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Pulsatile Insulin Secretion by Human Pancreatic Islets

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Insulin is secreted in discrete bursts. These pulses are also present when individual or groups of islets are perifused. Interpretation of the measured frequency and magnitude of pulsatile hormone secretion requires an examination of the sensitivity and specificity of the methods for pulse detection and validation of these for the experimental apparatus and hormone assay in which they are applied. In the present study we achieve these aims for a perfusion method for measurement of pulsatile insulin release by human islets. A deconvolution technique previously developed for measurement of pulsatile hormone secretion *in vivo* was specifically validated for *in vitro* pulse detection in the present study. Deconvolution analysis reliably (>90%) detected insulin pulses with an amplitude 20% or more above baseline and recovered quantitatively the insulin secretion profile, insulin secretion rate, and insulin pulse mass from single as well as multiple perifused islets. Cluster analysis was less sensitive, but was able to detect most (>80%) pulses with an amplitude of 40% or more above baseline. With this limitation, cluster analysis is potentially useful for groups, but not single perifused human islets. Analysis of single human islets showed that enhanced insulin secretion by increased glucose concentrations in the perfusate is achieved by enhancing insulin pulse mass with no change in pulse frequency. Perfused single or groups of human islets exhibited an interpulse interval (~6–8 min) comparable to that observed in humans *in vivo*. Dynamic *in vitro* perifusion should facilitate studies of the mechanisms driving pulsatile insulin secretion. (*J Clin Endocrinol Metab* 87: 213–221, 2002)

INSULIN IS SECRETED in a pulsatile manner (1–5). These pulses can be detected *in vivo* as well as *in vitro* in perifused islets of Langerhans (1, 6–11). Reliable detection and quantification of pulsatile hormone secretion require statistical methods that have been validated for the conditions in which they will be used. We previously validated the use of a deconvolution technique developed by Johnson and Veldhuis (12) for detection and quantification of pulsatile insulin secretion *in vivo* (5). In the present study we sought to extend this approach for the quantification of pulsatile insulin secretion by perifused human islets of Langerhans. Perifusion studies have the advantage that they allow analysis of the mechanisms that regulate insulin secretion in the absence of the complexities of feedback control (for example, by the changes in plasma glucose concentration). They also allow mechanistic studies of the factors that generate pulsatile insulin secretion (pacemaker activity) as well as coordinate this signal between islets (coordination).

In the present study we appraised the sensitivity and specificity of two statistically independent methods for pulse detection, cluster analysis (13) and deconvolution analysis (12). Cluster analysis was selected as a typical example of the most commonly used statistical algorithms for pulse detection. The deconvolution method used was selected because it provides a statistically based measure of pulse location as well as yields information on secretion rates and kinetics. Optimized pulse detection was then applied to examine pulsatile insulin secretion in response to a relevant stimulus (glucose) to explore the mechanism(s) driving enhanced insulin release.

Materials and Methods

Protocols

Protocol 1: sensitivity and specificity of pulse detection. The purpose of this protocol was to establish the criteria for valid insulin pulse detection from perifused human islets. Fluctuations in insulin concentration in perfusate collected from perifused islets include contributions from assay variance, variability arising from the perifusion apparatus, and genuine oscillations in the insulin secretion rate. Therefore, the influence of each of these components on two published methods for detection of pulsatile insulin secretion, cluster analysis and deconvolution, was examined in protocols 1a, 1b, and 1c. Protocol 1a was designed to evaluate experimental variability arising from the insulin assay, and protocol 1b to evaluate variability arising from the perifusion apparatus. These protocols were therefore concerned with minimizing false positives, *i.e.* maximizing specificity. Protocol 1c was designed to determine the limits of detection by cluster analysis and deconvolution for detection of true insulin pulses, *i.e.* maximizing sensitivity. To this end, protocols 1a–c were performed, in which the variance of each component outlined above was examined within the insulin concentration range (5–100 pmol/liter), which corresponds to those observed in the perfusate from human islets.

Protocol 1a: specificity; assay variance. Human insulin (Actrapid, Novo Nordisk A/S, Bagsvaerd, Denmark) was diluted to five concentrations (5, 20, 30, 60, and 100 pmol/liter) in Krebs-Ringer bicarbonate buffer (115 mmol/liter NaCl, 4.7 mmol/liter KCl, 2.5 mmol/liter $CaCl_2$, 1.2 mmol/liter $MgCl_2$, and 5 mmol/liter NaOH) supplemented with 0.2% human serum albumin. Forty aliquots were collected from each of the five insulin concentrations and stored at −20 C until assayed in duplicate.

Protocol 1b: specificity; perifusion apparatus variance. To determine variance in insulin concentration levels attributable to the perifusion system, the five insulin dilutions prepared in 1a were pumped through the perfusion system (duplicate experiments) in an identical manner to that in which islets were perfused. Specifically, the perfusate was equilibrated with 95% O_2/5% CO_2 and maintained at pH 7.4 at 37 C before being pumped

through the system at constant flow rate of 0.3 ml/min for 42 min. The effluent was collected at 1-min intervals and then stored at −20 C before assay.

Protocol 1c: sensitivity of true pulse detection. We simulated oscillations of insulin concentration (in a range comparable to those observed from human islets) in the effluent from the perifusion system by superimposing four amplitudes of insulin oscillation (7.5, 12.5, 22.5, and 50 pmol/liter) onto each of the 5 constant insulin infusions (total of 20 perfusions) described in 1b. In each experiment, 7 pulses were infused over a period of 42 min. Pulses were generated in a square waveform for 0.5 min superimposed on the constant insulin infusion. After the 0.5-min pulse, there was a 5.5-min period with the constant basal insulin infusion only, so achieving an interpulse interval of 6 min to mimic that observed in human islets. Perfusate effluent was collected at 1-min intervals for 42 min and stored at −20 C until assay in duplicate for insulin.

Apparatus. We used the ACUSYST-S perifusion system (Cellex Biosciences, Inc., Minneapolis, MN), which consists of a multichannel peristaltic pump, tubing sets, a heat exchanger, and perifusion chambers. The peristaltic pump draws the perfusate and delivers it through a heat exchanger, where it is warmed to 37 C. From the heat exchanger, the perfusate is delivered through islet chambers, which were loaded with Bio-Gel P_2 beads (Bio-Rad Laboratories, Inc. Hercules, CA). Output from the islet chambers is collected at 1-min intervals by a fraction collector. The volume of the islet chamber is 0.4 ml.

