Glucose but not KCl diminishes submembrane granule turnover in mouse beta-cells

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Abstract

KCl depolarization is widely used to mimic the depolarization during glucose-stimulated insulin secretion. Consequently, the insulin secretion elicited by KCl is often regarded as the equivalent of the first phase of glucose-induced insulin secretion. Here, the effects of both stimuli were compared by measuring the secretion of perfused mouse islets, the cytosolic Ca²⁺ concentration of single beta-cells and the mobility of submembrane insulin granules by TIRF microscopy of primary mouse beta-cells. Two cargo-directed granule labels were used namely insulin-EGFP and C-peptide-emGFP. The granule behaviour common to both was used to compare the effect of sequential stimulation with 40 mM KCl and 30 mM glucose and sequential stimulation with the same stimuli in reversed order. At the level of the cell secretory response, the sequential pulse protocol showed marked differences depending on the order of the two stimuli. KCl produced higher maximal secretion rates and diminished the response to the subsequent glucose stimulus, whereas glucose enhanced the response to the subsequent KCl stimulus. At the level of granule behaviour, a difference developed during the first stimulation phase in that the total number of granules, the short-term resident granules and the arriving granules, which are all parameters of granule turnover, were significantly smaller for glucose than for KCl. These differences at both the level of the cell secretory response and granule behaviour in the submembrane space are incompatible with identical initial response mechanisms to KCl and glucose stimulation.

Key Words

- ▶ beta-cells
- ▶ insulin granule
- ▶ insulin secretion
- ▶ plasma membrane
- ▶ TIRF microscopy

*Journal of Molecular
Endocrinology*
(2017) **59**, 311–324

Introduction

The hypothesis that the biphasic kinetics of insulin secretion is due to different pools of secretory granules appeared to be confirmed by the first TIRFM observations on insulin granule mobility and fusion, which were performed with insulin-secreting MIN6 cells (Ohara-Imaizumi *et al.* 2002a,b). This hypothesis provides that the initial fusion events upon glucose stimulation were due to a pool of granules already docked to the plasma membrane, whereas later fusion events involved

progressively more ‘newcomer’ granules, i.e. granules which are translocated from a reserve pool to the plasma membrane (Rorsman & Renstrom 2003). Subsequent studies with adenovirally transduced primary beta-cells essentially confirmed the first TIRFM observations (Ohara-Imaizumi *et al.* 2004, Nagamatsu 2006). The different behaviour of docked and newcomer granules was explained by different Ca²⁺ sensitivities (Ohara-Imaizumi *et al.* 2009).

However, later reports substantially modified this view. It was found that the newcomer granules do not represent a homogeneous population, but could be subdivided into more stationary and more mobile (restless) newcomers (Shibasaki *et al.* 2007). Newcomer granules need not stay attached to the plasma membrane before fusion but can fuse virtually without time delay (Takahashi *et al.* 2010). In contrast to the initial reports, the fusion of restless newcomer granules was found to dominate in both phases of glucose-induced insulin secretion, which led to the formulation of a revised model of insulin granule pools (Seino *et al.* 2011). This observation has led to the interesting hypothesis that the diminished first phase in type 2 diabetes may result from the loss of fusion competence of the docked granules, which can only incompletely be compensated for by accelerated fusion of newcomer granules (Gaisano 2014). It is also worth noting that granule transport is not unidirectional, since most of the granules which appear in the submembrane space return back into the cell interior after varying residence times (Kasai *et al.* 2008, Hatlapatka *et al.* 2011).

The latter observation raises the possibility that granules which are regarded as newcomers may have actually been present in the submembrane space for prolonged time, moving back and forth, in and out of the evanescent wave zone. However, it is difficult to reconstruct the pre-fusion history of the granules by visual inspection, in particular in well-granulated cells with a lot of granule path crossings. For this reason, we have developed an observer-independent evaluation programme to detect fusion events and to quantify the mobility of the entirety of submembrane granules (Matz *et al.* 2014). Using transiently transfected MIN6 cells, we noted that perfusion with 30 mM glucose or 40 mM KCl increased the granule turnover in the submembrane space, but the majority of the fusing granules did not fuse immediately after arrival but spent a time at the plasma membrane during which the lateral mobility strongly decreased (Schumacher *et al.* 2015).

In this study, TIRF microscopy (TIRFM) was used to study the pre-fusion behaviour of insulin granules in the submembrane space of primary beta-cells under conditions of sequential KCl and glucose stimulation of secretion. Apart from the problem of how to best transform the widely varying mobility of the fluorescent granules into quantitative data, the problem exists that the granule behaviour itself may be affected by the type of label. Early studies showed that the fluorescence reaction upon granule fusion was strongly affected by the type of granule label, even the amino acid sequence of

the linker region of otherwise identical fusion proteins left its mark (Michael *et al.* 2004). Assuming that at least part of the contradictions in TIRFM of insulin granules is due to the use of different labels, we have now used two cargo-directed labels, namely insulin-EGFP and C-peptide-emGFP (Watkins *et al.* 2002, Michael *et al.* 2007, Schumacher *et al.* 2015) in adenovirally transduced primary mouse beta-cells. The rationale was that only changes common to both labels reliably reflect the biological reaction pattern. In view of recent observations on different responses of MIN6 cells and primary mouse beta-cells (Schulze *et al.* 2016), the same sequence of KCl depolarization and glucose stimulation was used as in our earlier studies (Schumacher *et al.* 2015) to enable a comparison.

