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Detection of exocytosis at individual pancreatic β cells by amperometry at a chemically modified microelectrode

(insulin/hormone/peptide/secretion)

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ABSTRACT **Amperometry at a carbon fiber microelectrode modified with a composite of ruthenium oxide and cyanoruthenate was used to monitor chemical secretions of single pancreatic β cells from rats and humans. When the insulin secretagogues glucose, tolbutamide, and K^+ were applied to the cell, a series of randomly occurring current spikes was observed. The current spikes were shown to be due to the detection of chemical substances secreted from the cell. Chromatography showed that the primary secreted substance detected by the electrode was insulin. The current spikes were strongly dependent on external Ca^{2+}, had an average area that was independent of the stimulation method, and had an area distribution which corresponded to the distribution of vesicle sizes in β cells. It was concluded that the spikes were due to the detection of concentration pulses of insulin secreted by exocytosis.**

It is generally accepted that insulin is secreted from pancreatic β cells by exocytosis. Numerous lines of evidence provide strong support for this conclusion. For example, insulin is localized in β-cell vesicles (1), microscopic examination of stimulated β-cells shows vesicles that have fused with the cell membrane (2), and increases in cell capacitance follow stimulation of β cells (3, 4). However, insulin secreted from a β cell has not been measured with sufficient sensitivity, temporal resolution, and spatial resolution to detect the small concentration pulses that would be the consequence of insulin secretion by exocytosis. Thus, the time-resolved measurement of insulin secretion at the level of single cells would provide direct chemical evidence supporting the exocytotic theory of secretion. The ability to measure insulin at the level of single exocytosis events is also important because it could be useful in further unraveling the complexities of insulin secretion. Since defects in insulin secretion play a role in type II diabetes (5), these measurements may also have a significant medical impact.

Measurement of concentration pulses resulting from exocytosis requires the detection of around 10^6 molecules in the vicinity of a single cell with millisecond time resolution. Amperometry at carbon fiber microelectrodes has proven useful for these types of measurements (6–10). Amperometry has been applied to detection of exocytosis of catecholamine from single adrenal chromaffin cells (6–8), dopamine from single PC12 cells (9), and indoleamine from single mast cells (10). The data recorded from these measurements are a series of randomly occurring current spikes which have been demonstrated to be detection of packets of molecules released by exocytosis. These results demonstrate that amperometry can be a tool in the study of exocytosis of easily oxidized substances. Unfortunately, many substances, including insulin, are oxidized so slowly at common electrode materials that they are not detectable. To apply amperometry to detection of such substances requires an electrode that can catalyze oxidations better than the carbon fiber microelectrode. Chemical modification of electrodes has proven to be a powerful approach to enhancing the catalytic activity of electrode surfaces (11).

In this study, we have investigated the use of chemically modified carbon fiber microelectrodes for detection of insulin released from single rat and human pancreatic β cells. The chemical modification consists of an electrochemically deposited composite of ruthenium oxide and cyanoruthenate (Ru-O/CN-Ru) which catalyzes the oxidation of insulin (12, 13). In a previous study, we demonstrated that the electrode could be used to monitor chemical secretions from single human β cells (14). The signals recorded were series of current spikes suggestive of exocytosis. Here we report that the spikes measured at both rat and human β cells have the characteristics expected for exocytosis. Furthermore, we provide chromatographic evidence that the measured signals are primarily due to insulin and no other substance released from the cell.

EXPERIMENTAL PROCEDURES

Electrode Preparation and Testing. Glass-encased carbon fiber microelectrodes were prepared and modified with Ru-O/CN-Ru by previously described techniques (14, 15). Response times and calibration curves for the electrodes were obtained in a flow injection apparatus similar to that described elsewhere (14).

The number of electrons transferred per oxidation (n) was determined by chronoamperometry at a microelectrode (16). For this experiment, the steady-state current resulting from a potential step from +0.40 V (nonoxidizing potential for insulin) to +0.85 V (oxidizing potential for insulin) with the electrode immersed in a quiescent solution of 200 μM insulin was recorded. The current resulting from an identical experiment performed in background electrolyte was subtracted from the insulin current to obtain ΔI. n was calculated according to the following equation: $n = \Delta I/4rCDF$, where r is the radius of the electrode (disk carbon electrodes were used for these experiments), C is the concentration of insulin, D is the diffusion coefficient, and F is the Faraday constant. The diffusion coefficient for insulin used for these calculations was $1.98 \pm 0.02 \times 10^{-6}$ cm^2/s. This value was calculated by a method based on measuring the dispersion of an insulin concentration pulse as it flowed through a capillary tube (17).

