VISUALIZATION OF SECRETORY GRANULES IN PANCREATIC ISLETS AND β-CELLS USING TIRF AND CONFOCAL MICROSCOPY

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Insulin, a key hormone regulating glucose homeostasis, is released from β-cells in the pancreatic islets of Langerhans through Ca^{2+}-dependent regulated exocytosis of membrane bound insulin-containing secretory granules. The total number of granules present in a single β-cell has been estimated to about 13,000 (Dean, 1973, *Diabetologia* 9:115-119), which can be divided into at least three functionally distinct populations, the reserve, the readily releasable (RRP), and the immediately releasable pool (IRP) (Eliasson *et al.*, 1997, *J. Physiol.* 503:399-412). Granules in the two releasable pools are viewed as docked to the plasma membrane. The RRP requires ATP-dependent priming of the granules before fusion can occur whereas the IRP constitutes primed granules that can be rapidly released in an ATP- and temperature-independent manner in response to an elevation in intracellular Ca^{2+}. The sized of the IRP has been estimated to about 50 granules. To study secretion of granules located in the IRP we used confocal and total internal reflection fluorescence (TIRF) microscopy to monitor the distribution and movement of fluorescence labeled secretory granules. The confocal technique allowed us to detect granules throughout the β-cell while TIRF microscopy has the ability to focus on an extremely thin surface layer (< 0.15 μm) and therefore might be useful to monitor docked vesicles that fuse with the membrane in the process of exocytosis. The fluorescent techniques were also used to detect Ca^{2+} signals (Fluo-3) and membrane staining (di-2-ANEPEQ).

Experiments were performed using either cultured mouse (*Mus musculus*) pancreatic β-cells and islets or β-TC3 cells, an insulinoma cell line. The vesicles were labeled using either quinacrine or adenovirus-mediated infection of a syncollin-green fluorescent protein (Syn-GFP) gene construct that targets expression of the Syn-GFP protein to secretory granules. Freshly dissociated cells and islets were cultured at 37 °C for 1 to 4 days following viral infection. During subsequent experimentation at room temperature the cells were superfused with a control solution containing: 137 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes and 2 mM glucose. To trigger exocytosis increases in intracellular Ca^{2+} was achieved by rapidly (<50 ms) changing from the control to a depolarizing with iso-osmolar substitution of K^+ for Na^+ (45 mM KCl, 100 mM NaCl), or a solution to which 10 mM caffeine had been added. At room temperature, these manipulations would trigger the release of granules present in the IRP.

Figure 1A shows a confocal image of β-TC3 cells stained with quinacrine that is known to rapidly partition into the acidic environment of the secretory vesicles. This dye staining produced several bright spots of fluorescence separated by ~1-2 μm. Subsequent staining of the

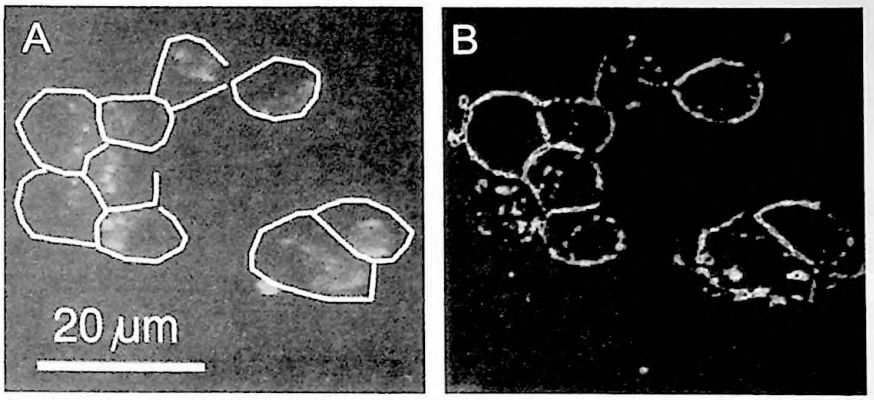

Figure 1. Confocal fluorescence images β-TC3 cells. Panel A: clustering of secretory granules labeled with quinacrine. Panel B: Subsequent stronger staining of cell membrane with di-2-ANEPEQ. The contours of cells detected in panel B are shown as solid white lines in panel A.

