Effect of pH and Insulin on Fibrillogenesis of Islet Amyloid Polypeptide *in Vitro*†

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ABSTRACT: Islet amyloid polypeptide (IAPP) is the constituent of amyloid deposits in pancreatic islets of type 2 diabetes in man, monkeys, and cats. This 37 amino acid peptide aggregates *in vitro* to form β-pleated sheet fibrils. Rodent IAPP has a different amino acid sequence and does not form amyloid either *in vitro* or *in vivo*. Fibrillogenic properties of human IAPP (hIAPP) were determined in vitro. The effect of pH and time course of fibril formation was studied by light scattering spectroscopy. Aggregation of $hIAPP_{1-37NH_2}$ and $hIAPP_{Tyr20-29}$ (0.25 mg/mL) was maximal at neutral/basic and acidic pH, respectively. The ultrastructure of $hIAPP_{1-37NH_2}$ fibrils (0.2 mg/mL) was examined using negative staining for electron microscopy. Short fibrils composed of 2 or more filaments were observed at pH 3−9 after 30 min incubation. At pH 7−9, IAPP fibrils formed a gel. After 6 months at pH 3, large sheets of parallel fibrils were seen. Specific binding of ^{125}I-$hIAPP_{1-37NH_2}$ to preformed IAPP fibrils detected by quantitative autoradiography and radioassay was maximal at pH 3. Binding was enhanced by insulin (3.7 nmol/L) and unaffected by glucose, calcium, glucagon, and apolipoprotein E. ^{125}I-$hIAPP_{1-37NH_2}$ bound specifically to islet amyloid in pancreatic tissue sections from type 2 diabetic patients. Conclusions: Binding to preformed IAPP fibrils is maximal at acid pH when hIAPP is largely in soluble form. IAPP is secreted together with insulin from the acidic secretory granules (pH 5.5) to the neutral pH of the extracellular space under normal conditions. These changes in pH together with increased accumulation of extracellular hIAPP in diabetes may promote amyloid formation.

Islet amyloid polypeptide (IAPP),[1] also known as "amylin", is the component peptide of amyloid deposits formed in pancreatic islets in type 2 (non-insulin-dependent) diabetes (Clark et al., 1987; Westermark et al., 1987). IAPP is a 37 amino acid peptide and is a normal component of islet β-cells: IAPP is colocalized with insulin in secretory granules and coreleased with insulin in response to β-cell secretagogues (Clark et al., 1989; Sanke et al., 1991). Species-specific differences in the amino acid sequence of IAPP (particularly in the amino acid region $IAPP_{20-29}$) determine its amyloidogenic characteristics (Figure 1). Rat IAPP (rIAPP) has 3 proline substitutions at residues 25, 28, and 29, which could explain the lack of fibril formation both *in vitro* and *in vivo* (Betsholz et al., 1989; Westermark et al., 1990). Primate and feline IAPP (lacking these proline residues) have the potential to aggregate *in vitro*, and diabetes-associated islet amyloid is present in monkeys, man, and cats (de Koning et al., 1993; Clark et al., 1990; Johnson & Stevens, 1973). However, since the amino acid sequence of IAPP is unchanged in diabetic patients, factors related to islet function in diabetes must also be important in amyloid formation from IAPP. Amyloid fibrils are characterized by a β-pleated sheet conformation of the component peptide as demonstrated by X-ray diffraction and CD spectroscopy (Glenner, 1980; Cort et al., 1994). The intrinsic β-structures of some amyloid peptides, including TTR, A4 peptide, and IgG, have been shown to be implicated in fibril formation (Benson et al., 1989; Halverson et al., 1990). Although IAPP is thought to be in β-pleated sheet conformation in fibrils (Glenner, 1988), the secondary structure of monomeric IAPP is not completely defined (Hubbard et al., 1991; Ashburn et al., 1992). Amyloid fibrils consist of one or more protofilaments; whereas transthyretin fibrils (TTR) are in tetrameric form (Blake et al., 1974), the structure of IAPP fibrils is unknown. Amyloid is formed in pancreatic islets in the extracellular spaces which are at neutral pH. In addition, IAPP fibrils have been identified at intracellular sites in cells overexpressing hIAPP, notably in β-cells of human insulinomas (O'Brien et al., 1994) and in islets of transgenic mice expressing the gene for hIAPP (hIAPP mice) (de Koning et al., 1994). However, IAPP fibrils are not present in insulin granules where the pH varies between 7.4 and 5.5 (Hutton et al., 1989; Clark et al., 1989). To determine the effects of pH and other factors present in pancreatic islets, such as glucose, insulin, and calcium, on fibrillogenesis of IAPP, formation of fibrils from synthetic $hIAPP_{1-37NH_2}$ and $hIAPP_{Tyr20-29}$ was examined by spectrophotometry, autoradiography, radioassay, and electron microscopy.

