Sustained Pulsatile Insulin Secretion From Adenomatous Human β-Cells

Synchronous Cycling of Insulin, C-Peptide, and Proinsulin

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The endocrine pancreas secretes insulin in a pulsatile fashion. This rhythm is generated at a site within the pancreas, although its precise location has not been determined. With an in vitro system, we tested the possibility that β-cells might generate spontaneous pulsatile insulin secretion in the absence of any external influence. Human insulinoma tissue from five patients was perifused for 7–10 h with RPMI-1640 medium and constant concentrations of glucose (5.5 mM). Insulin, C-peptide, and proinsulin were measured in the effluent collected at 3.3-min intervals. All three peptides demonstrated pulsatility of secretion in a similar, synchronous fashion that was sustained throughout each study. The Clifton cycle detection program demonstrated cycling in all five tumors, with an average period for all tumors of 28, 29, and 26 min for insulin, C-peptide, and proinsulin, respectively. Spectral analysis confirmed the regularity and consistency of the hormonal secretory patterns. Mean hormone concentrations secreted by different tumors varied, but insulin and C-peptide were secreted in a nearly 1:1 ratio. This study demonstrates *1*) that β-cells are able to generate spontaneous pulsatile insulin secretory activity, which is independent of innervation or the presence of other islet cells, and *2*) proinsulin secretion from the β-cell also has an inherent pulsatility. The synchrony observed in the cycles of proinsulin and its peptide products confirms their common secretory pathway in the β-cell. We conclude that the β-cell may be the originator of insulin cycling.
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Spontaneous fluctuations in serum concentrations of insulin have been demonstrated in several different species (1–4). As in the case of other hormones, these fluctuations are a result of pulsatile hormone secretion (5,6). In the isolated dog pancreas, insulin is secreted in a pulsatile fashion and is independent of fluctuations in glucose concentrations or CNS influence (5). Pulsatility of insulin secretion thus originates in the pancreas, but its mechanism is still not understood. It has been suggested that a pacemaker is located within the pancreas to generate and coordinate pulsatile secretion by the islets (7,8) utilizing a rich network of intrapancreatic neurons that innervate the endocrine pancreas. An anatomic site for this putative pacemaker has not been delineated (7,8).

Oscillatory phenomena also have been recognized within the pancreatic β-cell, including pulsatile electrical activity and ion fluxes (9–12). These observations raise the possibility that spontaneous secretory oscillations may also be generated within these cells. If so, the β-cell itself might be the site of origin of pulsatile secretion of insulin. This possibility was tested by examining insulin secretion from a pure β-cell preparation in vitro (13). With human insulinoma tissue, we report the existence of spontaneous pulsatile insulin secretion from human β-cells perifused in vitro.

RESEARCH DESIGN AND METHODS

Five patients (2 men and 3 women) 32–61 yr old presented with typical symptoms of hypoglycemia. The patients were found to have repeated hypoglycemic episodes, during which serum insulin and C-peptide concentrations were elevated. In all patients, a single tumor was removed at surgery, and a portion of these tumors was obtained for in vitro studies. Patients 4 and 5 were treated with diazoxide before surgery to prevent hypoglycemia while localizing studies were attempted. In each patient, the last dose was administered 40 h before the perifusion experiment. Pathological studies demonstrated β-cell tumors in each case. No somatostatin- or glucagon-containing cells were detected in any of these tumors by immunohistochemical methods.

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After excision at surgery, the tumors were maintained in sterile conditions and minced into fragments of 1–2 mm in diam. Between 3 and 30 mg of the fragments were placed in RPMI-1640 medium (Gibco, Grand Island, NY) containing 10% newborn calf serum (Irvine Scientific, Irvine, CA) and 5.5 mM glucose and cultured overnight in a CO_2 incubator. The next morning, the fragments were packed into a 1 cm diam column and sandwiched between two layers of swollen Cytodex 2 microcarrier beads (Sigma, St. Louis, MO) to a height of 0.7 cm (14). The column was perifused with RPMI-1640 culture medium and 5.5 mM glucose, which was continuously gassed with a mixture of 95% O_2/5% CO_2. Perifusion medium was pumped continuously with a peristaltic pump at a rate of 0.3 ml/min, and the effluent was collected (1 ml/fraction) for up to 10 h. An air trap was inserted immediately before the column. The column and the perifusion medium were maintained at 37°C in a water bath. Control studies were carried out to exclude a relationship between pulsatile insulin secretion and flow rate, temperature, or pressure changes; this involved weighing of effluent tubes, water bath temperature measurements, and continuous hydrostatic pressure measurements, respectively. Effluent tubes were frozen until hormone assay.

