ARTICLE

Glucose principally regulates insulin secretion in mouse islets by controlling the numbers of granule fusion events per cell

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Abstract

Aims/hypothesis In dispersed single beta cells the response of each cell to glucose is heterogeneous. In contrast, within an islet, cell-to-cell communication leads to glucose inducing a more homogeneous response. For example, increases in NAD(P)H and calcium are relatively uniform across the cells of the islet. These data suggest that secretion of insulin from single beta cells within an islet should also be relatively homogeneous. The aim of this study was to test this hypothesis by determining the glucose dependence of single-cell insulin responses within an islet.

Methods Two-photon microscopy was used to detect the glucose-induced fusion of single insulin granules within beta cells in intact mouse islets.

Results First, we validated our assay and showed that the measures of insulin secretion from whole islets could be explained by the time course and numbers of granule fusion events observed. Subsequent analysis of the patterns of granule fusion showed that cell recruitment is a significant factor, accounting for a fourfold increase from 3 to 20 mmol/l glucose. However, the major factor is the regulation of the numbers of granule fusion events within each cell, which increase ninefold over the range of 3 to 20 mmol/l glucose. Further analysis showed that two types of granule fusion event occur: 'full fusion' and 'kiss and run'. We show that the relative frequency of each type of fusion is independent of glucose concentration and is therefore not a factor in the control of insulin secretion.

Conclusions/interpretation Within an islet, glucose exerts its main effect through increasing the numbers of insulin granule fusion events within a cell.

Keywords Insulin · Islet · Secretion

Abbreviations
HPTS   Hydroxypyrene-3,6,8-trisulfonate
HRP   Horseradish peroxidase
SRB   Sulforhodamine B

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Introduction

Isolated single beta cells have a heterogeneous response to glucose. This has been recorded for glucose-stimulated protein biosynthesis as reported by amino acid incorporation [1, 2], glucose metabolism [3], calcium responses [4] and for insulin secretion [5]. As the glucose concentration is increased, the proportion of cells recruited to respond increases. For example, across the dose range 1 to 20 mmol/l, glucose induces a 14-fold increase in the number of cells incorporating leucine [1], and an eightfold increase in the number of cells secreting insulin [5]. These fold changes approximately account for the observed increase in insulin secretion in islets [6] and suggest that differential sensitivities to glucose of individual cells underlie the glucose dose dependence in islets [7]. In situ support for this is shown by a heterogeneous glucose

response of cells within islets in terms of leucine uptake [1] and heterogeneous activation of the insulin promoter [8].

However, direct comparison of insulin secretion shows there are differences in islets compared with single cells. Single cells secrete more basal insulin and respond to glucose with lower levels of secretion compared with islets [9, 10]. Single cells also show greater capacitance changes in response to stimulation than expected from the secretory response of cells within an islet [11]. The basis of these differences is likely to be cell-to-cell communication within the islet [10], with a major factor being gap junctional links between beta cells [12]. Such cell-to-cell coupling leads to coordination between beta cells with more homogeneous cellular responses as measured by NAD(P)H activity [13] and cell calcium signals [14, 15]. The prediction of this work is that, if we could measure the single-cell secretory responses within an islet, they should also be coordinated. However, to date in situ measurement of insulin secretion within islets has not been possible.

In the stimulus-secretion coupling pathway, calcium is the last step that triggers insulin granule fusion [16]. Measurement of the intracellular calcium response within islets, and in beta cell clusters, shows complexity in space, time and amplitude [4, 14, 17]. This makes it difficult to extrapolate from the observed calcium signals and estimate the secretory response. Therefore, while the calcium responses seem coordinated and relatively uniform within individual cells across an islet, this does not necessarily mean that insulin secretion is also uniform.

As well as driving the fusion of more granules, glucose may also regulate the dynamics of insulin granule fusion. Such complexities in the dynamics of the fusion pore that forms between the insulin granule and the cell membrane have been observed and have led to ideas that control of the fusion pore regulates the output of insulin and other granule contents, such as ATP [18, 19]. In chromaffin cells these differences in granule fusion dynamics play a direct physiological role in the regulation of adrenaline (epinephrine) secretion [20]. The possible relevance of granule fusion dynamics is, however, controversial in beta cells. Supporting evidence comes from studies on single cells [18, 19]. In contrast, recordings from intact islets suggest that complex granule fusion dynamics are rarely observed [21, 22]. If the fusion pore was under the control of glucose, this could exert an additional effect on the regulation of insulin secretion.

