Supplement to: Changes in insulin granule mobility and age contribute to changes in secretion after beta cell desensitization or rest.

Bastian Gaus[1], Dennis Brüning[1], Kathrin Hatlapatka[1], and Ingo Rustenbeck[1]

[1]Institute of Pharmacology, Toxicology and Clinical Pharmacy, Technische Universität Braunschweig, Braunschweig, Germany

RESEARCH DESIGN AND METHODS

Cell culture, plasmid construction and transfection

Insulin-EGFP (hIns-EGFP): The cDNA of human preproinsulin was cloned into the mcs of the expression vector pEGFP-N1 (Clontech, Mountain View, CA, USA) as described (Hatlapatka et al., 2011). Insulin-Timer (hIns-DsRed E5): The cDNA of DsRed E5 was excised with Age1 and Not1 from the expression vector pTimer (Clontech) and cloned into the mcs of the hIns-EGFP-plasmid from which the EGFP sequence had been excised (Hatlapatka, 2008). Insulin-secreting MIN6 cells (kindly provided by Jun-Ichi Miyazaki) were seeded on glass cover slips and cultured in DMEM medium (25 mM glucose), supplemented with 6 mM L-glutamine, 10% FBS and penicillin/streptomycin at 37°C and 5% CO_2. The cells were transfected in suspension using Lipofectamine 2000 (Invitrogen, Karlsruhe) according to the manufacturer's protocol and cultured on glass cover slips for 48 or 72 hours under the same conditions. The subsequent exposure to the test compounds, 500 µM tolbutamide or 1 µM clonidine, did not affect the viability of the MIN6 cells (Suppl. Fig. 1).

TIRF microscopy

The cover slip with the attached MIN6 cells was tightly screwed in a purpose-made perifusion chamber on the stage of an iMIC epifluorescence microscope under control of L.A. software (TILL Photonics, Gräfelfing, Germany). Temperature was maintained at 32.0 ± 0.1°C by overnight pre-warming the system with an environmental control chamber (Solent Scientific, Fareham, UK). The cells were perifused with HEPES-buffered Krebs-Ringer medium at a rate of 200 µl / min, which was continuously thermostated and equilibrated with 95% O_2 and 5% CO_2. Fluorescence in the evanescent field was excited by a 491 nm continuous-wave diode-pumped solid-state laser (100 mW Cobolt Calypso), run at 50%. The objective was a Zeiss α-Plan-Fluar (100x, 1.45 N.A.), the angle of incidence was 68° and the calculated decay constant (reduction of the initial intensity at the glass-membrane interface to 1/e = 37 %) of the evanescent field was 84 nm. One image pixel corresponded to 79 x 79 nm in the focal plane. Selection of the excitation and emission wavelengths was made with a quadruple line filter set for 405/491/561/630 nm lasers (AHF Analysentechnik, Tübingen, Germany). The exposure time was 50 ms per image, the cycle time for acquisition and storage was 125 ms. The fluorescent spots of the hIns-EGFP- or hIns-DsRed E5-labelled granules (separated at 560 nm) were localized and the mobility analyzed by an in-house written program using MATLAB 7.6.0 (The MathWorks, Natick, MA) as described (Matz et al., 2014).

Granule tracking and detection of exocytosis

The caging diameter describes the maximal distance between the positions of a granule in a running time window (Nofal et al., 2007). The caging diameter was determined for all identified granules with a residence time of > 1 s, since the calculation required a time window of 9 images (Suppl. Fig. 2). As a characteristic parameter to describe time-dependent changes within a granule population the half-maximal caging diameter (CD_{50}) was defined. To calculate this parameter a cumulative histogram of all CD values was generated and the maximal y-value was normalized to 1. After curve-fitting, the caging diameter corresponding to the half-maximal y-value was defined as CD_{50}. An increase of this parameter indicates a higher frequency of directed granule movements. To characterize the turnover of the granules in the submembrane space the arrivals and departures were determined. Both parameters gave closely matching data (Suppl. Fig. 3), therefore only the arrivals are depicted to characterize the mobility in the z-dimension. The exocytosis of the insulin granules was measured by a detection algorithm, which looked for a transient increase in fluorescence and a spreading cloud during a time window preceding a strong and lasting decrease in granule fluorescence (Matz et al., 2014). Also, a list of candidate granules was generated to permit the description of the pre-exocytotic behaviour. A problem in the analysis of age-dependent changes in the mobility of hIns-DsRed E5-labelled granules consists in the considerable overlap of the emission below and above 560 nm, i.e. green and red granules (Suppl. Fig. 4). This is the likely reason why the granule number and the cumulative granule number were higher, when green and red granules were added, than the corresponding values obtained with hIns-EGFP.

