Effects of Intravesicular H\(^+\) and Extracellular H\(^+\) and Zn\(^{2+}\) on Insulin Secretion in Pancreatic Beta Cells

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The effects of extracellular Zn\(^{2+}\) and pH and intravesicular pH on insulin and 5-hydroxytryptamine (5-HT) secretion from pancreatic beta cells were investigated. Insulin and 5-HT secretion from single cells was detected by amperometry as a series of current spikes corresponding to detection of multimolecular packets secreted by exocytosis. Spike width was used as a measure of the kinetics of clearance from the cell and the area of spikes as a measure of amount released. Changes in extracellular pH from 6.9 to 7.9 caused insulin spikes to become narrower with no change in area, whereas the same treatments had no effect on 5-HT secretion. Treatment of cells with Bafilomycin A\(_1\) or N-ethylmaleimide, both of which are expected to increase intravesicular pH by inhibiting V-type H\(^+\)-ATPase, had no effect on 5-HT secretion but caused insulin spikes to become more narrow. These results indicate that exposure to high pH, whether intravesicular or extracellular, accelerates release of insulin during exocytosis without affecting the amount of insulin released. Increasing extracellular Zn\(^{2+}\) concentration from 0 to 25 \(\mu\)M increased the width and decreased the area of insulin spikes without affecting 5-HT secretion. Zn\(^{2+}\) effects were likely exerted through a common-ion effect on Zn\(^{2+}\)-insulin dissociation. It was concluded that intravesicular storage conditions and extracellular ions can affect free insulin concentration in the vicinity of beta cells during secretion.

Insulin, produced and stored in pancreatic beta cells, is released by exocytosis in response to external stimuli, such as elevated glucose concentration. A greater understanding of exocytosis in beta cells is of intense interest because of the possible role of defective insulin secretion in type II diabetes (1, 2). Secretion by exocytosis can be considered to occur in several steps including vesicle docking, vesicle priming, vesicle fusion, and finally extrusion or clearance of vesicular contents into the extracellular medium (3). In this study, we examined the postfusion clearance of insulin from beta cell secretory vesicles during exocytosis.

Clearance of insulin from secretory vesicles is intimately related to the storage of insulin within a vesicle. Insulin is generally believed to be stored inside secretory vesicles as a solid hexamer bound with two Zn\(^{2+}\) ions per hexamer (4–6). This understanding is supported by a broad spectrum of evidence. It is known that in the presence of Zn\(^{2+}\), insulin will form tetramers and hexamers that bind Zn\(^{2+}\) in an insulin:Zn\(^{2+}\) ratio of 4:1 and 6:2, respectively (5), and that vesicles contain Zn\(^{2+}\) in 1–1.5-fold excess of that necessary to form Zn\(^{2+}\)-insulin hexamers (7). Zn\(^{2+}\)-insulin hexamers are insoluble below pH 7 (5, 6, 8, 9), and beta cell secretory vesicles possess a V-type H\(^+\)-ATPase that maintains the vesicle interior at or below pH 6 (10, 11). Electron micrographs and immunohistology also suggest the presence of solid insulin inside vesicles (12, 13). During exocytosis, secretory vesicles fuse with the plasma membrane, exposing the vesicular interior to the extracellular milieu, allowing release of stored insulin granules. It is apparent that insulin must dissolve to escape the vesicle. In addition, insulin must dissociate from Zn\(^{2+}\) because insulin monomer is the biologically active form (14, 15). Little is known about the mechanism or time scale of dissolution and dissociation, although one study has demonstrated that insulin is free from Zn\(^{2+}\) within 60 s of release (4). Additional investigation of this phenomenon has been hindered by the lack of methods for monitoring insulin secretion with sufficient temporal resolution to observe such effects.

Recently, amperometry with microelectrodes has been applied to the study of exocytosis at a variety of cell types, including adrenal chromaffin cells (16–19), PC12 cells (20), mast cells (21), neurons (22), and melanotrophs (23, 24). Secretion is detected as a series of current spikes that correspond to detection of concentration pulses generated by exocytosis. Analysis of current spikes can reveal subtle details about secretion. The area of current spikes (measured in coulombs) can be used to quantify the moles of detected hormone (or transmitter) released per vesicle using Faraday’s Law (19). The dynamics of vesicular release are reflected in the shape of the current spikes. Specifically, the width of spikes can be used as a measure of the rate of clearance from the vesicle because the width is determined by any slow kinetic step involved in clearing hormone from the vesicle and diffusional broadening of the secreted packet of molecules (25–28). Thus, the exquisite temporal resolution and sensitivity of amperometry for measuring actual secretory products has made it well suited for probing postfusion events in exocytosis (25–27, 29, 30).

