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Review

Visualising insulin secretion. The Minkowski Lecture 2004

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Abstract

Insulin secretion from pancreatic islet beta cells is a tightly regulated process, under the close control of blood glucose concentrations, neural inputs and circulating hormones. Defects in glucose-triggered insulin secretion, possibly exacerbated by a decrease in beta cell mass, are ultimately responsible for the development of type 2 diabetes. A full understanding of the mechanisms by which glucose and other nutrients trigger insulin secretion will probably be essential to allow for the development of new therapies of type 2 diabetes and for the derivation of "artificial" beta cells from embryonic stem cells as a treatment for type 1 diabetes. I focus here on recent developments in our understanding of beta cell glucose sensing, achieved in part through the development of recombinant targeted probes (luciferase, green fluorescent protein) that allow islet beta cell metabolism and Ca^{2+} handling to be imaged in situ in the intact islet with single cell resolution. Combined with classical biochemistry, these techniques show that the beta cell is uniquely poised, thanks to the expression of low levels of lactate dehydrogenase and plasma membrane lactate/monocarboxylate transporters, to channel glucose carbons towards oxidative metabolism, ATP synthesis and inhibition of AMP-activated protein kinase, a newly defined regulator of insulin release. I also discuss the molecular basis of the recruitment of secretory vesicles to the cell surface, analysed by the use of new imaging techniques including total internal reflection of fluorescence, as well as the "nanomechanics" of the exocytotic event itself.

Keywords ATP · Beta cell · Ca^{2+} · Exocytosis · GFP · Imaging · Islet · Luciferase · Metabolism · Secretion

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Abbreviations: AMPK, AMP-activated protein kinase · EGFP, enhanced green fluorescent protein · GK, glucokinase · K_{ATP}, ATP-sensitive K^+ channel · LDH, lactate dehydrogenase · MCT-1, monocarboxylate transporter-1 · mRFP, monomeric red fluorescent protein · myoVa, myosin Va · NPY, neuropeptide Y · PC, pyruvate carboxylase · SREBP1c, sterol regulatory element binding protein-1c · TCA, tricarboxylate/citrate cycle · TIRF, total internal reflection of fluorescence microscopy · tPA, tissue plasminogen activator

Introduction

Tight regulation of insulin release is essential for normal blood glucose homeostasis. Were daily insulin release to exceed ~2% of the total pancreatic content (more than 2500 units) then fatal hypoglycaemia would ensue. Conversely, whilst decreases in beta cell mass are apparent in late stage type 2 diabetes [1], defective insulin release from existing beta cells seems likely to be the underlying cause of this disease in most cases. Thus, hyperglycaemia is not usually evident before the loss of ~70% of beta cell mass in type 1 diabetes [2], while 40 to 50% of the beta cell mass remains intact after baboons are rendered diabetic with streptozotocin [3].

The study of glucose-regulated insulin secretion began in earnest in the 1960s with the establishment

of a radioimmunoassay for insulin [4]. More recently, studies on individual beta cells were made possible with the development of electrophysiological approaches including amperometry [5] and measurements of membrane capacitance [6, 7]. Though the latter techniques provide remarkable temporal resolution, they are, however, limited with respect to the spatial aspects of insulin release. Overcoming this limitation, molecular approaches now allow the expression in living beta cells and islets of a range of recombinant fluorescent and bioluminescent probes, often derived from lower organisms [8]. Combined with advances in imaging technologies, these tools enable the secretory event to be imaged in real time, allowing the intracellular signalling mechanisms that control it to be dissected.