Protocol 2: recovery of infused insulin pulses and insulin kinetics. The purpose of protocol 2 was to define the dynamics of islet secretion, based on the observed fluctuations of insulin concentration in the perifusate. The insulin output from the perifusion system is a function of both the actual secretion dynamics of the islet in the islet chamber and the damping of this signal in the subsequent drainage system as well as any losses of insulin via adsorption to the apparatus. Accordingly, we first delivered insulin pulses directly into the islet chamber in a profile that mimicked the secretory behavior of islet β-cells predicted by electrophysiology studies (6, 14, 15). This profile is shown in the *middle panel* of Fig. 4. Protocol 2 differs from protocol 1c as follows. First, in 1c, ranges of amplitudes of insulin concentration pulses were administered to allow us to establish the sensitivity of pulse detection. Second, in protocol 1c, insulin oscillations were presented upstream of the perfusion apparatus, whereas in protocol 2, they were delivered directly into the tissue chamber to mimic the actual secretion dynamics of islets in this system as closely as possible.

Protocol 3: pulsatile insulin release by human islets. To examine the pattern of insulin secretion from human islets in the above perfusion system, we studied insulin release by both single and groups (n = 5–20) of human islets.

Single humans islets were exposed to basal (5.5 mmol/liter) and stimulated (8, 16, and 24 mmol/liter) glucose concentrations (n = 4). Groups of 5–20 human islets were studied at basal (5.5 mmol/liter) and stimulated (16-mmol/liter) glucose (n = 20).

Human islets were isolated from pancreas tissue retrieved from four heart-beating organ donors by Department of Surgery, University of Leicester (Leicester, UK). The donors did not have a history of diabetes. After isolation, islets were cultured in RPMI 1640 medium containing 5.5 mmol/liter glucose, 2 mmol/liter glutamine, 10% FCS, 100 U/ml benzylpenicillin, and 0.1-mg/ml streptomycin. The islets were cultured in Leicester for 2 d before being sent to Edinburgh. Islets were then transferred to fresh RPMI 1640 medium (same composition as above) and cultured for 2 d before experiments were performed.

Assays

Insulin concentrations were measured in duplicate by two-site immunospecific insulin ELISA as previously described (11, 16). The assay uses two monoclonal murine antibodies (Novo Nordisk, Bagsvaerd, Denmark) specific for insulin. There is no cross-reactivity with proinsulin or split 32,33 and 31,32 proinsulins. The sensitivity of the assay is 5 pmol/liter, and the detection range is 5–2000 pmol/liter. The intra- and interassay CVs ranged from 2.0–2.3% and 3.7–4.5%, respectively. In the present studies all measured insulin concentrations were within this operating range of the insulin assay.

Calculations and statistical analysis

Deconvolution analysis calculates underlying insulin secretion rates and the position, duration, mass, and amplitude of insulin secretory bursts as well as the basal or nonpulsatile insulin secretion rate based on known or assumed half-life parameters (12, 17, 18). This deconvolution approach yields the number and locations in time, the individual amplitudes, and the half-durations of each hormone secretion episode. The individual amplitudes (A) and the half-durations (HD) of each pulse, in turn, provide the secretory burst mass per unit volume of distribution for each pulse. The mass is then simply provided by the equation: pulse mass = $(\sqrt{\pi})$(A)(HD)/2.354. The deconvolution algorithm employed is a maximum likelihood iterative reconvolution algorithm. This algorithm assumes that the secretion is modeled as the sum of a finite number of Gaussian-shaped secretion events. The concentration is then described as a convolution integral of the secretion and the elimination kinetics. This deconvolution program does not involve any smoothing. This deconvolution algorithm was specifically chosen because it is a maximum likelihood method, which correctly considers the observed experimental uncertainties of the data. A deconvolution algorithm formulated in this manner is actually a pulse detection algorithm, as the existence of any particular secretion event is evaluated by standard statistical techniques such as bootstrapping and Monte Carlo methods (19, 20). Alternate deconvolution algorithms based on Fourier methods do not provide an approach to assess the significance of individual secretion peaks, *i.e.* Fourier deconvolution methods cannot detect pulses, but maximum likelihood iterative reconvolution methods can. This algorithm and its validation have been discussed previously and will not be repeated here (12, 17, 18, 21). The weighted, nonlinear, least squares parameter estimation procedure is based upon a damped Gauss-Newton algorithm. As we have empirically observed that experimental uncertainties in hormone assays, such as the present insulin assays (data not shown), can be closely approximated by a Gaussian distribution, this method is a maximum likelihood method. The algorithm, associated methods for confidence interval estimation, and goodness of fit evaluation were discussed previously (9, 22–25).

To obtain the parameters required for deconvolution in this apparatus, a column of apparent half-life was estimated by monoexponential fitting of the observed insulin concentrations in serial 1-min samples after abrupt cessation of a continuous insulin infusion in two experiments, thereby correcting for secondary skewness imposed on the perifusion insulin waveform due to nonspecific time delay of column flow-through (26). The measured volume of distribution of insulin delivered in this manner to the perifusion system was 0.33 ml. The decay curve for insulin concentration after cessation of an insulin pulse conformed to monoexponential decay, supporting a single compartment model with a calculated half-life of 0.63 min.

Simulations were carried out based on the above reconvolution model (12, 17, 18, 21, 27, 28). In particular, the attached plot (Fig. 1) illustrates valid recovery of biomathematically simulated insulin pulse amplitudes extending over a 10-fold range and concomitantly shows recovery of the correct underlying mean interpulse interval (*solid line* and *dotted line*, respectively). Data in the plot are given as the mean ± SD (n = 10 simulations/data point). The reconvolution model was described previously (1, 7, 8, 16, 29). Simulations were performed to mimic pulsatile insulin release with the indicated mean amplitudes ± 30% coefficient of variaiton (*x*-axis), 8% superimposed additive sample experimental uncertainty, the perfusate insulin half-life, a 1-min descritzed sampling interval, a burst half-duration of 1.0 min, a mean interpulse interval of 7 min (±20%), and 0%, 30%, 70%, or 90% basal secretion (70% basal secretion data shown). The resultant synthetic time series were deconvolved with the analyst blinded to the simulation parameters. Amplitudes above 6 pmol/liter·min were detectable at $P < 0.01$ *vs.* baseline and sample noise.

