Effects of beta cell granule components on human islet amyloid polypeptide fibril formation

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Abstract Formation of amyloid-like fibrils in a solution of human islet amyloid polypeptide (hIAPP) with and without the presence of other β-cell granule components was studied in vitro. Insulin at less than equimolar concentration strongly inhibited hIAPP fibrillogenesis. Proinsulin had a weaker inhibitory effect while C-peptide, Ca^{2+} and Zn^{2+} each individually enhanced fibril formation. C-peptide combined with Ca^{2+} had an inhibitory effect. Since IAPP was found almost exclusively in the halo fractions of isolated islet secretory granules, primarily the concentrations of C-peptide, Ca^{2+} and possibly proinsulin may be crucial for the native state of IAPP. It is concluded that an imbalance between fibril formation enhancers and inhibitors may be of importance in the pathogenesis of amyloid in the islets of Langerhans.

Key words: Fibril; Islet amyloid polypeptide; Non-insulin-dependent diabetes mellitus; In vitro; Granule

1. Introduction

Islet amyloid polypeptide (IAPP) is a 37 amino acid residue polypeptide, mainly expressed in β-cells [1]. It is in human a product of a 69 amino acid precursor by cleavage at double basic amino acid residues [2–4]. IAPP is stored together with insulin, C-peptide and some other peptides in the β-cell secretory granules [5] and the components are released together at exocytosis. The normal function of IAPP is poorly understood but its structure supports a putative hormonal role [6]. A multitude of effects have been obtained experimentally and functions as glucoregulatory hormone, calcium regulating polypeptide or appetite regulator have been proposed (reviewed in [7]). Autocrine and paracrine effects have also been implicated. The role of IAPP in the development of diabetes is not known.

IAPP gives rise to amyloid deposits in human islets of Langerhans and deposition of amyloid is the most characteristic islet lesion in non-insulin-dependent diabetes mellitus (NIDDM) [8]. Amyloid forms by aggregation of IAPP into characteristic thin fibrils. The reason for islet amyloid development is incompletely understood but an obvious prerequisite for amyloid fibril formation is an amyloidogenic amino acid sequence, present in a central segment (positions 24–29) of IAPP in human, cat and raccoon but not in mouse, rat or hamster [6,9]. Thus, both full length human IAPP and a central fragment of human IAPP (hIAPP20–29) form fibrils with amyloid properties in vitro while corresponding rat IAPP peptides do not [10]. This fibril formation of hIAPP occurs rapidly and spontaneously in aqueous solutions.

With this knowledge, we hypothesized that there normally may exist a mechanism which hinders the formation of amyloid fibrils from IAPP in β-cells and in the islets of Langerhans, the only sites where it is expressed at high concentrations. Since IAPP is stored together with insulin, proinsulin and C-peptide in the secretory granules, the influence of these three latter polypeptides on fibril formation was studied in vitro. Both calcium and zinc are present at high concentration in the β-cell secretory granules and therefore the effect of these ions on fibril formation was included in the investigation. Finally, we determined the concentration of IAPP in the two β-cell granule compartments.

2. Materials and methods

2.1. Polypeptides and chemicals

Human full-length IAPP ('amylin') was from Peninsula (Belmont, CA). Human C-peptide was from Eli Lilly (Indianapolis, IN) and C-terminally acetylated human C-peptide was synthesized by Chiron Mimotopes Peptide Systems (Clayton, Victoria, Australia). Human insulin (humulin) and proinsulin were from Eli Lilly. Human C-peptide was dissolved (40 mg/ml) in distilled water. All other polypeptides were dissolved in dimethylsulfoxide (DMSO), IAPP at a concentration of 10 mg/ml and insulin, proinsulin and acetylated C-peptide at a concentration of 40 mg/ml. Zinc chloride and calcium chloride were dissolved in distilled water at a concentration of 100 mmol/l.

