High-frequency oscillations in circulating amylin concentrations in healthy humans

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AMYLIN IS A 37-AMINO ACID POLYPEPTIDE produced in pancreatic β-cells by posttranscriptional processing of pre-proamylin. The peptide is not a product of insulin biosynthesis and is coded by a different chromosome. The amylin precursor is transferred with proinsulin to the trans-Golgi-apparatus, where both precursors are processed into mature forms sharing the processing enzyme prohormone convertase (2). Amylin is co-localized with insulin in secretory granules and secreted in response to nutrient stimuli and other insulin secretagogues (10, 18).

It has been demonstrated that amylin, in addition to a native form, circulates in at least three different glycosylated forms, with O-glycosylations at position 6, position 9, or both (20, 32). The potential importance of these forms is so far not clear. Amylin exhibits a response to an oral glucose tolerance test similar to that of insulin (30). In healthy subjects, the circulating amylin-to-insulin molar ratio appears to be 1–2% in the fasting state, but this ratio has been found to be higher in a number of circumstances, e. g., recent data in rats have demonstrated that both the circulating and the islet amylin-to-insulin molar ratios increase in tolbutamide-treated euglycemic rats used as a model of the overworked β-cell (15). From an intracellular point of view, it is of interest that amylin secretion, in contrast to insulin, can be stimulated by glucose in the absence of Ca²⁺ in rat islet monolayer cultures (12). Thus regulation of the release of the two hormones appears to be at least partly independent.

It has been known for more than two decades that insulin is secreted in a high-frequency pulsatile pattern with a periodicity of 5–15 min (9, 14). Whether amylin is characterized by a similar release pattern is unknown. The present study was consequently undertaken to seek further insights into β-cell physiology and amylin secretion by examining secretory patterns of amylin in healthy humans during moderate hyperglycemia. To define high-frequency oscillations, time series were assessed by spectral analysis, autocorrelation analysis, deconvolution analysis, and approximate entropy (ApEn). Time series of amylin (nonglycosylated) and total amylin immunoreactivity (TAI; nonglycosylated plus glycosylated) were compared with those of insulin.

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RESEARCH DESIGN AND METHODS

Subjects and design. The protocol was performed in accordance with the Helsinki Declaration and was approved by the local Ethical Committee of Aarhus County. Eight healthy volunteers, four male and four female, mean (± SD) age 28.8 ± 4.7 yr and body mass index (BMI) 22.6 ± 3.4 kg/m², were studied. None had a family history of diabetes mellitus or took regular medication.

Protocol. Studies were performed after a 10-h overnight fast. At 0800, each subject was placed in a bed, and intravenous catheters were inserted in each antecubital vein for infusion and sampling purposes. After 30 min (t = 0), glucose infusion at a constant rate (2 mg·kg⁻¹·min⁻¹) was initiated. Glucose infusion was performed to stimulate amylin secretion and reach concentrations within the detectable range of the amylin assay. After another 60 min, steady state was assumed, and blood was collected every minute for 90 min in a heated hand vein to arterialize the blood. Further details on the sampling procedure have been described previously (24).

Blood samples were stored at −20°C and analyzed within a month.

Assays. Amylin and TAI concentrations in serum were measured in triplicate by use of two previously reported monoclonal antibody-based sandwich assays (20). Briefly, peptide is captured out of a 50-μl plasma sample (1-h incubation at room temperature) by an amylin-specific monoclonal antibody coated on the black 96-well plate. After the plate is washed, an alkaline phosphatase-conjugated monoclonal antibody specific to the COOH-terminal end of amylin is added to the well and incubated for 3 h at room temperature. After another wash, a signal is generated by adding the fluorescent substrate 4-methylumbelliferyl phosphate. Plates are read with a fluorescent plate reader (Dynatech, Chantilly, VA) and sample fluorescence is compared with that of a synthetic human amylin standard curve. Both assays use the same detection antibody but employ different capture antibodies. The amylin assay uses a detection antibody shown to detect nonmodified human amylin, but not the recently reported glycosylated forms (32). The TAI assay detects nonmodified amylin and the glycosylated forms. The cross-reactivities of both assays to proamylin, insulin, calcitonin gene-related peptides (I and II), and calcitonin are <0.05%. The intra- and interassay coefficients of variation for the assays are <10 and <15%, respectively (20).