The insulinoma tissue was equilibrated with the perifusion medium for 1.5 h before the start of sample collection. Sampling at 3.3-min intervals over 4–7 h ensured sufficient data points for optimal analysis. The viability of the preparation was documented with a terminal stimulus with 1 mM theophylline. This resulted in a significant twofold increase in mean insulin concentrations ($P < 0.005$).

Insulin immunoreactivity was measured by radioimmunoassay with an antibody obtained from Radioassay Systems Laboratories (Carson, CA; 15). Cross-reactivity with proinsulin was 60%. Intra- and interassay coefficients of variation (C.V.) for this assay were 6.5 and 8%, respectively. The limit of sensitivity was 30 fmol/ml. The radioimmunoassay for C-peptide was carried out with tyrosylated C-peptide as tracer and a goat antiserum raised against human C-peptide (generously provided by Bruce Frank, Lilly Research Lab.). The intra- and interassay C.V.s were 6.9 and 8.2%, respectively. The limit of sensitivity was 30 fmol/ml, with a cross-reactivity of 50% for proinsulin. Proinsulin was measured with a highly specific radioimmunoassay. This was carried out with a modification of a method reported previously (16). The antibody was raised in a goat and is specific for proinsulin, with <0.01% cross-reactivity with either insulin or C-peptide. Biosynthetic human proinsulin was used for preparing standards and tracer. Radioiodination was carried out with Iodogen (Pierce, Rockford, IL). The lower limit of sensitivity of this assay was 7 fmol/ml. Intra- and interassay C.V.s were 11.4 and 9%, respectively.

The data were analyzed for episodic secretion of insulin, C-peptide, and proinsulin with the cycle detection program developed by Clifton and Steiner (17). This program uses the assay C.V. as the basis of an iterative method to produce an adaptive threshold for identifying peaks and valleys in the data. The reliability of the detection process was determined by comparing the variance due to measurement error (assay variance) with the variance derived from experimental data fluctuation by analysis of variance (ANOVA). For each of 15 hormone profiles examined, there existed a statistically significant difference ($P < 0.001$ for 12 profiles; $P < 0.025$ for 3 profiles).

Unlike other hormones (17), it appears that insulin secretion is not only pulsatile but also periodic; that is, the pulse pattern repeats itself at regular intervals (2,3,6). To further characterize our data, we used spectral analysis (18). This method, well-known from engineering and studies of heart-rate phenomena, models the time-series data as a sum of cosine waves with periods $t/2$, $t/3$, $t/4$, . . . $2t/n$, where t is the total time interval and n is the sample size. The amplitude and phase of each sinusoid are estimated from the data by computing the discrete Fourier transform. The squared estimates of amplitude are smoothed and then plotted against the period. The collection of estimates, one for each period, is called the *spectral density function*. If the original data are truly periodic, then the plot of the spectral density function against frequency will show large amplitudes (peaks) for a few values of the period and be close to 0 elsewhere. The model for the data is then the sum of the cosine waves at these periods, with the computed amplitudes and phases. The apparent period and amplitude of the resultant waveform, defined by the rate at which the values cross the mean and the average maximum distance from the mean, are given by the Clifton method.

In some experiments, the mean hormone concentrations gradually decreased over the duration of the experiment. In this situation, we used linear regression to correct for the trends in the mean, i.e., "detrending." In the Fourier analysis, we also used linear filtering to remove components with periods ≥1 h, because the experimental design did not provide sufficient data for their evaluation. After the spectral density function was estimated with the program BMDPIT (19), we tested whether peak amplitudes were significantly different from 0 with a modification of Fisher's test (20). To illustrate the similarity in periodicity between peptides in each tumor, we first normalized the amplitude estimates for each of the three hormones by expressing them as percentage of total. We then averaged the values from each hormone for each value of the period to obtain an average spectrum. To test the synchrony between each of the three secreted peptides in time (phase), we modeled each tumor as the weighted sum of the two most significant components of the spectral density function with the coefficients from the Fourier analysis as weights. We estimated the pairwise phase difference to be the difference in time between the occurrence of the maxima in the models.