2.2. In vitro fibril formation

The in vitro fibril formation experiments were performed in 50 μl batches in small glass test tubes and all experiments were performed at least two times. The final concentration of IAPP was always 250 μmol/l. The peptides were mixed in two different ways. In one series of experiments, C-peptide and/or insulin was diluted with double distilled water to an appropriate concentration. IAPP was then added and mixed in vigorously. In another series of experiments with identical final polypeptide concentrations, polypeptide solutions and DMSO were first mixed and water was then added. The concentrations of insulin and C-peptide varied (Tables 1 and 2) while the final amount of DMSO and water was identical in all samples. When calcium or zinc chloride was included, polypeptide solutions and DMSO were first mixed, $CaCl_2$ or $ZnCl_2$ thereafter added and finally water. The final pH in all the mixtures was between 5 and 6, as estimated by spotting aliquots on pH paper. The mixtures were left at room temperature [10] and after incubation for 15 min, 1, 2 and 4 days and in some experiments 2 weeks, the mixtures were inspected for appearance of visible precipitate. Droplets (1 μL) were air-dried on glass slides, stained with alkaline Congo red and examined in a polarization microscope for green birefringence, typical of amyloid. Occurrence of amyloid-like material was semiquantitatively estimated according to a score from 0 to 4+.

In some preparations the diameter (arbitrary units) of 25 randomly chosen amyloid-like particles was measured in the light microscope with the aid of a scale in the eyepiece.

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Other droplets were diluted 1:10 with distilled water, placed on formvar-coated copper grids, negatively contrasted with 1% sodium phosphotungstate and studied in a Jeol 1200 electron microscope at 80 kV.

2.3. Analysis of IAPP–C-peptide aggregates

For further studies of the incorporation of C-peptide in the fibrils, reversed phase high performance liquid chromatography (RP-HPLC) was performed. Selected samples of IAPP/C-peptide mixtures were washed with distilled water, centrifuged and the small pellet lyophilized. The pellets were dissolved in 40 μl 70% formic acid and applied to a TSK ODS 120 T C_{18} HPLC column (LKB, Bromma, Sweden). The material was eluted from the column with a linear gradient of 70% acetonitrile in 0.1% trifluoroacetic acid and the eluate was monitored at 226 nm.

2.4. Glycation and solubility of IAPP-fibrils

Fibrils, made from IAPP and stored at room temperature for 1 and 5 months, respectively, were tested. In order to test whether glycation of IAPP affects fibril solubility, 5-month-old fibrils were glycated in vitro. For this, fibrils were incubated in the presence of 0.5 M glucose for 15 days at +37°C, as described [11,12].

IAPP fibrils (50 μg) were suspended for 5 h in 1 ml 6 M guanidine HCl in 0.1 M Tris HCl buffer, pH 8.0, containing 0.1 M dithiothreitol. The suspension was centrifuged, washed with distilled water and examined for the presence of amyloid-like fibrils.

2.5. Analysis of IAPP and insulin in islet β-cell granule compartments

Islets from 9 female FVB/N mice, 3–6 months old, were isolated by the collagenase method [13], and secretory granule-enriched fractions were prepared, lysed and separated by discontinuous sucrose gradient ultracentrifugation, into pH 5.4 soluble (halo) and insoluble (core) fractions as described [14]. The concentration of insulin in the fractions was determined by an insulin radioimmunoassay kit (Pharmacia, Uppsala, Sweden) with rat insulin as standard. IAPP was determined by a previously described radioimmunoassay [15], with rat/mouse IAPP as standard.

2.6. Statistics

When applicable, values are given as mean ± S.E.M. For comparison of means, the Mann-Whitney unpaired non-parametric test was used.

3. Results

3.1. IAPP fibrils

Dilution of the IAPP solution with water resulted in Congo red-positive material that was visible within 12 h. The fibrils tended to aggregate and after 48–96 h much of the material appeared in the light microscope as small often rounded particles which showed strong affinity for Congo red giving a bright green birefringence after such staining. Electron microscopically, amyloid-like, fine fibrils of about 75 Å in width were seen (Fig. 1). They were usually less than 500 nm long. Commonly, two or more fibrils were aggregated into small bundles. No amorphous material was seen electron microscopically.