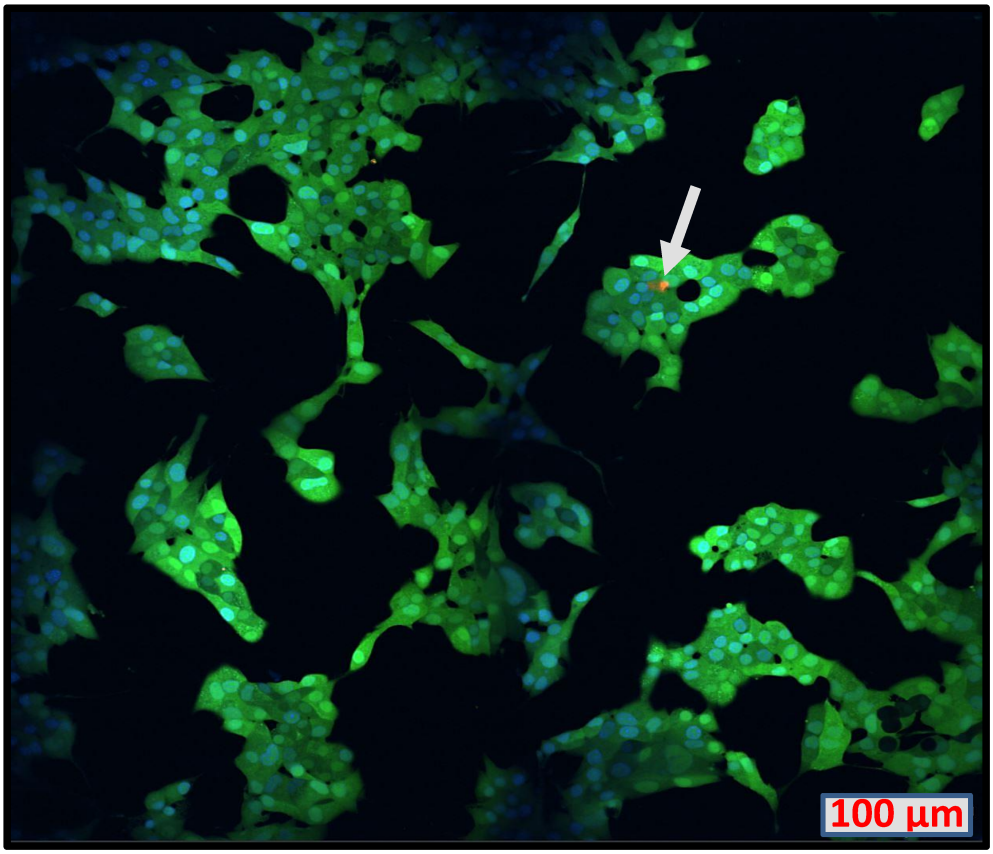
References

Hatlapatka, K., 2008. Fluorescent insulin fusion proteins for assessing the granulation state and exocytosis of insulin-producing cells. https://doi.org/10.24355/dbbs.084-200809020200-0

Hatlapatka K, Matz M, Schumacher K, Baumann K, Rustenbeck I. Bidirectional insulin granule turnover in the submembrane space during K(+) depolarization-induced secretion. Traffic 2011; 12:1166-1178.

Matz M, Schumacher K, Hatlapatka K, Lorenz D, Baumann K, Rustenbeck I. Observer-independent quantification of insulin granule exocytosis and pre-exocytotic mobility by TIRF microscopy. Microsc Microanal. 2014; 20:206-218.

Nofal S, Becherer U, Hof D, Matti U, Rettig J. Primed vesicles can be distinguished from docked vesicles by analyzing their mobility. J. Neurosci 2007; 27:1386-1395.