surface membrane with di-2-ANEPEQ (Fig. 1B) allowed us to determine the number and outline of cells and to place the quinacrine-fluorescence at one end of each cell (opposite to the nucleus). Confoal sectioning revealed ~20-100 fluorescence hot-spots in each cell, so that each hot-spot may be produced by a cluster of more than 100 secretory granules. Considering the resolution of the confocal technique ($(\Delta x, \Delta y, \Delta z) \sim$ (0.5 μm, 0.5 μm, 1 μm)) and density of vesicles, it is unlikely that this method would be able to detect single vesicles. The diameter of individual granules is 0.2-0.4 μm.

We therefore proceeded to use TIRF microscopy to focus on the space just below the membrane of β-cells and islets cultured on laminin-treated glass cover slips. The shallow depth of penetration from the surface of the glass cover slip may provide superior resolution ($\Delta x, \Delta y, \Delta z) \sim$ (0.5 μm, 0.5 μm, 0.15 μm)), but only if the cells adhere closely to surface. Staining of the cell membrane with di-2-ANEPEQ generally produced a well-defined, stable and fairly uniform fluorescence within the entire bright field image suggesting that the entire lower membrane surface was in close contact with the glass cover slip.

Cells were incubated with the Ca^{2+}-indicator dye Fluo-3AM (5 μM, 30 min) to ascertain elevated intracellular Ca^{2+} levels through activation of voltage gated Ca^{2+} channels by KCl depolarization or release of Ca^{2+} from intracellular stores triggered by extracellular application of caffeine. Figure 2A shows that the Ca^{2+}-induced TIRF fluorescence in β-TC3 cells was also fairly uniform within each cell but varied greatly from cell to cell. A fluorescent bead is seen in the right side of the panel. Beads that were added to the perfusion chamber settled on the surface of the cover slip within a few minutes and served to find the optimal focal plane and monitor the stability of the laser light (488 nm) used for fluorescence excitation. Figure 2C shows cellular Ca^{2+} transients produced promptly by rapid application of a depolarizing solution with 45 mM KCl. These Ca^{2+} signals were strong in some cells, but smaller or absent in others including some were bright cells (cell # 1, Fig. 2BC) that may be Ca^{2+}-overloaded. The TIRF images obtained with di-2-ANEPEQ and Fluo-3, suggest that the cells adhere well to the laminin-treated cover slips and that that some cells generate large and fast Ca^{2+} transients.