MATERIALS AND METHODS

Spectrophotometry. To examine the effects of pH on fibril formation in solution, lyophilized synthetic $hIAPP_{1-37NH_2}$ (Peninsula, St. Helens, U.K.) and $hIAPP_{Tyr20-29}$ (synthesized and purified to 93% purity by Star Biochemicals, Torrance, CA) were diluted to a concentration of 0.25 mg/mL in Tris-

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[1] Abbreviations: hIAPP, human islet amyloid polypeptide; rIAPP, rat islet amyloid polypeptide; UA, uranyl acetate; PTA, phosphotungstic acid; EM, electron microscopy; Tris-HCl, tris(hydroxymethyl)aminomethane; apoE, apolipoprotein E; TTR, transthyretin; TBT, Tris buffer Tween; IgG, immunoglobulin; hIAPP mice, transgenic mice expressing the gene for hIAPP.

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	N-IAPP	Islet amyloid polypeptide	C-IAPP
		1 20 29 37	
Human	TPIES:::HQVEKR	KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY	GKRNAVEVLKREPLNYLPL
Rat	--VG-GTNP--D--	----------R----L-PV-PP--------	----VA-DPN--S-DF-L-

FIGURE 1: Amino acid sequence of pro-IAPP in man and in rat. $IAPP_{1-37}$ is derived from pro-IAPP by proteolytic processing at both C- and N-terminal sites. $IAPP_{20-29}$ is the region responsible for the fibrillogenic potential of the molecule.

HCl + 0.01 M acetic acid, pH 2.5. The lowest concentration of IAPP that could be detected by measurement of light scattering at 372 nm on a double beam spectrophotometer was 0.12 mg/mL; however, better sensitivity was obtained with a concentration of 0.25 mg/mL. The concentration dependence of aggregation of $IAPP_{Tyr20-29}$ was examined over a range from 0.12 to 1 mg/mL at pH 2.5. The pH of the solution was changed stepwise from pH 2.5 by adding NaOH (0.1 M) sequentially into both the test and control cuvettes. Aggregation of IAPP into high macromolecular filamentous structures was monitored immediately after pH change. Solution of rIAPP was examined under the same conditions of pH and concentration. To determine the time course for aggregation, light scattering measurements were made in solutions of $hIAPP_{Tyr20-29}$, 0.25 mg/mL, pH 2.5, which were kept at room temperature with continuous agitation, and measurements were made at time 0, +10, +25, and +60 min.

Electron Microscopy. Synthetic IAPP peptides, as previously described, were made up in either Tris-HCl + acetic acid, pH 3.3; Tris-HCl, pH 7.4; or Tris-HCl + sodium acetate, pH 9, at a concentration of 0.2 mg/mL. The solutions were applied to carbon-coated Formvar on 400 mesh copper grids, blotted, negatively stained with 2% uranyl acetate (UA) or 1% phosphotungstic acid (PTA) in distilled water, washed, dried in air, and visualized under a JEOL electron microscope.

To determine changes in the fibril structure with time, solutions were examined after incubation at 37 °C for 30 min and at 4 °C for 24 h and 1 and 6 months. The same experiment was also performed by adding human insulin 500 mU/L (3.7 nmol/L) (Novo, Copenhagen, Denmark) to the solution, pH 7.4, at time 0. The peptide mixtures were incubated for 24 h at 37 °C or 1 month at 4 °C. Grids were prepared as above, and fibril preparations were immunogold labeled for insulin or IAPP by use of antisera against insulin (ICN, Thame, U.K.) and IAPP (Peninsula, U.K.). Antisera binding sites were identified with protein A-conjugated gold, and fibrils were negatively stained using UA or PTA as previously described.

Autoradiographic Binding Assay. Autoradiography was performed on IAPP fibrils formed by drying a 1 μL drop of synthetic $hIAPP_{1-37NH_2}$ (100 ng/μL in 0.01 M acetic acid) on poly(L-lysine)-coated glass microscope slides. The specificity of the binding was examined by comparison with background binding on the slide and dried insulin fibrils on the same slide. Bovine insulin (Sigma, Poole, U.K.) was converted into a fibril-containing gel following the method of Burke and Rougvie (1972).