Materials and methods

Chemicals

Collagenase NB8 for islet isolation was purchased from Serva (Heidelberg, Germany). Cell culture medium RPMI-1640 and ionomycin were obtained from Sigma (Taufkirchen, Germany), cell culture medium DMEM from Invitrogen (Karlsruhe, Germany) and fetal bovine serum (FCS Gold ADD) was from Bio & Sell (Nürnberg-Feucht, Germany). All other reagents of analytical grade were from E. Merck (Darmstadt, Germany).

Vector construction

The cDNA of human pre-proinsulin was cloned into the mcs of the expression vector pEGFP-N1 (Clontech) as recently described (Hatlapatka *et al.* 2011). This resulted in EGFP being connected to C-terminus of the human pre-proinsulin (hIns-EGFP) via the following linker: GDPPVATM. To generate insulin-timer, the cDNA of timer was amplified via PCR, creating restriction sites for AgeI and NotI. Afterwards, timer was cloned into the pEGFP-N1/hIns-EGFP vector to replace EGFP. mC-peptide-emGFP and mC-peptide-mCherry (Watkins *et al.* 2002) were originally obtained in pAdlox vectors, which were directly used for transfection.

For virus production, Bgl II and Not I were used to excise hIns-EGFP and insert it into the pShuttle vector contained in the AdEasy kit (Agilent). After homologous recombination in BJ5183Ad1 cells and linearization (Pac I) viruses were produced in Ad-293 cells. To produce viruses according to the same protocol, the sequence of mC-peptide-emGFP (mIns-c-emGFP) was excised together with the CMV-promotor region with Spe I and Not I,

inserted into the pShuttle vector and expressed using the AdEasy kit.

Cell culture, transient transfection and adenoviral transduction

For transient transfection experiments insulin-secreting MIN6 cells (kindly provided by Jun-Ichi Miyazaki) were used. They were cultured in DMEM medium (4.5 g/L glucose=25 mM, final glutamine concentration 6 mM) with 10% FCS and penicillin/streptomycin at 37°C and 5% CO₂. The cells were transfected in suspension using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol and cultured on glass coverslips. TIRFM was performed between 48 and 72 h after transfection.

Islets were isolated from the pancreas of NMRI mice (12–14 weeks old) by collagenase digestion and hand-picked under a stereomicroscope. Single beta-cells were obtained by incubating the islets in 5 mL Ca²⁺-free Krebs-Ringer medium for 10 min. In the beginning and at the end of this time, they were vortexed for one minute each. The beta-cells were cultured on glass coverslips in RPMI-1640 medium with 10% FCS and 5 mM glucose (10 mM glucose until attachment) at 37°C and 5% CO₂. After one day, the medium was removed and cells were treated with a mixture of 400 µL RPMI (10% FCS, 5 mM glucose) and 100 µL of the adenovirus, carrying the gene of interest (hIns-EGFP or mC-emGFP). Thereafter, 2.5 mL RPMI were added. 24 h later, the cells were washed with PBS (3 × 3 mL) to remove the extracellular virus particles and RPMI was refilled. TIRFM was performed after 2 or 3 days of viral infection (3 or 4 days of cell culture in total).

TIRF microscopy and granule tracking

The coverslip with the attached beta-cells was tightly screwed in a purpose-made perfusion chamber on the stage of an iMIC epifluorescence microscope under control of L.A. software 2.4.0.17 (TILL Photonics, Gräfelfing, Germany). Temperature was maintained at 32.0 ± 0.1°C by overnight pre-warming the system with an environmental control chamber. The cells were perfused with HEPES-buffered Krebs-Ringer medium equilibrated with 95% O₂ and 5% CO₂ at a rate of 200 µL/min. Imaging was only started after 40 min of perfusion to establish steady state conditions. Fluorescence in the evanescent field was excited by a 491 nm continuous-wave diode-pumped solid-state laser (75 mW Cobolt Calypso), run at 50% for the hIns-EGFP label or at 30% for the mC-peptide-emGFP

label. 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 75 × 75 nm in the focal plane. Selection of the excitation and emission wavelengths was made with a quadruple laser line filter set (AHF Analysentechnik, Tübingen, Germany). The duration of exposure was 50 ms per image, the cycle time for acquisition and storage was 125 ms. The fluorescent spots of the labelled granules were localized and the mobility was analysed by an in-house written programme using MATLAB 7.6.0 (The MathWorks, Natick, MA) as described (Matz *et al.* 2014).

During the 50-min perfusions, sequences of 200 images each were acquired at eight representative time points. The granule number at the beginning and the end of each sequence was counted as well as the cumulative number of all the granules that were identified during the sequencing. The granules were subdivided into short-term residents (presence for eight images or less, equivalent to ≤1 s) and long-term residents, i.e. those that were present throughout the entire sequence (≥25 s). The mobility in the z-dimension was described by the number of arrivals at and departures from the submembrane space, whereas the mobility in the x/y-dimension was described by the caging diameter. This parameter describes the maximal distance between the positions of a granule in a running time window of nine images. To characterize time-dependent changes within a population of granules, the caging diameter at half-maximal cumulative abundance (CD₅₀) was defined (Matz *et al.* 2014). The exocytosis of the insulin granules was measured by an automated detection algorithm, which looked for a transient increase in fluorescence intensity and a spreading cloud during a time window preceding a strong and lasting decrease in granule fluorescence (Matz *et al.* 2014).