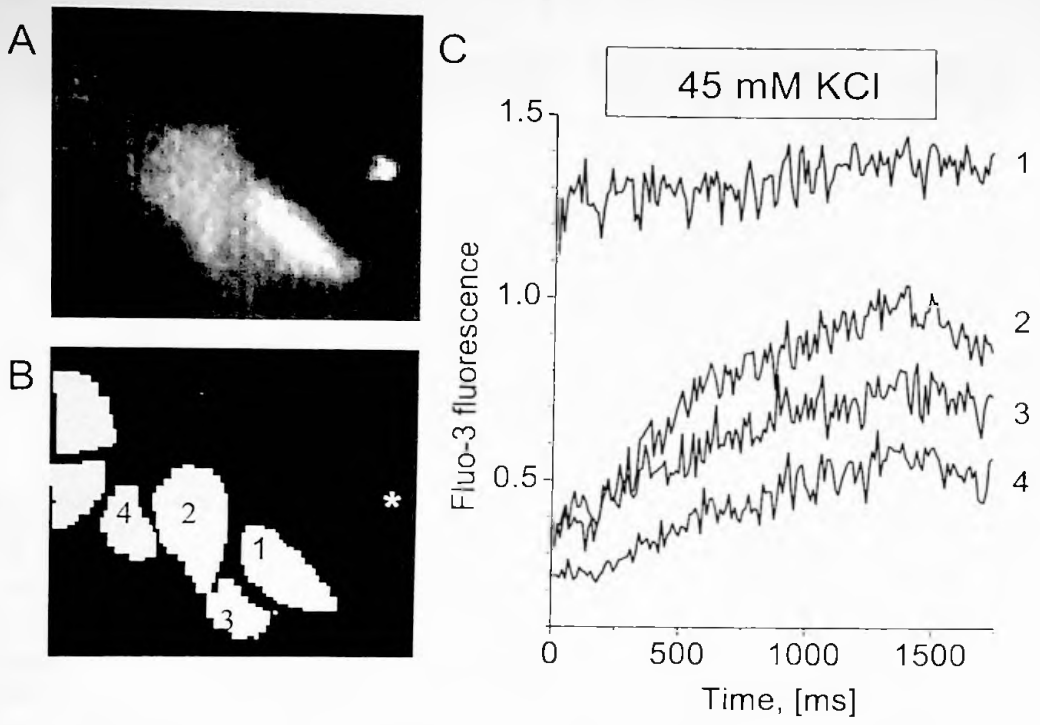

Figure 2. Detection of Ca^{2+}-dependent Fluo-3signals in β-TC3 cells using TIRF microscopy. Panel A: Image of cells and a fluorescent bead. Panel B identifies the cells by number and shows the location of a fluorescent bead (*). Panel C: Ca^{2+}-transients in cells exposed to 45 mM KCl for 1200 ms. The TIRF images were recorded at 734 Hz, but plotted after averaging over 8 frames.

The TIRF images obtained by fluorescence labeling of vesicles with GFP were quite different in that they contained a number of bright spots (Fig. 3CD) about as bright as the fluorescent beads, of which three are seen towards the right side of the panels. The spots produced by staining of vesicles could often be resolved at distances of 1-2 μm (Fig. 3BCD) as in the confocal sections (Fig. 1A). At other time they were found along contours too close together to be clearly distinguished. In cells that were stimulated repeatedly over a period of minutes, we observed that the intensity of the bright spots changed significantly, sometimes as much as 2 or 3 fold. Fig. 3E shows how the intensities of three bright spots changes over time so that point #2 went from being the weakest (panel C) to being the strongest (panel D).

Some well defined bright spots at distinct fixed locations showed repeated abrupt changes in fluorescence (5-10%) during brief (1 sec) activation by KCl depolarization (Fig. 3F). These changes may occur synchronously at different points, but it was also noticeable that the synchronized signals varied from spot to spot, and that a strong signal at one point (trace #1, last pulse), might be completely absent at a neighboring point (trace #2).

The present data are consistent with the notion that exocytosis in stimulated β-cells occurs at fixed locations where fluorescence labeled secretory granules produce changes in TIRF fluorescence as they dock at the membrane and then fuse to release their contents. The rapid changes are of the order of 5 to 10 %, while slower changes are much larger. This may indicate that the secretory sites resolved by TIRF microscopy harbor about 10-20 docked vesicles within