Glucose sensing: adenosine-triphosphate-sensitive K^+ channels

Glucose causes efficient increases in total intracellular ATP content (or an increase in ATP : ADP ratio) [9], which are reflected by measurable and sometimes oscillatory increases in concentrations of free ATP [10, 11]. These changes can be imaged in living beta cells or islets using recombinant targeted luciferase, an ATP-dependent photoprotein derived from the North American firefly, *Photinus pyralis*, and photon-counting imaging (Fig. 1). The demonstration of ATP-inhibitable K^+ currents in isolated beta cells [12] and their closure in intact cells by glucose [13] provided early evidence that such changes in free [ATP] may be critical for the response to glucose. A decade or so later, the channel-forming subunit (an inwardly rectifying K^+ channel, termed $K_{ir}6.2$) of an ATP-sensitive K^+ (K_{ATP}) channel was cloned from a beta cell cDNA library by Seino and colleagues [14], whilst an associated transmembrane protein of the "ABC cassette family", SUR1, which is capable of binding the sulphonylurea class of oral hypoglycaemic agents, was cloned by AguilarBryan et al. [15].

Closure of K_{ATP} channels [16], and a progressive depolarisation of the plasma membrane (from a resting potential of about −70 mV to 0 mV) [17], cause the beta cell to fire action potentials as glucose concentrations increase. These, in turn, open voltage-sensitive, L-type Ca^{2+} channels [18], and prompt the influx of Ca^{2+} ions. The resulting increase in intracellular free Ca^{2+} concentration [19, 20] is then the major stimulus that triggers secretory vesicle fusion with the plasma membrane [21, 22], whilst Ca^{2+} uptake by mitochondria also enhances mitochondrial ATP synthesis [23] to sustain glucose signalling [10, 11].

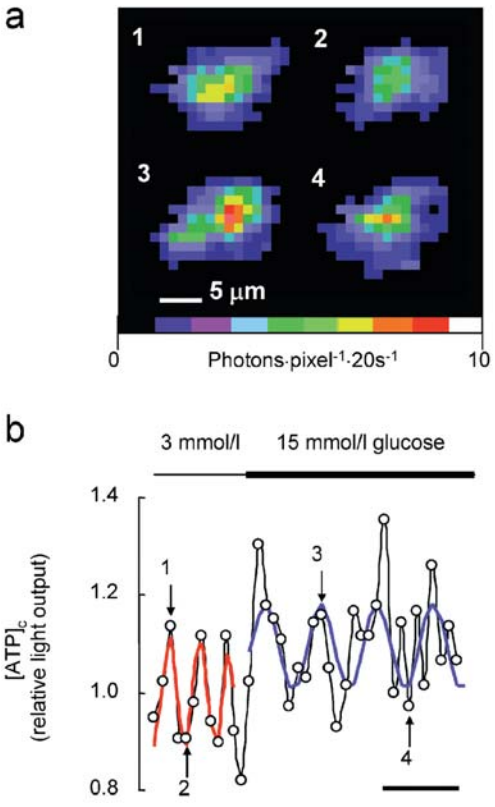

Fig. 1. Imaging ATP oscillations in a single living human beta cell. Human islets were infected, prior to photon-counting imaging of cellular bioluminescence, with an adenovirus expressing cytosolically targeted firefly luciferase, a reporter in free ATP concentration. Other details, see [38]. Images (**a**) were collected at the time points shown in (**b**). Horizontal bar: 2 min

Glucose sensing: metabolic specialisation of the beta cell

It is generally accepted that the intracellular metabolism of glucose and ATP synthesis, rather than the binding of the sugar to a specific "receptor", explains the triggering of insulin secretion (the "fuel hypothesis") [24, 25, 26]. The mammalian beta cell enjoys an unusual metabolic configuration, which tunes its glucose sensing to the insulin requirements of the whole animal. Firstly, glucose uptake is catalysed by the high K_m (i.e. low-affinity) liver-type glucose transporter, Glut2 [27]. Secondly, the first committed step in glycolysis, glucose phosphorylation, is catalysed in the beta cell by the low-affinity type IV hexokinase, better known as glucokinase (GK) [28]. Together, these "sensors" ensure that glucose phosphorylation increases sigmoidally as blood glucose concentrations rise over the physiological range (3.5–8 mmol/l). Correspondingly, inactivating mutations of GK in humans cause MODY2 [29], whilst beta-cell-specific inactivation of the *GK* gene causes decreased sensitivity to glucose [30] and impaired insulin release in vivo [31].