Colocalization of granule labels

In colocalization experiments the fluorescence emission was separated by a dichroic beamsplitter (Dichrotome, TILL Photonics) and projected side by side on the same camera chip. This offset between green and red granules could arise from a different decay constant of the evanescent field, due to its dependence on the excitation wavelength, or from the different projection of the fluorescence emission on the camera chip. The red label hIns-dimer was used to clarify this issue. The position of the red image and the faint green image showed the same

offset when excited at 561 nm only or when alternating between 491 and 561 nm. Thus, the offset was caused in the emission pathway. Shifting the green image by three pixels to the right and by six pixels upwards gave an exact colocalization, which was maintained for the entire length of the image sequences (Supplementary Fig. 1, see section on supplementary data given at the end of this article). This offset correction was used in all colocalization experiments.

Microfluorimetric measurements of the cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

Single islet cells were loaded at 32°C with Fura-PE3/AM at a concentration of 2 μM for 20 min, and then the coverslip with the attached cells was inserted into the perfusion chamber. The experiments were performed under the same conditions as for the TIRF measurements except for the following: Fura fluorescence was excited at 340 and 380 nm using a Polychrome V coupled to the iMIC epifluorescence microscope, the emission ($>460\text{ nm}$) was collected by a Zeiss Fluor objective (40 $\times$, 1.3 N.A.) and registered by a cooled CCD camera (Sensicam QE, pco, Regensburg, Germany) under control of L.A. software (TILL Photonics). For the calculation of Ca^{2+} concentrations minimal and maximal ratio values ($R_{\min}$ and $R_{\max}$, respectively) values were determined by perfusion of the beta-cells with Krebs-Ringer medium containing 500 μM EGTA and 2.5 μM ionomycin, followed by 10 mM Ca^{2+} and 2.5 μM ionomycin (Supplementary Fig. 2). For the K_d of Ca^{2+} binding, a value of 224 nM was assumed.

Insulin secretion

Batches of 50 cultured NMRI mouse islets were introduced into a purpose-made perfusion chamber and perfused with a HEPES-buffered Krebs-Ringer medium (115 mM NaCl, 4.7 mM KCl, 2.6 mM CaCl_2 , 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 20 mM NaHCO_3 and 10 mM HEPES, 2 mg/mL BSA) saturated with 95% O_2 and 5% CO_2 , which contained the respective secretagogue. Corresponding to the TIRFM measurements, the temperature was set at 32°C and for control experiments at 37°C. The insulin content in the fractionated efflux was determined by ELISA (Mercodia, Uppsala, Sweden).

Statistics

Graphpad Prism4 software (GraphPad) was used for statistic calculations. If not stated otherwise,

'significant' refers to $P < 0.05$, and 't-test' to the unpaired, two-sided t-test.

Results

Insulin secretion

In contrast to MIN6 cells (Schumacher *et al.* 2015), transduced primary beta-cells were not sufficiently stable for continuous perfusion experiments of 90 min duration at 37°C (40-min equilibration plus 50-min experiment). This became clear by the impaired ability of these cells to decrease the elevated cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) levels after stimulation (data not shown). At 32°C, the control of $[\text{Ca}^{2+}]_i$ was unimpaired. To confirm

Figure 1

Insulinotropic effect of successive stimulation with 40 mM KCl and 30 mM glucose at 37°C (A) and 32°C (B). 50 cultured mouse islets were perfused with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose. From 40 to 50 min, the K^+ concentration was raised to 40 mM and from 60 to 70 min, the glucose concentration was raised to 30 mM (open circles). The reversed sequence of exposure is indicated by closed circles. Values are means $\pm$ S.E.M. of three experiments each.

Figure 2

Pattern of $[Ca^{2+}]_i$ increase in adenovirally transduced or non-transduced beta-cells elicited by stimulation with 40 mM KCl or 30 mM glucose. Single mouse beta-cells cultured for 1d (A) or 4d (B) were loaded with Fura 2/AM and perfused at 32°C with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose. From 10 to 20 min, the K^+ concentration was raised to 40 mM and from 30 to 40 min, the glucose concentration was raised to 30 mM (black traces). The reversed sequence of exposure is indicated by dark grey traces, the control (5 mM glucose throughout) by light grey traces. Values are means $\pm$ s.e.m. of three experiments each. Mouse beta-cells that had been transduced by hIns-EGFP (C) or mC-peptide-emGFP (D) had spent a total time of 4 days in culture and were loaded with Fura and perfused as described previously. To check for possible functional impairment by fluorescence excitation in the TIRF mode, single mouse beta-cells labelled with hIns-EGFP were loaded with Fura 2/AM and perfused with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose for a 40-min equilibration period (E and F). Then, two image sequences were acquired in the TIRF mode at 0 and 9 min; thereafter, the Fura ratio was measured in the epifluorescence mode from 10 min on for further 22 min. This way the $[Ca^{2+}]_i$ increase in response to 40 mM KCl (E) or 30 mM glucose (F) was measured under the same conditions as applied to the TIRF registrations of the granule mobility. The cell that was exposed to the TIRF excitation is indicated by the black trace, neighbouring unexposed cells by the grey trace. Values are means $\pm$ s.e.m. of three experiments each.

that TIRF measurements at this temperature generate physiologically meaningful data, the secretory response at 32°C was compared with that at 37°C. The stimulation protocol was the same as that for the $[Ca^{2+}]_i$ measurements and TIRFM of labelled granules.

