Biophysical Journal Letter:

D. Michael et. al "Fluorescent Cargo Proteins in Pancreatic Beta Cells: Design Determines Secretion Kinetics at Exocytosis"

Supplemental Material:

Materials and Methods

Tissue Collection and Cell Culture

Adult (8-12 weeks) male rats (Sprague-Dawley, Charles River, Wilmington, MA) were anesthetized (50 mg/kg pentobarbital, intraperitoneal) and sacrificed according to procedures approved by the USC animal use committee in accordance with IUCAC standards. The pancreas was inflated via the pancreatic duct with 10 ml of cold digestion solution: Hank's balanced salt solution (HBSS) containing collagenase (0.233 mg/ml, Liberase RI, Roche, Indianapolis, IN) and DNase I (0.1 mg/ml, Roche, Indianapolis, IN). The excised pancreas was transferred to a glass vial (20 ml capacity) containing 5 ml of cold digestion solution, and immediately incubated (static, 33 min) in a water bath at 37°C. After incubation, the vial was shaken vigorously, and the contents were washed at least 4 times with cold HBSS. The tissue was centrifuged (200 g, 1 min), and the resulting supernatant was discarded. Islets were enriched by centrifugation (15 min, 200 g) in a discontinuous BSA gradient (35%, 31%, 27% and HBSS). Islets were washed once more, picked by hand using a pipette tip (200 µl capacity) and collected in a microcentrifuge vial (~300 islets/vial). For dispersion, islets were incubated at 37°C for 5 minutes in Ca^{2+}-free solution (0.5 ml, HBSS without Mg^{2+} or Ca^{2+} containing 1 mM EGTA) and thereafter, for 5 minutes in HBSS (0.5 ml) containing neutral protease (0.03 units/ml, Sigma, St. Louis, MO). Upon removal of the enzyme solution, islets were resuspended in culture medium (~1.33 µl/islet RPMI 1640 without FBS, 8 mM glucose) and dispersed into small clusters of cells using gentle trituration with a pipette tip (1.0 ml capacity). Cells were plated (40 µl/dish) on culture dishes coated with a laminin-rich matrix (S1) and allowed to attach for ~30 minutes. Thereafter, cells were cultured (RPMI

1640, 10% FBS, 8 mM glucose) at 37°C with 5% CO_2 and used within 5 days. RIN cells (generous gift of Peter C. Butler, University of California, Los Angeles) were cultured in RPMI 1640 with 10% FBS and 11 mM glucose.

For experiments with mice, we used gender- and age-matched CPE +/+ and CPE -/- pairs of mice (30 weeks old) for both electron- and fluorescence microscopy. The CPE -/- mouse has been characterized and a manuscript has been accepted (S2). To isolate islets, we used a protocol identical to the one described above for rats, except that the pancreas was incubated at 37°C for only 25 minutes.

Vectors and Adenovirus

Beginning with the plasmid pGEM4Z containing a DNA insert encoding for rodent amyloid polypeptide (rIAPP), the rIAPP insert was PCR-modified to eliminate the stop codon and to introduce an EcoR1 restriction site. The amplified fragment was restriction digested with EcoR1 and HindIII and subcloned into pEGFP-N2 (BD Biosciences, San Jose, CA), preserving the reading frame. Syncollin-EGFP chimera was obtained as a generous gift from Dr. J. Michael Edwardson, Department of Pharmacology University of Cambridge, UK, made as previously described (S3). The rIAPP-EGFP and syncollin-EGFP chimera cDNAs were then subcloned into the pShuttle adenoviral shuttle vector and recombinant adenoviruses generated as described (S4-6). The resulting recombinant adenoviruses were then amplified and purified as previously described (S5; S6). The construction of adenovirus containing insulin C-peptide fused to emerald GFP has been described (S7). C-peptide fused to TIMER protein was made similarly using the exact same sites within the C-peptide coding region. Briefly, the gene for TIMER was constructed in pDsRed1-C1A by site-directed mutagenesis to give the S197T "Bright" and V105A "TIMER" mutations, based on the published information (S8). PCR products of the TIMER template were cut with SmaI and BstEII, then placed into the corresponding sites within the C-peptide coding sequence of "Adlox.Insulin II," such that three alanine codons flank either end of the TIMER insert, all in frame with the C peptide coding segment. The entire C-TIMER construct was confirmed by DNA sequencing and used to make recombinant adenovirus, as described (S7).

DNA Manipulations

For site-directed mutagenesis and deletion of plasmid DNA, the QuikChange II kit (Stratagene, La Jolla, CA) was used according to standard protocols. The following primers (Qiagen, Valencia, CA) were used: C-to-S mutation- CTTACTCCTGGAATTCAGCAGTCGACGGTACCG (forward) and CGGTACCGTCGACTGCTGAATTCCAGGAGTAAG (reverse); linker deletion- CTGGATTTCTTACTCCTGATGGTGAGCAAGGG (forward) and CCCTTGCTCACCATCAGGAGTAAGAAATCCAG (reverse). Sequence changes were confirmed by bidirectional automated sequencing of ~700 base pairs overlapping the target (USC Microchemical Core facility). For the deletion, double endonuclease digestion (EcoRI and NotI) and agarose gel electrophoresis confirmed that a unique EcoRI site was removed and that the size of the vector was appropriate, consistent with deletion of only the intended region.