^{125}I-$hIAPP_{1-37}$ (radiolabeled Tyr; Peninsula Laboratories, St. Helens, U.K.) was added to each slide to cover the dried fibrils (3 μL drop, 0.006 μCi, specific activity of approximately 1000 Ci/mmol) and left to incubate overnight at 4 °C in a wet chamber. Sections were washed 3 × 5 min in Tris buffer and 5 min in Tris buffer/0.2% Tween 20 (TBT) to reduce background binding, dipped in photographic emulsion K5 (Ilford, Knutsford, U.K.) (diluted 1:2 in dH_2O) at 40 °C, and left to dry at room temperature in the dark. Slides were put in a light-tight box at 4 °C for 3 days, developed in D19 (Kodak, New Haven, CT), fixed in Hypam (Ilford, U.K.), washed, stained with congo red, and visualized under light microscopy.

Quantitation of the autoradiographic silver grains was performed using a computerized image analyzer (IBAS Kontron, Messergeraete, Germany). Areas (10 × 0.3 mm^2) were selected at the cardinal points of the dried ring of insulin or IAPP fibrils for quantitation (Figure 4a). Adjacent regions of the same total area outside the rings were examined to quantitate nonspecific background labeling, which was subtracted from the counts on the fibril ring ($n \geq 3$ for each experimental condition).

The effect of pH on the binding of IAPP to preformed fibrils was examined by diluting the ^{125}I-$hIAPP_{1-37}$ into Tris-HCl, pH 7.4; Tris-HCl + acetic acid, pH 3; or Tris-HCl + NaOH, pH 13 and 8.4. The effects of glucose (5−15 mM) and calcium (4.5−9 mM) were also determined.

To determine if IAPP would bind to amyloid deposits formed *in vivo*, histological sections of pancreas from nondiabetic and islet amyloid-containing type 2 diabetic subjects obtained at post-mortem were dewaxed, rehydrated, and incubated for 4 days in ^{125}I-$hIAPP_{1-37}$, 4.7 Ci/mmol in Tris-HCl, pH 7.4, which covered the tissue section (100 μL). Sections were washed in TBT, coated in photographic emulsion, and left for 3 days at 4 °C before developing and fixing the emulsion and staining with congo red. Specificity of binding of ^{125}I-$hIAPP_{1-37}$ for islet amyloid was determined by incubation with sections of heart containing amyloid deposits formed from TTR.

Radiobinding Assay. The sensitivity of the autoradiographic binding assay was further increased by performing the test in plastic tubes and detecting the radioactivity in a γ counter. $hIAPP_{1-37NH_2}$ (400 ng in 4 μL) was allowed to dry at the bottom of plastic tubes, and 250 μL of ^{125}I-$IAPP_{1-37}$ (0.026 μCi, specific activity of approximately 1000Ci/mmol) was added and incubated at 4 °C overnight. Tubes were then washed 3 × 5 min each with TBT, and radioactivity was measured in a γ counter. Each sample was measured in duplicate. Nonspecific background (tracer binding to empty tubes) was subtracted from binding under test conditions. The maximal binding measured represented approximately 10% of added total counts. Binding of ^{125}I-$hIAPP_{1-37}$ to insulin fibrils (prepared as above) dried in tubes was used to assess the specificity of the binding assay.

The time course of binding was determined by incubation from 30 min to 24 h. The effects of pH (3−13), insulin (50−500 mU/L), glucose (2.5−15 mM), calcium chloride (2.25−4.5 mM), human glucagon (22.5−180 mM), and a mixture of isoforms of human apolipoprotein E (ApoE) (2 ng/mL) (Scripps Laboratories, San Diego, CA) were examined by adding test solutions to the ^{125}I-$hIAPP_{1-37}$ ($n \geq 3$ in each experimental condition). The effects of increasing the concentration of IAPP in the tube on binding