To gain an understanding of the control glucose exerts on insulin secretion in an islet we need methods to study single-cell secretion from beta cells within an intact islet. The only method currently available is an indirect fluorescence assay that uses two-photon microscopy to track the entry of extracellular fluorescent dye into each granule as it fuses with the cell membrane [22, 23]. This method has been successfully used to measure the responses to high levels of stimulation

(forskolin and high glucose [22]). However, it has not been employed to measure the response to more physiologically relevant stimuli and has not been completely validated to determine if it is a method that can be used to accurately determine the single-granule basis of glucose-dependent insulin secretion.

In this study we have employed two-photon microscopy to measure single insulin granule fusion within single beta cells in intact islets. We have shown that the time course and extent of single granule fusion events is consistent with measures of insulin secretion from whole islets. We then studied the patterns of granule fusion across a range of glucose concentrations and determined that although cell recruitment is a factor, the major influence of glucose is to increase the numbers of granule fusion events within each single beta cell. In a development of the method we measure 'kiss-and-run' fusion events and show that this type of fusion is present but independent of glucose concentration.

Methods

Experimental solutions Experiments were performed in Na-rich solution (NaCl 140 mmol/l, KCl 5 mmol/l, $MgCl_2$ 1 mmol/l, $CaCl_2$ 2.5 mmol/l, $NaHCO_3$ 5 mmol/l, HEPES 5 mmol/l, 3–20 mmol/l glucose as indicated), adjusted to pH 7.4 with NaOH.

Islet preparation CD1 mice were obtained from a University of Queensland breeding colony. The mice were humanely killed according to local University of Queensland animal ethics procedures (approved by the University of Queensland, Anatomical Biosciences Ethics Committee). Isolated mouse pancreatic tissue was prepared by a collagenase (type IV) (Worthington, Lakewood, NJ, USA) digestion method in Hanks buffer (Sigma-Aldrich, Castle Hill, NSW, Australia), adjusted to pH 7.4 with NaOH. Isolated islets were maintained (37°C, 95/5% air/CO_2) in RPMI-1640 culture medium (Sigma-Aldrich) supplemented with 10% FBS (Gibco, Life Technologies, Mulgrave, VIC, Australia), and 100 U/ml penicillin/0.1 mg/ml streptomycin (Invitrogen, Life Technologies, Mulgrave, VIC, Australia).

Islet imaging Isolated islets of Langerhans were cultured for 2–3 days and then, prior to imaging, were bathed in an Na-rich solution containing 3 mmol/l glucose for 30 min (37°C, 95/5% air/CO_2). Two-photon imaging was performed at 34°C, with exocytic events recorded as the entry of extracellular dye into each fused granule (Fig. 1).

Two-photon imaging We used a custom-made, video-rate, two-photon microscope with a ×60 oil immersion objective (NA 1.42, Olympus; Olympus Australia, Mt Waverley, VIC,
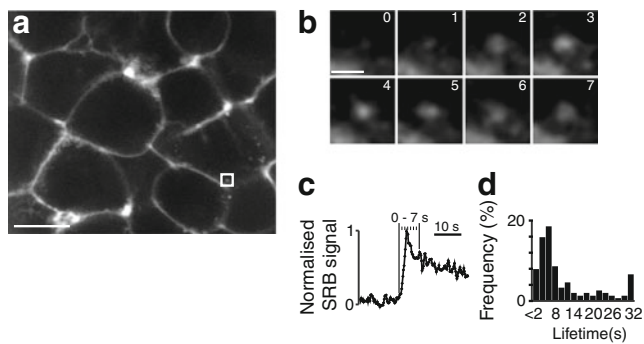
Fig. 1 Two-photon detection of single granule fusion events. (**a**) A two-photon cross-section through the core (three cells deep into the islet) of a single islet. Extracellular SRB dye outlines each cell and when stimulated with glucose (8 mmol/l) granule fusion leads to the appearance of bright spots of fluorescence (see fluorescent spot in the region bounded by a box). Scale bar, 10 μm. (**b**) These fusion events arise suddenly and remain for many seconds, as shown in the time-series images (enlarged from the square region highlighted in the low-magnification image) and taken at 1 s intervals as indicated on the graph in (**c**). Scale bar, 1 μm. (**c**) The plot of time against the SRB fluorescence in a region of interest placed over the granule. (**d**) A histogram plotting frequency against event lifetime shows a distribution of lifetimes. See also ESM Figs 1 and 2

Australia), providing an axial resolution (full width, half maximum) of ~1 μm. We imaged exocytic events using sulforhodamine B (SRB; 800 μmol/l) as a membrane-impermeant fluorescent extracellular marker excited by femtosecond laser pulses at 950 nm, with fluorescence emission detected at 550–650 nm. Hydroxypyrene-3,6,8-trisulfonate (HPTS) was imaged using excitation at 950 nm and emission detected at 420–520 nm.

