Extracellular pH Is Required for Rapid Release of Insulin from Zn−Insulin Precipitates in β-Cell Secretory Vesicles during Exocytosis

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Pancreatic β-cells secrete insulin in response to elevated glucose concentrations. Developing a greater understanding of insulin secretion is of intense interest since type II diabetes is associated with insufficient insulin secretion.[1] Many details of the secretion process are not well understood. One of the most enigmatic aspects of secretion is exocytosis, which is the last step in secretion. In exocytosis, vesicles containing insulin fuse with the plasma membrane, which exposes the vesicle interior to extracellular space and allows insulin to escape.[2,3] The vesicle membrane is eventually reclaimed by the cell by endocytosis. The final step of exocytosis is clearance of stored insulin from the vesicle interior. Electron micrographs of insulin secretory vesicles show a dark, crystalline core surrounded by a clear halo.[3,6] The halo presumably contains soluble components while the dark core contains crystalline Zn−insulin.[3] The insulin is likely stored as a hexamer unit associated with two Zn ions.[4,5] Release of insulin requires dissolution of the granule and dissociation of the Zn−insulin complexes. The time scale of this process is unknown, although previous measurements have found that insulin is free of Zn no more than 60 s after release.[4] Furthermore, the important factors that control release of insulin from the Zn−insulin precipitates are not clear. We have tested the hypothesis that sudden exposure of the vesicle interior to extracellular pH during exocytosis causes rapid dissolution and dissociation of insulin from the solid Zn−insulin. This hypothesis was developed on the basis of the facts that the intravesicle pH is around 5.5−6.0[7] and Zn−insulin hexamers prepared in vitro are insoluble below pH 7.0.[8,9]

To test this hypothesis, we measured insulin secretion from single β-cells with high time resolution at different extracellular pH's. Insulin was measured by amperometry at a carbon fiber microelectrode modified with a composite of ruthenium oxide and cyanoruthenate (Ru−O/CN−Ru) as described elsewhere.[10,11] Amperometry has previously been demonstrated to be a suitable method for detecting minute quantities released during single exocytosis events.[12−18] To measure insulin secretion, the electrode was positioned about 1 μm away from a single β-cell adhered to a culture plate. The β-cells used in these experiments were isolated from rats using a collagenase digestion as described elsewhere.[19] Amperometric detection was accomplished in a two-electrode cell using a battery to apply voltage and measuring the current with a Keithley 427 current amplifier. The data were low pass filtered at either 33 or 333 Hz and collected at a rate of at least 3 times the filter bandwidth. Cells were stimulated to secrete insulin by application of 200 μM tolbutamide dissolved in Kreb's Ringer buffer (KRB) to the cell with a micropipet positioned about 30 μm from the cell. Flow rates through the pipet were about 1 nL/s. For control experiments, we measured 5-hydroxytryptamine (5-HT) secretion from β-cells using a carbon fiber microelectrode. Normally, 5-HT is not present in β-cells at levels that are high enough to be detected by amperometry;[10] however, β-cells will accumulate 5-HT into secretory vesicles.[20−22] The accumulated 5-HT is released by exocytosis, which is detectable as current spikes at unmodified carbon fiber microelectrodes.[23,24] Thus, detection of 5-HT can serve as an indicator of vesicle fusion and opening at β-cells. For these experiments, β-cells were allowed to accumulate 5-HT by incubating them for 16 h in culture media containing 1 mM 5-HT.

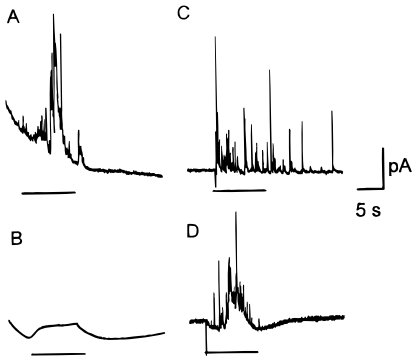

Figure 1. Comparison of insulin and 5-HT secretion from single pancreatic β-cells at different extracellular pH's following stimulation for 10 s with 200 μM tolbutamide: (A) insulin measurement at pH 7.4, (B) insulin measurement at pH 6.4, (C) 5-HT measurement at pH 7.4, and (D) 5-HT measurement at pH 6.4. All measurements of 5-HT were made at cells that had been allowed to accumulate 5-HT overnight. Lines below traces indicate application of stimulus. Current bar is 5 pA for insulin measurements and 2.5 pA for 5-HT measurements. Data were low pass filtered at 33 Hz. The dip in current for trace D and the bump in B were artifacts of the stimulation. The slow drift in traces A and B is characteristic of the modified electrode.

