The Cytokine Interleukin-1β Reduces the Docking and Fusion of Insulin Granules in Pancreatic β-Cells, Preferentially Decreasing the First Phase of Exocytosis*§

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The prediabetic period in type I diabetes mellitus is characterized by the loss of first phase insulin release. This might be due to islet infiltration mediated by mononuclear cells and local release of cytokines, but the mechanisms involved are unknown. To determine the role of cytokines in insulin exocytosis, we have presently utilized total internal reflection fluorescence microscopy (TIRFM) to image and analyze the dynamic motion of single insulin secretory granules near the plasma membrane in live β-cells exposed for 24 h to interleukin (IL)-1β or interferon (IFN)-γ. Immunohistochemistry observed via TIRFM showed that the number of docked insulin granules was decreased by 60% in β-cells treated with IL-1β, while it was not affected by exposure to IFN-γ. This effect of IL-1β was paralleled by a 50% reduction in the mRNA and the number of clusters of SNAP-25 in the plasma membrane. TIRFM images of single insulin granule motion during a 15-min stimulation by 22 mM glucose in IL-1β-treated β-cells showed a marked reduction in the fusion events from previously docked granules during the first phase insulin release. Fusion from newcomers, however, was well preserved during the second phase of insulin release of IL-1β-treated β-cells. The present observations indicate that IL-1β, but not IFN-γ, has a preferential inhibitory effect on the first phase of glucose-induced insulin release, mostly via an action on previously docked granules. This suggests that β-cell exposure to immune mediators during the course of insulitis might be responsible for the loss of first phase insulin release.

Accumulating evidence over the past 30 years suggests that β-cells are destroyed by an autoimmune process in type 1 diabetes mellitus (T1D)1 (1). The prediabetic period in humans is characterized by the presence of islet cell autoantibodies (2), which seem to have a close correlation with histological evidence of autoimmunity (3). There are already subtle changes in β-cell function during this period, including both disproportionately elevated proinsulin/insulin levels (4, 5) and a preferential loss of the first phase insulin secretion in response to an intravenous glucose challenge (6–9). Of note, β-cell suppression precedes β-cell death in T1D, as suggested by results obtained in islets isolated from prediabetic non-obese diabetic mice (10, 11) or from a patient who died immediately after diagnosis of T1D (12). This initial β-cell functional suppression might be due to exposure to cytokines. Indeed, our previous observations (13) indicate that islet exposure to cytokines under in vitro conditions reproduce the disproportionately elevated proinsulin/insulin levels observed in prediabetic patients. Moreover, microarray analysis of purified β-cells or insulin-producing INS-1 cells cultured in the presence of IL-1 β or IL-1β + IFN-γ showed inhibition of the expression of several genes involved in the exocytosis of insulin granules, including SNAP-25, VAMP-2, and Rab3 (14, 15). This raised the intriguing possibility that cytokines could also contribute for the defective first phase insulin release observed in prediabetic patients.

The biphasic kinetics of insulin release is probably explained by the presence of a small population of docked granules, released during the first phase of insulin secretion, and the subsequent arrival of “newcomer” granules targeted to the plasma membrane and released during the second phase of insulin secretion (16, 17). The three SNAREs (N-ethylmaleimide-sensitive fusion protein attachment protein receptors), namely SNAP-25, syntaxin 1, and synaptobrevin (VAMP) are expressed in pancreatic β-cells and play a crucial role for granule fusion with the cell membrane (18–20). To better understand the mechanisms involved in insulin granule exocytosis, we have recently developed an approach based on a green fluorescence protein (GFP)-tagged insulin granule system, combined with total internal reflection microscopy (TIRFM) (21). This system allows us to observe the motions of single insulin granules during approach, docking, and fusion with the membrane and also to localize endogenous t-SNAREs such as SNAP-25 and syntaxin-1 in the cell membrane (22, 23). We have presently utilized this system to characterize insulin granule motion in β-cells treated for 24 h with cytokines. The

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§ The on-line version of this article (available at http://www.jbc.org) contains supplemental Quick Time movies S1–S3.

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data obtained suggest that IL-1β reduces the docking/fusion of insulin granules in pancreatic β-cells, preferentially decreasing the first phase of exocytosis, along with a decrease in SNAP-25 mRNA expression and SNAP-25 clusters on the plasma membrane.