Images (resolution of 10 pixels/μm) were analysed with the Metamorph program (Molecular Devices, Sunnyvale, CA, USA). Exocytic event kinetics were measured from regions of interest (0.78 μm^2, 78 pixels) centred over individual granules. Traces were rejected if extensive movement was observed.

Electron microscopy Islets were washed in warmed PBS, then washed in 2.5% (wt/vol.) glutaraldehyde and fixed using a pressurised microwave system. Fixed islets were processed into epoxy resin. Pelleted islets were sliced at 60 nm (ultra-microtome) and viewed using a JOEL1010 electron microscope (Joel Australasia, Frenchs Forest, NSW, Australia).

Insulin secretion Four islets were counted, placed in a chamber in extracellular solution containing 3 mmol/l glucose at 37°C, with O$_2$/CO$_2$ (95/5%), for 30 min. They were then stimulated by the addition of glucose to the extracellular solution containing 0.2% BSA. The supernatant fraction was sampled and insulin measured using a homogeneous time-resolved fluorescence assay (Cisbio, catalogue number 62INSPEB; Genesearch, Arundel, QLD, Australia) and an anti-insulin antibody plate reader (Tecan, Infinite F200 Pro;

Tecan, Port Melbourne, VIC, Australia). Insulin concentrations were calculated using a standard curve.

Statistical analyses All numerical data are presented as mean ± SEM. Statistical analysis was performed using Microsoft Excel and GraphPad Prism. Data sets with just two groups were subjected to a two-tailed, unpaired Student's *t* test. Islets from at least three animals were used in each experiment.

Results

Single granule fusion events in islets show glucose dependence Isolated mouse pancreatic islets were bathed in Na-rich solution containing the fluorescent dye SRB and imaged with two-photon microscopy. Figure 1a shows the dye outlining each cell. We recorded three cell layers into the islet: a depth in a rodent islet at which most cells are beta cells [24]. With our imaging parameters, a two-photon section records an area that, on average, includes 24 cells and a section depth of 1 μm. We recorded at 34°C and tracked SRB entry into each granule as it fused with the cell membrane (Fig. 1b). Control experiments show the recorded events had the same diameter as expected for insulin granules and co-localisation of extracellular dye within fused granules with insulin (Electronic supplementary material [ESM] Figs 1 and 2).

Histogram plots of the apparent granule lifetimes measured from the middle of the rising phase of the SRB signal until the time when the fluorescence signal was indistinguishable from the baseline fluorescence (i.e. the point of granule collapse) revealed modal granule lifetimes of 4–6 s (Fig. 1d, *n* = 121 exocytic events). This suggests that our temporal sampling (3–5 frames per second) resolves the real time course of granule fusion events. Previous work measured the opening phase of the fusion pore at 1.8 s [22], which is a measure of the time taken for dye to enter the granule lumen. Our slightly longer durations reflect measurement of the total granule lifetime, which includes the decay phase. We note here that these measures of fluorescent dye changes do not reflect the much faster kinetics of fusion-pore opening.

The key distinguishing characteristic of beta cell secretion is its glucose dependence. To measure this we switched from using 3 mmol/l glucose to solutions containing more glucose. Each islet was exposed to only one concentration of glucose and two-photon microscopy then recorded each granule fusion event over 20 min. Figure 2 shows typical examples of responses obtained at glucose concentrations between 3 and 20 mmol/l (3 mmol/l glucose, eight islets; 6 mmol/l glucose, 12 islets; 8 mmol/l glucose, eight islets; 15 mmol/l, ten islets; and 20 mmol/l glucose, 12 islets). The figure shows the cells outlined by the extracellular dye with the regions where each
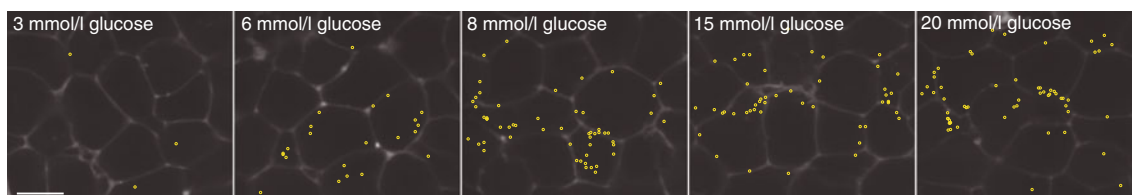
Fig. 2 Glucose dose dependently increases the number of granule fusion events. Examples of image areas taken from separate islets challenged with 3, 6, 8, 15 and 20 mmol/l glucose. In each case the image shows the islets at the beginning of the 20 min recording period. Image analysis was used to identify each fusion event and the position of each one is indicated by the yellow circles on the images. Scale bar, 10 μm