Islet Isolation and Cell Culture. Human islets of Langerhans were obtained from Washington University Medical School, St. Louis. Islets were dispersed into individual cells and maintained in culture exactly as described (14). Rat islets were isolated from 200- to 220-g male Sprague–Dawley rats by collagenase digestion (18). Isolated rat islets were allowed to recover overnight in an incubator at 37°C and 5% CO_2. For dispersion, the islets were digested with 0.8 ml of 0.05%

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Abbreviations: KRB, Krebs–Ringer buffer; Ru-O/CN-Ru, ruthenium oxide/cyanoruthenate composite.

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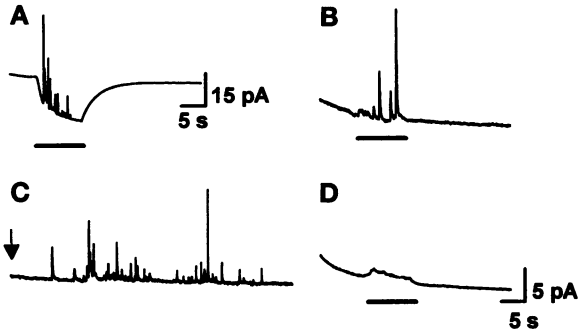

FIG. 1. Current recordings from stimulation of human β cells with 200 μM tolbutamide (*A*), 64 mM K^+ (*B*), 16 mM glucose (*C*), or KRB (*D*). Recordings in *A–C* were obtained at different cells; the recording in *D* was obtained from the same cell as in *B*. Horizontal bars indicate stimulation except for the glucose case (*C*), where the arrow indicates the end of a 30-s stimulation. Scale bars in *D* also apply to *B* and *C*.

trypsin/0.53 mM EDTA at 37°C for 60 s. Three milliliters warmed RPMI 1640 medium was added to stop the digestion. The islets were then washed and suspended in 1.5 ml of modified CMRL 1066 medium. The cells were placed in 35-mm tissue culture dishes at 10^5 cells per dish.

Single-Cell Measurements. Single cell-measurements were performed in a manner similar to that described (6, 7, 14). For single-cell measurements, the cell culture medium was replaced with Krebs–Ringer buffer (KRB; ref. 14). The cells were maintained at 37°C during recordings. To perform measurements, a working microelectrode was brought to within 1 μm of a cell by use of a micropositioner (Burleigh PC-1000). Substances were applied to individual cells by pressure ejection of solutions from the tips of micropipettes positioned ≈30 μm from the cells (14). Stimulants were dissolved in KRB. For K^+ stimulations, 60 mM KCl was added to KRB with an equivalent amount of NaCl removed. For glucose stimulations, the total glucose concentration in KRB was 16 mM. Unless stated otherwise, K^+ and tolbutamide stimulations were of 10-s duration and glucose stimulations were of 30-s duration. For recordings, the sensing electrode was at +0.85 V unless noted otherwise. Between measurements, the sensing electrode was at +0.40 V to prevent rapid degradation of the electrode (14).

HPLC Analysis of Islet "Releasates." Releasates from islets were analyzed by HPLC. The HPLC system utilized SSI 222D pumps (Fisher Scientific) and a standard injection valve equipped with a 50-μl injection loop (Valco Instruments, Houston). Flow rates were 1.00 ml/min. Separations were performed with a Keystone Scientific C_8 column packed with 5-μm porous particles. Unless stated otherwise, the mobile phase was 72% (vol/vol) 50 mM K_2SO_4/10 mM KH_2PO_4 (adjusted to pH 2 with H_2SO_4) and 28% (vol/vol) acetonitrile. Some HPLC experiments were also done with a PerSeptive Biosystems hydrophobic interaction chromatography column. A thin-layer electrochemical cell was used for detection and was built in-house according to a design similar to that reported previously (19). The detector electrode was a glassy carbon disk (3 mm in diameter) modified under the same conditions as used for the microelectrode.

To analyze releasates, 100 islets which had been incubated in RPMI 1640 medium for 1–5 days were placed in 100 μl of KRB with 3 mM glucose and incubated at 37°C and 5% CO_2 for 20 min. This medium was removed and directly injected into the HPLC system to obtain a prestimulation background. The medium was replaced with 100 μl of KRB with 16 mM glucose and 200 μM tolbutamide and allowed to incubate for 20 min. This solution was removed and directly injected into the HPLC system to obtain stimulated-islet chromatograms.