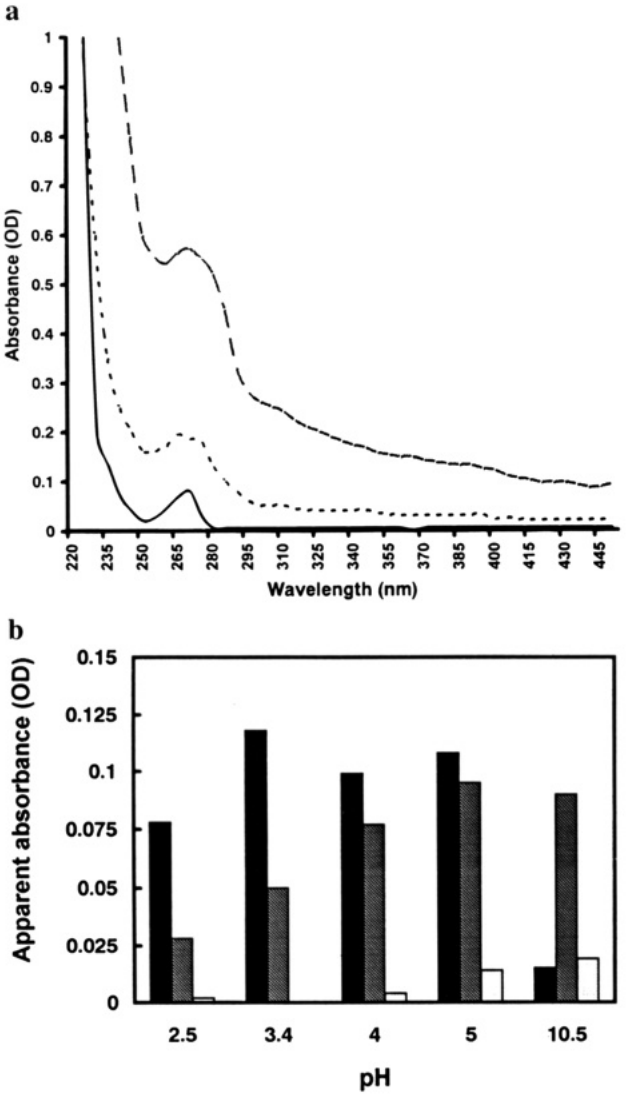

FIGURE 2: Absorbance measurements for $hIAPP_{Tyr20-29}$ (— —), $hIAPP_{1-37NH_2}$ (- - -), and $rIAPP_{1-37}$ (—) (0.25 mg/mL). (a) Absorbance measurements at 220−445 nm for solutions IAPP at pH 2.5. The peptides showed a characteristic absorbance band at 260−290 nm. Light scattering measurements were made at 372 nm. The lowest sensitivity of detection was estimated at 0.025 OD. (b) Effect of pH on aggregation of IAPP. Light scattering was measured at 372 nm (apparent absorbance) at pH 2.5−10.5. No significant light scattering was recorded for rat IAPP at any pH (white). Aggregation of $hIAPP_{Tyr20-29}$ was present at pH 2.5−5 (black), and aggregates resolubilized at pH 10.5. Aggregation of $hIAPP_{1-37NH_2}$ (grey shaded) was maximal above pH 4 and was not detectable at pH 2.5.

were examined, and changes in the surface area of the preformed fibrils were determined by vortexing the tube containing the 4 μL drop before drying.

RESULTS

Spectrophotometry. Characteristic absorbance peaks for the aromatic groups of the peptides were observed under all conditions of pH (Figure 2a). No aggregation, as detected by light scattering, was observed with the control rIAPP solution at any value of pH. Fibrillogenesis of both $hIAPP_{1-37NH_2}$ and $hIAPP_{Tyr20-29}$ was affected by changes of pH. At pH 2.5, little or no light scattering was detected from $hIAPP_{1-37NH_2}$, and light scattering increased with pH, reaching a maximum at pH 4−5. Aggregates of $hIAPP_{1-37NH_2}$ formed at pH 7.4 could not be resolubilized by changing