Serum insulin concentration was measured in duplicate by a two-site immunospecific insulin ELISA, as previously described (1). The detection range was 5–600 pmol/l. At medium (150 pmol/l), medium-high (300 pmol/l), and high (350 pmol/l) insulin concentrations, the interassay coefficients of variation were 4.5, 4.9, and 5.5%, respectively, and the intra-assay coefficients of variation were 2.8, 2.6, and 2.4%, respectively. There was no cross-reactivity with proinsulin, split 32,33 and des-31,32 proinsulin, C-peptide, insulin-like growth factor (IGF)-I, IGF-II, and glucagon. The antibodies cross-react 30 and 63% with split-65,66 and des-64,65 proinsulin, respectively.

Data analysis. Autocorrelation and cross-correlation analysis and spectral analysis were performed with the software SPSS version 8.0 (SPSS, Chicago, IL), which was also used for detrending and random shuffling procedures. Spectral analysis, autocorrelation analysis, and cross-correlation all require stationary time series (4). Despite the constant glucose infusion rate, minor trends were observed in some data sets. Before autocorrelation analysis and spectral analysis, data were therefore stationarized by subtraction of the seven-point equal-weighted centered moving average from the original data set, and the analyses were applied to the residuals. This procedure serves only detrending purposes and does not smooth out high-frequency variations in the data. The choice of 7 min as the length of the moving average was based on the knowledge of insulin pulse frequency reported to be 5–15 min (14, 24). To ensure that observed high-frequency oscillations were not a product of the detrending procedure performed, autocorrelation and spectral analysis were applied to first-difference derivatives as well.

Autocorrelation and cross-correlation. Autocorrelation analysis was performed on the stationarized time series without prior smoothing to allow detection of high-frequency pulsatility. By autocorrelation analysis, the data from the observed time series are correlated pointwise to an exact copy lagged by 1-min intervals. An autocorrelation coefficient was considered significant if the first positive peak after the first trough exceeded the 95% confidence interval or if the autocorrelogram itself had a cyclic pattern and one of the subsequent peaks exceeded the confidence limit.

To determine the frequency of oscillations by autocorrelation, data were pooled from the eight subjects. M values were transformed to Z values by Fisher’s Z transformation, and the mean values were calculated and transformed to m values by the inverse procedure (19). 95% Confidence intervals for autocorrelation analysis were calculated as ±2.217/√N (7).

Cross-correlation analysis was applied to the original pooled data set as well as to the residuals to be able to evaluate synchronicity in trends as well as in high-frequency oscillations in amylin and insulin concentration time series. 95% Confidence intervals for cross-correlation were calculated as ±2.217/√N (7).

Spectral analysis. The principle of spectral analysis is to break down a time series intocosine waves of different periods and quantify each cosine wave in terms of the amplitude that allows the best fit to the observed data. Spectral analysis was performed using the Tukey-Hanning window. Different window lengths were tested. Very erratic spectra were obtained using a short window length, whereas smoother but less well-defined spectra with broad peaks were obtained by application of a longer window length (11). To achieve a balance between the stability and fidelity, a window length of 9 was used. A spectral density plot was created using the time domain, and the peak value was determined. To assess the significance of the spectral density peak, a method of random shuffling spectral analyses was applied. Each time series was subjected to computerized random shuffling, and after each time, spectral analysis was performed. The spectral density value at each frequency of these 100 random series was determined. The distribution of the spectral densities at each frequency after random shuffling was in good accordance with normal distribution. The means ± SD at each frequency tested were calculated, and confidence intervals were estimated on the basis of a χ² distribution, as given by Jenkings and Watts (11) and further described by Chatfield (4). Spectral analysis was considered significant if the dominant peak exceeded the confidence interval.