1d-cultured islets perfused at 37°C with 5 mM glucose showed a fast and strong increase in insulin secretion when the K^+ concentration in the medium was raised to 40 mM for 10 min. During the intermediate wash-out phase of 10 min, basal secretion levels were re-established and the subsequent increase for 10 min of the glucose concentration to 30 mM led to a secretion response that

was 43% (AUC) of the preceding KCl stimulus. When glucose was the first stimulus, its effect made up 56% of the subsequent KCl effect. The insulinotropic effect of KCl was not significantly different when preceding or following the glucose stimulation (Fig. 1A).

At 32°C, essentially the same pattern of secretion resulted. Overall, the response was diminished, more so for glucose than for KCl. This was particularly remarkable for the glucose stimulation after KCl. At 37°C, the AUC was 74% of the glucose effect as the initial stimulus, and at 32°C, it amounted only to 37%. The peak of the secretory response to KCl stimulation was practically unchanged

Figure 3

Colocalization of two insulin granule labels in transiently transfected MIN6 cells. MIN6 cells were transiently transfected with hIns-EGFP and mC-peptide-mCherry (left), hIns-tdimer and mC-peptide-emGFP (middle) and mC-peptide-emGFP together with mC-peptide-mCherry (right). Note the discordant labelling and the prominent red granules in the middle image. For the complete set of spectrally separated TIRF images, colour overlays and intensity overlays, see Supplementary Fig. 3A to C.

Figure 4

Beta-cells adenovirally transduced with hIns-EGFP (A) and mC-peptide-emGFP (B). The images show the same cell in transmitted light, epifluorescence and TIRF mode (from left to right). The upper row shows the cell at the beginning, the lower at the end of the 50-min perfusion comprising the acquisition of eight image sequences in the TIRF mode. In the TIRF mode, a moderate decrease in the submembrane granule number and fluorescence intensity is recognizable, also a moderate increase in the background fluorescence. This did not affect the precision of granule identification and tracking.

at the lower temperature, but the decrease thereafter was more marked (Fig. 1B).

Cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$)

To verify that the signals for granule mobility and fusion were not affected by the adenoviral transduction and/or the subsequent culture period the $[\text{Ca}^{2+}]_i$ of single islet cells was measured at 32°C after 1 or 4 days of cell culture or after 3 days of adenoviral transduction, i.e. after 4 days of culture. The duration of cell culture left its mark in that the increase of $[\text{Ca}^{2+}]_i$ was generally diminished (Fig. 2A vs B). Non-transduced cells showed the same response pattern as cells transduced either with hIns-EGFP or with mC-peptide-emGFP (Fig. 2B vs C or D). The $[\text{Ca}^{2+}]_i$ increase by 40mM KCl showed the typical initial overshoot and was less dependent on the sequence of stimulation than

the $[\text{Ca}^{2+}]_i$ increase by 30mM glucose. The steady state $[\text{Ca}^{2+}]_i$ after 10min of the first stimulation phase was not different between KCl and glucose, whereas the peak after 2–3 min of stimulation was always clearly higher with KCl (Fig. 2A, B, C and D).

Finally, it was tested whether the fluorescence excitation during TIRF measurements affected the $[\text{Ca}^{2+}]_i$ increase by KCl or by glucose. After acquiring two image sequences under the same conditions as for the measurement of granule number and mobility (see below), the fluorescence excitation was switched to conventional epifluorescence and the Fura fluorescence ratio was measured during stimulation with either 40mM KCl or 30mM glucose. The $[\text{Ca}^{2+}]_i$ increase in response to KCl and glucose appeared unchanged in comparison with neighbouring cells unexposed to TIRF fluorescence excitation; however, the resting $[\text{Ca}^{2+}]_i$ levels were moderately increased (Fig. 2E and F).

Insulin granule labelling

To explore the feasibility of co-labelling the granules with insulin and C-peptide, the degree of colocalization was determined by transient cotransfection of MIN6 cells with hIns-EGFP and mC-peptide-mCherry. Only about two-thirds of the granules showed both green and red fluorescence (yellow in the overlay, Fig. 3). In the next step, the contribution of the fluorescent protein to the discordance was checked by replacing the EGFP moiety of hIns-EGFP by tdimer, the tandem dimer derivative of DsRed. hIns-tdimer was transfected together with mC-peptide-emGFP. The degree of co-labelling was even more decreased and the red fluorescent granules were particularly prominent because of their larger size and/or intensity (Fig. 3, see also Supplementary Fig. 3). This suggested that not only the properties of the physiological granule cargo protein, but even more so the properties of the fluorescent protein contributed to the heterogeneity of granule labelling.