We have extended amperometry to detection of insulin exocytosed from pancreatic beta cells (31, 32). For this application, a carbon-fiber electrode modified with ruthenium oxide/cyanoruthenate (Ru-O/CN-Ru), a catalyst that promotes oxidation of insulin, is used for detection. This method has allowed the first direct measurement of exocytosis of insulin from single beta cells with high temporal resolution (32). In using amperometry to study postfusion events in insulin release, we have

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found it useful to measure 5-HT \(^1\) release as a control. 5-HT can be “loaded” into beta cells by incubating the cells in medium containing both 5-HT and 5-hydroxytryptaphan. Substantial evidence now exists that demonstrates that 5-HT is loaded primarily into beta cell secretory vesicles and that 5-HT is co-secreted with insulin by exocytosis (34–36).\(^2\) 5-HT can be detected at bare carbon electrodes and insulin can be detected only at Ru/O-CN-Ru-modified electrodes. Thus, it is possible to measure, in separate experiments, secretion of 5-HT from loaded cells and secretion of insulin from cells that have not been allowed to accumulate 5-HT.

In an initial study on postfusion clearance of insulin, we found that exposure to a pH gradient between the vesicle and the extracellular environment is required to achieve rapid release of free insulin from solid Zn\(^{2+}\)-insulin granules (37). In the present study, we extended this investigation to a variety of extracellular pH values. In addition, we examined the effect of intravesicular pH and extracellular Zn\(^{2+}\) on insulin secretion. The study reveals strong effects of the extracellular and intravesicular environments on the rate and amount of free insulin released during exocytosis.

**MATERIALS AND METHODS**

Electrode Preparation and Testing—Carbon fiber microelectrodes were constructed as described previously (38, 39). Finished electrodes consisted of a 9 \(\mu\)m carbon fiber (P-55S, Amoco Performance Products) sealed with epoxy (Miller Stephenson, Danbury, CT) in the tip of a glass pipette. The total electrode diameter at the tip was \(\sim 30\) \(\mu\)m, and the electrode was polished at a 30–45° angle using a pipette beveler (BV-10, Sutter Instruments). For detection of insulin, electrodes were chemically modified as described previously to produce a mixed valent Ru/O-CN-Ru film (32), whereas 5-HT detection was performed with bare carbon fiber microelectrodes (34).\(^2\) Tests of the electrode response to insulin and 5-HT were performed by positioning the tips of electrodes approximately 10 \(\mu\)m from micropipette tips (outer diameter, 30 \(\mu\)m) under a Krebs-Ringer buffer solution on the stage of a microscope. Solutions were pressure-ejected from the tips of the pipettes at 9 p.s.i. for 5 s while the amperometric current was recorded.

Data Collection and Analysis—Amperometry was performed using a battery to apply potential to a sodium-saturated calomel electrode. Currents were monitored at the working electrode using an AI-403 current amplifier and Cybercarrp 320 signal conditioner (Axon Instruments, Foster City, CA). For detection of 5-HT, the potential at the working electrode was 0.65 V, whereas for detection of insulin, the potential was 0.85 V. All voltages are versus a sodium-saturated calomel electrode. When using the modified electrode, the potential was held at 0.40 V between recordings to improve electrode stability as described elsewhere (32). Data were slow pass filtered at 300 Hz for insulin measurements and at 1000 Hz for 5-HT. These filter settings were found to not affect the peak widths relative to higher settings. Data were collected at a rate that was three to five times the filter frequency using a personal computer (Gateway 2000 P5–166) via a data acquisition board (DigiData 1200B, Axon Instruments). The area and frequency using a personal computer (Gateway 2000 P5–166) via a data acquisition board (DigiData 1200B, Axon Instruments). The area and frequency using a personal computer (Gateway 2000 P5–166) via a data acquisition board (DigiData 1200B, Axon Instruments).

**RESULTS**

Effect of Zn\(^{2+}\) on Insulin Detection—We have previously used the Ru/O-CN-Ru electrode to detect insulin and insulin secretion (31, 32). An issue that has not been addressed but that is important in the interpretation of these data is the effect of Zn\(^{2+}\) complex formation on insulin detection. Fig. 1 illus-

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\(^1\) The abbreviation used is: 5-HT, 5-hydroxytryptamine.