Deconvolution analysis. Plasma amylin and insulin concentration time series were analyzed by deconvolution analysis to quantify basal secretion, interpulse interval, secretory burst mass, and amplitude. Deconvolution was performed with an iterative multiparameter technique by use of the following assumptions: 1) Hormones are secreted in a finite number of bursts with 2) an individual amplitude, 3) a common half-duration superimposed on a basal time-invariant secretory rate, and 4) a biexponential disappearance rate of amylin and insulin (24). The disappearance model for amylin consisted of estimated half-lives of 3 and 13 min, with...
a fractional slow component of 28% (5). A variety of half-lives were tested, and whereas calculated total release and distribution were affected by kinetics, detected frequency was not. The heterogeneity of TAI, consisting of different forms of amylin with different kinetics, does not allow deconvolution to be carried out on TAI concentration time series. For insulin, the estimated half-lives were 2.8 and 5 min, with a fractional slow compartment of 28% (24).

ApEn. Regularity of serum amylin and insulin concentration time series was assessed by the model-independent and scale-invariant statistic ApEn. ApEn measures the logarithmic likelihood that runs of patterns that are close (within r) for m contiguous observations remain close (within the tolerance width w) on subsequent incremental comparisons. A precise mathematical definition has been prescribed by Pincus (21). ApEn is to be considered a family of parameters dependent of the choice of the input parameters m and r, and it is to be compared only when applied to time series of equal length. In this study, ApEn was calculated with r = 20% of the SD in the individual time series and m = 1, parameter choices that are standardly utilized in virtually all endocrinological applications of ApEn. A larger absolute value of ApEn indicates a higher degree of process randomness. ApEn is rather stable to noise that is withheld within the tolerance width w. There is a precedence for detrending the time series by first differencing before ApEn calculation. To evaluate the effect of trends in the time series, ApEn was calculated on the original data sets as well as on detrended data. Observed ApEn values were compared with ApEn calculations performed on randomly shuffled series, and ApEn was considered as significant when the observed value differed from maximally random by two or more SDs of ApEn.

Statistical analysis. All data are given as means ± SD. Comparisons of data for amylin, TAI, and insulin were carried out by the Wilcoxon signed-rank test. Bivariate correlations were calculated by Spearman's ρ.

RESULTS

Plasma amylin and insulin concentrations. In one of the study subjects, almost all measured values of amylin fell below the detection limit, and this time series was consequently excluded from further calculations. Therefore, seven amylin, eight TAI, and eight insulin concentration time series were analyzed. Average concentrations of amylin, TAI, and insulin are given in Table 1. During modest hyperglycemia (plasma glucose 5.9 ± 0.3 mM), the molar ratios of amylin to TAI and of amylin to insulin were 0.36 ± 0.10 and 0.06 ± 0.02, respectively. No systematic changes in these ratios were observed over time. There was a significant bivariate correlation between mean amylin concentration, mean TAI concentration, and mean insulin concentration when the study subjects were compared. Representative time series of amylin, TAI, and insulin for one study subject are shown in Fig. 1.

Deconvolution analysis. Secretary burst mass of amylin and insulin was 1.63 and 26.4 pM/burst, respectively, and the interpulse interval was estimated to be ~6 min. Data from the deconvolution analysis for amylin and insulin are given in Table 1. Autocorrelation and cross-correlation. The results for autocorrelation analysis of the individual time series are given in Table 1. The estimated frequency was significantly slower for insulin than for amylin and TAI.

Table 1. Characteristics of high-frequency oscillations analyzed by deconvolution analysis, autocorrelation analysis, spectral analysis, and approximate entropy (amylin vs. insulin, P = 0.042, TAI vs. insulin, P = 0.027). By autocorrelation analysis of the pooled data set, amylin and TAI were characterized by a pulsatile pattern, with a significant correlation at the lag time of 4 min (r = 0.25, P < 0.001, and r = 0.25, P < 0.001, respectively) indicating an interpulse interval in this range. The autocorrelogram of insulin showed a similar pattern, with a shoulder at 5 min and a peak at 9 min (lag time 9 min, r = 0.08, P = 0.05; Fig. 2).