Consequently, the localization of two monomeric labels, emGFP and mCherry, was compared when both were fused to mC-peptide. Here a high, but not complete degree of co-labelling of similarly sized granules resulted (Fig. 3). So to minimize the effect of the fluorescent protein and to avoid possible interactions by co-expression, it was decided to perform two separate sets of experiments, each with hIns-EGFP and mC-peptide-emGFP, the latter differs from EGFP by just three amino acids.

Table 1 Comparison of typical parameters of insulin granules in beta-cells labelled by hIns-EGFP or by mC-peptide-emGFP.

Cell and granule parameters	hIns-EGFP	mC-peptide-emGFP	Difference
Cell footprint area (μm^2)	102 $\pm$ 13	168 $\pm$ 21	$P=0.0085$
Granule number per cell	175 $\pm$ 16	201 $\pm$ 26	n.s.
Granule number per μm^2	1.7 $\pm$ 0.19	1.2 $\pm$ 0.13	$P=0.048$
Total granule number per 25s	2314 $\pm$ 207	2681 $\pm$ 269	n.s.
Total granule number per $\mu\text{m}^2 \times 25\text{s}$	22.7 $\pm$ 2.2	16.0 $\pm$ 1.8	$P=0.028$
Granule turnover per 25s	22.7:1.7 = 13.4	16.0:1.2 = 13.3	
Short-term resident granules per 25s (as % of total)	1874 $\pm$ 176 (79.8 $\pm$ 0.8%)	2153 $\pm$ 234 (80.3 $\pm$ 0.7%)	n.s. n.s.
Long-term resident granules (as % of number per cell)	75 $\pm$ 11 (43.1 $\pm$ 2.2%)	61 $\pm$ 14 (31.9 $\pm$ 2.7%)	n.s. ($P=0.0024$)
Arrivals (as % of number per cell)	6.3 $\pm$ 0.3%	7.0 $\pm$ 0.4%	n.s.
Caging diameter	82.5 $\pm$ 1.5 nm	85.5 $\pm$ 1.5 nm	n.s.

Data were taken from the first sequence of each experiment. Significances were calculated with the unpaired, two-tailed *t*-test. Data means $\pm$ s.e.m. of 22 (hIns-EGFP) or 18 (mC-peptide-emGFP) cells.

Insulin granule mobility

Even though the TIRF images of the hIns-EGFP- and mC-peptide-emGFP-transduced beta-cells appeared virtually undistinguishable (Fig. 4), the mean cell area (measured as cell footprint in the TIRF mode) of the mC-peptide-emGFP-expressing beta-cells was significantly larger than that of the hIns-EGFP-expressing beta-cells (Table 1). Since the number of granules was not different on a per-cell basis, the mean granule density (granule number per μm^2) was significantly higher in hIns-EGFP-transduced cells (Table 1). This difference did not result in a higher granule turnover as can be seen from the number of granule arrivals and the percentage of short-term resident granules. Nominally, each granule was exchanged 13 times during 25 s, the duration of one image sequence. This number was the same for either label (Table 1).

The same experimental protocol used to measure secretion and $[\text{Ca}^{2+}]_i$ was used to measure the granule mobility in the submembrane space. During a 50-min perfusion, eight sequences of 200 images each were acquired at characteristic time points. The value of the first sequence was normalized to 100% for each single experiment. With both labels, the perfusion with 5 mM glucose (baseline condition) led to significant changes (Fig. 5). At the beginning of the last sequence (49 min), the granule number per cell footprint was significantly reduced (to 68% with hIns-EGFP and to 39% with mC-peptide-emGFP, $P=0.04$, *t*-test, for the difference). The total number of granules identified per image sequence was also diminished. Furthermore, the number of short-term resident granules and of arriving granules showed a reversible stepwise increase upon exchange of the perfusion medium. These two parameters are intrinsically related to each other and describe the mobility in the

z-dimension. The mobility in the *x/y*-dimension is described by the caging diameter, here only the C-peptide label showed a stepwise increase (Fig. 5).

To explore the reason for this unexpected behaviour, the experiments were repeated with the acquisition of only three image sequences, two at the beginning and one at the end of the perfusion (at 0, 9 and 49 min). The less-frequent image acquisition protocol resulted in less reduced granule number per cell and total granule number per image sequence, but this was not accompanied by changes in the granule mobility parameters (Supplementary Fig. 4). Since the values of the latter parameters were essentially unchanged as compared to the initial values, even though clear stepwise increases had occurred in between (Fig. 5), these changes reversed, and there was no lasting damage. However, to specifically characterize the changes in granule mobility upon insulinotropic stimulation, it was necessary to calculate net values by subtracting the values of the control perfusions from the corresponding values of the test perfusions.

The calculation of net values showed that the number of granules per cell footprint was unchanged after the intermittent stimulation by 40 mM KCl and by 30 mM glucose, each with a duration of 10 min and separated by a 10 min wash-out phase (Fig. 6). The same was true when the sequence of exposure was reversed. While this result was the same for both granule labels, their reaction differed somewhat in that mC-peptide-emGFP gave more marked responses, in particular to the KCl stimulus (Fig. 6). Since the response of both labels showed a similar pattern, means of the net values of both labels were calculated for all of the mobility parameters (Fig. 7).