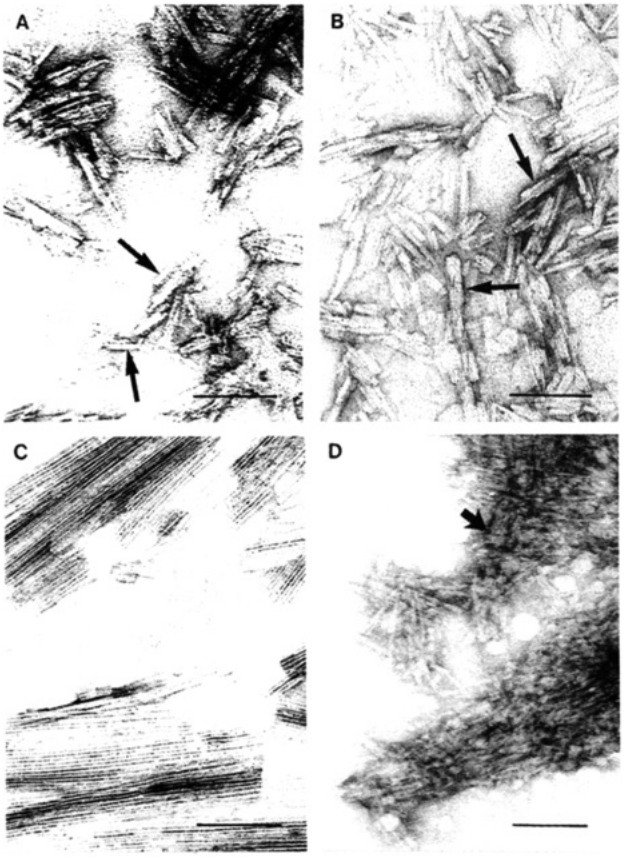

FIGURE 3: Fibrils of IAPP formed under different conditions of pH negatively stained with 0.1% PTA and examined by electron microscopy. (A) Fibrils of hIAPP prepared at pH 3 after 24 h incubation. Short filaments are visible either singly or arranged in pairs (arrows). Fibril diameter was 4−8 nm. Scale bar = 100 nm. (B) Fibrils of hIAPP prepared at pH 3 after 1 month; no increase in the amount of fibril aggregates was noted. However, the fibrils were in bundles of more than 2 protofilaments with little change in the length (arrows). Scale bar = 100 nm. (C) Fibrils of hIAPP prepared at pH 3 after 6 months; filaments were elongated (up to 2 μm), forming large sheets 50−250 nm wide, composed of numerous filaments (3−4 nm in diameter). Scale bar = 100 nm. (D) Fibrils of hIAPP prepared at pH 7.4 after 1 month; large aggregates of protofilaments which were either single or in pairs were irregularly arranged in clumps (arrow). Scale bar = 100 nm.

the pH to 3. Aggregation of $hIAPP_{Tyr20-29}$ was maximal at pH 2.5−5 (Figure 2b). These aggregates were solubilized at alkaline pH. $IAPP_{Tyr20-29}$ prepared at pH 10 showed aggregation when pH was changed to 3. Reduction of the concentration from 1 to 0.5 mg/mL decreased the spectroscopic measurements by half. No differences in light scattering were observed when samples were preincubated from 1 to 60 min before spectroscopic measurements.

Microscopic Observations of Fibrillogenesis. Fibrils were visible by electron microscopy at all pH conditions and at 30 min following preparation of the test solutions. There were only small changes in the fibril structure with longer incubation times. The appearance of the fibrils prepared at pH 3 was different from those made at pH 7.4 and at pH 9. At 30 min, pH 3, fibril aggregates were short (60 nm) and composed of 1 or 2 protofilaments (3−8 nm in diameter) arranged in parallel. This was similar at 24 h (Figure 3A). Following a 1 month storage of the solution at 4 °C, there was no apparent increase in the amount of fibril aggregates on the microscope grids. However, the fibrils were in bundles of up to 10 protofilaments with little change in the

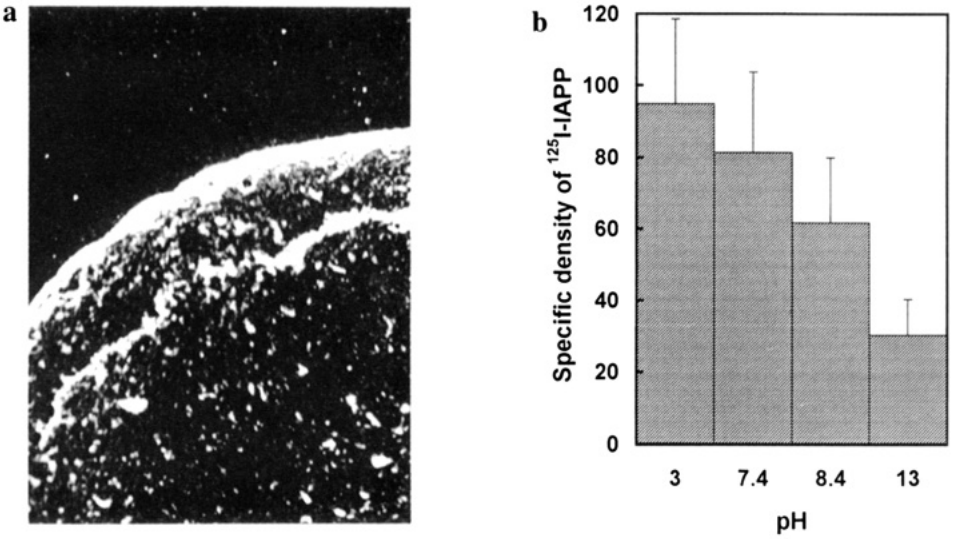

FIGURE 4: Autoradiographic binding assay. (a) Computer image of the specific binding of radiolabeled IAPP to preformed IAPP fibrils at pH 3: silver grains are localized over the ring of IAPP fibrils with low background autoradiography. Original magnification 80×; reduced to 65% of original for publication. (b) Effect of pH 3−13 on the specific binding of radiolabeled IAPP to preformed IAPP fibrils on slides. The specific density of autoradiographic grains on the IAPP fibril ring was obtained after subtraction of background. IAPP binding was maximal at pH 3 and decreased as pH was increased. $n = 6$, mean ± SEM.

length (Figure 3B). After storage of the solution for 6 months at pH 3, fibrils were elongated (up to 2 μm), forming large sheets 50−250 nm wide, composed of numerous protofilaments (3−4 nm in diameter) (Figure 3C).