To ensure that any oscillations observed in these experiments were indeed a result of secretory fluctuations, control studies were carried out in which the three peptides were perifused through the system with identical techniques but in the absence of islets; then the effluent was assayed for hormone concentrations. No consistent pattern of oscillations was observed when the data were subjected to the analysis described above.

RESULTS

Mean concentrations of immunoreactive insulin secreted by the five tumors ranged from 11.5 to 539 fmol · ml^{-1} · mg^{-1} wet wt of tissue (Fig. 1; Table 1). In tumors 2, 3, and 5, mean hormone concentrations tended to decrease with time, requiring a detrending procedure to enable analysis of pul-

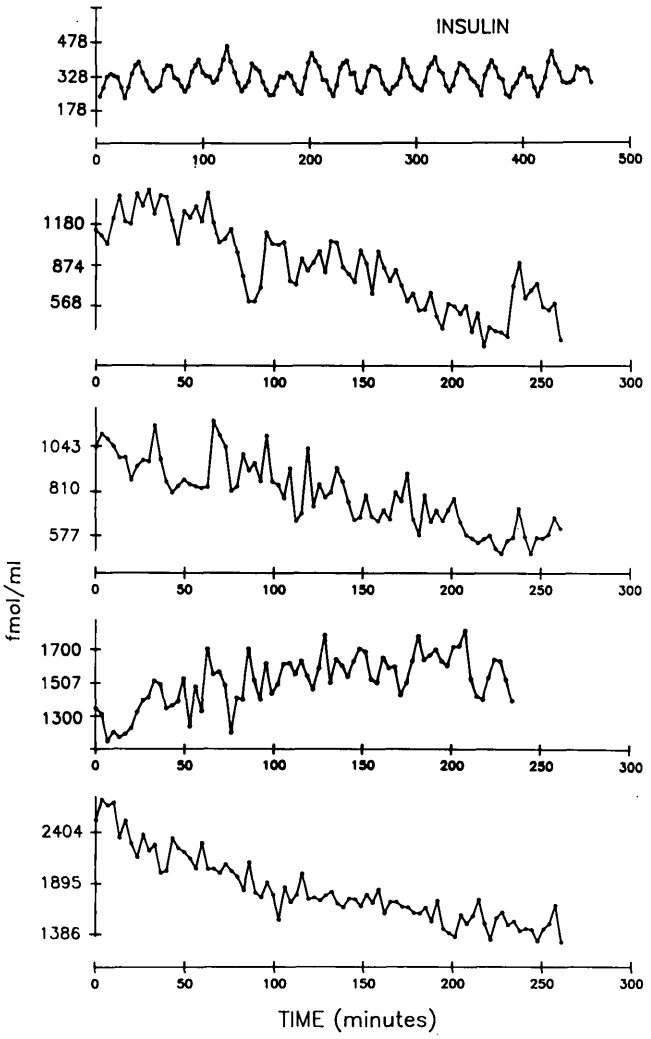

FIG. 1. Secretory pattern of immunoreactive insulin from fragments of 5 human β-cell adenomas perifused with RPMI-1640 and 5.5 mM glucose. Secretory pattern of each tumor are shown separately in consecutive order, beginning from *top*. Note that scales on *y-axes* vary.

satility. ANOVA confirmed the existence of significant pulsatility in all tumors ($P < 0.005$ or less). The periods determined by the Clifton cycle detection program ranged between 17 and 45.7 min, and the amplitude ranged from 4.9 to 87.4 fmol · ml^{-1} · mg^{-1} wet wt (Table 2).

Mean concentrations of immunoreactive C-peptide ranged from 7.1 to 614.4 fmol · ml^{-1} · mg^{-1} (Fig. 2; Table 1). The C-peptide–insulin molar ratio was 0.93:1 (Table 1). C-peptide oscillations were detected in each of the tumors ($P < 0.025$ or less by ANOVA). Periods were 25.9–39.1 min and amplitude was 3.3 to 309.4 fmol · ml^{-1} · mg^{-1} (Table 2).

Mean concentrations of immunoreactive proinsulin ranged from 1.5 to 80 fmol · ml^{-1} · mg^{-1} (Fig. 3; Table 1). Proinsulin pulsatility was also observed in all tumors ($P < 0.025$ or less by ANOVA). Analysis of pulsatility revealed a period that ranged between 17.4 and 39.1 min, and amplitude was 1–28.5 fmol · ml^{-1} · mg^{-1} (Table 2).