When a β-cell is stimulated with 200 μM tolbutamide in pH 7.4 media, a series of current spikes is observed at the electrode as shown in Figure 1A. We have previously shown that these spikes correspond to detection of insulin released by exocytosis.[11] Measurements made with a bandwidth of 333 Hz show

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that the spikes have an average width at half-height of 37 ± 27 ms ($n = 78$). (The width of the insulin spikes is not limited by the response time of the modified electrode since we have measured it to be <2 ms.) The average area under the spikes corresponds to 1.6 amol of insulin.[11] This amount is in agreement with previous estimates of insulin vesicular content.[11,25] The insulin detected is free insulin, dissociated from Zn^{2+}, since we have found that the electrode does not detect solubilized Zn−insulin complexes (data not shown). This observation is in agreement with previous studies on the reduction of insulin which showed that complexing of Zn^{2+} with insulin inhibits the electroactivity of insulin.[26] The narrow spikes and the amounts detected suggest that a large portion of the Zn−insulin complex dissolved and dissociated rapidly after vesicle opening. When a similar experiment is performed with cells bathed in pH 6.4 media, no current spikes are detected, as illustrated in Figure 1B, even though flow injection experiments showed that insulin is detectable at this pH. To determine if vesicle opening still occurred at the lower pH, we compared secretion of 5-HT from β-cells at pH 6.4 and 7.4 as illustrated in Figure 1C,D. No statistically significant differences in the number or area of spikes were observed for 5-HT at different pH's. These results demonstrate that lower pH did not inhibit vesicle fusion and pore opening, but rather hindered release of detectable insulin from the granule. Thus, the data show that a crucial step to releasing free, uncomplexed insulin from Zn−insulin precipitates following vesicle opening is exposure of the precipitates to extracellular pH.

An interesting question that is not easily answered from these results is the fate of insulin following stimulation at pH 6.4. Since it is known that insulin release is not eliminated at low pH,[27] it is possible that the granule dissolved and/or dissociated slowly so that free insulin was released over a relatively long time. Insulin released slowly would be too dilute to be detected by the electrode.

In contrast to insulin, 5-HT is stored in a form (presumably in solution) that readily escapes from the vesicle at either pH. This point is further illustrated by comparing the peak shapes for insulin and 5-HT measured at pH 7.4 as illustrated in Figure 2. Spikes due to 5-HT have a width at half-height of 7.0 ± 7.1 ms ($n = 102$) when measured with a bandwidth of 333 Hz, which is significantly less than the 37 ± 27 ms for insulin ($p < 0.005$). (The width of spikes due to 5-HT was apparently limited by the bandwidth of the measurement.) The larger width of the insulin spikes compared to the 5-HT spikes suggests that the dissolution/dissociation process determines the spike width and is the rate-limiting step to releasing free insulin during exocytosis. Furthermore, 14% of the 5-HT current spikes have a "foot" prior to the rapid upstroke as illustrated in Figure 2A,B. Current spike shapes such as this have also been observed in the measurement of catecholamine and indoleamine secretion from adrenal chromaffin[15] and mast cells,[17] respectively. The foot has been attributed to detection of material that "leaks" from a fusion pore prior to complete opening of the vesicle. In contrast to the foot observed on current spikes due to 5-HT, a foot was never observed on current spikes due to insulin detection as shown by the spikes in Figure 2D−F. Thus a

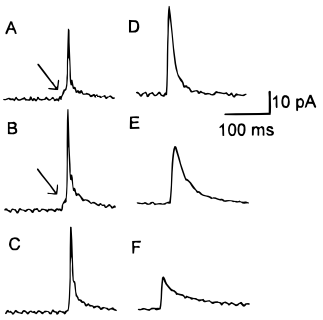

Figure 2. Comparison of spike shapes for insulin and 5-HT at pH 7.4. (A−C) Spikes due to detection of 5-HT. Arrows indicate a "foot" in traces A and B. (D−F) Spikes due to detection of insulin. All measurements were made using stimulation conditions as in Figure 1. Data were collected at 1500 Hz and filtered at 333 Hz.

detectable quantity of insulin, which is stored in a solid form, does not leak out of the vesicle core during the brief time the fusion pore is open.

From the combination of these results with previous observations, the following picture emerges concerning insulin storage and release. β-Cells store about 1.6 amol of insulin in a vesicle with a diameter of about 342 nm.[28] The ability to pack and store this amount of insulin is facilitated by the inclusion of Zn^{2+} and the maintenance of a vesicular pH that is below that required to dissolve the Zn−insulin precipitate.[29] After vesicle fusion, dissolution *and* dissociation of the solid Zn−insulin granule occurs on the time scale of 37 ms in a process that is dependent upon exposure of the granule to extracellular pH. The possible change in intravesicle pH during fusion pore formation is not sufficient to cause substantial release of stored insulin.

These results are reminiscent of those from mast cells and adrenal chromaffin cells. Mast cells have vesicles that are large enough to be observed by light microscopy. At low extracellular pH, it was observed that the granule in the vesicle remained intact.[30] Electrochemical measurements of catecholamine release at low pH also showed a retardation of the clearance of secretory products.[31] In both of these cases, however, the secreted products are stored in a condensed matrix of ionic polymer which undergoes conformational changes with increases in pH. For example, in adrenal cells, catecholamine was stored in a matrix of chromogranin A which expands, dissociates, and loses affinity for catecholamine with a pH change from 5.5 (intravesicle pH) to extracellular pH. Thus, the use of pH gradients to drive release of vesicular contents following fusion is common in exocytosis; however, the chemical changes that follow the pH changes vary with cell type and secretory product.

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