Significant cross-correlation was found when analysis was performed on the undetrended data, with a maximal correlation found at time lag 0 (amylin vs. TAI, r = 0.47, amylin vs. insulin, r = 0.30, TAI vs. insulin, r = 0.41, all P < 0.001). When analysis was performed on the residuals after subtraction of the 7-point moving average, amylin vs. TAI showed a significant cross-correlation at time lag 0 (r = 0.47, P < 0.001). Amylin vs. insulin also exhibited a peak at time lag 0, but the correlation was not statistically significant (P = 0.06). In contrast, a significant cross-correlation between TAI and insulin was observed at time lag 0, indicating simultaneous peaks of the hormones (r = 0.12, P < 0.001).

Spectral analysis. Results for the spectral analysis of the individual time series are given in Table 1. As for autocorrelation analysis, the estimated frequency was significantly slower for insulin than for amylin and TAI (amylin vs. insulin, P = 0.017, TAI vs. insulin, P = 0.018). An example of spectral analysis for one individual is shown in Fig. 3. A distinct peak exceeding the confidence interval is seen in the spectral analysis of the observed time series. Frequency estimations carried out on first-difference derivatives gave almost identical results, indicating a stability toward the procedure of detrending.
Table 1 lists the ApEn values obtained when the defined input parameters are applied. Also, the number of time series significantly different from a random seed is shown. When applied to the raw data, 6 (out of 7), 7 (out of 8), and 7 (out of 8) of the amylin, TAI, and insulin time series, respectively, were tested as significantly different from random. When applied to first-difference derivatives, only 4, 3, and 1, respectively, were found to be significantly different from random. This might indicate that even the minor trends observed in these data sets may influence the ApEn calculations.

DISCUSSION

Our study demonstrates for the first time regular cyclic oscillations of amylin concentrations in healthy humans exposed to a modest and constant intravenous glucose challenge. Although autocorrelation and spectral analysis are designed to detect regularly recurring variations in data series, deconvolution analysis (which is independent of this assumption) also disclosed only a 15% coefficient of variation of amylin interpulse intervals. This finding strongly suggests that amylin during glucose stimulation, like insulin, is secreted in a pulsatile manner. Dependent on the mathematical model employed, the pulse frequency was found to be between 4 and 6 min. Similar to insulin, the mechanisms of the regulation of pulsatile amylin secretion are not well understood, but intracellular metabolic events in the β-cell (25), the intrinsic neural network within the pancreas (28), and a metabolic feedback loop between the liver and the islets (31) probably all contribute to the dynamic pulsatility of amylin as well as insulin release.

Amylin has been shown to exert actions on gastric emptying (16), satiety (34), and the secretion of glucagon (8). Because the effects on gastric emptying and satiety appear to be centrally mediated, it is not likely that the oscillatory pattern of amylin concentrations is of significant relevance for those actions. After administration of an amylin antagonist, augmented insulin secretion has been shown in perfused isolated rat islets (33) and in isolated rat β-cells during an arginine challenge (3). These findings strongly suggest the possibility that amylin influences insulin secretion. Indeed, at high doses, amylin has been found to suppress

![Fig. 1. Representative concentration profiles of amylin (A), total amylin immunoreactivity (TAI) (B) and insulin (C) during constant glucose infusion (2 mg·kg⁻¹·min⁻¹). Blood was sampled every minute for 90 min.](http://ajpendo.physiology.org/)

![Fig. 2. Autocorrelation analysis of amylin (A), TAI (B), and insulin (C) performed on residuals after subtraction of a 7-point moving average. Data are pooled from 8 subjects. Dotted line indicates 95% confidence limits.](http://ajpendo.physiology.org/)
The amylin concentration at the β-cell level is clearly much higher than the concentrations measured peripherally, and thus it is tempting to suggest a possible paracrine regulatory role of amylin on insulin secretion. Corelease of an inhibitor of insulin secretion may serve as one of several mechanisms to prevent inappropriate insulin release during glucose stimulation. It has previously been demonstrated that not only insulin, but also glucagon and somatostatin are released in a pulsatile manner from isolated canine pancreas (28). Like these hormones, amylin may thus contribute to the overall control of glucose homeostasis.