exocytic event occurred identified by yellow circles. As expected, increasing numbers of fusion events were observed at higher glucose concentrations. Fusion events at the edge of the cells were due to fusion with the cell membrane lying perpendicular to the two-photon cross-section. Fusion events that occurred apparently in the middle of the cells were due to fusion with cell membrane lying above, or below, the plane of focus.

Simple geometry (assuming spherical cells, see ESM text) shows that the area of membrane sampled was the same regardless of where in the cell the cross-section was made. In this way cells 'cut' in smaller cross-section have the same membrane area as those 'cut' in larger cross-section. We therefore sampled from similar areas of cell membrane wherever the two-photon optical slice passed through the cells. There is an exception to this, because it assumes the two-photon depth passes fully through a cell. A two-photon section at the very top or bottom of a cell might have an excitation depth that only partially penetrates the cell and the sampled membrane area would be consequently smaller. We estimate these partial sections accounted for only around 10% of all the cell cross-sections and therefore were a minor component in our measurements. In support of these calculations direct measurement shows no correlation between the apparent perimeter of a cell in the two-photon slice and the number of exocytic events observed within that cell (ESM Fig. 3).

A histogram of the total number of fusion events (Fig. 3a) shows a dose-dependent 15-fold increase from low to high glucose. The average time course of the cumulative appearance of exocytic events (Fig. 3a) shows that increasing glucose concentration decreases the latency to the first event and increases the number of exocytic events over time (Fig. 3a). Together, these data are consistent with the expected glucose-driven fusion of insulin granules in beta cells.

We note here that, across all the glucose concentrations, our observations of exocytosis are consistent with the fusion of a population of granules of the same size. We did not see larger fusion events that might indicate compound exocytosis. This is consistent with previous observations that compound exocytosis is rare [22] and only significantly occurs in the presence of other stimuli such as glucagon-like peptide 1 (GLP-1) [25] or carbachol [26].

Increasing glucose concentration drives increases in the number of responding cells and granule fusion events within a cell We used the spatial and temporal resolution of our recordings to determine the nature of the glucose-driven increase in granule fusion events at the cellular level. First, we analysed the numbers of responding cells. From our images many cells showed insulin granule fusion events and, as the
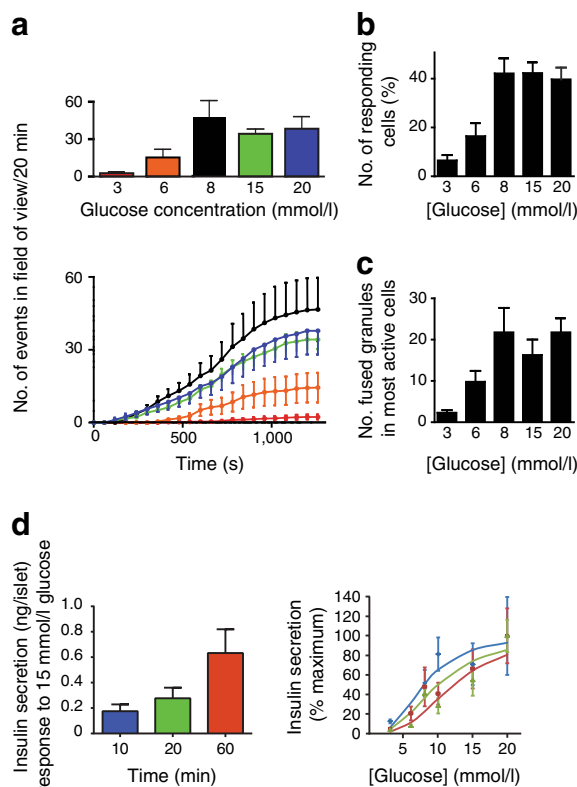