Cluster analysis, in contrast, determines statistically significant up- or downstrokes in the serial insulin concentrations, providing information only about the frequency and amplitude of the oscillations in the data series without any assumptions or knowledge of the insulin half-life in the system of study or underlying secretory burst waveform. The *t* statistics used for evaluating significant up- and downstrokes in the

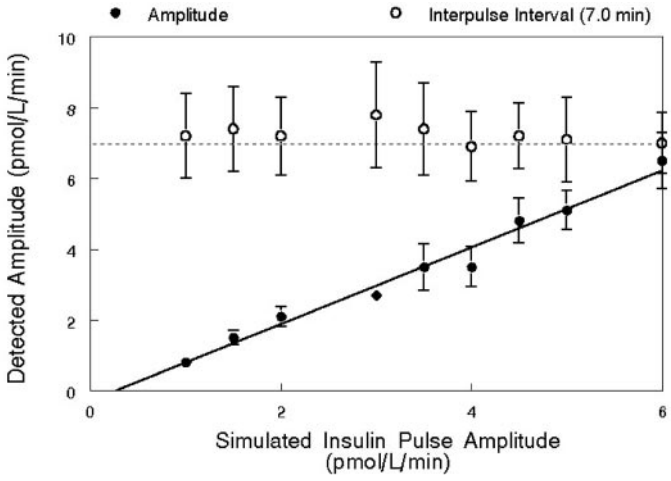

FIG. 1. The valid recovery of biomathematically simulated insulin pulse amplitudes is shown extending over a 10-fold range, and concomitantly, the recovery of the correct underlying mean interpulse interval is illustrated (*solid line* and *dotted line*, respectively). Data in the plot are given as the mean ± SD (n = 10 simulations/data point).

insulin-time series were taken as 2.0, based on prior simulation studies. The corresponding estimated cluster sizes of 2 and 2 in the nadirs and peaks were defined using signal-free insulin profiles (below).

Results

Protocol 1: sensitivity and specificity of pulse detection (Fig. 2)

Protocol 1a and 1b: detection of false positive pulses due to assay variance alone or variance due to assay and perfusion apparatus. Despite the low coefficient of variation of the current insulin assay, fluctuations in the insulin concentration obtained by multiple replicates from the same sample of buffer can give the appearance of pulses when plotted sequentially, as shown in Fig. 2 (*top panel*). When using cluster analysis to examine these data, the criteria required to avoid false detection of insulin oscillations can be ascertained by inspection of Table 1. Use of a *t*-score of 2 or greater resulted in detection of less than 2 false positives in 40 assay replicates regardless of the cluster size selected to define a pulse. Use of a cluster size of 2 × 2 and a *t*-score of 2 resulted in detection of a mean of 0.8 false positives (never more than 1) in the 6 assay profiles (40 samples/assay) examined.

When the same insulin concentration profiles were subject to pulse detection using deconvolution (using a single half-life of 0.63 min and a volume of distribution of 0.33 ml, as established in protocol 2), a mean of 1.3 false positives was detected for the 40 samples assayed at each concentration.

A representative insulin concentration profile arising from a constant insulin infusion is shown in Fig. 2 (*middle panel*). Cluster analysis applied to these data using a *t*-score of 2 and a cluster size of 2 × 2 detected two or three false positive pulses per 40-min perfusion. Deconvolution of the same 40-min insulin concentration series provided comparable rates of false positives (mean, 2.5 pulses/40-min profile) during a constant insulin infusion. In conclusion, either cluster analysis or deconvolution when optimized reveals occasional pulses using a high density (every 1 min) sampling schedule. An insulin pulse interval of approximately 20 min under

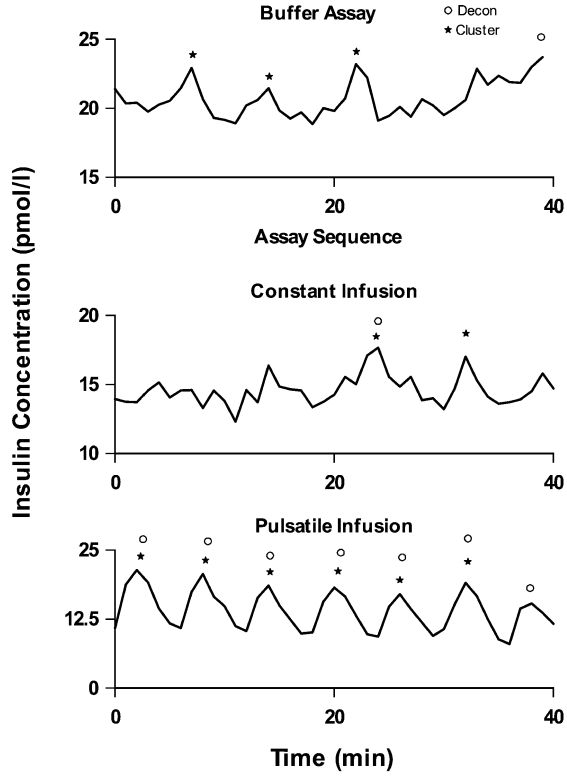

FIG. 2. *Top panel*, Forty assay replicates from the same buffer sample plotted in sequence of assay; *middle panel*, 40 insulin concentrations (mean of duplicates) obtained sequentially from the perfusion apparatus after perfusion of the same buffer shown in *top panel* at a constant rate through the perfusion apparatus; *bottom panel*, 40 insulin concentrations (mean of duplicates) obtained in sequence from the perfusion apparatus during a pulsatile insulin infusion. The site of a pulse is identified by a *circle*; a cluster is indicated by a *star*.

these conditions should be interpreted with caution, because it may simply reflect false positive pulse events.