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trates current recorded during injection of Krebs-Ringer buffer solutions that contained 30 μM insulin (A) or 30 μM insulin along with 15 μM Zn²⁺, which promotes the formation of Zn²⁺-insulin complexes (B). As the data show, addition of Zn²⁺ abolished the signal obtained from detection of insulin. The presence of Zn²⁺ did not affect the detection of other, non-Zn²⁺ binding compounds, such as 5-HT (Fig. 1, C and D). These results strongly suggest that the electrode detects free insulin and not insulin complexed with Zn²⁺.

Spike Width Analysis—Fig. 2 compares high resolution recordings of spikes resulting from detection of insulin and 5-HT. Several differences in spike shape were observed for detection of 5-HT and insulin. The most marked difference in shape of the spikes was that the average width at half-height of 5-HT spikes was 5.3 ± 0.4 ms, which is significantly lower (p < 0.0005) than the 30 ± 2.1 ms obtained for insulin spikes. As mentioned under “Introduction,” the width at half-height of a current spike is a measure of the time required for detected material to extrude from a vesicle, diffuse to the electrode, and be detected. Thus, some of the difference in width could be attributed to the difference in diffusion coefficient for 5-HT and insulin. The random walk model, which predicts widths based solely on diffusion, indicates that the insulin spikes should be three times the width of 5-HT spikes, yet we observed a 6-fold difference. The model also predicts that the widest diffusion-limited spikes, which would occur for a release site that is the maximum possible distance from the electrode, should be 22 ms for 5-HT and 58 ms for insulin. Experimentally, we found that none of the 5-HT spikes were broader than this maximum value. In contrast, 45% of the insulin spikes were over the maximum diffusion-limited width. Taken together, these results indicate that release of 5-HT is indistinguishable from diffusion control and that a factor other than diffusion contributes to slowing down the kinetics of release of insulin.

Another difference in shape was that 14% of the 5-HT spikes had a small current increase or “foot” prior to the rapid increase to the spike apex, as shown in Fig. 2C. In contrast, such features were not observed on spikes due to detection of insulin at physiological pH. Prespike features like those seen for 5-HT detection have also been observed in the detection of catecholamine release from adrenal chromaffin cells (16, 25) and 5-HT release from mast cells (21). This shape has been attributed to leakage of material from a fusion pore that forms prior to complete opening of the vesicle (16, 21, 25). The lack of a foot for detection of insulin suggests that insulin does not leak from a fusion pore in quantities that are detectable.

Effects of Extracellular pH on Insulin Extrusion—To evaluate the effect of extracellular pH on exocytosis, single cell recordings of insulin secretion were made following tolbutamide stimulation when the extracellular pH was 6.40, 6.90, 7.05, 7.20, 7.40, and 7.90. Similar experiments were performed for detection of 5-HT secretion from loaded cells at pH 6.40, 7.10, and 7.40. Fig. 3 summarizes the spike widths and areas that were recorded in these different experiments. As mentioned under “Introduction,” the area of isolated current spikes is considered a direct measure of the amount of product (insulin or 5-HT in this case) detected from a single vesicle (19). Spike area for insulin detection is not significantly changed by increasing extracellular pH above 6.9 (Fig. 3A); however, no spikes were detected at pH 6.4. In contrast, the width at half-
Effects of Intravesicular pH on Insulin and 5-HT Extrusion—To investigate the effect of the vesicular proton pump and lowered vesicular pH on insulin and 5-HT secretion, the effect of Bafilomycin A1 and N-ethylmaleimide, compounds known to inhibit V-type H+–ATPase activity and raise vesicular pH to cytoplasmic pH ($44, 45$), on spike shapes was evaluated. Fig. 4 compares typical current traces recorded at untreated cells and cells incubated with 1 μM Bafilomycin A1 or 50 μM N-ethylmaleimide. As observed from an inspection of these plots, the number of spikes per stimulation, duration of secretory activity, and area of the spikes are not significantly different for any of these treatments.

Although overall secretory activity for insulin was unaffected by ATPase inhibition, insulin spike shape was affected as illustrated in Fig. 5, A–C, and summarized in Fig. 5D. The spike width was significantly decreased ($p < 0.001$) by treatment with both agents. Furthermore, after treatment with Bafilomycin A1, 10% of the detected spikes exhibited a foot, and after treatment with N-ethylmaleimide 18% of spikes had this feature. Spike samples shown in Fig. 5, B and C, are typical of those that had a foot. As stated above, no spikes obtained from untreated cells had such prespike features. In contrast, the spike width for 5-HT was unaffected (Fig. 5D), and there was no effect on the percentage of 5-HT spikes with feet.