EXPERIMENTAL PROCEDURES

Islet Cell Preparation, Adenovirus Infection, and Exposure to Cytokines—Pancreatic islets of Langerhans were isolated from male Wistar rats by collagenase digestion, as described (17). Isolated islets were dispersed in calcium-free Krebs-Ringer buffer (KRB) containing 1 mM EGTA and cultured on fibronectin-coated (Roken Co., Ltd., Tokyo, Japan) high refractive index glass (Olympus, Tokyo, Japan) in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen), 200 units/ml penicillin, and 200 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO2. For labeling the insulin secretory granules, pancreatic β-cells were infected with recombinant adenovirus AdEx1CA insulin-GFP as described previously (17). Two days after infection, they were exposed for 24 h to IL-1β (100 units/ml; kindly provided by Dr. C. W. Reynolds, NCI, Bethesda, MD) and/or IFN-γ (2000 units/ml; Invitrogen, Baesley, Scotland). For determination of mRNA expression (see below) and insulin content, rat pancreatic β-cells were purified by autoflow-culture-activated cell sorting, cultured overnight, and exposed for 24 h to IL-1β and/or IFN-γ, and insulin was measured by radioimmunoassay as described previously (14, 24).

Immunohistochemical Analysis—Single cells cultured on high refractive index glass and/or without cytokines were fixed, permeable with 2% paraformaldehyde, 0.1% Triton X-100, and processed for immunohistochemistry as described previously (22). They were labeled with monoclonal anti-insulin antibody (Sigma) and anti-SNAP-25 antibody (Wako Co., Ltd., Osaka, Japan) and then processed with goat anti-mouse IgG conjugated to Alexa Fluor-488 (Molecular Probes, Eugene, OR). Immunofluorescence was detected with TIRFM. This procedure allowed us to evaluate the number of docked insulin granules and SNAP-25 clusters.

TIRF Microscopy for Observing the Immunofluorescence and for Monitoring GFP-tagged Insulin Granule Motion—The Olympus total internal reflection system was used with minor modifications as described previously (21). Briefly, light from an argon laser (488 nm) was introduced to an inverted microscope (IX70, Olympus) through a single-mode fiber and two illumination lenses; the light was focused at the back focal plane of a high aperture objective lens (Apo 100×/OH4; NA 1.65, Olympus). To observe the fluorescence image of Alexa Fluor-488, we used a 488-nm laser line and a long pass 515-nm filter. To monitor the motion of the single insulin granules, the adenovirus-infected and cytokeratin-treated cells on the coverslip (Olympus) were mounted in an open chamber and incubated for 30 min at 37 °C in KRB containing 110 mM NaCl, 4.4 mM KCl, 1.45 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 4.8 mM MgCl2, 10 mM HEPES (pH 7.4), and 0.3% bovine serum albumin. Cells were then transferred to the thermostat-controlled stage (37 °C), and stimulation with glucose was achieved by addition of 52 mM glucose-KRB into the chamber (final concentration: 22 mM glucose). For observation of GFP, we used a 488-nm laser line for excitation and a 515-nm long pass filter for the barrier. Diodomethane sulfur immersion oil was used to make contact between the objective lens and the coverslip, and the measured penetration depth was about 45 nm.

Acquiring the Images and Analysis—Images were collected by a cooled charge-coupled device camera (Micromax, MXM-512-BFT, Princeton Instruments; operated with Metamorph 4.6, Universal Imaging, Downingtown, PA). Images were acquired every 300 ms. The analyses, including tracking (the single projection of different images) and area calculations were performed using Metamorph software. To analyze the data, fusion events were manually selected, and the average fluorescence intensity of individual granules in a 1 × 1-µm square placed over the granule center was calculated. The number of fusion events was manually counted while looking about 30,000 frame time lapses. Sequences were exported as single TIFFF files and further processed using Adobe Photoshop 6.0, or they were converted into Quick Time movies (see supplemental material). This procedure allowed us to dynamically evaluate single insulin granule motion.