The periods of all three (pro)insulin peptides were similar within each tumor studied. The overall mean ± SE periods for all five tumors, estimated by the Clifton cycle detection program for insulin, C-peptide, and proinsulin, were 27.9 ± 5.1, 29.0 ± 2.5, and 26.0 ± 4.1 min, respectively.

Spectral analysis confirmed the hypothesis of regular periodicity in hormone secretion for all tumors and of consistency between hormones in a single tumor. There were from one to three strong components in each tumor, consisting of a low-frequency component with a period of 27–38 min, a mid-range frequency component with a period of 17–22 min, and a high-frequency component with a period of 7–8.5 min. The presence or absence of these components was consistent within tumors, although the relative amplitude of the peaks varied between peptides in some tumors. Figure 4 is a plot of the average spectra from the three hormones for each tumor with the normalized values. Figure 4 illustrates this consistency, showing definite peaks at those frequencies, which were common to all peptides. In tumor 1, there was a single peak corresponding to a period of 27 min for each hormone, which shows as a single peak on the average spectra. The average spectrum in tumor 2 (Fig. 4) also showed a single dominant cycle with a 33-min period. In tumor 3, there were two components of equal amplitude, with periods of 33 and 17.5 min, respectively, and also a low-amplitude, high-frequency component with a period of ~8 min. In tumor 4, there was a dominant cycle with a period of 34 min and two smaller-amplitude cycles with periods of 20 and 7.4 min. There was less agreement between the peptides for tumor 5 than there was for the others, so there are lower peaks in the average spectra. The three major peaks were at periods of 33, 19, and 8.25 min. Tumors 4 and 5 demonstrated somewhat less regularity than the other three, possibly explained by prior exposure to diazoxide, which appears to have a similar effect to reduce regularity

TABLE 1
Mean hormone concentrations secreted by insulinomas (fmol · ml^{-1} · mg^{-1})

Tumor	Insulin	C-peptide	Proinsulin	Immunoreactivity	
				C-peptide/insulin	Proinsulin/insulin
1	11.5 ± 1.8	7.1 ± 1.4	1.9 ± 0.35	0.62	0.20
2	87.9 ± 15.2	90.1 ± 25.3	1.51 ± 0.5	1.02	0.02
3	65.6 ± 14.2	53.8 ± 13.4	2.04 ± 0.4	0.82	0.03
4	539 ± 56.4	561 ± 64.3	80.0 ± 12.4	1.04	0.15
5	525 ± 142	614 ± 167	10.6 ± 1.4	1.17	0.02
Total				0.93 ± 0.2	0.08 ± 0.09

Values are means ± SD except as noted. Secretion was measured per milligram wet weight of tissue after perifusion (tumor 1, 28.2 mg; tumor 2, 9.9 mg; tumor 3, 12.7 mg; tumor 4, 2.8 mg; tumor 5, 3.6 mg).

TABLE 2
Analysis of pulsatility with Clifton cycle detection program

	Pulsatility (fmol · ml^{-1} · mg^{-1})				
	Mean	Amplitude	Period (min)	Cycles (*n*)	Amplitude/mean (%)
Tumor 1					
Insulin	11.5	4.9	29.1	15	42.7
C-peptide	7.1	3.3	27.4	16	47.1
Proinsulin	1.9	1	31.1	14	51.1
Tumor 2					
Insulin	87.9	13.4	45.7	5	15.3
C-peptide	90.1	66.9	39.1	6	74.2
Proinsulin	1.5	1	39.1	6	68.2
Tumor 3					
Insulin	65.6	25.6	29.3	8	39.0
C-peptide	53.8	30.3	26.4	9	56.3
Proinsulin	2	1	24	10	49.5
Tumor 4					
Insulin	539	87.4	17	13	16.2
C-peptide	561	163.8	26.4	8	29.2
Proinsulin	80	28.5	18.3	12	35.6
Tumor 5					
Insulin	525	80.3	18.5	17	15.3
C-peptide	614	309.4	25.9	12	50.4
Proinsulin	10.6	3	17.4	18	28.3

in isolated rat islets (21). Analysis of cycle phase, comparing pairs of peptides in each tumor, revealed differences less than one sampling period (<3.3 min) in 13 of 15 comparisons, with the exception of C-peptide in tumor 5, which was between one and two sampling intervals out of phase with the other two peptides.