Protocol 1c: sensitivity of true positive pulse detection; optimal cluster size and t-score for cluster analysis. A representative insulin concentration profile obtained during a pulsatile insulin perifusion (seven pulses infused) is shown in Fig. 2 (*bottom panel*). As cluster analysis (like many pulse detection algorithms) permits a range of sensitivity for pulse detection, we established first in protocols 1a and 1b acceptable sensitivity (*t*-scores) and cluster sizes to avoid false positives (Tables 1a and 1b). In Table 1c, the outcomes of varied *t*-scores and cluster sizes are summarized for all infusions (four amplitudes imposed on five basal concentrations) examined in protocol 1c. The use of cluster analysis with a *t*-score of 2 and a cluster size of 2 × 2 detected 64% of pulses. Although a *t*-score of 1 and a cluster size of 1 × 1 detected 99% of pulses, these criteria led to six false positive pulses over the same period during a constant infusion (Table 1b). Therefore, the most appropriate cluster criterion for detection of insulin pulses from human islets in this perifusion system is a *t*-score of 2 and a cluster size of 2 × 2. The use of these criteria provided detection of 64% of true pulses (mean detection rate of all amplitudes infused) during a pulsatile infusion while yielding 2.7 false positives, on the average, during a constant insulin infusion. Having estab-

TABLE 1. Cluster analysis for Protocol 1

Protocol 1A					Protocol 1B					Protocol 1C				
Peak v nadir	t-score				Peak v nadir	t-score				Peak v nadir	t-score			
	1	2	3	4		1	2	3	4		1	2	3	4
1 × 1	2.3	0.3	0	0	1 × 1	6	1.7	0.4	0.1	1 × 1	6.9	4.3	2.1	1.1
2 × 2	3.3	0.8	0.17	0	2 × 2	4.4	2.7	1.1	0.6	2 × 2	5.2	4.5	3.9	3
3 × 3	2.7	1.5	0.17	0.17	3 × 3	3.3	2.5	1.6	0.7	3 × 3	4.1	3.7	3.2	2.7
4 × 4	2.3	0.8	0.3	0.17	4 × 4	2.5	1.9	1.2	0.5	4 × 4	2.1	1.9	1.5	1.1
5 × 5	0.8	0.5	0	0	5 × 5	1.5	0.8	0.5	0.5	5 × 5	1.4	1.1	0.9	0.6
6 × 6	0.17	0.17	0	0	6 × 6	0.9	0.8	0.4	0.2	6 × 6	0.8	0.6	0.4	0.4
7 × 7	0.17	0	0	0	7 × 7	0.7	0.5	0.5	0.2	7 × 7	0.5	0.4	0.3	0.2
8 × 8	0	0	0	0	8 × 8	0.3	0.4	0	0.1	8 × 8	0.3	0.3	0.3	0.2

Number of pulses detected by use of Cluster analysis in insulin concentration profiles from Protocol 1 using cluster sizes from 1 × 1 to 8 × 8 and t-scores from 1 to 4. Protocol 1a shows the mean number of false positive pulses detected from duplicate measurement of 40 aliquots obtained from five buffer preparations containing insulin at 5, 20, 30, 60, and 100 pmol/liter. Protocol 1b shows the mean number of false positive pulses detected when the same buffer preparations were pumped through the perifusion system at a constant rate and 40 consecutive aliquots collected at 1-min intervals for subsequent assay in duplicate. Protocol 1c shows the mean number of true pulses identified (of seven) during a pulsatile insulin infusion (pulse amplitudes of 7.5, 12.5, 22.5, and 50 pmol/liter superimposed on each of the basal insulin concentrations of 5, 20, 30, 60, and 100 pmol/liter to reproduce the range of oscillations observed from human islets). The most acceptable assessment of pulsatile insulin detection was obtained with a cluster size of 2 × 2 and a t-score of 2.

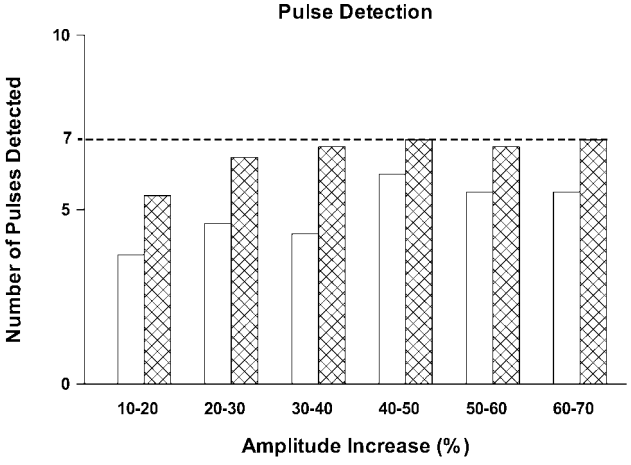

FIG. 3. The sensitivity of pulse detection by cluster analysis (□) and deconvolution (▨) for detecting infused pulses as a function of the percent increment in amplitude above baseline. Seven pulses (*broken line*) were actually infused.

lished the most optimal criterion for pulse detection by cluster analysis for perfused human islets, we then sought to use these to compare this approach to the selected deconvolution method.

Cluster analysis vs. deconvolution (Fig. 3). The larger a pulse as a percent increment above baseline, the more likely it is to be identified by any statistical method of pulse detection. We examined sensitivity for pulse detection by cluster analysis and deconvolution over pulse increments of 10–70% above baseline. When the mean amplitude of pulses was 40% or greater above baseline, cluster analysis detected approximately 80% of pulses. However, when the amplitude of pulses was 10–20% above baseline, cluster analysis detected approximately 50% of pulses.

Deconvolution detected 94% of pulses (mean detection rate of all amplitudes infused). Deconvolution detected approximately 100% of true pulses when the amplitude of the insulin pulse above baseline was 20% or more and about 80% of pulses with a pulse amplitude of 10–20% above baseline. In conclusion, over the whole range of amplitude, deconvolution was more sensitive (6% false negatives *vs.* 34% false negatives with cluster analysis) and comparably specific (2.5 *vs.* 2.7 false positive/40 insulin samples during constant infusion) than cluster analysis.