mRNA Isolation and RT-PCR—mRNA isolation and RT-PCR were performed as described previously (25). The number of cycles was selected to allow linear amplification of the cDNA under study. For semiquantitative PCR, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as control. We have previously shown (26) and confirmed in the present experiments that a 6–24 h exposure to IL-1β and IFN-γ do not affect GAPDH mRNA expression in purified β-cells. Thus, GAPDH expression was as follows after, respectively, 6 and 24 h treatments (optical density values; means ± S.E. of four experiments): 6 h, control, 8.6 ± 0.5; IL-1β, 8.4 ± 0.8; IFN-γ, 8.5 ± 0.4; IL-1β + IFN-γ, 8.2 ± 0.9; 24 h, control, 6.2 ± 0.6; IL-1β, 7.1 ± 0.8; IFN-γ, 6.1 ± 0.8; IL-1β + IFN-γ, 6.7 ± 1.2. The primer sequences were as follows: GAPDH, 5′ TTC CTC AAG ATT GTC AGC AA 3′ (forward) and 5′ AGA TCC ACA ACG GAT ACA TT 3′ (reverse); SNAP-25, 5′ GAT GTG GCC GAG ACC TTG TT 3′ (forward) and 5′ GAG TCA GGC TTC ATG ATA 3′ (reverse); and VAMP-2, 5′ TCA GCA ATT AAG CGG AG 3′ (forward) and 5′ CTA AGG GTA TAT CTT 3′ (reverse).

Statistical Analysis—Data are presented as means ± S.E., and comparison between groups was performed by t test or by Wilcoxon Rank Sum Test or by analysis of variance followed by Fisher’s test and regression analysis.

RESULTS AND DISCUSSION

IL-1β, but Not IFN-γ, Reduced the Number of Insulin Granules Docked to the Plasma Membrane—To examine the docking status of insulin granules by cytokines exposure, rat primary pancreatic β-cells were exposed to IL-1β or IFN-γ for 24 h, immunostained with anti-insulin antibody, and then examined by TIRF microscopy. TIRF imaging depicts the single insulin granules docked to the plasma membrane, enabling accurate docked insulin granule counting (17). We defined “docked granule” when the vesicle of was located within 100 nm of the plasma membrane as reported somewhere (27). As shown in Fig. 1, the number of docked insulin granules in β-cells treated with IL-1β was markedly decreased (248.9 ± 35.1 per 200 µm2) in control versus 92.6 ± 31.0 in IL-1β; n = 11 cells; p < 0.0001), but there was no difference between IFN-γ-treated (240.6 ± 59.1 per 200 µm2) and control cells. We have shown previously that a 24-h exposure of β-cells to IL-1β does not affect insulin content and cell viability (24, 28, 29), and in experiments performed in parallel with the determinations of mRNA expression (see below), we observed that a 24-h exposure to IL-1β + IFN-γ also failed to affect β-cell insulin content: control β-cells, 28 ± 9 ng of insulin/106 cells; IL-1β + IFN-γ, 25 ± 5 ng of insulin/106 cells (n = 4). Thus, the decreased number of docked insulin granules in IL-1β-treated β-cells is not due to a decrease in insulin content but is probably caused by IL-1β-mediated impairment of insulin granule docking.
Expression

It is well known that SNARE proteins play an important role in docking/fusion of insulin granules, and we have recently demonstrated by TIRF imaging analysis that syntaxin 1 and SNAP-25 are distributed in numerous release-ready granules (22, 23). Against this background, we presently analyzed quantitatively and spatially the expression of SNAP-25 protein on the plasma membrane. As shown in Fig. 2, the number of SNAP-25 clusters per 200 μm² counted using TIRF images; n = 14 cells.

**Fig. 2.** TIRF images and the quantitative analysis of SNAP-25 clusters on the β-cell plasma membrane. Pancreatic β-cells were treated as described the legend of Fig. 1 and immunostained for SNAP-25. The upper panel shows the typical TIRF images of SNAP-25 clusters on the plasma membrane. The lower panel shows the number of SNAP-25 clusters per 200 μm² counted using TIRF images; n = 14 cells.

**IL-1β Decreased SNAP-25 Clusters and SNAP-25 mRNA Expression**—It is well known that SNARE proteins play an important role in docking/fusion of insulin granules, and we have recently demonstrated by TIRF imaging analysis that syntaxin 1 and SNAP-25 are distributed in numerous release-ready granules in the intact plasma membrane of MIN6 cells and rat primary pancreatic β-cells (22, 23). Against this background, we presently analyzed quantitatively and spatially the expression of SNAP-25 protein on the plasma membrane. As shown in Fig. 2, the number of SNAP-25 clusters on the plasma membrane of pancreatic β-cells treated with IL-1β decreased to about half of the control levels (225.1 ± 58.8 per 200 μm² in control versus 136.1 ± 24.3 per 200 μm² in IL-1β; p < 0.0001, n = 14 cells). In contrast, IFN-γ treatment did not affect the number of SNAP-25 clusters (218.7 ± 40.4 per 200 μm²). In line with these observations, a 24-h exposure of β-cells to IL-1β, but not to IFN-γ, induced a 50% decrease in SNAP-25 mRNA expression (Fig. 3A). This inhibitory effect of IL-1β was not potentiated by the concomitant presence of IFN-γ (Fig. 3A).