In general, the average period estimated by the Clifton program (Table 2) approximates fairly closely the periodicities obtained by spectral analysis (Fig. 4). In tumors 1 and 2, the single low-frequency peak observed on spectral analysis approximated the average period determined by the Clifton analysis; in tumor 3, the average period (26 min)

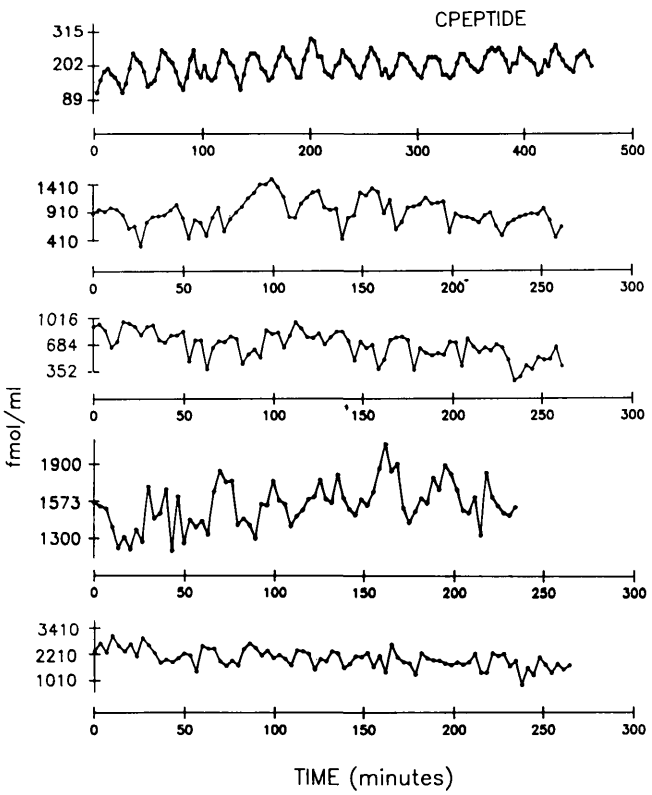

FIG. 2. Secretory pattern of immmunoreactive C-peptide from fragments of 5 human β-cell adenomas. Secretory pattern of each tumor is shown separately in consecutive order, beginning from *top*. Note that scales on *y-axes* vary.

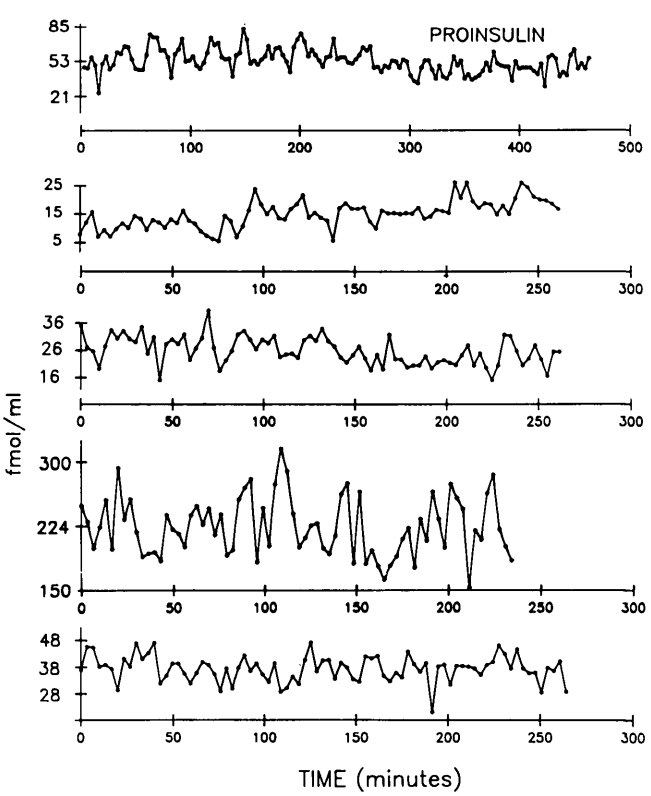

FIG. 3. Secretory pattern of immunoreactive proinsulin from fragments of 5 human β-cell adenomas. Secretory pattern of each tumor is shown separately in consecutive order beginning from *top*. Note that scales on *y-axes* vary.