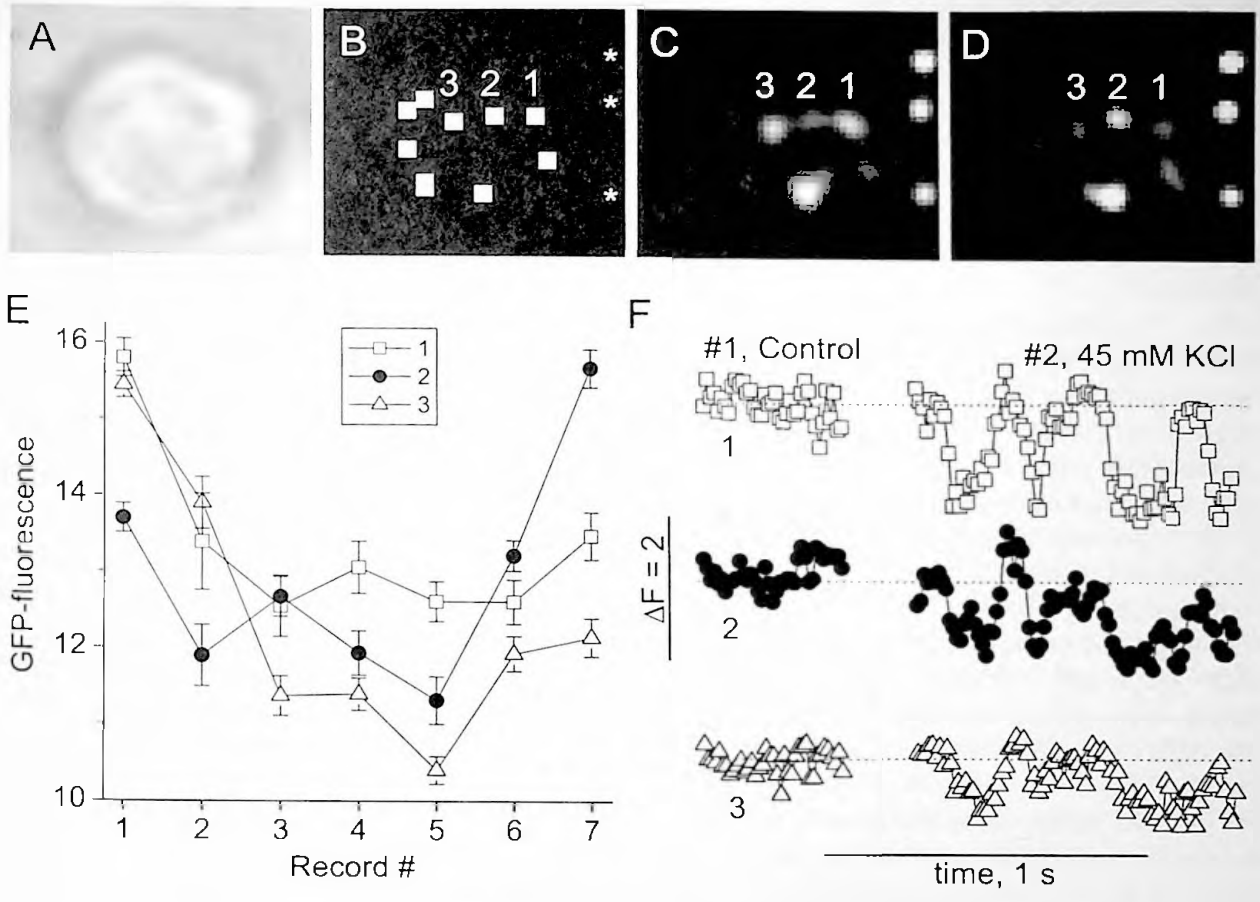

Figure 3. Detection of secretion in a single mouse pancreatic β-cell using Syn-GFP and TIRF microscopy. A: Bright field image of the cell about 12 μm in diameter. B: Diagram showing location of "secretory sites" (■) and fluorescent beads (*). Panels C (record #2) and D (record #6) show changes in fluorescence images also graphed in panel E for the three sites identified by number. Panel F: rapid changes in fluorescence evoked by KCl-depolarization. Note that the traces in this panel were shifted from levels in panel E to appear aligned and in sequence. Each plotted point corresponds to 8 averaged frames recorded at 734 Hz.

that the secretory sites resolved by TIRF microscopy harbor about 10-20 docked vesicles within ~0.15 μm of the membrane. It is tempting to speculate that the docking of a single vesicle produces an abrupt increase in fluorescence, and vesicle fusion an abrupt fall, and that these processes are stochastic but to some degree synchronized by repetitive firing of action potential producing localized Ca^{2+} signals near the membrane. While such detail may require both technical improvement and more extensive experimentation, the present data make it plausible that TIRF microscopy used in conjunction with Syn-GFP may be developed a much-needed tool to routinely study the control of insulin secretion at the cellular level. We thank Dr. Li Ma for preparing the viral vector for expression of Syn-GFP. Supported by NIH RO1 16152, AHA 808116U, and the American Diabetes Association.