RESULTS

Detection of Secretion from β Cells. When tolbutamide, K^+, or glucose was applied to single human β cells, each of these stimulations produced a series of current spikes which appeared to occur at random times at the electrode (Fig. 1). Similar results were obtained with rat β cells. The time course and frequency of the spikes varied with the stimulation conditions (Table 1). Spikes generally occurred only after a stimulation and rarely were observed before a cell was stimulated. In addition, application of buffer to a cell produced no spikes (Fig. 1*D*).

The variability in time course of spike occurrences with different stimulation methods corresponds well with what is known about insulin secretion. The K^+ and tolbutamide stimulations caused secretion to occur almost immediately after their application to the cell. Furthermore, spikes were observed to cease soon after the stimulation were ended. These results are consistent with the ideas that (*i*) tolbutamide and K^+ act by directly depolarizing the cell and (*ii*) the steps leading to exocytosis following depolarization are rapid. In contrast, glucose stimulation was relatively slow to initiate secretion, but secretion was longer lasting. The slow step in stimulus–secretion coupling is apparently associated with the formation of internal messengers responsible for initiating depolarization (20). However, once the messenger is present, it can persist even after glucose is lowered back to nonstimulus levels.

We previously observed that spikes resulted from glucose stimulations at human β cells only when the electrode potential was sufficient to oxidize insulin (14). We have found that K^+ and tolbutamide stimulations produce current spikes with similar voltage dependencies (Fig. 2). These results imply that the spikes are due to the anodic detection of a substance with an oxidation potential similar to insulin. These results, taken with those elsewhere (14), demonstrate that the spikes detected following application of tolbutamide, K^+, and glucose to rat and human cells correspond to detection of chemicals

Table 1. Summary of current spike observations for three different stimulation conditions

β-Cell origin	Stimulus	No. of cells used	Time to initiation of spike activity,* s	Duration of spike activity,† s	Frequency‡, Hz	Spike area,‡ pC (no. of spikes)
Human	Glucose (16 mM)	10	60–240	180–300	6–10	0.23 ± 0.087 (61)
	K^+ (60 mM)	13	0–2	5–9	1–3	0.29 ± 0.16 (58)
	Tolbutamide (500 μM)	12	0–2	4–9	2–4	0.29 ± 0.097 (216)
Rat	Glucose (16 mM)	4	40–60	120–200	5–10	0.20 ± 0.094 (63)
	Tolbutamide (200 μM)	18	0–2	3–9	1–3	0.23 ± 0.088 (528)

*Time required from start of stimulus to observation of first spike. Range is given.
†Time from first observed spike to last spike for a given stimulation. Range is given.
‡No. of peaks observed during the spike activity divided by the duration of spike activity. Only peaks with heights 5 times the peak-to-peak noise level were used.
‡Mean ± SD is given. No effort was made to reject spikes that were not well resolved from neighbors.

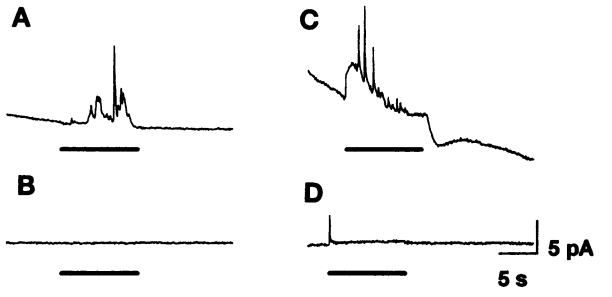

FIG. 2. Current recordings from stimulation of a rat β cell with 200 μM tolbutamide measured with an electrode at +0.85 V (*A*) and +0.40 V (*B*), and from stimulation of a human β cell with 64 mM K^+, measured with an electrode at +0.85 V (*C*) and +0.40 V (*D*). Horizontal bars indicate stimulation.

secreted from the cell. In other words, the current spikes are not electrical artifacts or non-faradaic current spikes.

Although the patterns of spikes vary with stimulations, the area under isolated spikes does not vary significantly with stimulation (Table 1). This result is expected if the spikes are associated with the detection of exocytosis events, because the area of the spike, which has units of coulombs, represents the charge passed to oxidize the compound(s) detected. The charge passed is directly proportional to the number of moles oxidized, according to Faraday's law. If a spike represents the detection of the contents of a vesicle, the mean value observed should be independent of the stimulation method because acute stimulations should not affect the vesicular content.