Similarly, IL-1β treatment decreased VAMP-2 mRNA expression, and this inhibitory effect was also not potentiated by IFN-γ (Fig. 3B). Thus, IL-1-β treatment reduced SNAP-25 mRNA expression and the number of SNAP-25 clusters, along with the reduction of docked insulin granules. The exact molecular regulation of the different stages of membrane fusion still remains to be clarified and it is difficult to precise the mechanism by which the IL-1β-induced reduction in SNAP-25 mRNA and protein expression might lead to a preferential decrease in granule docking. Nevertheless, it appears that SNAP-25 plays a crucial role in the docking status (23), and it has been reported recently that SNAP-25 is a strictly required molecule in evoked fusion events (30, 31). Of note, IL-1β inhibits the expression of mRNAs encoding for other proteins potentially involved in this process, including VAMP-2 (present data) and Rab3 (14, 15). Additional experiments are now required to clarify how these diverse effects of IL-1β will culminate in the observed modifications of exocytosis.

**TIRF Imaging Analysis of Single Insulin Granule Motion**—Since the frequency of granule fusion and the demand for granules change continuously in response to external signals, releasable granules must be replenished in concert with fusion.
events. The present observations suggest that IL-1β affects at least one crucial step in this delicate balance, namely the docking of insulin granules. In the present study, we explored the effects of IL-1β on insulin granule fusion at the cellular level. To obtain a dynamic TIRF image of insulin exocytosis from cytokine-treated β-cells, rat primary β-cells were first infected with Adex1CA insulin-GFP to label the insulin granules and then exposed to IL-1β or IFN-γ for 24 h. Fig. 4 shows the real-time TIRF images of a single insulin granule motion when stimulated by 22 mM glucose for 15 min (for control, see movie S1). As shown in the histogram of number of fusion events per min (Fig. 4; Control), the fusing granules originated mostly from previously docked granules during the first phase of glucose-stimulated insulin release (0–5 min), while during the second phase (>5 min) those fusing granules arose mostly from newcomers as previously described (17, 21). This pattern was, however, modified following exposure of β-cells to IL-1β (Fig. 4; IL-1β-treated; see movie S2). There were rare fusion events during the first phase (movie S2), and the histogram of fusion events (Fig. 4) indicates a clear reduction in the number of fusion events during the first phase. Moreover, there was a marked reduction in the fusion from previously docked granules in IL-1β-treated β-cells, while the fusion from newcomers during the later second phase was preserved. We quantitatively analyzed the data shown in Fig. 4. The results showed that the total number of fusion events in IL-1β-treated cells during the first phase (0–5 min) was significantly decreased (27.3 ± 6.9 in control versus 6.5 ± 5.2 in IL-1β-treated cells in 0–300 s, n = 4, p < 0.005). Remarkably, very few fusion events occurred from previously docked granules in IL-1β-treated cells (19.1 ± 4.2 in control versus 1.6 ± 1.1 in IL-1β-treated cells, n = 4, p < 0.0005). On the other hand, during the second phase (>5 min), there was no significant difference of total number of fusion events between control and IL-1β-treated cells despite a trend for reduced values in IL-1β-treated cells (control, 65.2 ± 21.0: IL-1β-treated, 34.4 ± 18.1 during 301–1020 s, n = 4). Of note, IFN-γ-treated β-cells have a similar TIRF image as compared with that of controls (see movie S3). Although the number of fusion events in IFN-γ-treated β-cells appeared to be increased during the first phase (Fig. 4), there was no statistically different number of total fusion events between control and IFN-γ-treated β-cells. There was also no significant difference of total number of fusion events during the second phase (65.2 ± 21.0 in control versus 48.5 ± 9.1 in IFN-γ-treated cells during 301–1020 s, n = 4). As a whole, our observations suggest that IL-1β has a preferential inhibitory effect on the first phase of glucose-induced insulin release, mostly via an action on previously docked granules. This bears similarity to the pattern of insulin release observed in prediabetic subjects. The present data, together with our previous observations (13), raise the possibility that β-cell exposure to immune mediators during the course of insulin is responsible for the key metabolic modifications observed in this period, namely disproportionately elevated proinsulin release and loss of first phase insulin release.

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