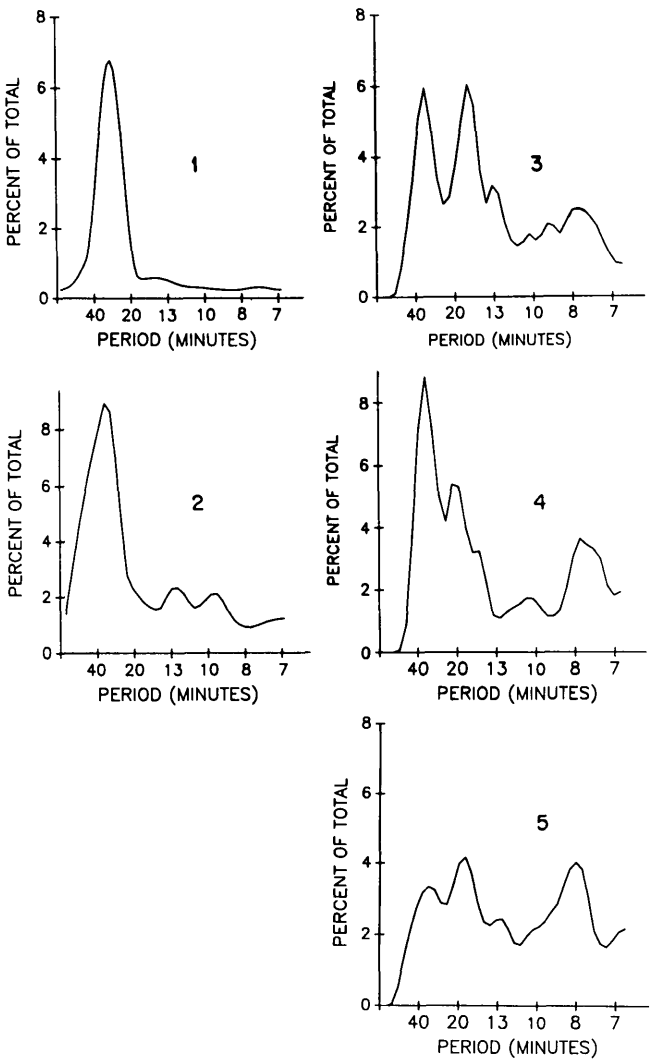

FIG. 4. Results of spectral analysis for each tumor. Each curve represents average of spectra for insulin, C-peptide, and proinsulin for that tumor.

obtained by the latter was found to be similar to the average periods of the two major components observed in spectral analysis (25 min); and in tumors 4 and 5, the periods found by the Clifton program tended to be shorter—this was reflected in spectral analysis by the appearance of high-frequency components of greater magnitude. These contributed to the shortening of the apparent period (derived by Clifton analysis) in these two tumors, despite the presence of low-frequency components similar to those found in the other tumors.

DISCUSSION

Insulin was secreted by these tumors in an apparent cyclic fashion, which was sustained during the entire perifusion period (Fig. 1), despite a constant concentration of glucose in the medium. Secretion of C-peptide with similar pulsatility lends strong support to this finding. Different tumors exhibited various secretory patterns. However, both the Clifton cycle detection program and spectral analysis demonstrated consistency in the secretory patterns of insulin and C-peptide within each tumor. In addition, spectral analysis confirmed the regularity of the secretory oscillations. The characteristics of the insulin and C-peptide pulsations, which demonstrate that they have similar cycles, might be expected from two molecules that are processed and released together from the same secretory granules of the β-cell. Phase analysis indicated that these cycles are almost synchronous, although a slight phase shift of up to 3 min cannot be excluded by these data. The molar relationship between the mean insulin and C-peptide concentrations also suggests equimolar release of these peptides in pulsatile fashion. Note that the period of the insulin cycle observed in these tumors is longer than that observed in healthy human subjects in vivo but similar to that observed in vagotomized patients (3). The absence of central innervation in both cases could conceivably account for this similarity.