3.2. Effects of C-peptide

When IAPP was added to any of the tested concentrations of C-peptide, a fine precipitate was formed rapidly. When stained with Congo red, large homogeneous and, compared to pure IAPP-fibrils, more weakly stained aggregates appeared, which exhibited a green birefringence (Table 1). Congo red-positive material appeared more rapidly as compared with pure IAPP and was visible already after 1 h. The amount of such material increased with increasing C-peptide concentration. Both C-peptide preparations behaved identically. Electron microscopically, fibrils appeared that often were longer as compared to the fibrils formed from pure IAPP. Furthermore, large aggregates of sometimes parallel fibrils were commonly seen (Fig. 1B). Some amorphous material also appeared. The two different ways of mixing the peptides gave identical results. C-peptide alone did not give rise to fibrils.

3.3. Effects of insulin and proinsulin

Dilution with water of the insulin solution resulted in a visible precipitate, except at the lowest tested insulin concentration. The precipitate did not bind Congo red and showed white but no green birefringence. When IAPP was added to a solution containing 170–1400 μmol/l insulin, no amyloid-like material was found light or electron microscopically (Table 2). Even at lower concentrations, insulin significantly inhibited IAPP fibril formation (Table 2). In the mixtures of proinsulin and IAPP a small amount of amyloid-like material was seen but much less than in the IAPP–C-peptide mixtures or when IAPP alone was diluted with water. The mixtures containing IAPP (250 μmol/l), insulin (700 μmol/l) and C-peptide (650 μmol/l) behaved like those containing only IAPP and insulin. Thus, in the presence of insulin, C-peptide did not enhance fibril formation.

The above results did hold true for a period of 2 days to more than 1 week of incubation at room temperature. However, when the polypeptide mixtures were allowed to incubate for more than 2 weeks at room temperature, some material stained like amyloid by Congo red appeared.

3.4. Effects of zinc and calcium

Zinc chloride concentration-dependently enhanced formation of amyloid-like material (Table 3). When IAPP was incubated with 100–500 μmol/l of zinc chloride, more and larger Congo red-positive particles appeared compared to material formed from IAPP alone (diameters of amyloid-like particles at 0 and 200 μmol/l of Zn^{2+} were 0.50 ± 0.08 and 1.25 ± 0.14 arbitrary units, respectively, $P < 0.001$). Electron microscopi-

Table 1
Effect of different islet β-cell granule components on the formation of amyloid-like fibrils from a solution of human IAPP

IAAP	Proinsulin	C-peptide	Amyloid score
250	900	–	0
250	450	–	2+
250	225	–	2+
250	–	1300	3–4+
250	–	650	3–4+
250	–	–	3+

All values are μmol/l.

Table 2
Effect of various concentrations of insulin on the formation of amyloid-like fibrils from a 250 μmol/l solution of human IAPP

Insulin concentration (μmol/l)	0	17	35	85	170	700	1400
Amyloid score	3+	1+	1+	1+	0	0	0

Table 3
Effect of various concentrations of zinc chloride on the formation of amyloid-like fibrils from a 250 μmol/l solution of human IAPP

$ZnCl_2$ concentration (μmol/l)	0	25	50	100	200	500	1000
Amyloid score	2+	2+	2+	3+	4+	3+	2+