Solutions prepared at pH 7.4 and at pH 9 contained large aggregates of protofilaments which were usually single and irregularly arranged in large clumps (Figure 3D). These aggregates were formed in a gel. They formed immediately, did not increase with time, and were similar at both pH 7.4 and pH 9. The IAPP fibrils formed in the presence of insulin at pH 7.4 were similar in appearance to those observed in the absence of insulin and did not change with incubation up to 1 month. Immunogold labeling for both insulin and IAPP showed irregular localization of insulin over the fibrils which were labeled for IAPP.

IAPP Binding to Preformed Fibrils. (i) Autoradiography. The autoradiographic binding method on glass slides permitted quantitative measurement and localization of binding of hIAPP to preformed fibrils by counting the autoradiographic silver grains. The preformed fibrils of $hIAPP_{1-37NH_2}$ or insulin on the glass slides showed the characteristic birefringence in polarized light after congo red staining. Quantitation of the autoradiographic grains on the background (glass slide) and test fibrils was made under identical conditions in each experiment due to clear demarcation of the amyloid deposit as a visible ring of fibrils which formed during evaporation (Figure 4a). Binding of ^{125}I-$hIAPP_{1-37}$ was specific for IAPP, and no significant binding was detected on insulin fibrils under any conditions. Binding was significantly increased at acid pH (pH 3) compared to pH 8.4 ($p < 0.001$) (Figure 4b) but was not affected by addition of glucose or calcium.

^{125}I-$hIAPP_{1-37}$ showed specific binding at pH 7.4 to histological sections of tissue from type 2 diabetic patients containing islet amyloid; there was no binding to connective tissue or the exocrine or endocrine pancreatic cells in these sections, and autoradiographic grains were restricted to congo red positive islet amyloid and did not bind to histological sections of cardiac tissue containing amyloid formed from TTR.

(ii) Radiobinding Assay. The radiobinding assay method in test tubes showed good reproducibility: very low levels of binding (<2%) were accurately determined following addition of small amounts of ^{125}I-$hIAPP_{1-37}$ (±20 000 cpm). The binding was related to the concentration of the IAPP and surface area of the preformed fibrils shown by increased binding when the drop was dispersed by vortexing. Binding was also proportional to the concentration of labeled peptide added to the tube. Binding was time dependent: little specific binding occurred at 30 min incubation and increased over a 24 h period (Figure 5a). Binding was not maximal at 24 h, but it was not possible to examine binding at longer periods due to the instability of the tracer at these dilutions and the increase of the nonspecific background. The pH dependence of binding of ^{125}I-$hIAPP_{1-37}$ was similar to that observed with autoradiography (Figure 5b); maximal binding occurred at acid pH. Binding was not affected by calcium, glucose, glucagon, or ApoE. Binding was increased by up to 4 times in the presence of insulin at a concentration of 500 mU/L ($p = 0.019$) (Figure 5c).

DISCUSSION

Spectrophotometry. These spectrophotometric results suggest that fibrillogenesis of $hIAPP_{1-37NH_2}$ is affected by pH and that $hIAPP_{1-37NH_2}$ at 0.25 mg/mL is in a soluble form at acid pH. $hIAPP_{1-37NH_2}$ at the same concentration is in a macromolecular form at pH 7.4. It has been suggested that initial stages of fibrillogenesis of IAPP involve formation of oligomers rather than dimers (Cort et al., 1994). However, this cannot be confirmed since small aggregates such as dimers cannot be detected with this spectrophotometric technique. Fibril formation of other amyloid peptides is also pH dependent due to conformational changes of the peptides; the A4 peptide (fragment 1−40) forms fibrils more readily at neutral pH compared to acid conditions (Fraser et al., 1991, 1992). The change in light scattering produced by IAPP was observed instantly after changing the pH, and the amount of light scattering was not increased by extending the period of incubation up to 60 min, indicating that synthetic hIAPP forms fibrils immediately in appropriate conditions. This