The parameters reflecting the *z*-direction mobility, namely the total number of granules, the short-term

Figure 5

Number and mobility of submembrane insulin granules during continuous perfusion with 5 mM glucose (control condition). Single mouse beta-cells labelled with hIns-EGFP (filled circles) or mC-peptide-emGFP (open circles) were perfused with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose. After a 40-min equilibration period, the 50-min measurement period started. At eight time points during this period, sequences of 200 images each were acquired. The panels show the granule number in the first image of each sequence (upper left), the total number of granules identified per sequence (upper right), the percentage of short-term-resident granules (middle left) the percentage of long-term-resident granules (middle right), the number of newly arrived granules (lower left) and the CD50 values of the caging diameter (lower right). The dotted lines indicate the switching between medium reservoirs to exactly mimic the test conditions. All values were normalized with respect to the first sequence and are means $\pm$ s.e.m. of six experiments each.

resident granules and the arriving granules gave a consistent pattern. Glucose when applied as the first stimulus led to a decrease of each of them, whereas KCl when applied as the first stimulus did not lead to

changes vs the initial values. This resulted in significant differences between glucose and KCl in the course of the first stimulation and/or the subsequent wash-out because the effect of glucose persisted during this time (Fig. 7). The

Figure 6

The effect of KCl and glucose on the number of submembrane granules before and after correction for the changes during control condition. Single mouse beta-cells labelled with hIns-EGFP (left panels) or mC-peptide-emGFP (right panels) were perfused with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose. After a 40-min equilibration period, the 50-min measurement period started. From 10 to 20 min, the K⁺ concentration was increased to 40 mM, and from 30 to 40 min, the glucose concentration was increased to 30 mM (upper panels). In a separate set of experiments, the sequence of exposure to the stimuli was reversed (lower panels). At eight time points during these experiments, sequences of 200 images each were acquired. All panels show the granule number in the first image of each sequence. The gross number is given by open circles, and the net number after subtraction of the control values (continuous presence of 5 mM glucose, see Fig. 5) is given by filled circles. All values were normalized with respect to the first sequence and are means $\pm$ S.E.M. of six experiments each.

response pattern during the second stimulation was more uniform in that both glucose and KCl induced significant increases in the total granule number, the number of short-term residents and of arriving granules. Likewise both decreased the number of long-term resident granules (Fig. 7). The caging diameter, a parameter describing the x/y -dimension mobility, showed a significant difference between glucose and KCl during the wash-out after the first stimulation.

In these experiments, a total number of 36 events were detected, which fulfilled the criteria of exocytosis. hIns-EGFP-labelled cells contributed 20 exocytoses originating from 9 cells and mC-Peptide-emGFP-labelled cells contributed 16 exocytoses originating from 6 cells. There was no increased occurrence during the phases of stimulation.

Discussion

Macroscopic insulin secretion

The TIRFM of insulin granules has different perspectives. One is to characterize the molecular interactions between the insulin granule and its attachment site at the plasma membrane (Gandasi & Barg 2014), another is to study the release of granule content after fusion pore opening (Ma *et al.* 2004, Michael *et al.* 2006). Finally, granule number and mobility in the submembrane space can be

analysed to clarify how pre-fusion events contribute to the pattern of secretion. It is the latter aim to which this study is directed.

It is widely believed that the secretion elicited by 30 or 40 mM KCl is an appropriate surrogate for the first phase secretion or at least for the role of depolarization in the course of glucose stimulation (Straub & Sharp 2004). So the response to this purely depolarizing stimulus was compared with the response to a maximally effective concentration of glucose (30 mM) during the same perfusion. The perfusions were also performed with reversed sequence of exposure, because the effect of an insulintropic stimulus is known to be influenced by type of preceding stimulation, specifically glucose enhances the response to a second stimulus, whereas a non-nutrient stimulus is inhibitory (Nesher & Cerasi 1987, 2002).

The overall pattern of secretion was quite similar at 37°C and at 32°C, so the TIRF measurements, which had to be conducted at 32°C, can be regarded as physiologically relevant. The following important differences in the insulin secretory response to KCl stimulation vs glucose stimulation were found in the present study. At 37°C, the ability of glucose to stimulate insulin secretion was moderately reduced by a preceding KCl stimulation, and at 32°C, it was strongly reduced. At 37°C, the efficiency of KCl was virtually unchanged when it was preceded by glucose, and at 32°C, it was significantly more effective than the initial KCl depolarization, even though the peak

Figure 7

Submembrane granule number and mobility during stimulation with 40 mM KCl followed by 30 mM glucose (open circles) or during stimulation with 30 mM glucose followed by 40 mM KCl (closed circles). Comparison of the net values. For both sequences of stimulation, mean values were calculated from the net values of both labels as depicted in Fig. 6. Significant differences (P ranging between 0.012 and 0.042, unpaired two-sided t -test) are indicated by asterisks. The total number of granules, the short-term-resident granules and the arriving granules reflect the mobility in the z -dimension, and the caging diameter reflects the mobility in the x/y -dimension. The values are means $\pm$ s.e.m. of ten (closed circles) or nine (open circles) cells, respectively.

secretion rates were the same. As a general rule, it can be concluded that a purely depolarizing stimulus diminishes the insulinotropic effect of a subsequent glucose stimulus. Glucose stimulation enhances the effect of a subsequent depolarization.