This study has demonstrated for the first time that the β-cell can generate spontaneous pulsatile insulin secretion and therefore provides important new information concerning potential secretory mechanisms. First, because insulinoma tissue studied in vitro is not innervated, the oscillatory secretion pattern observed cannot be dependent for its generation on neural connections such as the system of intrapancreatic ganglia, which may constitute the pancreatic "pacemaker" (7,8). This however does not rule out an important coordinating or modulatory role of a local nervous system in pulsatile insulin secretion in the whole pancreatic organ (7,8). Second, our study also takes advantage of the fact that there were only β-cells present in the tissues that were perifused and therefore demonstrates that cyclical insulin secretion occurs in the absence of non–β-cells of the islets. A paracrine mechanism for insulin pulsatility is therefore also unlikely (22). Third, our observation that β-cells generate a cyclic, apparently periodic rhythm, suggests that models for the pancreatic pacemaker that have relied on this assumption are probably accurate. Others have questioned this assumption as a result of the irregular oscillating activity observed in vivo (6). The possibility was therefore raised that the pancreatic pacemaker does not generate insulin oscillations that are strictly periodic. As an alternative, it was suggested that irregular oscillatory activity may occur, which is transformed from random biological variability into a preferred frequency range by intrapancreatic regulatory pathways, thus generating "pseudoperiodic" fluctuations (6). Our results suggest that the underlying rhythm of insulin secretion is likely to be a regular, periodic oscillation.

Thus, this study provides evidence that the β-cell may be the ultimate source of cyclic secretory activity in the absence of external influence. It is possible, but unlikely, that the pulsatile insulin secretion observed in this study is a peculiarity of tumor cells only. On the basis of these findings, we would predict that the β-cells of the normal isolated islet might also secrete insulin in pulsatile fashion, despite removal of the islets from the pancreatic organ. Supporting this contention, oscillatory insulin secretory patterns have been reported in normal isolated islets of mice (23,24) and rats (14,25).

Proinsulinlike immunoreactivity was also secreted by these tumors in easily measurable concentrations. This gave us the opportunity to study the secretory pattern of the prohormone and the insulin and C-peptide released from the β-cells of this tumor. Proinsulin was secreted in the same rhythmic fashion and demonstrated the same periodicity as insulin and C-peptide. Phase estimation demonstrated that,

in these tumors, proinsulin is secreted in similar fashion to insulin and C-peptide, with phase shifts of <3 min in all instances. Thus, these studies confirm the cosecretion of these three peptides. To our knowledge, this is the first description of in vitro dynamic secretory studies of proinsulin and thus the first demonstration of pulsatile proinsulin secretion from the β-cell. Studies of this kind have been limited in the past by the difficulty in obtaining human islet tissue and the unavailability of radioimmunoassays for the proinsulin of other species in which islets were more easily obtained.

In normal β-cells, insulin secretion occurs by a sequential process, the first step of which is packaging of proinsulin into secretory granules after traversing the Golgi apparatus (26). Enzymatic processing of proinsulin occurs within these secretory granules where proinsulin is almost completely converted into insulin and C-peptide. All three molecules are then secreted by the β-cell during exocytosis (27). Although proinsulin is the major component in immature, clathrin-coated secretory granules, it comprises a very small proportion of the peptides stored in mature secretory granules (26), and accounts for <20% of circulating insulin immunoreactivity, once secreted by the β-cell into the circulation (28). In contrast, proinsulin is a major component of β-cell secretion in patients with insulin-secreting tumors, and its measurement in serum has been used as a diagnostic test for this disorder (28). Various mechanisms for enhanced secretion of proinsulin in tumors have been suggested: increased turnover of the synthesis-secretion process, with rapid transit of secretory granules to the plasma membrane and secretion before enzymatic processing is complete (29,30); or secretion of proinsulin by a different intracellular pathway than insulin, possibly not involving typical secretory granules (30). Although not providing a mechanism for increased proinsulin secretion rates, our study utilizes this abnormality to demonstrate that proinsulin is directly secreted from the β-cell together with insulin and C-peptide. Considering the similarity in secretory rhythm, it is therefore likely that the proinsulin and its cleavage products are secreted by the same secretory granule pathway.

In conclusion, this study demonstrates that the β-cell secretes insulin in a sustained pulsatile fashion independent of other islet, exocrine, or neural cells. Application of this model to studies of the metabolic, ionic, or electrical concomitants of insulin secretion at the level of the β-cell, will allow direct comparison with the pulsatile secretory process and thereby may advance our understanding of insulin secretion.

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