Fig. 3 The glucose-dependent increase in secretion is predominantly due to recruitment within focal regions of exocytosis. (**a**) Data from all experiments (in Fig. 2): the bar chart counts the total average number of fusion events and the line graph plots the cumulative average time course of the appearance of the fusion events (error bars are SEM). (**b**) The mean ± SEM of the apparent number of responding cells within the two-photon section. (**c**) The total number of exocytic events, recorded over the 20 min period after glucose application, within the four best responding cells at each glucose concentration. (**d**) Measures of glucose-stimulated insulin secretion show a time-dependent increase in insulin secreted (bar chart) and a rightward shift in the dose-response curve at increasing times in glucose (line graph). Blue, 10 min; green, 20 min; and red, 60 min

glucose concentration increased, the number of recruited cells increased (Fig. 3b). As our two-photon slices sample less than 10% of the total area of the cell membrane we suggest that the apparently non-responding cells are likely to be responding in regions outside of our sampled area. A quantitative assessment of granule numbers therefore requires a method to identify all granule fusion events within one cell, something that is not technically possible at the moment. Nevertheless, our aim here was to understand the targets for glucose-induced recruitment of granules. It is clear that the observed increase in the number of cells is a factor in the glucose-driven response, with an approximately fourfold change across the glucose concentrations tested.

Second, focusing on the responding cells it is clear that glucose exerts a strong effect to increase the number of granule fusion events within each cell (Fig. 3c). We showed that the number of fused granules is glucose dependent, with a ninefold change (between 3 and 8 mmol/l glucose; 6, 8, 15, 20 mmol/l glucose all significantly different from 3 mmol/l glucose, Student's t test $p < 0.01$).

How do our measures of numbers of granule fusion events compare with the amount of insulin secreted? We used an homogeneous time-resolved fluorescence (HTRF) assay to measure insulin secretion from four islets, in response to a range of glucose concentrations at 10, 20 and 60 min (Fig. 3d, $n = 4$–6 animals). The bar chart shows the maximal amount of insulin secreted in response to 15 mmol/l glucose at each of the times. The line graph normalised the responses (to maximal) and fitted a sigmoidal function. The estimated EC_{50} values for glucose were 7.95, 9.97 and 12.24 mmol/l at the times 10, 20 and 60 min, respectively. This left shift in the EC_{50} at shorter time-frames of stimulation has been observed before [17] and is likely to reflect larger differences in the glucose dependence of the second phase of the response.

We can now directly compare measured insulin secretion with our observations of the number of granule fusion events as a means to validate our two-photon assay. Our two-photon slice samples a volume of 2,000 μm^3 (~40×50×1 μm) and the approximate volume of an average islet is 1.6×10^6 μm^3 [27]. Our sample volume therefore represents ~1/800 of the total volume of an average islet. At 15 mmol/l glucose the mean number of granule fusion events in our two-photon sample volume is 33.1 within the 20 min recording (±4.26, mean ± SEM, $n = 10$ islets). Scaled up this gives a total number of fusion events of 26,400 per islet. The amount of insulin contained in a single granule is 1.6×10^{-18} mol per granule [28] (9.25×10^{-15} g/granule). Thus, based on our measures of granule fusion and the estimated amount of insulin in a granule we calculate that the total amount of insulin secretion is 0.244×10^{-9} g/islet. This is in close agreement with our measures of insulin secretion, which were 0.175×10^{-9} g/islet with 15 mmol/l glucose at 10 min and

0.277×10^{-9} g/islet at 20 min; this validates our two-photon assay as an accurate measure of secretory output.

Single granule fusion events show complex spatial and temporal patterns within beta cells across an islet Having shown that the main drivers for glucose are increased recruitment of cells and increased exocytic responses within cells, then how is this manifest across an islet? To address this we conducted a detailed analysis of the timing and spatial position of each granule fusion event across cells within an islet ($n = 4$–6 animals and 8–10 islets for each glucose concentration). At 3 mmol/l glucose we observed occasional fusion events. These occurred throughout the recording period and were found in cells across our two-photon section (Fig. 4). Measurement of the average perimeter of the cells responding to 3 mmol/l, show they were the same size as those cells that responded to 20 mmol/l glucose. This suggests that across the glucose dose range we are likely to be recording insulin granule fusion within beta cells (ESM Fig. 4).