Whilst the proximal aspects of glucose metabolism, catalysed by Glut2 and Gk, are closely similar in the

beta cell and liver (but different from the majority of cells), there are marked differences in the distal end of the glycolytic pathway in these two cell types. Firstly, beta cells express vanishingly low levels of lactate dehydrogenase (LDH) activity and of the plasma membrane monocarboxylate (lactate) transporter-1 (MCT-1) [32, 33, 34, 35]. These specialisations ensure that: (i) close to 100% of glucose-derived pyruvate enters the tricarboxylate/citrate (TCA) cycle and is either broken down to H_2O and CO_2 yielding ATP [32] (75%), or assimilated into newly synthesised proteins [36]: this feature is important for the normal stimulation of insulin secretion by glucose since overexpression of LDH leads to a right shift in the dose response to the sugar [37, 38], and islet levels of LDH are increased in a model of type 2 diabetes, namely 85 to 95% pancreatectomy [39]; (ii) circulating lactate and pyruvate do not stimulate insulin secretion during exercise [35] (Fig. 1a). Correspondingly, glucose-induced increases in [ATP] are significantly smaller in pancreatic alpha than beta cells [40] (unpublished observations, M. Ravier and G.A. Rutter), which possess relatively high levels of LDH. In contrast, levels of mitochondrial glycerolphosphate dehydrogenase are elevated in beta cells [32, 36, 41], providing a mechanism for the re-oxidation of glycolytically derived NADH to NAD^+.

Pyruvate enters mitochondrial metabolism in beta cells by one of two routes, oxidative decarboxylation by pyruvate dehydrogenase [42], or carboxylation to oxaloacetate catalysed by pyruvate carboxylase (PC) [43]. In contrast to the situation in the liver, where PC activity is required for gluconeogenesis, the absence of phosphoenolpyruvate carboxykinase and glucose 6-phosphatase means that this pathway is inactive in beta cells (Fig. 2). Instead, PC permits input of carbon atoms into the citrate cycle ("anaplerosis"), thus compensating for the subsequent "cataplerosis" (i.e. net loss of carbon atoms from the cycle), for example for amino acid biosynthesis [36]. Moreover, the generation by mitochondria of citrate and the production in the cytosol of NADPH by the action of malic enzyme may also be important for glucose signalling [44, 45], although presently the downstream mechanisms are unclear. On the other hand, by increasing cytosolic malonyl-CoA levels, glucose appears to inhibit the β-oxidation of fatty acids, and the consequent accumulation of acyl-CoA in the cytosol may enhance insulin release [46]. Finally, mitochondria provide a source of cytosolic glutamate [47], which is proposed to further enhance secretion [48, 49] perhaps by rendering the vesicle lumen more alkaline [50]. An action of glutamate on intravesicular glutamate receptors, thus generating local increases in the Ca^{2+}-mobilising second messenger inositol 1,4,5-trisphosphate, has also been proposed very recently (F. Nicolleti, G.A. Rutter et al., unpublished results).