Effects of Extracellular Zn2+ Concentration on Insulin and 5-HT Extrusion—In addition to the effects of pH, the effects of 0, 5, 15, and 25 μM extracellular Zn2+ on secretion measurements were examined. Fig. 6 illustrates typical secretion measurements of insulin and 5-HT under these different conditions. As extracellular Zn2+ concentration increased, the area of insulin spikes decreased while the half-width increased, as summarized in Fig. 7. These combined effects caused spike amplitudes to decrease and result in detection of smaller numbers of spikes as illustrated in Fig. 6. The smaller number of spikes is presumed to result from fewer spikes being above the noise level because of the decreased amplitudes. This effect is maximized at 25 μM Zn2+, at which no insulin spikes were detected (data not shown). In contrast, when measuring 5-HT release, the area and half-width of the spikes were not significantly different at any given Zn2+ concentration (see Figs. 6 and 7).

**DISCUSSION**

Dynamics of Exocytosis Events—Although 5-HT is released simultaneously with insulin, it escapes from the vesicle at a significantly higher rate, as evidenced by the narrower spikes and the presence of feet, which suggest leakage out of vesicles during fusion pore formation. 5-HT release is indistinguishable from simple diffusion out of the vesicle, whereas insulin release is slower, suggesting that a factor other than diffusion controls postfusion release. These results may be explained by considering differences in storage of the two secretory products. Although the exact form that 5-HT is stored in the vesicles is unknown, it is reasonable to expect that it is stored in solution because 5-HT is highly soluble at the vesicular pH. In contrast, insulin is stored as a solid complex of Zn2+-insulin (4, 5, 12). In addition, the electrode only detects free insulin and not Zn2+-bound insulin, as illustrated in Fig. 1. Therefore, it seems likely from these considerations that the dissolution and dissociation of Zn2+-insulin complex controls the rate of insulin extrusion during exocytosis.

Effects of Extracellular and Intravesicular pH—In our initial work, we demonstrated that a change in extracellular pH from 7.4 to 6.4 could hinder insulin clearance to such an extent as to make it undetectable by amperometry while having no effect on 5-HT secretion (37). The data in Fig. 3, obtained over a wider pH range, show that decreasing extracellular pH from 7.9 to 6.9 increases the time required for insulin clearance (increasing spike width) but does not affect the amount of insulin released during an exocytosis event (no change in area). 5-HT secretion is unaffected in this pH range (Fig. 3), proving that this effect is specific to insulin extrusion and is not an effect on vesicular fusion. Further investigation has revealed that increasing vesicular pH with V-type H+–ATPase inhibitors, which is expected to change the storage conditions for insulin, causes insulin to be more rapidly extruded (as demonstrated by narrower spikes) and even leak out of fusion pores (as demonstrated by the presence of feet). This effect suggests that some dissolution of the insulin granule occurs inside the vesicle when the vesicular pH is raised. Therefore, under normal conditions, low pH inside the vesicle maintains insulin in a solid state that does not escape as readily during fusion pore formation or vesicle fusion. These results further emphasize the importance of the pH gradient between the vesicle and extracellular media in rapidly changing insulin from a solid, storage state to a dissolved, releasable form. These results also highlight the difference between postfusion release of other hormones and insulin. For example, in catecholamine release from adrenal chromaffin cells, increasing the pH has the effect of increasing the time course of postfusion extrusion (25). Furthermore, the effects of pH on catecholamine release are not nearly as dramatic as those observed here. These differences can be attributed to differences in storage of the compounds. In the case of catecholamine, release requires the unraveling of a protein gel rather than dissolution of a solid-state hormone (25).
Effect of Zn$^{2+}$ on Insulin Extrusion—In addition to dissolving, the insulin-Zn$^{2+}$ complex must ultimately dissociate after vesicle fusion because the active form of insulin is a monomer (14, 15). We have postulated that availability of extracellular Zn$^{2+}$ may affect the release of free insulin from beta cells through a common-ion effect. Extracellular Zn$^{2+}$ concentrations in the range of 5–25 $\mu$M markedly decreased area and increased width of insulin spikes without affecting 5-HT spikes. The lack of an effect on 5-HT detection demonstrates that free insulin concentrations in the vicinity of a beta cell during secretion. The possible physiological significance of the Zn$^{2+}$ effect is not clear because the actual concentration of Zn$^{2+}$ in the interstitial space of an islet is not known; however, Zn$^{2+}$ concentration in serum is approximately 15–25 $\mu$M (47–50). It has previously been demonstrated that in vivo insulin is available as free monomer within a few seconds of entering the portal vein, presumably because the large dilution of insulin drives dissociation (4); therefore, it seems unlikely that extracellular Zn$^{2+}$ could affect the endocrine function of free insulin.