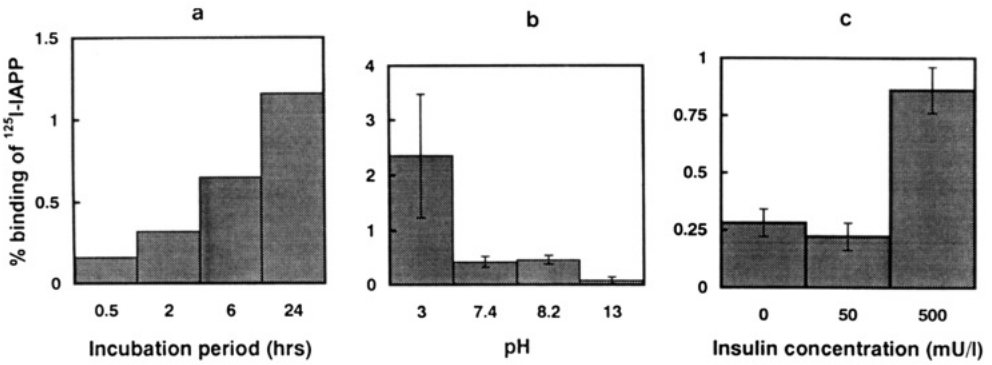

FIGURE 5: Radiobinding assay demonstrating binding of radiolabeled IAPP to preformed IAPP fibrils. (a) Time course of binding. IAPP binding increased from 0.5 to 24 h. No detectable binding occurred within 30 min. (b) Effect of pH 3−13. IAPP binding was maximal at pH 3 and significantly reduced at pH 7.4 and 8.2. No binding was detected at pH 13. $n = 4$, mean ± SEM. (c) Effect of insulin on IAPP binding. Binding was unaffected by addition of human insulin, 50 mU/L, in the solution of radiolabel. However, binding was increased 4-fold in the presence of insulin, 500 mU/L. $n = 4$, mean ± SEM.

contrasts to fibril formation from A4 protein which is not instant and progressively increases over periods of days (Ma et al., 1994). The conditions for fibril formation from $hIAPP_{Tyr20-29}$ are optimal at acid pH, indicating that the behavior of this peptide fragment from the most hydrophobic part of hIAPP is different when isolated from the intact molecule. Furthermore, fibrils formed from this fragment appear to be less stable than those formed from the whole molecule.

IAPP is colocalized with insulin in β-cell granules (Clark et al., 1989). This cellular compartment has a low pH, changing from pH 7.4 to pH 5.5 during maturation of the granules prior to release (Hutton et al., 1989). The absence of aggregates or fibrils in β-cell granules even when IAPP fibrils are present in other sites (de Koning et al., 1994) suggests that an acidic milieu has a stabilizing effect on IAPP. When β-cell granules containing IAPP are released into the extracellular compartment, the change in pH to 7.4 would promote IAPP fibril formation. However, since amyloid is not a common feature of nondiabetic subjects, factors other than pH and amino acid sequence but related to β-cell dysfunction in diabetes must be involved. The concentration of IAPP either in the granule or in the extracellular space under normal conditions is unknown, but on the basis of measurements of secretion and islet content, IAPP concentration is less than 10% of the insulin concentration (Sanke et al., 1991). Release of IAPP from rat islets has been estimated to be 0.01 pM/h (Stridsberg et al., 1993), and IAPP, like insulin, diffuses rapidly into the islet capillary. However, if clearance from the islet space becomes impaired, high concentrations would be achieved which are likely to promote fibril formation. Impaired clearance has been suggested as a factor in amyloid formation between cells in islets of hIAPP mice (de Koning et al., 1994).

Microscopic Observations of Fibrillogenesis. Short fibrils of IAPP were formed within 30 min of preparation of the solutions at all pH examined. These were in the form of two or more protofilaments 5 nm in diameter arranged in a parallel array. There was no evidence for elongation of single fibrils over periods of hours as observed for A4 peptide fibrils *in vitro* (Tomski & Murphy, 1992). However, large sheets of fibrils were formed over periods of months at pH 3: these sheets consisted of large numbers of parallel protofilaments, suggesting that interfilament binding can occur under suitable conditions. Intracellular amyloid fibrils are arranged in parallel in β-cells of human insulinomas and in islets of hIAPP mice (Clark et al., 1991; de Koning et al., 1994). This may reflect the pH or the time over which the fibrils are formed at these intracellular sites. Feline $IAPP_{20-29}$ has been shown to form a 30 nm wide ribbon which could be similar to these fibril sheets (Westermark et al., 1990).

