Nielsen L, Khurana R, Coats A, Frokjaer S, Brange J, Vyas S, Uversky VN, Fink AL. Effect of Environmental Factors on the Kinetics of Insulin Fibril Formation: Elucidation of the Molecular Mechanism. *Biochemistry*. 2001; 40:6036–6046. [PubMed: 11352739]
46. Andreetto E, Yan LM, Tatarek-Nossol M, Velkova A, Frank R, Kapurniotu A. Identification of Hot Regions of the Ab–IAPP Interaction Interface as High-Affinity Binding Sites in both Cross- and Self-Association. *Angew Chem Int Ed*. 2010; 49:3081–3085.

Abbreviations

IAPP	islet amyloid polypeptide
Aβ	amyloid- β
T2DM	type-2 diabetes mellitus
AD	Alzheimer's disease
ATR-FTIR	attenuated total reflection Fourier-transform infrared spectroscopy

AFM	atomic force microscopy
PC	phosphatidylcholine
PE	phosphatidylethanolamine
SM	sphingomyelin
PI	phosphatidylinositol
PS	phosphatidylserine
PA	phosphatidic acid
PG	phosphatidylglycerol
LUV	large unilamellar vesicle
GUV	giant unilamellar vesicle
<i>N</i>-Rh-DHPE	<i>N</i> -(lissamine-rhodamine-B-sulfonyl)-1,2-dihexadecanoyl- <i>sn</i> -glycero-3-phosphoethanolamine

Highlights

- Isolation and characterization of membrane lipids from a β -cell line of rat.
- Interaction studies of IAPP, A β , and both peptides with isolated β -cell membranes.
- Detection of IAPP-A β heterocomplex formation in the peptide mixture.
- Different properties of the heterocomplexes compared to the individual peptides.
- IAPP-A β heterocomplexes still permeabilize the isolated β -cell membranes, at a drastically reduced rate, however.

Figure 1. Phospholipid content of the cellular lipids isolated from the rat insulinoma-derived INS-1E β -cell line by chloroform/methanol extraction. The lipid mixture was analyzed by mass spectrometry at the Kansas Lipidomics Research Center Analytical Laboratory. PC: phosphatidylcholine, PE: phosphatidylethanolamine, SM: sphingomyelin, PI: phosphatidylinositol, PS: phosphatidylserine, PA: phosphatidic acid, and PG: phosphatidylglycerol.

Figure 2.

(a) Temperature-dependence of the symmetric CH₂-stretching mode wavenumber, $\tilde{\nu}_{\text{sym}}(\text{CH}_2)$ of the isolated β -cell membrane system. Black lines indicate subsequent changes in slope that can be ascribed to onsets of phase transitions. (b) Fluorescence microscopy image of an *N*-Rh-DHPE labeled giant unilamellar vesicle (GUV) composed of isolated β -cell membrane lipids at 25 $^\circ\text{C}$. The vesicle, which exhibits a diameter of $\sim 26 \mu\text{m}$, shows a homogeneous lipid distribution. The scale bar corresponds to 10 μm .

Figure 3.

Shift of the amide-I' band of (a) IAPP ($c = 3 \mu\text{M}$), (b) A β ($c = 3 \mu\text{M}$), and (c) IAPP + A β ($c = 3 \mu\text{M}$ each) with time after injection into an ATR cell containing a membrane of the isolated β -cell lipids at $T = 25^\circ\text{C}$. Left: Primary ATR-FTIR spectra after buffer subtraction and baseline correction. Right: Area normalization of the spectra shown on the left.

Figure 4.

Time-dependent fluorescence microscopy images of the interaction of 5 μM C-terminally labeled IAPP (IAPP-K-Bodipy-FL, green) with *N*-Rh-DHPE labeled GUVs composed of the isolated β -cell membrane lipids (red) filled with buffer containing the fluorophore ATTO647 (blue). IAPP immediately adsorbs to the GUV, which readily leads to membrane leakage and GUV disintegration. The scale bars represent 10 μm .

Figure 5.

Interaction of 5 μM N-terminally labeled A β (Fluorescein-A β , green) with N-Rh-DHPE labeled GUVs composed of the isolated β -cell membrane lipids (red) filled with buffer containing the fluorophore ATTO647 (blue). Fluorescence microscopy images show initially a homogeneous distribution of A β within the buffer solution. Membrane leakage is observed after 40 h, however. Arrows point at A β aggregates formed in solution. The scale bars represent 10 μm .