Another interesting observation in our study was that both amylin and TAI were secreted in an oscillatory manner. This finding suggests, but does not prove, that amylin is released from the β-cells in both nonglycosylated and glycosylated forms. If glycosylation were solely a postsecretory event, one would expect the oscillatory pattern to be less pronounced for the TAI concentration time series, which reflects both nonglycosylated and glycosylated forms (20, 32).

Various techniques of time series analysis were applied to evaluate whether the observed time series were regular and pulsatile. Each method makes assumptions about the nature of the pulsatility and is differently affected by noise and changes in interpulse interval and pulse amplitude. Spectral analysis and autocorrelation analysis both require stationarity in the data and a fairly constant interpulse interval and pulse amplitude. Deconvolution analysis does not make these assumptions and can be applied to the original data sets. The latter method may lead to over- or underestimation of the number of pulses in the case of noise or missing data points. The pulse frequency is therefore presumably most correctly estimated by the former mentioned methods, but no single method is universally superior in addressing the question of pulsatility.

The interpulse interval of insulin observed in previous studies is reported to be between 4 and 13 min. This rather large variation may in part be due to differences in study conditions, and most previous studies have been carried out under basal conditions without glucose stimulation (9, 14). It has previously been suggested that a glucose challenge increases insulin pulse frequency (23), but it should be noticed that the issue is controversial (29). The pulse frequency found in our study is comparable to that in previous studies carried out during glucose infusion (23).

Another important parameter determining the pulse frequency is the sampling frequency. One-minute data have been shown to yield a higher calculated pulse frequency than 2-min sampling when estimated by pulse detection (22). Noise has a tendency to disable autocorrelation and cross-correlation analysis. It is therefore sometimes preferential to smooth the data before the analysis (14, 19). However, even a mild smoothing procedure like a 3-point moving average impairs the possibility to detect pulsatility below and near the frequency of 3 min. Consequently, we chose not to perform any smoothing before correlation analyses. It was therefore not surprising that not all concentration time series revealed significant autocorrelation.

ApEn is a complementary approach to time series analysis. It has been applied to various hormone concentration sequences and has been shown to be capable of discerning normality from pathophysiology (17). In the present study we found a difference in ApEn when applied to raw data compared with stationarized data, indicating an apparent higher regularity in the not detrended data. This may in part be explained by minor trends in the concentration time series, but the actual impact of this finding is uncertain.

Immunohistochemical studies provide evidence of colocalization of amylin and insulin in the same secretory granules (6), and the significant cross-correlations between amylin and TAI and between TAI and insulin secretion in perfused rat pancreas (26). The amylin concentration at the β-cell level is clearly much higher than the concentrations measured peripherally, and thus it is tempting to suggest a possible paracrine regulatory role of amylin on insulin secretion. Corelease of an inhibitor of insulin secretion may serve as one of several mechanisms to prevent inappropriate insulin release during glucose stimulation. It has previously been demonstrated that not only insulin, but also glucagon and somatostatin are released in a pulsatile manner from isolated canine pancreas (28). Like these hormones, amylin may thus contribute to the overall control of glucose homeostasis.
would strongly support cosecretion of the hormones. The fact that cross-correlation analysis was significant when applied to the original data set, as well as to the residuals after detrending, indicates synchronicity of high-frequency oscillations as well as in response to the glucose challenge. The calculated difference in frequency between amylin and insulin indicated by autocorrelation and spectral analysis may therefore be a statistical estimation phenomenon rather than a physiological one. However, separate secretory pathways and regulatory mechanisms also have to be considered.

Our study illustrates that it is possible, despite a longer half-time and a less precise assay of amylin when applied to the original data set, as well as to the statistical estimation phenomenon rather than a physiological one. However, separate secretory pathways and regulatory mechanisms also have to be considered.

In conclusion, the present study substantiates that amylin and glycosylated amylin are secreted in a pulsatile manner after elevation of plasma glucose by glucose infusion. Whether basal amylin concentrations also exhibit an oscillatory pattern is not known. Disorderly insulin secretion has been demonstrated in overt diabetes mellitus and, in the prediabetic state, whether disorderly amylin release may be a predictor of β-cell failure.

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References