Protocol 2: recovery of infused insulin pulses

In this protocol we tested the recovery of insulin secretion kinetics during known pulsatile insulin infusions given directly into the islet chamber so as to reproduce as closely as possible the presumed pattern of insulin secretion by human islets. As deconvolution, but not cluster, analysis measures secretion, we employed deconvolution only in protocol 2. Figure 4 allows direct comparison of the known insulin delivery rates (*middle panels*), the insulin concentration profile that resulted from this (*top panels*), and the calculated insulin secretion rates by use of deconvolution of these concentration data (*bottom panels*). Deconvolved insulin secretion closely approximated known insulin delivery rates into the islet chambers. In Fig. 5 the insulin concentration profile arising from the pulsatile perfusion is compared with the insulin concentration fit obtained by deconvolution (*top panel*) with the corresponding secretion rate shown in the bottom panel. Deconvolution of the insulin concentration profiles also yielded excellent recovery of the known insulin delivery rate (92.4 ± 4.9%) as well as the known insulin infused pulse mass (94.5 ± 1.6%). The deconvolution program ascertained that 93 ± 2.5% of insulin delivery was derived from pulses, whereas in this protocol 100% of insulin was delivered in pulses.

Protocol 3: pulsatile insulin release by human islets

Recovery of insulin secretion. Recovery of insulin secretion from perifused human islets using deconvolution of the insulin concentration profile was 102 ± 1.4% for single islets and 104.8 ± 1.4% for multiple islets (Fig. 6).

Pulse frequency single vs. multiple islets. Perifusion experiments (5 independent channels per experiment) were performed on 4 occasions with single islets and on 20 occasions

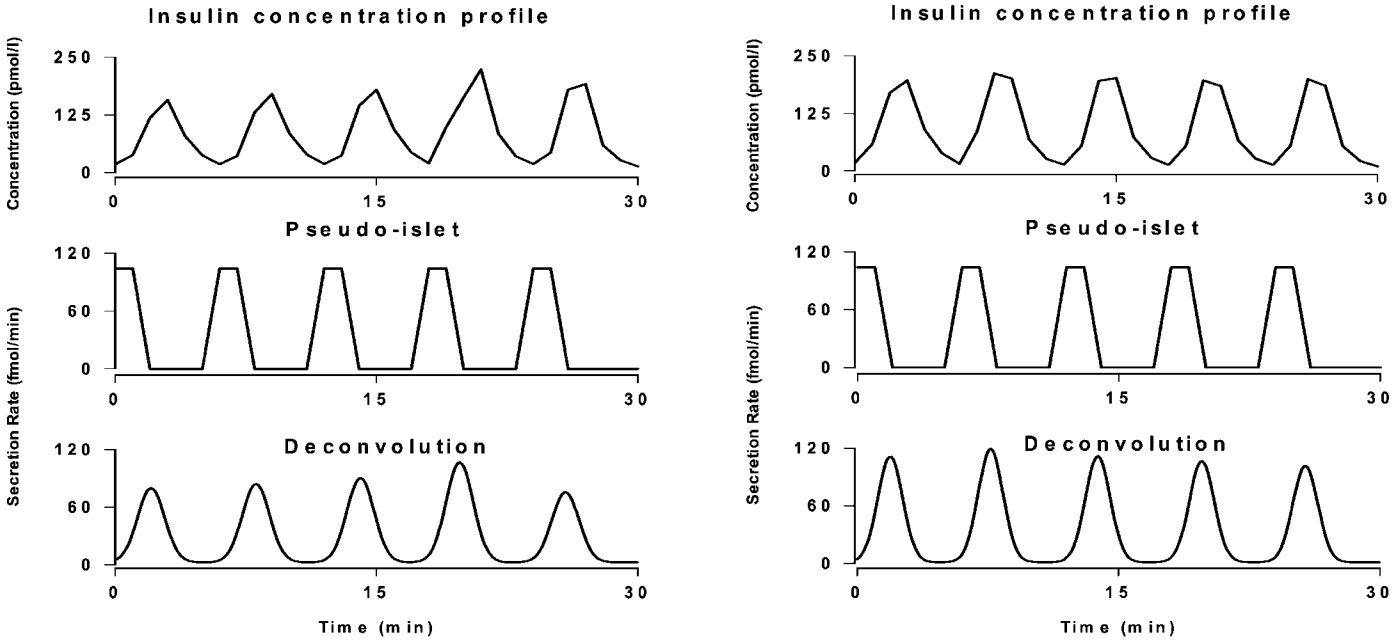

FIG. 4. The insulin concentration profile (*top panels*), the insulin secretion profile infused to mimic a pseudoislet (*middle panels*), and the deconvolved insulin secretion rates (*bottom panels*) in two studies described in protocol 2.

with multiple islets. Pulse detection by deconvolution *vs.* cluster analysis provided a comparable measure of pulse interval (6.7 ± 0.5 *vs.* 8.0 ± 1.5 min; $P = 0.12$, deconvolution *vs.* cluster) when multiple islets (5–20 islets) were perfused regardless of the mean insulin concentration in the islet effluent (mean insulin concentration, 26.1 ± 3.9 pmol/liter; range, 10.4–48.8 pmol/liter). This is consistent with the 30% or higher percent increment in amplitude of pulses arising from multiple islets. Although there is no known pulse interval with real islets (in contrast to the pseudoislet studies in protocol 2), the likely pulse interval from electrophysiological studies is about 6 min (14, 15, 30), which agrees well with these data. In contrast, when single human islets were perifused, the pulse interval with deconvolution remained comparable to that seen in multiple islet perfusions, whereas cluster analysis detected fewer pulses and consequently a longer pulse interval (6.9 ± 1.3 *vs.* 11.9 ± 1.0 min; $P = <0.01$, deconvolution *vs.* cluster). These findings are consistent with the data for pulse detection summarized in Fig. 3. In the basal unstimulated state, the mean percent pulse increment arising from single islets was 15% above baseline (predicting 80% sensitivity for pulse detection by deconvolution *vs.* 50% by cluster analysis), whereas in the stimulated state the percent increment was 25%, predicting detection of 90–150% of pulses by deconvolution and 65% of pulses by cluster analysis.

In stimulated multiple islets the percent increment in pulse amplitude was approximately 50%, predicting detection rates of 100% by deconvolution and 80% by cluster analysis. The insulin concentration range in the effluent from the perifused multiple human islets was consistently within the range selected for the validation studies (5–100 pmol/liter). Studies of single islets occasionally provided basal insulin concentrations of about 3–5 pmol/liter. As deconvolution, but not cluster, analysis provides adequate sensitivity for insulin pulse detection in both unstimulated and stimulated human islets, we used this method to examine the effects of increased glucose concentrations on pulsatile insulin release by humans. Also, as multiple (n = 5–20) islets consistently yield insulin concentrations of more than 5 pmol/liter even in the basal state, whereas single islets occasionally provide lower insulin concentrations, we conclude that the former are more appropriate for studies undertaken under basal unstimulated conditions.