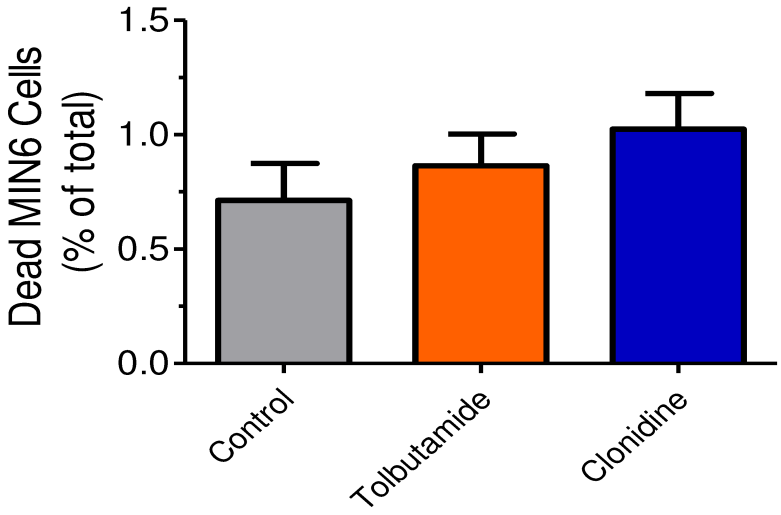


Figure 1. Viability of MIN6 cells after culture in the presence of 25 mM glucose (control) or, additionally, 500 µM tolbutamide or 1 µM clonidine. The fluorescence micrograph shows the cells after control culture.