Pattern of $[Ca^{2+}]_i$ increase by KCl and glucose

The secretion by KCl depolarization is regarded to be largely, if not entirely the consequence of Ca^{2+} influx via voltage-dependent Ca^{2+} channels and the resulting $[Ca^{2+}]_i$ increase (Straub & Sharp 2004, Seino *et al.* 2011). For the glucose-induced secretion pattern, the $[Ca^{2+}]_i$ increase by Ca^{2+} influx is regarded to exert an indispensable trigger function but not to shape it, since glucose generates additional signals from its metabolic breakdown (Henquin 2000, Panten *et al.* 2013). In the present study, the initial overshooting $[Ca^{2+}]_i$ increase by KCl resembled the secretion pattern, whereas the plateau-like $[Ca^{2+}]_i$ increase by glucose did not resemble the secretion pattern. However, when glucose was preceded by KCl, the diminished insulinotropic efficiency of glucose was reflected by a clearly reduced $[Ca^{2+}]_i$ increase.

The $[Ca^{2+}]_i$ measurements also demonstrated that the response to the insulinotropic stimuli was not altered by adenoviral transduction. Overall, the transduction as such left no specific mark, but in agreement with earlier observations (Gilon *et al.* 1994), the cell culture duration affected the glucose-induced $[Ca^{2+}]_i$ increase, whereas the KCl-induced increase was more robust. The fluorescence excitation of the granule labels in the TIRF mode did not influence the $[Ca^{2+}]_i$ increase during stimulation, but the level of resting $[Ca^{2+}]_i$ was increased. This may have contributed to the stepwise changes of the granule mobility parameters during control perfusions when image sequences were only separated by a short time interval.

Granule mobility and fusion

The changes in the granule number and mobility visible during the control perfusion (5 mM glucose throughout) are best explained by the fluorescence excitation causing photobleaching and reversible functional alterations. This interpretation is supported by control experiments in which less frequent and fewer fluorescence image

acquisitions resulted in smaller decreases in parameter values over the same time interval. Granules labelled with C-peptide-emGFP were more susceptible than those labelled with hIns-EGFP (Videos 1 and 2), but with longer intervals between image sequences a clear recovery occurred with both labels. Overall, their response pattern was qualitatively similar, but the co-labelling experiments using different combinations of fluorescent proteins caution against premature generalizations.

Video 1

Insulin granule mobility in continuously perfused mouse beta-cells. Single mouse beta-cells labelled with hIns-EGFP were perfused with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose at 32°C. This sequence of 200 images at the beginning of the experiment shows the mobility of insulin granules in the submembrane space. View Video 1 at <http://movie-usa.glencoesoftware.com/video/10.1530/JME-17-0063/video-1>.

Video 2

Insulin granule mobility in continuously perfused mouse beta-cells. Single mouse beta-cells labelled with mC-peptide-emGFP were perfused with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose at 32°C. This sequence of 200 images at the beginning of the experiment shows the mobility of insulin granules in the submembrane space. Note the more marked bleaching as compared with hIns-EGFP-labelled granules. View Video 2 at <http://movie-usa.glencoesoftware.com/video/10.1530/JME-17-0063/video-2>.

In view of the nonstationary baseline under control condition, the calculation of net values was necessary to specifically identify the effects of high glucose and KCl depolarization (Fig. 5). It is common practice in the TIRFM of secretory granules to refer to the pre-stimulatory values as control. In light of the above, this may be insufficient, in particular, when the fluorescence is excited for prolonged time periods (Ohara-Imaizumi *et al.* 2002b, Shibasaki *et al.* 2007). The problem is likely made worse when the stimulus is applied as a concentrated bolus to statically incubated cells on the microscope stage (Ohara-Imaizumi *et al.* 2002b, 2004, Shibasaki *et al.* 2007, Gheni *et al.* 2014).

Consideration of the mean net values of both labels (Fig. 7) leads to the following conclusions. In contrast to what one would expect from the pool size hypothesis

(Rorsman & Renstrom 2003), KCl did not lead to a stronger reduction of submembrane granules per cell footprint than glucose. However, differences emerged when considering the mobility in the z-dimension. The mobility in the z-dimension, or in other words the granule turnover in the submembrane space, is determined by the arrival, residence time and departure of the granules and is also reflected in the total (i.e., cumulative) number of granules identified during one image sequence (Matz *et al.* 2014, Schumacher *et al.* 2015). When glucose was the first stimulus, the turnover was decreased, and when KCl was the first stimulus, it was slightly (nonsignificantly) increased, resulting in significant differences between these stimuli. A similar decrease of turnover was seen when the glucose concentration was raised from 5 to 15 mM (Brüning & Rustenbeck, unpublished observation). This may indicate that nutrient stimuli prolong the residence of the granules at the plasma membrane, possibly by amplifying signals (Schulze *et al.* 2017), and thus, increase the probability of docking and ultimately exocytosis.