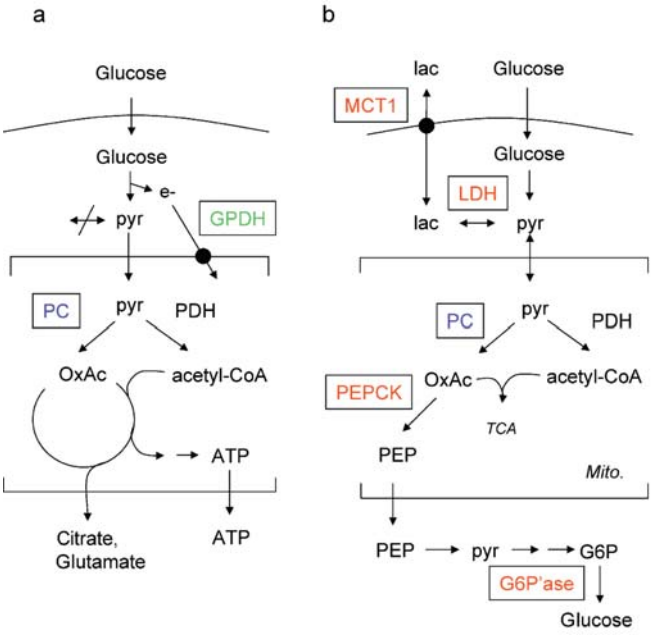

Fig. 2. Metabolic specialisations of (**a**) beta versus (**b**) liver cells. In both cell types pyruvate (pyr) derived from glucose can be oxidatively decarboxylated by pyruvate dehydrogenase (PDH) or carboxylated by pyruvate carboxylase (PC), furnishing oxaloacetate (OxAc) for replenishment of carbon atoms (anaplerosis) in the citrate/tricarboxylate (TCA) cycle, and efficient generation of reducing equivalents for ATP synthesis by the respiratory chain. In the near-complete absence of lactate dehydrogenase (LDH) (**b**), electrons generated at the glyceraldehyde dehydrogenase step of glycolysis are fed into the mitochondrial respiratory chain when NADH is oxidised to NAD^+ by mitochondrial glycerol phosphate dehydrogenase (GPDH), the levels of which are 60-fold higher than in the hepatocyte [32, 41]. In the liver cells, high levels of the plasma membrane monocarboxylate transporter (MCT-1) and LDH permit conversion of exogenous lactate to pyruvate for subsequent gluconeogenesis, excluded in the beta cell by the absence of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphate dehydrogenase (G6P′ase)

Alternative beta cell glucose sensors: AMP-activated protein kinase

Recent findings from this [51, 52, 53, 54] and other laboratories [55, 56, 57] have suggested that AMP-activated protein kinase (AMPK) may be a key regulator of insulin secretion.

Composed of α, β, and γ subunits, AMPK is a heterotrimeric enzyme complex [58], which is strongly stimulated by 5′AMP, both allosterically and through the effects of an upstream protein kinase [59]. AMPK was first identified as an activity which, through the phosphorylation and inactivation of key biosynthetic enzymes [60], was responsible for rapidly shutting down fatty acid synthesis in the face of fuel depletion [58]. Subsequent cloning of the catalytic subunit of the enzyme [61] revealed that mammalian AMPK was a close homologue of the sucrose non-fermenting factor-1 complex in yeast and plants [62, 63]. Multiple