Effects of glucose on insulin secretion. The insulin concentration profile arising from perfusion of multiple human islets revealed distinct oscillations that were detected as insulin pulses (Fig. 7). Oscillations in insulin concentration were less obvious in unstimulated single islets, but became more distinct with increased glucose. After the perfusate glucose concentration was increased from 5.5 to 8, 16, and 24 mmol/liter (8, 16, and 20 considered together), the insulin secretion rate per islet increased (2.8 ± 0.2 to 4.9 ± 0.6 fmol/islet·min; $P < 0.05$), and this was achieved by an increase in pulse mass (6.5 ± 2.7 to 19.5 ± 7 fmol/pulse; $P < 0.05$) with no change in pulse frequency (8.1 ± 0.9 to 6.3 ± 0.9 min; P = NS).

Based on single human islets, the calculated proportions of insulin secreted in distinct pulses *vs.* in basal (nonpulsatile) insulin release were 23.3 ± 9.3% *vs.* 56.2 ± 7.2% (basal *vs.* stimulated with glucose). The mean calculated proportion of insulin secreted in discrete bursts with multiple islet perifusions was 32.2 ± 3.9%.

Discussion

In vivo, most insulin is secreted in discrete secretory bursts (5). Regulation of insulin secretion is achieved physiologically through the mechanism of modulation of the amplitude of insulin pulsatility (6, 31–33). Abnormal pulsatile insulin secretion is an early defect in patients developing type 1 or

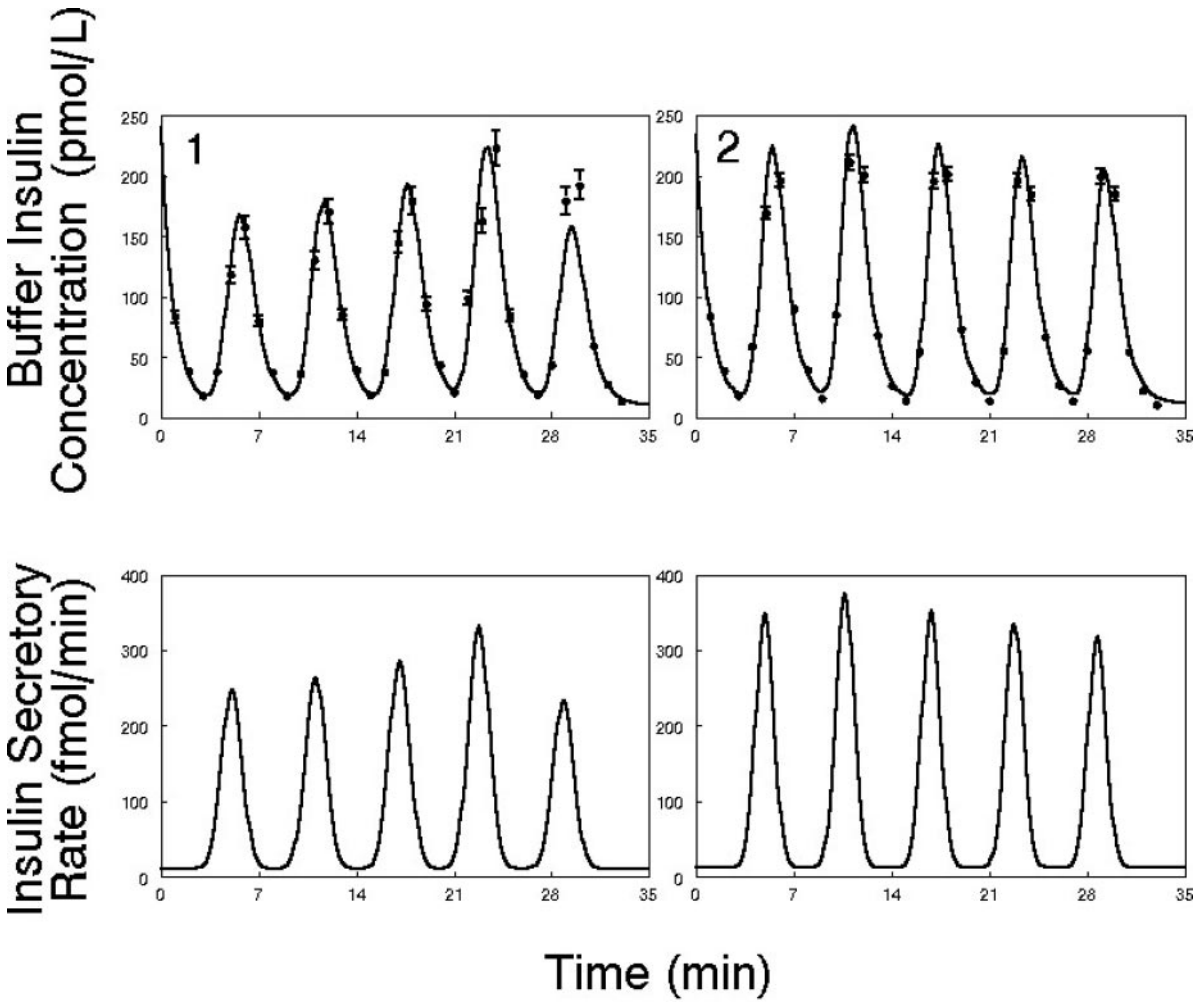

FIG. 5. The best-fit insulin concentration profiles derived during deconvolution (*smooth curve*, *top panel*) and measured insulin concentration for the pseudoislet experiments (protocol 2). The corresponding insulin rates (*bottom panel*) are also shown.

type 2 diabetes (6, 34). Accordingly, an understanding of the mechanisms underlying the generation, coordination, and regulation of insulin secretory bursts is potentially of importance. Estimation of pulsatile insulin secretion *in vivo* in humans and large animals presents particular experimental and analytical challenges, including the delivery of insulin into the portal vein and liver, wherein it undergoes extraction before delivery to the usual sampling site, the systemic circulation (5, 35, 36). Estimation of pulsatile insulin secretion in rodents is complicated by the limitations of blood volume. To overcome these problems, pulsatile insulin secretion has frequently been examined in isolated perifused islets (1, 6–8, 10, 11). However, the quantification of pulsatile insulin release from isolated islets is not without technical challenges. The quantity of insulin released by isolated perfused islets is very small, so such studies are susceptible to the difficulties of distinguishing variability of concentration data due to noise in the assay system from small fluctuations due to episodic secretion at or near the limits of the capacity of assay resolution. Furthermore, any perfusion system is vulnerable to experimentally uncontrolled fluctuations in output associated with mechanical facets of the apparatus.