Ca^{2+} Dependence of Signals. External Ca^{2+} is required to support exocytosis when β cells are stimulated with depolarizing secretagogues (21). To ascertain the Ca^{2+} dependence of the signals observed, cells were stimulated in Ca^{2+}-free medium. Stimulations were performed by applying 200 μM tolbutamide with 2.4 mM Ca^{2+} and 200 μM tolbutamide without 2.4 mM Ca^{2+} to the same cell. Fig. 3 shows the results obtained from this type of experiment. Experiments performed with K^+ stimulations produced similar results. Table 2 summarizes the Ca^{2+}-dependence data for both types of stimulation. In general, current spikes occurred only when Ca^{2+} was present in the stimulation medium. Application of only Ca^{2+} (i.e., regular KRB containing 2.4 mM Ca^{2+}) to the cells produced a small background shift which was present when no cells were near the electrode. From these results, it is possible to conclude that the spikes observed are strongly dependent on external Ca^{2+} in the medium.

Chromatographic Analysis of Islet Releasates. Although it is clear that the electrodes used in these experiments detect insulin (13, 14), it is not clear that the secreted molecules which are detected as current spikes are only insulin. β cells secrete other substances in addition to insulin. The released compounds may be contained within the same vesicles as insulin or may be released from other vesicles. Some of the compounds which may be coreleased with insulin, such as C-peptide, ATP, and proinsulin, have been shown to be undetectable at the electrode (14).

To identify the secreted substance(s) detected at the electrode, HPLC analysis of releasates from islets of Langerhans was performed. The electrode used for detection was identical to that used for single-cell measurements except that it was larger, to accommodate the HPLC system. Fig. 4 illustrates results from this experiment. The large peak occurring early in the chromatogram is due to the injection of KRB onto the column. Clearly the dominant change in the chromatogram after incubation of islets with tolbutamide is the appearance of peaks due to rat insulins I and II. No other peaks are observed to change significantly. Equivalent results were obtained with mobile phases of different organic content (5% and 50% acetonitrile were used), different chromatography columns (hydrophobic interaction stationary phase), and human islets. These results demonstrate that insulin was the only substance that was secreted at a high enough level to be detected at the electrode.

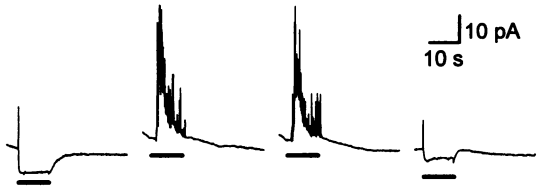

FIG. 3. Ca^{2+} dependence of tolbutamide-induced current spikes. The same cell was used for all recordings. The cell was in Ca^{2+}-free KRB. The horizontal bars indicates stimulation. In all cases the stimulating solution contained 200 μM tolbutamide. The tolbutamide was dissolved in (from left to right): Ca^{2+}-free KRB, KRB (includes 2.4 mM Ca^{2+}), KRB (includes 2.4 mM Ca^{2+}), and Ca^{2+}-free KRB. Five minutes was allowed between each stimulation.

The Ru-O/CN-Ru electrode is not especially selective for insulin; however, it is not surprising that it can be successfully used for monitoring insulin in these experiments when one considers the unique environment around a β cell. The most obvious chemical change outside a β cell following stimulation is the release of vesicular contents. Although many substances are present in vesicles, the dominant compounds are insulin and C-peptide. Indeed, 80% of the protein in a vesicle is an equimolar mixture of insulin and C-peptide (22). The most likely proteinaceous interferent is amylin, which has been shown to be present at up to 10% of the level of insulin in a vesicle (23). As a disulfide-containing peptide, it is detectable at the electrode with a similar sensitivity to insulin. However, amylin, which has a retention time of 12.2 min on the HPLC column used for these experiments, was not secreted at a high enough level to be detected.

DISCUSSION

Current Spike Shapes. Fig. 5 shows examples of spikes that were resolved from neighboring spikes. The spikes typically had rise times of 25–40 ms. Given that the filter setting was 20 Hz, it is likely that the spikes were changing faster than could be detected with this system. Attempts to use wider bandwidths for the measurement resulted in signal/noise ratios too low to allow observation of the peaks. Qualitatively, the fast rise and drawn-out decrease of the spikes are consistent with idea of rapid opening of the vesicle followed by diffusion from the point of exocytosis to the electrode (24). Quantitative analysis of the peak shapes will require an improved detection system that does not distort the peaks.