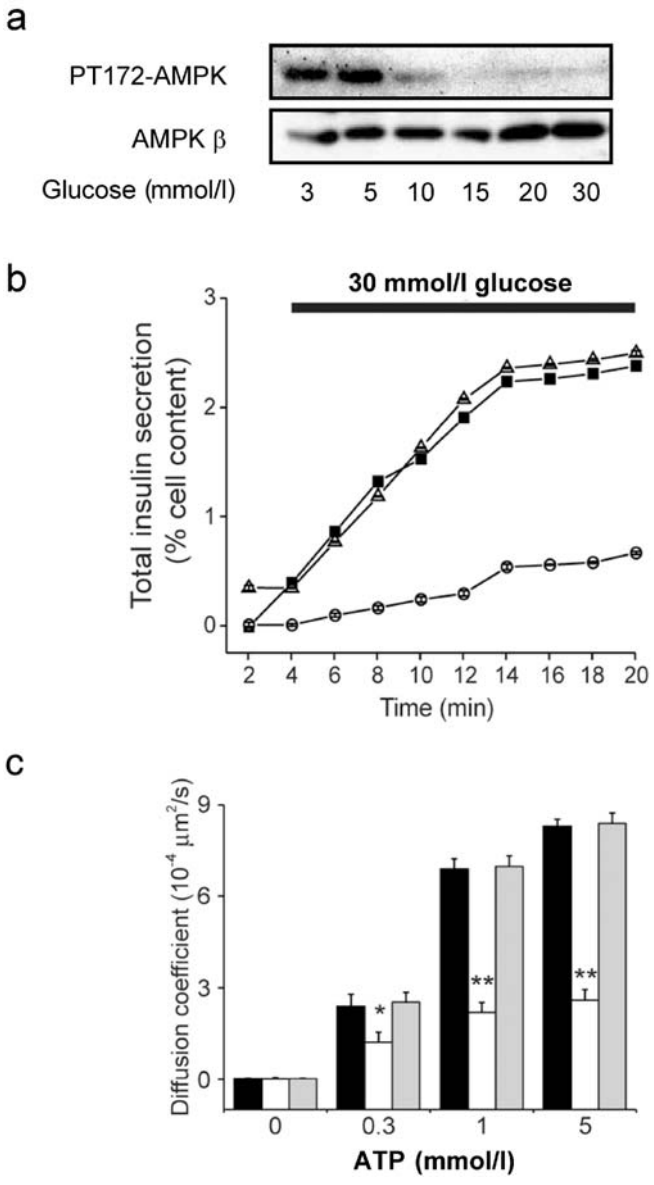

Fig. 3. Role of AMP-activated protein kinase in the control of insulin secretion. **a.** Changes in the phosphorylation state of AMPK α-subunits at threonine-172 in response to alterations in glucose concentration. MIN6 cells were incubated for 1 h in modified Krebs–Ringer medium prior to cell lysis, SDS-polyacrylamide gel electrophoresis and western (immuno)blotting with anti-(phosphor-172) AMPK antibodies (Prof. D.G. Hardie, Department of Biochemistry, University of Dundee, Scotland). **b.** Impact of expressing dominant–negative (open triangles) or constitutively active (circles) forms of AMPK on insulin secretion from MIN6 cells. **c.** Impact of forced changes in AMPK activity on insulin secretory vesicle movement in permeabilised cells. Black bars: control (null) adenovirus; open bars: AMPK constitutively active; grey bars: AMPK dominant–negative. $^{**}p<0.01$ for the effect of AMPK constitutively active. Modified from [52] (**a**) and [53] (**b, c**)

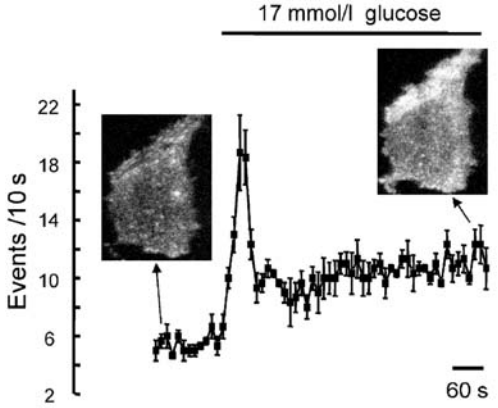

Fig. 4. Phases of insulin release imaged in single beta cells. MIN6 cells expressing the vesicle-anchored fluorescent probe phluorin [121] were imaged using TIRF microscopy initially at 3 mmol/l glucose and then at 17 mmol/l glucose as shown. Note the two distinct phases of the response to glucose

isoforms of each AMPK subunit exist [64]. Islets and beta cells express α1- and α2-containing AMPK complexes [51], both of which are acutely inhibited by elevated glucose concentrations in beta cell lines including HIT-T15 [55], MIN6 [51] and INS-1 [65], as well as in primary rodent and human islets [54] (Fig. 3a). Overexpression of a constitutively active form of AMPK leads to a near-complete suppression of glucose-stimulated insulin secretion (Fig. 3b) [52, 53] as also observed after the treatment of islets with the antidiabetic agent (and inhibitor of respiratory chain complex 1) metformin [54, 56]. Importantly, whilst high doses (>200 μmol/l) of the drug were required for rapid effects, physiological concentrations (20 μmol/l) were effective after longer incubation times (16 h). To a large part at least, the effects of activated AMPK can be attributed to a suppression of glucose oxidation [52, 56, 57] and consequently Ca^{2+} influx. The mechanisms through which AMPK inhibits glucose metabolism have yet to be fully elucidated, but the activation of fatty acid oxidation and also a Randle effect [66] may be involved (though see [67] for a contrary view).