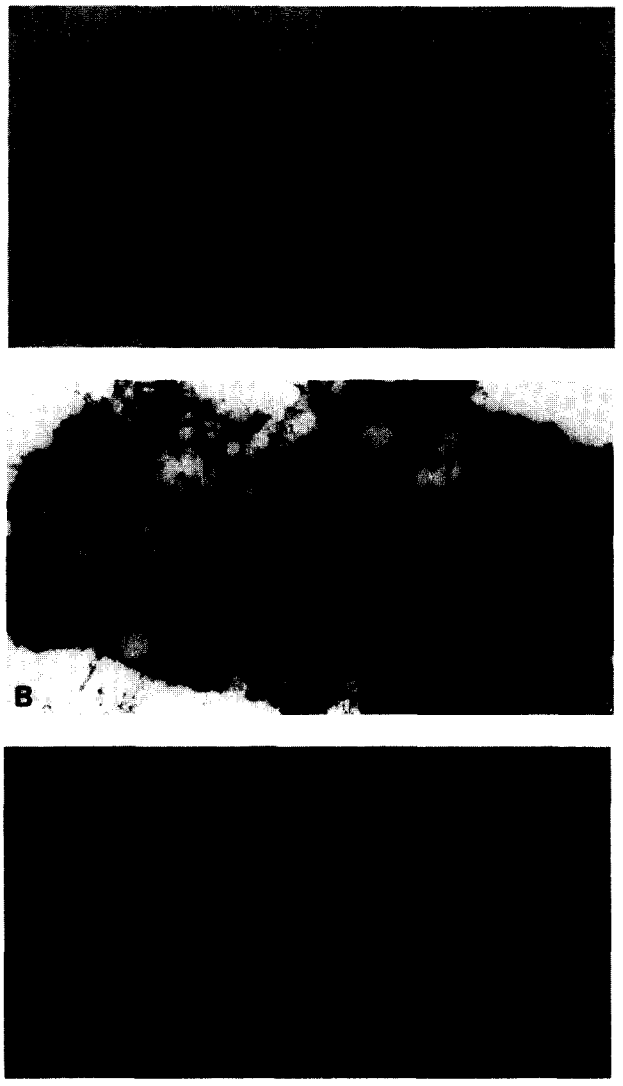

Fig. 1. Fibrils formed from human IAPP. Bar = 200 nm. A: Amyloid-like fibrils formed from IAPP. The fibrils are thin, rigid and short. B: Fibrils formed from IAPP in the presence of C-peptide. The thin fibrils are aggregated into thick bundles. C: Fibrils formed from IAPP in the presence of zinc chloride. The fibrils are long and even.

cally, the fibrils varied in length but many were much longer (often >2 μm) than those formed from IAPP in absence of zinc (Fig. 1C). Similar effects, including the occurrence of large Congo red-positive particles, were seen with calcium chloride which also enhanced fibril formation at the tested concentrations (5, 10 and 20 mmol/l). In contrast, when IAPP was incubated with C-peptide (650 μmol/l) and calcium chloride (10 or 20 mmol/l), a significant inhibition (amyloid score 1+) of the formation of amyloid-like fibrils occurred. The mixing of insulin (700 μmol/l), C-peptide (650 μmol/l) and calcium chloride (5, 10 or 20 mmol/l) with IAPP completely abolished fibril formation.

3.5. Analysis of the IAPP–C-peptide aggregates

When the amyloid-like precipitate, which appeared after mixing C-peptide with IAPP, was dissolved in formic acid and run on RP-HPLC, two peaks of similar size were obtained (not shown). The peaks corresponded exactly to the elution positions of IAPP and C-peptide, respectively.

3.6. Solubility of IAPP fibrils

All IAPP-fibrils tested were insoluble in 6 M guanidine HCl. In this respect there was no difference between fibrils that were one month old, five months old or glycated in vitro.

3.7. IAPP in secretory granules

The total amount of IAPP was 30-fold higher in pH 5.4 soluble fractions of secretory granules compared to the insoluble fractions. The amount of immunoreactive insulin was 4-fold higher in the fraction containing secretory granule cores compared to the soluble fractions (Table 4). As seen, the molar concentration of immunoreactive insulin in the soluble fractions is 10 times higher than that of IAPP. It should also be noted that the extractable amount of IAPP is about 2% of insulin on a molar basis.