Glucose at 6 mmol/l induced many more fusion events. These were clustered in time, giving rise to oscillations in activity. Importantly, these oscillations recruited multiple cells, all of which showed exocytic events within narrow time periods (Fig. 4). Finally, 15 mmol/l glucose induced many more events. These findings provide evidence for coordinated oscillatory activity across multiple cells within the islet.

Single granule fusion kinetics are not glucose dependent The analysis presented so far has counted the number of fusing granules and has not taken into account the possible dynamics of the granule fusion pore. To directly assess this we developed a method to quantify fusion pore closure. As this is the essential first step to granule recovery in the kiss-and-run model, our method is a direct test for these granule behaviours. Our method employs a technique to monitor granule acidification. As has been shown previously, recaptured granules rapidly re-acidify after fusion pore closure [29]. In our experiments we included two extracellular dyes, SRB and HPTS, the latter a pH-sensitive extracellular dye [30]. These dyes move into the fused granules and any subsequent acidification, tracked as a selective decrease in the HPTS fluorescence, was used as an index of granule recapture (Fig. 5a). In most fusion events HPTS fluorescence closely followed the time course of SRB fluorescence. In fewer than 10% of all granule fusion events HPTS fluorescence decreased significantly away from the SRB fluorescence (Fig. 5a), indicating acidification and granule recapture. Across a range of glucose concentrations there was a slight but not significant (Student's t test) trend to a greater proportion of granule recapture at higher glucose concentrations (Fig. 5b; 6 mmol/l, 11 islets, 166 events; 8 mmol/l, eight islets, 383 events; 15 mmol/l, ten islets, 341 events; 20 mmol/l, 11 islets, 420 events).
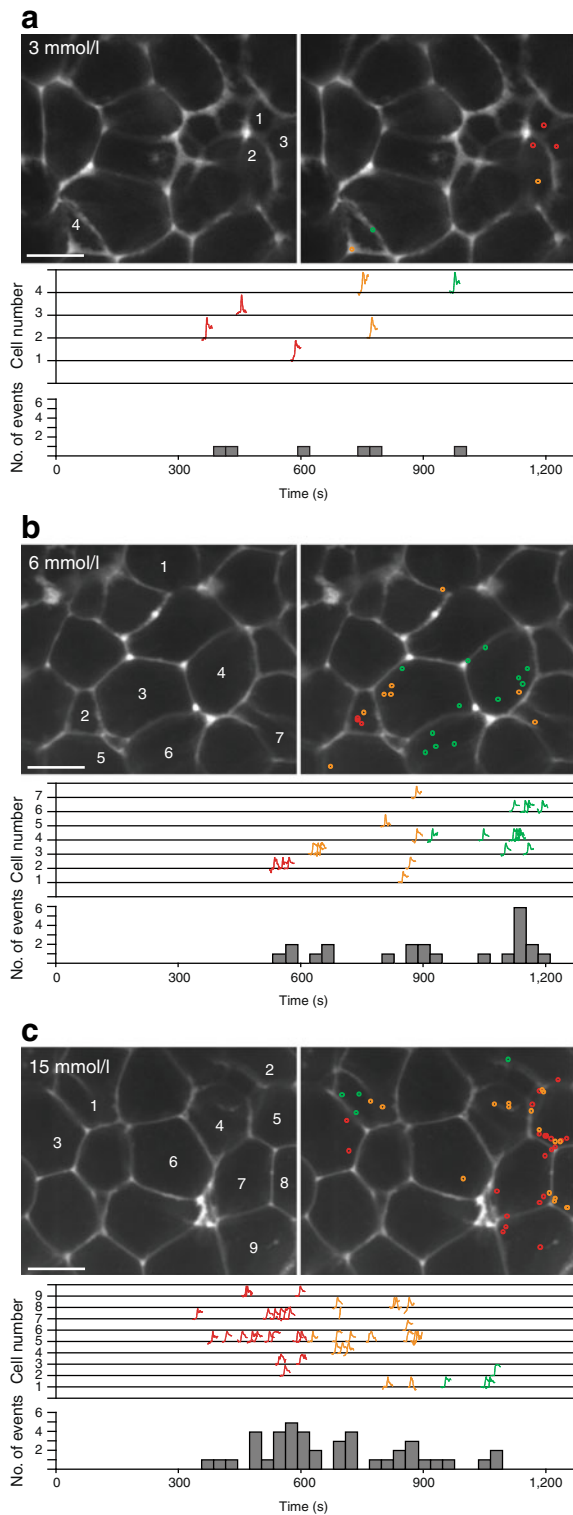
Fig. 4 Spatial and temporal dependence of fusion events on glucose. Typical examples of the fusion events induced by (**a**) 3 mmol/l, (**b**) 6 mmol/l, and (**c**) 15 mmol/l glucose. In each case glucose was added at time 0. In each panel the upper two images are the same fields of view. The left image numerically identifies each responding cell. The right image identifies each fusion event, observed over a 20 min time period, with colour-coded circles: red, 5–10 min; orange, 10–15 min; and green, 15–20 min. The graph for each glucose concentration shows a brief time sample (just before the event and a few seconds after) of the average fluorescent changes in 0.8 μm regions of interest placed over each fusion event, colour coded as before. The histogram for each glucose concentration shows the number of fusion events occurring in 30 s time bins. These observed spatial and temporal glucose-dependent changes in exocytosis further confirm that we recorded the responses of beta cells. Scale bars, 10 μm