Table 2. Summary of Ca^{2+} dependence for tolbutamide and K^+ stimulations

β-Cell origin	Stimulation conditions*	No. of cells	No. of current spikes per stimulation†
Human	Tolbutamide/Ca^{2+}	16	33 ± 10
	Tolbutamide/no Ca^{2+}	6	0.2 ± 0.2
	Ca^{2+}	6	1.5 ± 0.9
	K^+/Ca^{2+}	8	14 ± 2
	K^+/no Ca^{2+}	8	0.9 ± 0.7
Rat	Tolbutamide/Ca^{2+}	6	9.6 ± 1.9
	Tolbutamide/no Ca^{2+}	6	0.2 ± 0.2

*Stimulation buffer consisted of Ca^{2+}-free KRB with 3 mM glucose plus the agents indicated (concentrations: tolbutamide, 200 μM; Ca^{2+}, 2.4 mM; K^+, 60 mM). Ca^{2+} and K^+ were added as the chloride salts.

†Peaks greater than 5 times the peak-to-peak noise level were counted. Mean ± SEM is given.

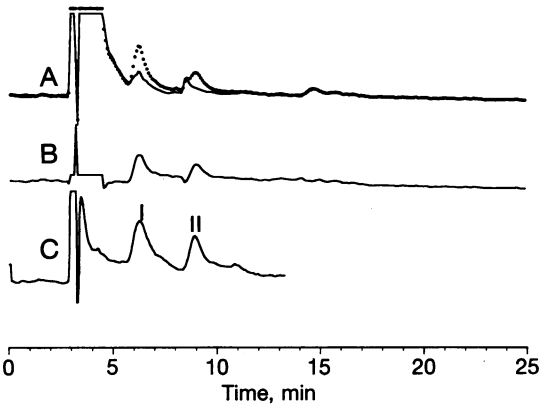

FIG. 4. (*A*) Chromatograms from releasates of islets of Langerhans. Solid line is the prestimulation chromatogram and the dotted line is the stimulated-islet chromatogram. (*B*) Subtraction of prestimulation from stimulated islet chromatogram in *A*. (*C*) Chromatogram of standard rat I and II insulin (3 μM in KRB) as indicated.

Current Spike Areas. The spike area, which has units of charge, can be converted to the number of moles oxidized by Faraday's law. Use of Faraday's law requires knowledge of the number of electrons transferred during the oxidation (n); since the electrochemical mechanism for oxidation of insulin at this electrode is not known, it was necessary to measure n. Using the measured n of 1.1 ± 0.1 (four measurements), we calculated that the average area for isolated spikes at human cells corresponds 1.7 amol, or 1.0×10^6 molecules, of insulin. For rat cells, the average spike area corresponds to 1.6 amol, or 9.6×10^5 molecules of insulin. [In a previous report (14), we had estimated 4 electrons transferred per oxidation, which led to a lower estimate of insulin per vesicle. The higher value in the present case is more accurate since n was measured.] A previous estimate for the maximum amount of insulin per vesicle in rats was $8–9.6 \times 10^5$ molecules (25). Thus, our values show good agreement with this previous estimate.

Distribution of Spike Areas. To further tie the current spikes to the vesicular contents of β cells, it is useful to examine the distribution of spike areas as shown in Fig. 6. In constructing this distribution, only spikes that appeared well-resolved from neighboring spikes and had a smooth shape, suggestive of single events, were used. In addition, only spikes with a signal/noise ratio > 5 were used.

With a knowledge of vesicle sizes and their distribution, and the assumption that the concentration of insulin is constant in vesicles, it is possible to write a probability density function that predicts the frequency of spike area occurrences of a given charge (7). The equation is a complex function of mean vesicle radius, standard deviation of the vesicle diameters, charge of the spike, and concentration in the vesicle. (See ref. 7 for the entire equation.)