Figure 6.

Effect of a mixture of IAPP-K-Bodipy-FL and Fluorescein-A β ($c = 5 \mu\text{M}$ each, green) on the integrity of GUVs composed of isolated β -cell membrane lipids (red) filled with buffer containing the fluorophore ATTO647 (blue). Already after 30 min an accumulation of the peptides at the GUV was obtained, which led to membrane permeabilization with time. Arrows indicate IAPP/A β aggregates in solution. The scale bars represent 10 μm .

Figure 7. Localization of IAPP-K-Bodipy-FL ($c = 5 \mu\text{M}$, green) and DAC-A β ($c = 5 \mu\text{M}$, gray) in the presence of GUVs composed of isolated β -cell membrane lipids at $t = 30 \text{ min}$ and $t = 2 \text{ h}$. Accumulation of both peptides at the lipid interface is visible after 30 min and increases with time.

Figure 8.

ThT fluorescence assay of (a) IAPP ($c = 3 \mu\text{M}$) and (b) IAPP + A β ($c = 3 \mu\text{M}$ each) in the presence of lipid vesicles of different compositions (black: without vesicles, red: lipid vesicles consisting of DOPC/DOPG 7:3 (molar ratio), green: lipid vesicles comprising 15% DOPC, 10% DOPG, 40% DPPC, 10% DPPG, 25% cholesterol, blue: lipid vesicles prepared of the isolated β -cell membrane lipids). Data are the average ($\pm$ standard deviation) from six assays.

Figure 9.

Time-dependent AFM images of the aggregation of IAPP + Aβ ($c = 3 \mu\text{M}$ each) on mica, imaged in phosphate buffer. The same scan area was followed over time and the AFM images are shown with a vertical color scale from dark brown to white corresponding to an overall height of 8 nm. To guide the eye, one fibril that recurs in all images is marked with a blue rectangle. The scale bar included corresponds to 1 μm for all images.

Figure 10.

AFM images of the dried samples of IAPP (a), IAPP + A β (b), and A β (c) aggregated in phosphate buffer on mica for 4 h (IAPP, $c = 3 \mu\text{M}$), 24 h (IAPP + A β , $c = 3 \mu\text{M}$ each), and 72 h (A β , $c = 3 \mu\text{M}$) at room temperature. On the left hand side of each panel, the whole scan area is shown whereas the concomitant section profile of the marked detail of the AFM image is given at the right hand side. The horizontal black line in the zoomed AFM image is the localization of the section analysis, indicating the vertical distances between pairs of arrows. For IAPP, protofibrils and mature fibrils with mean heights of $0.67 \pm 0.15 \text{ nm}$ ($n = 216$) and $5.21 \pm 1.62 \text{ nm}$ can be detected, respectively (panel a). In contrast, the equimolar mixture of IAPP and A β aggregates much slower and thinner fibrils with a mean height of $3.52 \pm 0.68 \text{ nm}$ ($n = 155$) are observed after 24 h. The protofibrils exhibit a mean height of

0.81 ± 0.14 nm ($n = 132$, panel b). Finally, only a few oligomers and fibrils were observed after 72 h for the aggregation of A β . The scale bar included corresponds to 1 μ m.

Table 1

Lag time and apparent growth constant k_{app} for the fibril formation of IAPP and IAPP + A β in the presence of different lipid vesicle compositions.

peptide(s)	lipid vesicles	lag time / h	$k_{\text{app}} / \text{h}^{-1}$
IAPP			
	-	4.21 ± 0.24	0.85 ± 0.14
	DOPC/DOPG (7:3)	1.39 ± 0.10	2.56 ± 0.04
	anionic raft membrane	0.25 ± 0.19	5.72 ± 0.05
	β -cell membrane	0.35 ± 0.30	3.27 ± 0.08
IAPP + A β			
	-	3.81 ± 0.57	0.80 ± 0.35
	DOPC/DOPG (7:3)	4.28 ± 0.09	1.68 ± 0.05
	anionic raft membrane	0.35 ± 0.17	3.94 ± 0.05
	β -cell membrane	0.22 ± 0.17	4.16 ± 0.04