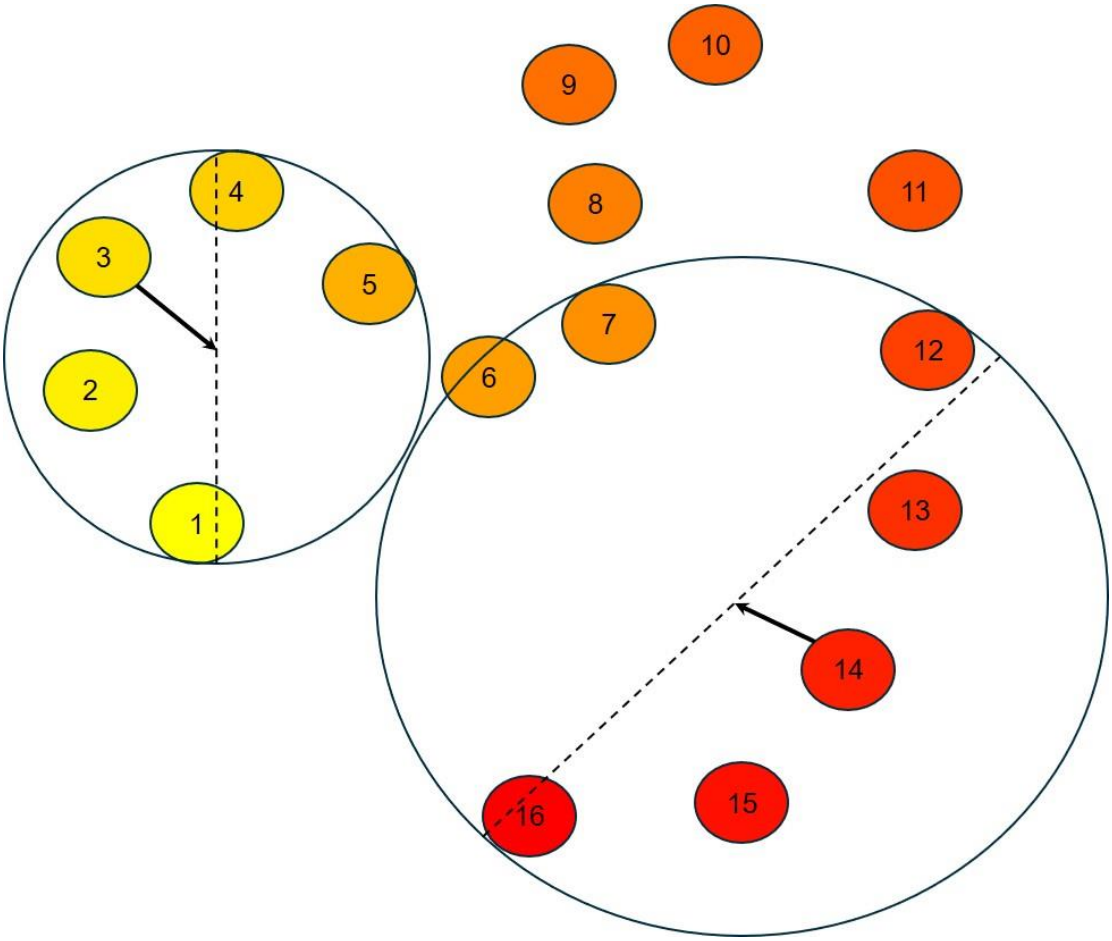


Figure 2. Principle of the caging diameter as parameter to describe the granule mobility parallel to the plasma membrane. In this exemplary plot, the granule is located at 16 different positions at 16 time points in a sequence. The caging diameter is the diameter of the circle containing all granule positions within the given time window. Setting the time window at ± 2, a smaller caging diameter results at granule position 3 than at granule position 14.

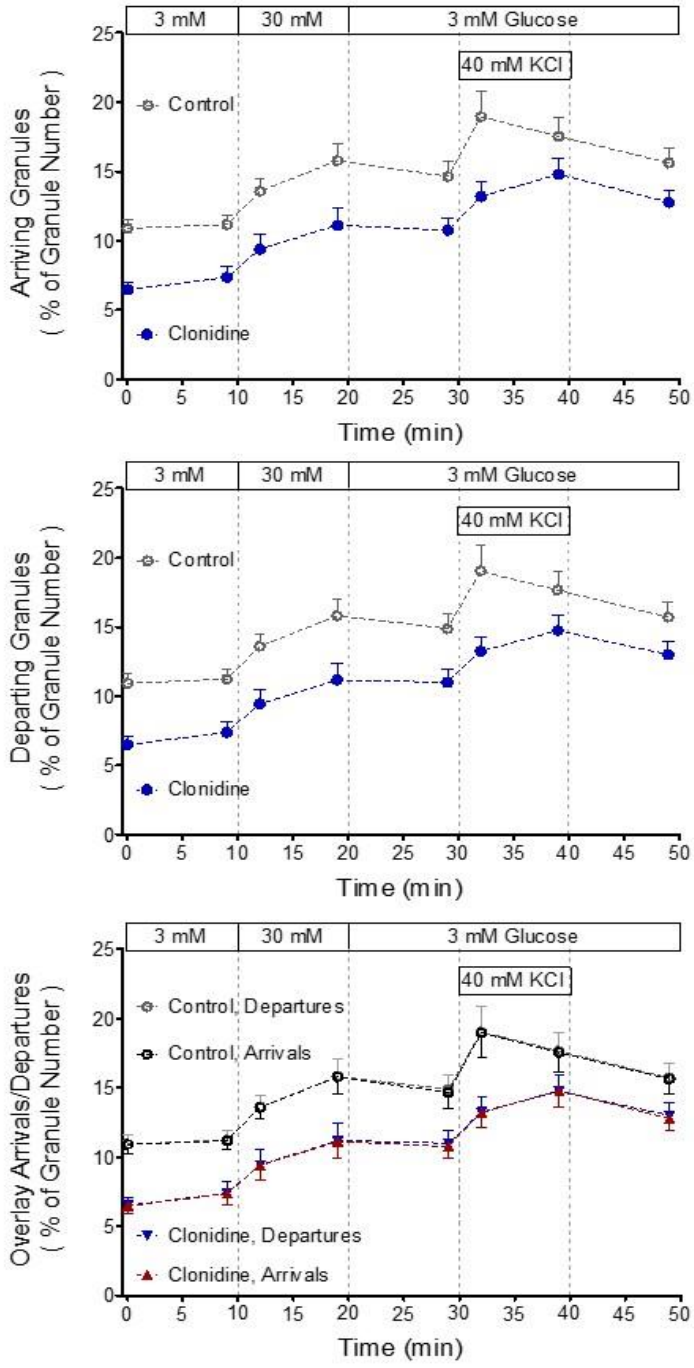


Figure 3. Comparison of the number of arriving and departing granules. When the tracking algorithm could not locate an identified granule in the preceding images, an arriving granule was defined, when the granule track could not be continued, a departing granule was defined. So, the numbers of arrival and departure were separately determined. Nevertheless, both numbers were closely matching. For details of the search algorithm see Matz et al., 2014.