In the present study we established the conditions in which pulsatile insulin secretion by single or multiple perifused human islets can be measured in a (commercially available) perifusion system using a well characterized ELISA for human insulin. These investigations document optimal criteria for pulse detection from human islets using two statistically independent methods for pulse detection, cluster analysis and a multiparameter deconvolution method. The analyses further define the sensitivity of the two techniques for pulse detection. The deconvolution technique effectively quantitated insulin secretion rates and accurately identified the number, location, and magnitude of insulin secretory bursts that caused an increment in amplitude exceeding 20% of baseline. Deconvolution provided a similar estimate of the frequency of insulin pulses whether islets were perifused singly or in groups. Cluster analysis provided a reasonable estimate of insulin pulse number and location so long as the amplitude of the oscillations was 40% or more above baseline and groups, rather than single islets, were studied. Our appraisal highlights a cautionary note, inasmuch as multiple assay replicates *per se* and/or a perifusion system may generate spurious pulses in insulin concentration series (protocol 1a or protocol 1b) in the absence of genuine biological secretory bursts. As the human insulin assay used in the

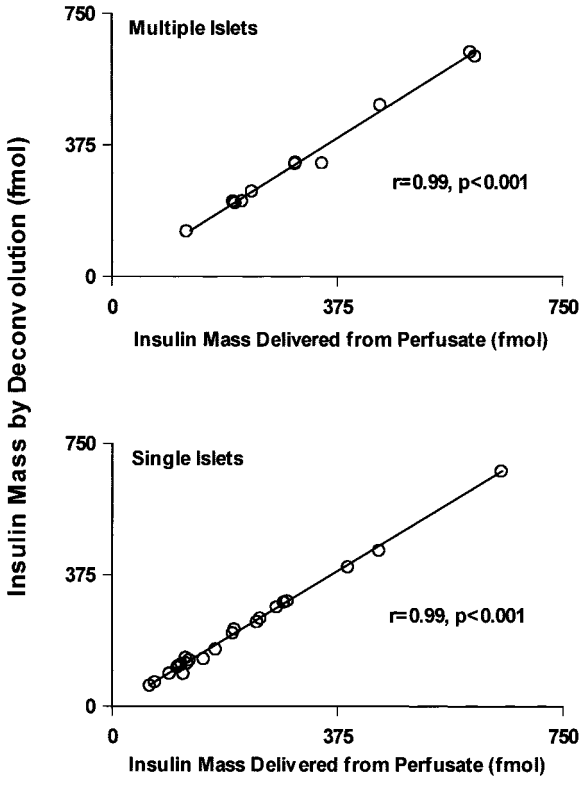

FIG. 6. The relationship between the insulin mass calculated by the product of the insulin concentration and the buffer flow rate (*x*-axes) and the insulin mass obtained by deconvolution (*y*-axes). The relationships when multiple islets were perfused in each chamber (*top panel*) and single islets were perfused per chamber (*bottom panel*) are shown.

current studies has a lower coefficient of variation and is more sensitive and specific than available rodent insulin assays or prior human insulin assays, these problems could be expected to be magnified when studying rodent islets or human islets using conventional RIAs.

Application of the newly validated techniques in human islets (protocol 3) provided some interesting physiological insights. The frequency of detected insulin pulses from human islets was one pulse approximately every 6 min, which is comparable to that observed *in vivo* when sampling directly from the portal vein in dogs or humans (5, 26, 36, 37). Sampling from the systemic circulation in humans with conventional immunoassays initially yielded a lower pulse frequency (*e.g.* interpulse interval of 15–20 min) (29, 34). However, subsequent systemic sampling studies have measured insulin concentration(s) by ELISA and applied deconvolution analysis validated for application *in vivo* (32, 37, 38). These methods disclosed insulin pulse frequency comparable to that observed in the current and earlier studies of single perfused islets. In fact, examination of Fig. 2 reveals how small amplitude pulses compared with the baseline present in the systemic circulation *in vivo* are vulnerable to an underestimate of the pulse frequency, particularly when discrete peak detection analysis is used. In the current studies the observed frequency of pulsatile insulin release by deconvolution was comparable whether islets were perfused as single islets or in groups of islets. The frequency is consistent with prior studies of perfused groups of islets (8, 14). Using rodent islets, it has been necessary to perifuse groups of islets to allow adequate detection of insulin release or enlarged single mouse islets from genetically obese mice (6, 7, 34). Here we find that stimulated single human islets (16 mmol/liter glucose) consistently yield sufficient insulin for reliable detection, although occasionally in the basal state single islets secrete insufficient insulin for reliable pulse detection, particularly with cluster analysis. We therefore recommend multiple islets per channel (5–20) for studies designed to examine insulin secretion under basal conditions or for studies of the inhibition of insulin secretion.

The current studies are also consistent with prior *in vivo* (and *in vitro*) studies that suggest that regulation of the rate of insulin secretion is predominately achieved through modulation of the pulsatile component of secretion. Here we report that stimulation of insulin secretion in individual human islets by an increased concentration of glucose is achieved mechanistically via specific enhancement of the insulin pulse mass with no change in pulse frequency. Measurement of pulsatile insulin *in vivo* by sampling from the portal vein to avoid hepatic insulin extraction reveals that almost all insulin is secreted in the pulsatile mode (5, 36, 37). However, in the present studies *in vitro* examination of the insulin released by perifused human islets reveals that the proportion of insulin secreted in discrete bursts is about 30–50% (or more). This apparent difference might be due to the inefficiency of insulin delivery from islet β-cells *in vitro* into the perifusion system compared with the delivery of insulin to the portal vein *in vivo*. In the latter context, β-cells discharge insulin vesicles into capillaries, which have a relatively high blood flow moving past all of the β-cells before the collection of this blood into the pancreatic veins and the portal vein. In contrast, *in vitro*, the capillaries in the islets are lost, and insulin secreted by β-cells (located predominately in the core of the islet) would be delivered into spaces within the islet, from which it could diffuse to the Bio-Gel in which the islet is embedded and then to the buffer to be delivered from the perifusion apparatus. Insulin might transiently adhere to Bio-Gel beads and the walls of the collecting vessels before appearing in the eluate. These factors would be expected to result in a damping of the waveform of the insulin released by the islet, which would contribute to the predicted effect of underestimating the proportion of insulin released in discrete bursts. Moreover, the removal of the islets from the pancreatic neural network and other paracrine influences may influence the secretory behavior of these cell clusters.