Regulation of Insulin Extrusion during Exocytosis—Our observations show that free insulin concentrations in the vicinity of beta cells are affected by the intravesicular pH and extracellular pH and Zn$^{2+}$ concentrations. These results have been interpreted as effects on the rate of dissolution and dissociation...
in driving postfusion release of insulin from beta cells. The effects of H\(^+\) and Zn\(^{2+}\) on insulin release that were observed are a natural manifestation of the complex mechanism of insulin synthesis and storage. The fact that the quanta of insulin release and the rate of insulin release can be altered also raises the possibility that insulin secretion can be regulated by the intravesicular and extracellular ionic environment. Such postfusion control of release has recently been proposed as a novel regulatory mechanism in a variety of cell types (52). Postfusion regulation of insulin release would seem to hold little relevance to the endocrine action of insulin given the large distance between the release site and the site of action. However, several lines of evidence support the notion of an autocrine and/or paracrine role for insulin within an islet. Specifically, insulin has been demonstrated to (a) bind to islet cells (59), (b) affect insulin secretion (61, 62), and (c) activate insulin receptors in beta cell lines (60, 33). Therefore, if the intravesicular or extracellular ionic environment does mediate postfusion regulation of insulin release, then its effects would likely be exerted at the level of autocrine or paracrine signaling of insulin.

Fig. 7. Comparison of insulin and 5-HT secretory area and width at half-height at different extracellular [Zn\(^{2+}\)]. A, effect of extracellular [Zn\(^{2+}\)] on mean spike area for insulin and 5-HT. The asterisk (*) indicates difference from 0 μM [Zn\(^{2+}\)] at a significance of \(p < 0.10\), and ** indicates difference at \(p < 0.02\). B, effect of extracellular [Zn\(^{2+}\)] on mean half-width of spikes for insulin and 5-HT. The asterisk (*) indicates difference from 0 μM [Zn\(^{2+}\)] at a significance of \(p < 0.001\), and ** indicates difference at \(p < 0.01\). For detection of insulin, numbers of spikes (n) were 76, 68, and 22, whereas for detection of 5-HT, n values were 198, 75, and 70 for 0, 5, and 15 μM extracellular zinc, respectively. For all experiments, at least five different cells were used.

as Zn\(^{2+}\)-insulin hexamers during exocytosis. This interpretation ignores the possible role of vesicle matrix proteins, such as chromogranin, in storing and extruding free insulin. In adrenal chromaffin cells and mast cells, glycoproteins, such as chromogranin, in the vesicle matrix have been shown to be critical in storing and then extruding the secretory products (51–54). These studies have shown that matrix proteins help release the secretory products in a pH-dependent mechanism that involves ion exchange and phase transition of the storage proteins (51–54). Chromogranin is found in the secretory vesicles of beta cells (55–57) and therefore could play a similar role in extruding insulin. Our data cannot exclude such a possibility; however, it seems unlikely, given the differences in storage of insulin in beta cells and histamine or catecholamines in the other cell types. First, insulin is stored as a solid (4, 5, 6) and therefore is not associated with the chromogranin. Second, histochemical evidence shows that chromogranin in beta cell secretory vesicles is concentrated in the halo region separate from insulin in the dense core (57). Finally, in beta cell vesicles, insulin and C-peptide make up 80% of the protein content, and membrane-bound proteins make up 10%, leaving just 10% of the total protein as matrix proteins (58). Such a distribution would seem to preclude a dominant role for chromogranin in storing and dispersing insulin, especially given the importance of Zn\(^{2+}\)-insulin complex formation in storage.

This study has revealed some of the chemical events involved in regulating insulin release, and the potential importance of matrix proteins in controlling this process. Further studies are needed to determine the role of matrix proteins in insulin release and to understand how they interact with other regulatory mechanisms, such as intravesicular and extracellular ionic environment.
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