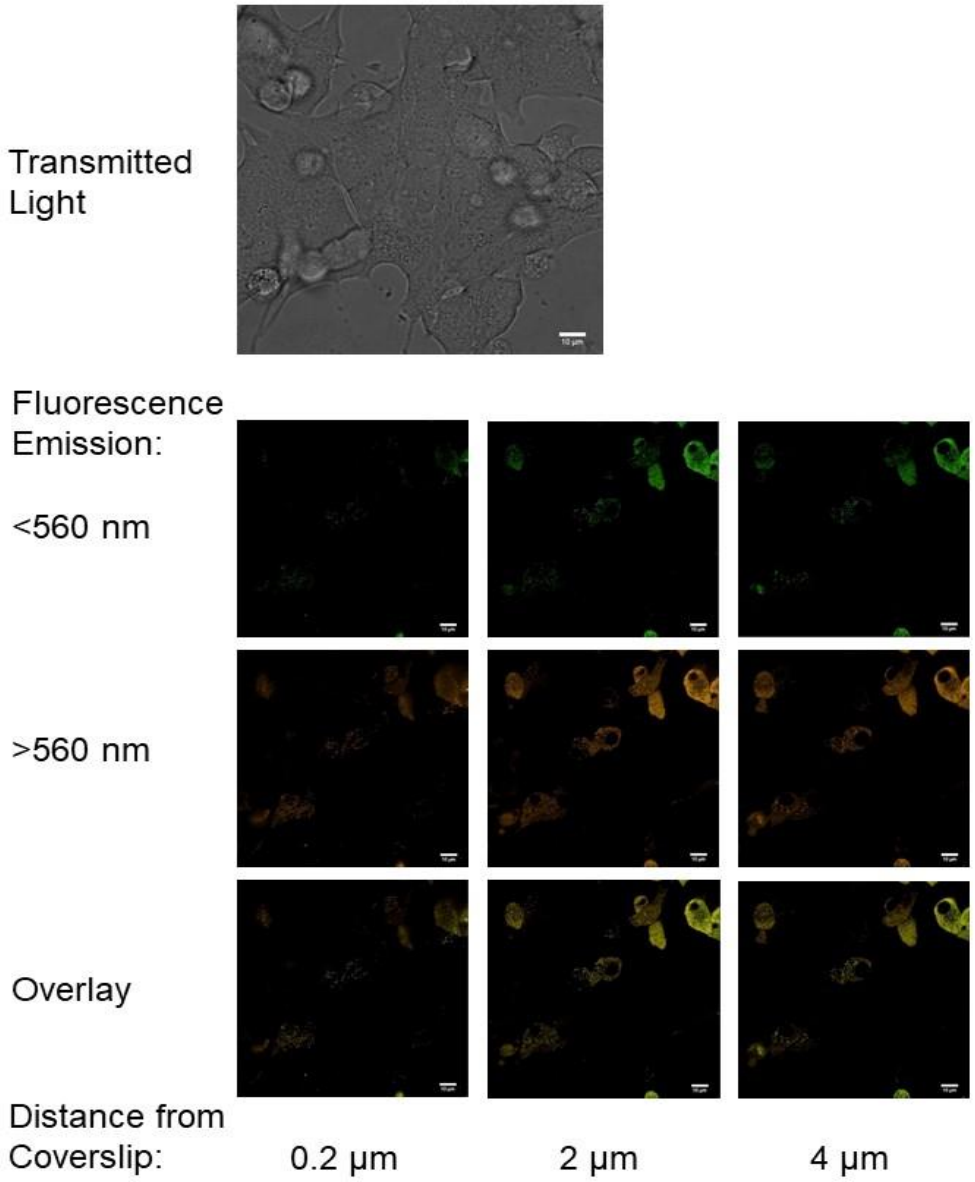


Figure 4. Comparison of the fluorescence emission below and above 560 nm (green and red, respectively) of MIN6 cells transfected with hIns-DsRed E5 (timer) and cultured in the presence of clonidine. The majority of the granules emits in both channels and no systematic difference in distribution is immediately visible. Specifically, the red emission is present in the superficial plane (0.2 µm) as well as in the central planes (2 and 4 µm). A quantitative evaluation with granule identification and tracking is necessary to detect differences in the mobility and distribution of submembrane granules according to their age.