DNA Delivery

Following overnight incubation after plating, cells were transduced with adenovirus ($5x10^7$ plaque forming units/ml in RPMI 1640 with 10% FBS and 8 mM glucose) containing the gene of interest. Following incubation (4 hours), the medium was removed and replaced with RPMI 1640 (10% FBS, 8 mM glucose). Cells were imaged between 16 and 72 hours following transduction.

For plasmid transfection, RIN cells were grown to 80-90% confluence and transfected with plasmids using a standard protocol (Metafectene, Biontex, Munich, Germany; 1 μg plasmid DNA: 5 μl of reagent).

Solutions and Chemicals

Extracellular solution was composed of [mM]: 136 NaCl, 3.6 KCl, 2.5 $CaCl_2$, 1 $MgSO_4$, 1 L-glutamine, 4 glucose and 10 HEPES (pH 7.4, 305 mOsm). For potassium stimulation, an equal quantity of KCl replaced NaCl to yield a final potassium concentration of 50 mM. All solutions were prepared with water from a commercial

purification system (Nanopure Infinity, Barnstead Thermolyne, Dubuque, IA). All salts for solutions were obtained from Sigma Aldrich with the highest purity available (St. Louis, MO).

Total Internal Reflection Fluorescence Imaging

An Olympus IX 70 (Olympus, Melville, NY) inverted microscope was configured for prismless total internal reflection fluorescence (TIRF) microscopy (S9) using a high numerical aperture objective lens (α-PlanFLUAR, 100x, 1.45 NA, Zeiss, Thornwood, NY). The beam from an argon ion laser (Coherent, Innova 5, Santa Clara, CA) was coupled to a single mode fiber optic (ThorLabs, Newton, NJ) and collimated for subsequent focusing at the back focal plane of the objective, yielding a small spot of TIR illumination in the specimen plane. Images were collected using a charge coupled device camera (either a Cascade or Cascade 512B (only Fig. 1A), Roper Scientific, Tucson, AZ) under computer control (Metamorph, Universal Imaging Corporation, Downingtown, PA). Images were acquired repetitively at one of two rates (~2-5 Hz or 140 Hz: images were acquired at low temporal resolution unless otherwise indicated). The exposure time was adjusted to optimize contrast and to maximize use of the camera's dynamic range (typically 66 ms for slow acquisition and 3 ms for fast acquisition). A mechanical shutter (Vincent Associates, Rochester, NY) gated the excitation light: for slow acquisition, the sample was illuminated only during the exposure time while for fast acquisition, the sample was illuminated throughout the imaging period. Some focal drift occurred while imaging at elevated temperatures and was compensated by adjusting the offset of a piezoelectric positioner (PiFoc, Physik Instrumente, Walbronn, Germany). Imaging experiments occurred between 35 and 37°C with continuous perfusion of the cells (~1 ml/min). Pulled glass pipettes (~3 μm tip diameter) were positioned near cells for transient application of test solutions.

Data Analysis

For primary cultured cells, detailed analysis was restricted to cells with a rapid (<5 s from application) and vigorous (>20 vesicles) response. In contrast, with RIN cells,

we analyzed all cells that showed a response. To quantify the size of individual fluorescence spots, we fit their fluorescence profiles to a Gaussian curve using a software algorithm written in MatLAB (Mathworks, Natick, MA), and the diameter was taken as two times the standard deviation (*i.e.* 2σ). All data are reported as mean±SE. In Figures 1, 2 and 3, all fluorescence intensity traces for single vesicles were corrected for local background. Error bars show SE for each point.

Detailed Figure Legends

Figure 1. As indicated, images were captured at two different frame rates. (**A**) *Montage*- consecutive images for a representative vesicle labeled with C-emGFP. Beginning at the seventh frame, the fluorescent spot exhibited a rapid increase in intensity before dispersing and collapsing into the background – a series of events consistent with exocytosis. Normalized fluorescence, corrected for local background, is plotted below the montage (average of 5 vesicles, one cell). The intensity plot simultaneously shows average fluorescence, as functions of time and position, for each of 18 adjacent rows in a matrix centered on a single vesicle; each row is 135 nm (1 pixel) tall and 400 nm (3 pixels) wide. (**B-E**) Four different patterns of fluorescence change occurred at exocytosis: disappearance (**B**), dimming (**C**), transient brightening (**D**) and persistent brightening (**E**). *Montages*- consecutive images for a single representative vesicle. Above each montage is the name of the fluorescent cargo protein used to label the vesicle; it also indicates the preferred pattern of fluorescence change for vesicles labeled with each fluorescent cargo protein. (*Top trace*) For a group of representative vesicles, individual fluorescence intensity traces were first corrected for local background, then normalized and aligned, and finally averaged (n=[100,14], [25,10], [100,28], [103,29],: [vesicles, cells]) to produce the trace shown. For each point, the vertical bars represent SE. (*Middle trace*) Whole-cell fluorescence change is shown for a single representative cell expressing the fluorescent cargo protein indicated above the montage. (*Bottom trace*) Average fluorescence is shown for a region of interest located 3 microns from the closest edge of the cell. [For *single vesicle* traces, fluorescence was averaged in a 1 μm x 1μm box centered on the vesicle. Similarly, for *adjacent region*