Prior investigation has demonstrated multiple differences between the effects of 40 mM KCl and 30 mM glucose. The plasma membrane depolarization by 40 mM KCl is stronger and lacks action potentials, the associated Ca²⁺ influx is continuous and the macroscopic insulin secretion reaches higher initial peak values but quickly recedes without recovery (Willenborg *et al.* 2012, Belz *et al.* 2014). Thus, the previously published observation that KCl preferentially induced the fusion of granules pre-existent in the submembrane space, whereas glucose induced the fusion of granules that had newly arrived at the submembrane space (Seino *et al.* 2009) appears quite plausible. However, this earlier observation seemingly contradicts the present data that glucose diminishes the granule turnover as compared with KCl. This discrepancy may result from the different granule populations under study. Here, we have quantified the entirety of the granules in the submembrane space, whereas the earlier study considered only those granules that fused with the plasma membrane.

The difference between KCl and glucose was also visible with the caging diameter, the parameter which describes the mobility in the x/y-dimension (lateral mobility) (Matz *et al.* 2014). Again, KCl stimulation was associated with a higher mobility as compared with glucose. This effect, which appeared only during the wash-out phase may be secondary to the changes in turnover, since prolonged residence of the granules at the plasma membrane is associated with a decrease in lateral mobility

(Schumacher *et al.* 2015). The 'cages,' functionally defined here to account for induced restrictions in the lateral mobility of the granules, likely involve the cortical actin web (Mziaut *et al.* 2016).

Concordant with the observations on the mobility and fusion of granules in chromaffin cells (Duncan *et al.* 2003), it has been recently shown that ageing of insulin granules is associated with changes in mobility (Ivanova *et al.* 2013, Hoboth *et al.* 2015). In contrast to the chromaffin cell granules, however, which showed increased mobility with age, insulin granule aging by a few hours diminished the mobility of insulin granules. How this property relates to the turnover in the submembrane space or to the higher release probability of newly synthesized insulin (Schatz *et al.* 1975, Gold *et al.* 1982, Halban 1982) is unclear.

In our preceding investigations using MIN6 cells under highly similar conditions, both glucose and KCl increased granule turnover. However, only glucose did so in a temperature-sensitive manner, in parallel with the much higher temperature sensitivity of the glucose-induced insulin secretion (Schumacher *et al.* 2015). In these experiments, the calculation of net values was not deemed necessary, because the changes during the control perfusions were much less extensive than during the stimulation phases. In MIN6 cells, the number of exocytoses of hIns-EGFP-labelled granules was about 5 times higher than that in the present experiments; however, similar to the present experiments, the fusion rate was not increased when the macroscopic insulin secretion was increased by glucose or KCl (Schumacher *et al.* 2015). The following factors may underlie this discrepancy:

(1) Method of detection: Our detection algorithm requires the combined occurrence of a fluorescence increase shortly before a lasting decrease together with the appearance of a spreading cloud of fluorescent material (Matz *et al.* 2014) (see Video 3). This may be overly restrictive. Alternatively, the observer-based

evaluation may be overly permissive. (2) The label: With labels based on EGFP and emGFP, the increase of fluorescence is caused by fluorescence dequenching when the intragranular pH value increases after fusion pore opening (Michael *et al.* 2006). However, this process may be protracted, diminishing the peak of fluorescence. Also, the granule fusion pore may have a filtering effect on the release of granule cargo (Takahashi *et al.* 2002, Collins *et al.* 2016), thereby preventing the generation of a fluorescent cloud. (3) The dissociation of the islet: The coordinated interaction of the beta-cells within the islet markedly enhances the insulinotropic efficacy of stimuli as compared with dissociated beta-cells (Salomon & Meda 1986, Bavamian *et al.* 2007, Chowdhury *et al.* 2013); thus, the rate of insulin granule exocytoses may reflect the macroscopic secretion pattern when the beta-cell under study is within an islet or a cell cluster (Low *et al.* 2013, Almaca *et al.* 2015), but not when it is dissociated and cultured for several days. The role of the islet microenvironment for the process of exocytosis itself is emphasized by the observation that within islets, beta-cell exocytosis is directed towards the vasculature (Low *et al.* 2014).

Therefore, to investigate the relation between rates of exocytosis and submembrane granule number and turnover under conditions pertaining to the kinetics of macroscopic insulin secretion, it seems essential to use entire islets or cell clusters in an appropriate environment and to combine cargo-directed with extracellular labels.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-17-0063>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by grants from the Deutsche Forschungsgemeinschaft (DFG Ru 368/5-2) and the Deutsche Diabetes Gesellschaft.

Acknowledgements

Skillful technical assistance by Claudia Bütetisch and Carolin Rattunde is gratefully acknowledged.

Video 3

Granule mobility and exocytoses in continuously perfused mouse beta-cells. Single mouse beta-cells labelled with hIns-EGFP were perfused with HEPES-buffered Krebs-Ringer medium containing 5 mM glucose at 32°C. This sequence of 200 images shows exocytotic events, characterized by the sudden increase in fluorescence of a single insulin granule, accompanied by the spreading cloud of fluorescence. View Video 3 at <http://movie-usa.glencoesoftware.com/video/10.1530/JME-17-0063/video-3>.

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Received in final form 18 July 2017

Accepted 1 August 2017

Accepted Preprint published online 1 August 2017