4. Discussion

Most amyloid fibril proteins are truncated forms derived from larger normal or mutant precursors where the precursor itself rarely is amyloidogenic. In islet amyloid, so far only full length, non-mutant IAPP has been identified. Formation of amyloid can be regarded as a form of off-pathway aggregation of proteins, possibly from a folding intermediate [16]. Aggregation of proteins during the folding process is believed to be normally abolished by chaperones. Human IAPP has an unusually strong tendency to form amyloid-like fibrils spontaneously from aqueous solutions [10]. Therefore, a mechanism that hinders fibrillogenesis is probably present in the endoplasmic reticulum and β-cell secretory granules where IAPP must be present at fairly high concentration.

This study clearly shows that several islet β-cell granule components affect fibril formation of IAPP. At the pH, at which the *in vitro* experiments were performed, IAPP is positively and C-peptide negatively charged, respectively, while insulin is close to its isoelectric point. A binding capacity of IAPP to C-peptide was therefore expected and C-peptide not only enhanced the fibrillogenesis but was incorporated in the fibrils as revealed by the HPLC study. Furthermore, Ca^{2+} and Zn^{2+} each individually strongly enhanced formation of amyloid-like material. However, C-peptide combined with Ca^{2+} acted as an inhibitor of fibrillogenesis. Insulin, alone or in combination with other granule components, also turned out to be a potent inhibitor of fibrillogenesis. In the pancreas in vivo, the IAPP:insulin molar ratio is below 10% (which is in accordance with the present study) and even when present at a molar concentration much below that, insulin almost completely abolished fibril formation in vitro. These results, taken together, indicate that a complex balance between different granule components may exist that hinders IAPP amyloid formation. Hypothetically, a

Table 4
Extractable amount of IAPP and insulin in the soluble and non-soluble fractions of mouse beta cell granules

	soluble	non-soluble	*P* value
IAPP	364 ± 25	12 ± 2	>0.001
Insulin	3820 ± 496	14,320 ± 1230	>0.01

All values are pmol (mean ± S.E.M.).

disturbance in such a balance may lead to fibrillogenesis. If such a disturbance really occurs in islets with amyloid deposits is still to be shown.

Presence of calcium and zinc gave rise to longer IAPP fibrils and strongly increased formation of big amyloid-like particles. In the present study, this effect of Zn^{2+} was most evident at a concentration of 200 μmol/l which was close to equimolarity with IAPP. Zinc, as well as aluminium and iron, has also been shown to promote the Alzheimer β-protein aggregation in vitro [17]. Similar to what was found with IAPP, Zn^{2+} induces large amyloid-like particles from β-protein in vitro and zinc has therefore been proposed to play a role in the pathogenesis of amyloid brain lesion [18].

The exact site of normal storage of IAPP in the secretory granules has not been studied in detail previously. It has been suggested that IAPP most probably occurs in the translucent halo [19,20] although in one immune electron microscopy study the strongest IAPP immunoreactivity was found on the granule cores [5]. This latter finding could possibly be due to a preparation artefact but it could not be ruled out that IAPP is stored together with insulin on the granule core surface. The results of the present study clearly show that almost all immunoreactive IAPP goes with the soluble phase of the granules, i.e. the halos. Thus, our results show that IAPP is in close contact with several components that may affect fibril formation. Although most insulin is found within the granule cores, the concentration of insulin and proinsulin-forms in the halo is probably high enough to completely abolish fibril formation under normal condition. It should be emphasized that calcium when combined with C-peptide also inhibited IAPP fibrillogenenesis.

One property that native islet amyloid shares with Aβ-amyloid in neuritic plaques in Alzheimer's disease, is its unusual resistance to solvents like 6 M guanidine HCl and 8 M urea (solvents that dissolve other types of amyloid fibrils). It has been hypothesized that this resistance could be due to some secondary phenomenon like cross-linking or glycation of the protein. However, the present study indicates that the insolubility is a property of the IAPP-fibril as such.

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