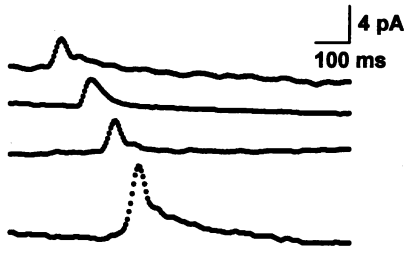

FIG. 5. Examples of isolated spikes recorded at single β cells. In all cases, the data were collected at 220 Hz. From top to bottom, the current spikes are from a human β cell after glucose stimulation, a rat β cell after glucose stimulation, a human β cell after tolbutamide stimulation, and a rat β-cell after tolbutamide stimulation.

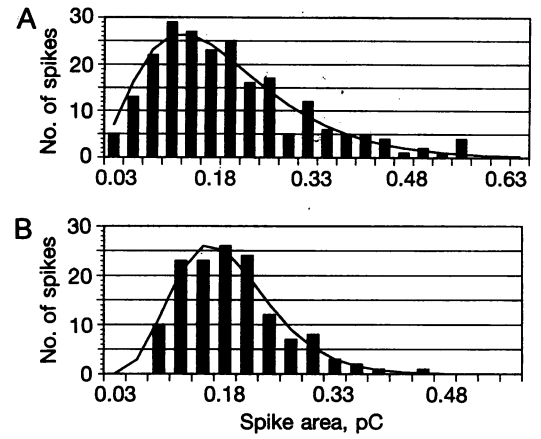

FIG. 6. Distribution of spike areas measured by amperometry at single human (*A*) and rat (*B*) β cells. Data are combined from tolbutamide and glucose stimulations (222 spikes in *A* and 140 spikes in *B*). Solid line is the best fit from the probability density function with 118 mM (*A*) or 74 mM (*B*) as the insulin concentration in vesicles.

Vesicle sizes in human β cells have been measured by electron microscopy and found to have an average diameter of 300 ± 61 nm (26). By using this value, the equation predicting the frequency of areas was fit to the actual data and a good correlation was obtained (correlation coefficient, $r = 0.9732$) as shown in Fig. 6. Similarly, the spike area distribution from rat β cells could be fit with good correlation ($r = 0.9779$) by using a diameter of 342 ± 43 nm (25). Thus, the distribution of spike areas that is observed can be attributed to the distribution of vesicle sizes that are expected to be encountered.

The equation used for making the correlations has a single adjustable parameter, the concentration of insulin in the vesicle. The concentration calculated to give the best fit for human cells was 118 mM insulin, and for rat cells it was 74 mM. Previous studies had estimated an insulin concentration of 41.6 mM inside vesicles of rat insulinomas (22). Semiquantitative immunohistochemical studies indicate that insulinomas have lower concentrations of insulin per vesicle than primary β cells (27). Given this difference, the estimates of insulin concentration in the vesicle are in remarkable agreement.

Detection of Exocytosis. The current spikes that have been measured are clearly due to the detection of a chemical that is secreted from the cell. The chromatographic results show that the primary substance secreted by the cells which can be detected by the electrode is insulin. Combined, these facts lead us to the conclusion that the current spikes are due to the detection of packets of insulin. The strong Ca^{2+} dependence, stimulation by known secretagogues of insulin, random occurrence of the spikes during a stimulation, constant area of spikes with stimulation, an area that corresponds to expected amounts of insulin in the vesicle, and a distribution of spike areas corresponding to vesicle diameter distributions combine to provide a powerful argument that the current spikes represent detection of exocytosis events occurring at the cell.

We have directly measured the quantized secretion of peptide, insulin. The advantages of measurements made at this level are clear. Basic information, such as the rate of exocytosis (Table 1), delays in stimulus–secretion coupling (Table 1), and insulin content per granule, is readily obtained. In addition, the kinetics of single exocytosis events are amenable to study. For example, previous work with adrenal chromaffin cells (8) and mast cells (10) has revealed a small increase in current, termed a "foot," prior to the rapid rise of the full spike. The foot has been attributed to leakage of hormone during opening of a fusion pore. The fact that catecholamine can leak out has further been related to the storage of hormone within the

granule (24). We have not observed a foot on any of the spikes for insulin. Since formation of a fusion pore is likely to be similar for β cells and adrenal chromaffin cells, the lack of a foot is indicative of relatively slow kinetics of insulin dissolution and extrusion from the interior of the granule. Single-cell resolution will allow cell-to-cell heterogeneity to be further studied. Large variations in the number of spikes and the time to initiate spikes following glucose stimulation were observed with this method (Table 1). β cells have shown considerable heterogeneity in their electrophysiology (20). Further experiments should allow correlation of heterogeneous electrophysiological and secretion patterns.

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