IAPP (0.2 mg/mL) formed a gel at neutral pH. Gel forming properties have been described for both $hIAPP_{1-37}$ and $hIAPP_{20-29}$ in high concentrations (3−10 mg/mL) at both acid and neutral pH. These gels contained filaments arranged in parallel (Westermark et al., 1990). Extracellular islet amyloid consists of short fibrils arranged in a random fashion which corresponds to the appearance of the fibrils formed at neutral pH. The lack of effect of insulin on aggregation of IAPP *in vitro* suggests that insulin is not an essential component for fibrillogenesis. However, insulin was detected by immunogold labeling in the IAPP fibril aggregates but not as a regular structural part of the IAPP fibrils as has been described for the interaction of A4 peptide and α_1-antichymotrypsin (Fraser et al., 1994). Insulin has been reported to be a component of islet amyloid (Westermark et al., 1983). It is likely that insulin attaches transiently to the fibrils as the secreted peptide traverses the amyloid deposits which are situated between the β-cells and capillaries but does not form a stable complex with IAPP.

IAPP Binding to Preformed Fibrils. This experimental protocol examines binding under "seeded" conditions with much smaller amounts of peptide than is required for absorbance studies described here and by other groups (Come et al., 1993). This method is suitable for use with other amyloid proteins which aggregate *in vitro* to assess the effects of agents on binding to preformed fibrils.

Autoradiographic and radioassay methods indicate that IAPP binds specifically to IAPP fibrils and does not form a complex with insulin fibrils. This process of IAPP binding could be in the form of extension of existing fibrils or formation and deposition of new fibrils. The presence of a nidus is known to promote interaction of added peptides to fibrils (Jarrett & Lansbury, 1993), which may require different conditions to that of the initiation of fibrillogenesis. Increased binding of IAPP at acid pH suggests that the structure and charge of the IAPP molecule are appropriate for association of monomers with existing fibrils. However, the behavior of the relatively hydrophobic β-sheet region, $IAPP_{24-27}$, determines the aggregation properties of the molecule (Glenner, 1988; Westermark et al., 1990). Spectroscopic observations indicate that there is less aggregation

of $IAPP_{1-37}$ at acid pH. This suggests that binding is greater when IAPP is in a soluble form. At more alkaline pH, the reduction of the binding may be due to inappropriate molecular conformation of IAPP for fibril extension or caused by combination of 2 or more molecules of IAPP in the liquid phase to form an oligomer which itself has reduced binding capacity. It is possible that the disulfide bridge could be involved in IAPP molecular interaction. However, this is unlikely since fibrils are formed from $IAPP_{20-29}$ which does not contain cysteines and the proposed interaction of transthyretin via disulfide bridges seems uncertain (McCutchen & Kelly, 1993).

Calcium concentrations are high in β-cell granules (1−10 mmol/L), and high glucose levels associated with glycosylation of many structural proteins are characteristic features of diabetes. The lack of effect of calcium, glucose, and glucagon demonstrates that these agents are not major factors for islet amyloid formation in diabetes. Binding of IAPP to existing fibrils was increased by high concentration of insulin (3.7 nmol/L). This concentration is similar to that achieved in the islet during β-cell secretion. However, no effect of insulin on fibril formation or structure was detected with electron microscopy, suggesting that insulin does not form a permanent complex with IAPP or islet amyloid: electron microscopy failed to detect specific immunoreactive sites for insulin on IAPP fibrils, and insulin has only rarely been detected in islet amyloid deposits (Westermark & Wilander, 1983).

Autoradiographic observations on tissue sections indicate that hIAPP binds specifically to islet amyloid and not to TTR. This shows that structural features exist which are specific for islet amyloid deposits to allow fibril extension from monomeric IAPP. ^{125}I-A4 has been shown to bind to cerebral tissue containing neuritic plaques with an apparently reversible reaction, but binding of the labeled A4 was not shown to be specific by testing on other forms of amyloid (Maggio et al., 1992). Continuous extension of fibrils from monomers explains the progressive nature of islet amyloidosis. These autoradiographic studies indicate the high affinity of hIAPP for amyloid. Soluble hIAPP binds to amyloid which has been formed *in vivo* and contains IAPP fibrils surrounded by glycosaminoglycans, amyloid P protein, and other components such as apolipoprotein E (Young et al., 1992; Westermark et al., 1975; Clark et al., 1994). Furthermore, formalin fixation and heat associated with the tissue processing do not destabilize the configuration of the binding sites for fibril formation. Further understanding of the behavior of hIAPP in the process of amyloid fibril extension and fibrillogenesis will indicate methods of reducing amyloid formation in diabetes.

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