traces, fluorescence was averaged in a 1 μm x 1μm box centered 3 μm from the edge of the cell.]

Figure 2. Electron micrographs show distinct morphologies for insulin vesicles in (**A**) CPE +/+ mice and (**B**) CPE -/- mice (matched for both age [30 weeks] and gender). These electron micrographs are from intact pancreatic slices that contained no fluorescent cargo proteins. (**C** and **D**) Membrane fusion caused persistent brightening of rIAPP-EGFP-labeled vesicles when expressed in either CPE +/+ mice or CPE -/- mice. Single vesicle traces were prepared as described in Figure 1 (**C**- [23,7], **D**- [50,13]: [vesicles, cells]).

Figure 3. *Top* Pre-pro-islet amyloid polypeptide was fused to EGFP through a variable sequence of amino acids (hatched region). The black arrows indicate cleavage sites. Protein fragments are as follows: Signal- signal peptide; F1- processing fragment 1; IAPP- mature IAPP, F2-processing fragment 2. For each fluorescent cargo protein, the sequence of amino acids introduced by cloning is indicated above the montage. (**A**) Rodent islet amyloid polypeptide (rIAPP) was fused to EGFP through a sequence of 18 amino acids. (**B**) A serine residue replaced the lone cysteine residue within the sequence linking rIAPP to EGFP (asterisk). (**C**) rIAPP was fused directly to EGFP. (**D**) Human IAPP was fused to EGFP through a short sequence of amino acids [see ref. 3 in main article (Barg 2002)]. For each panel, the montage displays consecutive images for a single representative vesicle. Normalized fluorescence intensity traces were prepared as described in Figure 1 (**A**- [31,7], **B**- [32,10], **C**- [31,13], **D**- [25,3]: [vesicles, cells]).

References

(S1) Beattie, G. M., V. Cirulli, A. D. Lopez, and A. Hayek. 1997. Ex vivo expansion of human pancreatic endocrine cells. *Journal of Clinical Endocrinology and Metabolism.* 82:1852-1856.

(S2) Cawley, N. X., J. Zhou, J. M. Hill, D. Abebe, S. Romboz, T. Yanik, R. M. Rodriguiz, W. C. Wetsel, and Y. P. Loh. 2004. The Carboxypeptidase E Knockout Mouse Exhibits Endocrinological And Behavioral Deficits. *Endocrinology.* Published online: 10.1210/en.2004-0847

(S3) Hodel, A., and J. M. Edwardson. 2000. Targeting of the zymogen-granule protein syncollin in AR42J and AtT-20 cells. *Biochemical Journal.* 350:637-643.

(S4) He, T. C., S. B. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. Vogelstein. 1998. A simplified system for generating recombinant adenoviruses. *Proceedings of the National Academy of Sciences of the United States of America.* 95:2509-2514.

(S5) Lingohr, M. K., L. M. Dickson, J. F. McCuaig, S. R. Hugl, D. R. Twardzik, and C. J. Rhodes. 2002. Activation of IRS-2-mediated signal transduction by IGF-1, but not TGF-alpha or EGF, augments pancreatic beta-cell proliferation. *Diabetes.* 51:966-976.

(S6) Wrede, C. E., L. M. Dickson, M. K. Lingohr, I. Briaud, and C. J. Rhodes. 2002. Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). *Journal of Biological Chemistry.* 277:49676-49684.

(S7) Watkins, S., X. Geng, L. Li, G. Papworth, P. D. Robbins, and P. Drain. 2002. Imaging secretory vesicles by fluorescent protein insertion in propeptide rather than mature secreted peptide. *Traffic.* 3:461-71.

(S8) Terskikh, A., A. Fradkov, G. Ermakova, A. Zaraisky, P. Tan, A. V. Kajava, X. N. Zhao, S. Lukyanov, M. Matz, S. Kim, I. Weissman, and P. Siebert. 2000. "Fluorescent timer": Protein that changes color with time. *Science.* 290:1585-1588.

(S9) Axelrod, D. 2001. Selective imaging of surface fluorescence with very high aperture microscope objectives. *J Biomed Opt.* 6:6-13.