The presence of pulsatile insulin secretion arising from single perifused islets at a frequency comparable to that seen *in vivo* has been observed previously (1, 7, 19, 37) and reveals that the pacemaker function for pulsatile insulin secretion may be present in the islets themselves. Indeed, individual β-cells show oscillatory membrane depolarization (and therefore presumably insulin secretion), although this frequency is higher than that in isolated islets (39). It is assumed that the coordination of this pulsatile secretion by the cells within an islet is obtained through tight gap junctions between β-cells (39). The question also arises as to how groups of islets show coordinate pulsatile insulin release when perifused? It has been suggested that coordinate pulsatile insulin

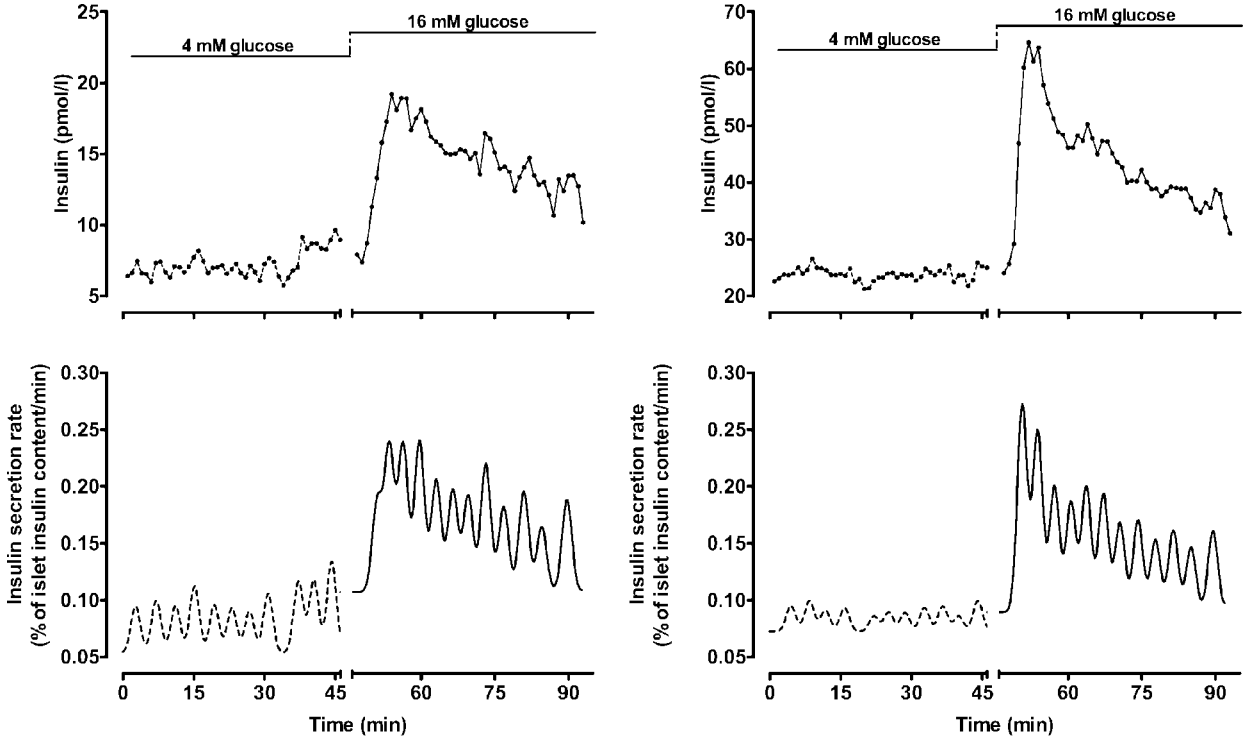

FIG. 7. Insulin concentration profile (*top panels*) and deconvolved insulin secretion rate (*bottom panels*) for two channels of six human islets perfused at 4 and 16 mmol/liter glucose.

secretion *in vivo* is accomplished by neural interconnections between islets scattered in the pancreas (40, 41). However, these are obviously lost in isolated islets undergoing perifusion. It has been proposed that oscillations in glycolysis may be the pulse generator for pulsatile insulin release by islets. (42). Although this mechanism might contribute to coordination of the pulsatile insulin secretion *in vivo* where there is an oscillating glucose concentration, it still does not explain how islets perfused at a constant glucose concentration have coordinated secretion. Possible explanations for this phenomenon include inhibition of insulin secretion by a product of the islet during a secretory burst. Delivery of this product into the surrounding buffer could entrain surrounding islets. Both insulin (43) and islet amyloid polypeptide (44) could meet this criteria. Alternatively, there might be some degree of electrophysiological coupling of islets compacted together in an ionic solution. However, the mechanism that subserves the coupling of pulsatile insulin secretion by islets in a perfusion chamber may have little mechanistic importance for islet to islet coordination of islets scattered in the exocrine pancreas *in vivo*.

In summary, in the present study we have established the conditions for reliable detection of pulsatile insulin secretion from human islets in an *in vitro* perifusion system. Application of the system to human islets reveals that, in general, perifused islets behave remarkably like islets *in vivo*. Thus, we confirm that insulin is released in pulses at an interval of about 6 min, and that regulation of insulin secretion (stimulation by increased glucose concentration) is achieved predominately by amplification of the magnitude of these insulin bursts. The minimum proportion of insulin released by isolated islets that can be confidently ascribed to discrete insulin bursts is lower than that we have previously observed *in vivo*. This could reflect some degree of anatomical disruption of the islet and/or decreased resolution of pulses *per se*. The present system should allow more detailed examination of the factors that generate and regulate discrete insulin bursts by human islets.

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