endocytic structures and in electron micrographs was present in various small endocytic compartments and in whole granules which were positively identified as insulin granules by the simultaneous presence of insulin crystals (Fig. 5c, $n = 3$ islets).

Discussion

Our two-photon imaging gave direct insights into the glucose dependence of single-cell secretory responses within intact islets. Past work looking at factors upstream in the stimulus-secretion cascade within intact islets has provided contrasting views. There is evidence for heterogeneous cell responses in terms of upregulation of insulin expression [8] and protein synthesis [1]. In contrast, the NAD(P)H [13] and calcium responses [14] to glucose appear much more homogeneous. We now show that increasing glucose concentration does recruit cells across the islet, providing evidence for heterogeneity. However, the dominant effect is an increase in the secretory response within cells and for the coordination of responses across cells. Furthermore, our data argue against the post-fusion behaviour of granules as a factor in glucose control. Our data add further evidence for the importance of cell-to-cell coordination within an islet and highlight recruitment of full granule fusion within single cells as the major factor under the control of glucose.

Identification of beta cells Many methods have been used in the study of exocytosis in beta cells. The main advantage of the method we use is the ability to record many exocytic events from many cells within intact islets. Our recordings are consistent with recording exocytosis of insulin-containing granules. Beta cells do contain synaptic-like microvesicles [31], but these are much smaller (<100 nm diameter) than insulin granules and even if fusion was detectable they would be distinct from the ~400 nm diameter granules we record (ESM Fig. 1). We might occasionally record from alpha cells. However, this is unlikely given their location on the periphery of rodent islets, inhibition of their responses with glucose and our data showing we record from cells of a consistent cell size (ESM Fig. 4). Indeed our estimates of beta cell area are almost

Whole-granule recapture within intact islets has been controversial and to further our analysis we performed electron microscopy on fixed islets. To assay for recapture we bathed living islets in horseradish peroxidase (HRP), stimulated with glucose and then washed with fresh extracellular buffer without HRP before fixing. HRP would therefore only be present in
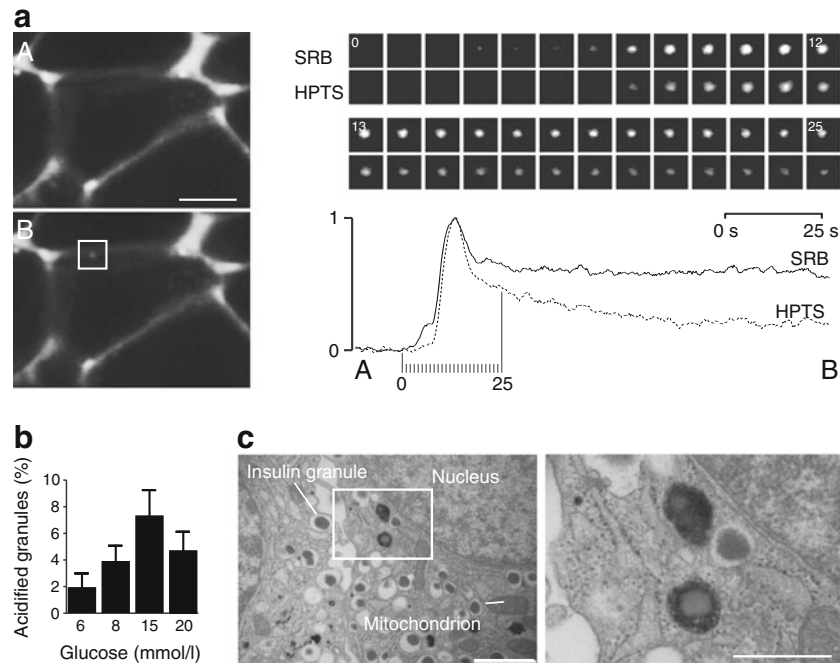
Fig. 5 Granule recapture measured in live cell and electron microscopy experiments. (**a**) SRB and HPTS dyes enter simultaneously into fusing granules. In most cases the dyes behaved with an identical kinetics. However, in a minority of granules the HPTS fluorescence was seen to decrease compared with SRB, which is indicative of granule acidification. An example of a low-magnification image (A, before the event, and B, at the end of the event) is shown. The white square outlines a single event and the image sequences for both SRB and HPTS are shown (frames separated by 1 s) and plotted as graphs of fluorescence intensity over time. Scale bar, 10 μm. (**b**) To identify acidified granules we placed a criterion of a separation of the SRB and HPTS plateau levels of more than three times the standard deviation of the signal noise. (**c**) Example electron micrographs (low power and high power from boxed region) show evidence for HRP uptake in granules that also contain an insulin crystal. Left scale bar, 1 μm, right scale bar, 500 nm

identical to previous measures [32] and, as alpha cells are smaller, these are unlikely to contaminate our recordings.

The temporal and spatial occurrence of exocytosis reported here is also consistent with expected beta cell responses (Figs 2 and 4). The very close agreement between the amount of insulin secreted, based on calculations of the number of fusion events, and the actual amount of insulin secreted is a validation that our assay accurately records the fusion of insulin granules.

Single granule kinetics The evidence from single cells is that fusion of insulin granules is not necessarily all or none and that, once opened, the fusion pore is capable of closure [18, 33]. The implication of these behaviours is that they might differentially control insulin secretion, with only partial release occurring before the fusion pore closes. The prevalence of these alternative behaviours in native islets is controversial, with two reports suggesting pore closure occurs only rarely, if at all [21, 22].

Our data provide strong new evidence to support the notion that the fusion pore can close in intact islets. We show that, after fusion, granules can re-acidify, and second we show HRP uptake in granules that contain insulin crystals. Quantification of granule acidification shows granule recapture is rare and occurs in fewer than 10% of all fusion events. Furthermore, this proportion does not change significantly across a range of glucose concentrations. If anything, there is a small increase in recaptured granules at higher glucose concentrations (Fig. 5). This trend is contrary to expectations as high glucose leads to an increase in insulin secretion and transient fusion would decrease insulin secretion. It is important to emphasise that our assay only records acidification in those granules where the pore has closed for a sufficiently long time for the proton pump to change granule acidity [34]. We therefore cannot measure fusion pore flickering or fusion pore diameters.

Our findings argue against a role for glucose in controlling granule dynamics. However, different forms of granule fusion may be a component in the differential release of low-molecular-weight substances [35] and may, therefore, be part of mechanisms to couple cell-to-cell behaviour. Alternatively, different fusion behaviour may be associated with pathological responses [36].

Does our work have any implications for diabetes? One interesting outcome from our work is that it is glucose concentrations across a range from 3 to 8 mmol/l (Fig. 3) that appear to exert the major stimulatory effect on insulin granule exocytosis. This range is very close to the range of fasting to maximal glucose levels in both mouse (5–10 mmol/l) [37] and humans after a meal (5–8 mmol/l) [38] and indicates that normally the beta cells are operating within their optimal

range of glucose concentrations. In contrast, in untreated type 2 diabetes, fasting blood glucose has been measured as 9 mmol/l, rising to >11 mmol/l after a meal [39]—a range of glucose concentrations that our data indicate would have a minimal effect in stimulating further insulin secretion. This shift away from the optimal stimulatory range of glucose is therefore likely to be a further factor in the development of the disease.

Finally, our work directly quantifies glucose-induced cell recruitment vs an increased cellular response and shows the latter is more significant within the context of the islet. It is likely that a similar situation exists in human islets. However, given the differences in human vs rodent islet architecture [24], innervation [40], cell-to-cell communication [41] and GLUT2 expression [42], we would anticipate that the details of the response would be different. It will be interesting to apply our techniques to the measure of insulin secretion in human islets.

Contribution statement All authors made substantial contributions to the conception and design of the experiments. JTW, JMM, OHD, JB, AR and MZ performed research. GM and RGP developed the methods. HYG and PT designed the research. All authors were involved in drafting the manuscript and all approved the final version.

Duality of interest The authors confirm there is no duality of interest associated with this manuscript.

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