Glucose-stimulated insulin secretion is biphasic: visualisation in living beta cells

A cardinal feature of glucose-stimulated insulin secretion both in vitro and in vivo is its biphasic nature [68], which is evident both in isolated islets, and in single beta cells examined with vesicle-targeted fluorescent probes (Fig. 4). Until very recently, the mechanisms involved in biphasic insulin release were incompletely understood. Three theories have been proposed: (i) time-dependent changes in the concentration of signalling molecules [69]; (ii) physical recruitment of vesicles from a reserve to a readily releasable pool [70]; and (iii) recruitment of vesicles to a readily releasable pool by other mechanisms (e.g. covalent modification of a vesicle protein) [71]. Arguing against (i), increases in free [ATP] or intracellular

Ca^{2+} concentration are usually monophasic or oscillatory [72]. As regards (ii) and (iii), electron microscopy showed that the morphologically docked pool comprised several hundred vesicles, a quantity significantly greater than the maximum number of vesicles in the readily releasable pool (~15) [71], although only a tiny fraction of the total beta cell vesicle complement (~13,000). Nevertheless, recent data in neurones [73] suggest that readily releasable vesicles are not localised close to the presynaptic membrane, but are instead dispersed through the vesicle cluster. If this were the case in beta cells, then translocation would still be required even for vesicles in the readily releasable pool.

By labelling the secretory vesicle membrane with a chimeric construct encoding the membrane-resident protein phogrin (phosphatase on the granule of insulinoma, also called IA-2β [74]) fused to enhanced green fluorescent protein (EGFP), we aimed to image and quantify vesicle movement [75] in single living beta cells. Phogrin, a single transmembrane-spanning vesicle protein, is well suited to the task of localising EGFP to secretory granules, since it possesses multiple and partially redundant targeting sequences [76]. Fusion of EGFP to the C-terminus of phogrin also allows the photoprotein to face the cell cytosol, rather than the lumen, thus avoiding fluorescence quenching at low intraluminal pH. In contrast, the fusion of insulin with EGFP [70] is more problematic [77], unless steps are taken to reduce expression levels (T. Tsuboi, G.A. Rutter, unpublished results). The use of a fluorescent marker combined with confocal microscopy [75, 78] provides advantages over the use of earlier microscopic techniques such as differential interference contrast [79, 80], since the latter may be complicated by interference from other organelles (e.g. mitochondria, lysosomes). In addition, "evanescent wave" microscopy (also called total internal reflection of fluorescence or TIRF microscopy) [53, 81, 82], in combination with fluorescent probes, allows selective analysis of fusion events at the cell surface (see below). Using the phogrin.EGFP chimera, it was possible to show that at low (sub-stimulatory) glucose concentrations vesicles displayed only short oscillatory ("jiggling") movements about a fixed point [75]. However, when the glucose concentration was raised, much longer (several μm) vesicle excursions occurred, frequently towards the plasma membrane. Using TIRF microscopy, others [70] have recently suggested that first-phase secretion involves predocked vesicles, whilst sustained release involves "newcomers" to the membrane.

Mechanisms involved in the regulation of vesicle recruitment to the plasma membrane

Our early studies [75] showed that vesicle movement was sensitive to microtubule disruption with nocodazole, but barely affected by the disruption of actin filaments with colchicine. Moreover, simultaneous imaging of vesicles and microtubules suggested that the vast majority of long excursions occurred along microtubules [83]. Correspondingly, inhibition of vesicle recruitment to the cell surface through the expression of an inactivating (dominant-negative) mutant of kinesin lacking the motor domain [78] did not affect the initial phase of glucose-stimulated secretion in MIN6 cells (measured after 20 min), but completely blocked further release of the hormone (measured at 90 min). These findings demonstrate that whereas vesicle recruitment may not be the sole mechanism involved in the second phase of insulin secretion, it is an essential prerequisite for sustained release of the hormone. Interestingly, kinesin inhibition, achieved with either the dominant–negative mutant [78] or by RNA interference [83], led to an essentially complete cessation of vesicle movement, suggesting that anterograde movements of vesicles predominate in the beta cell.

Increases in ATP concentration activate kinesin-dependent vesicle movement in permeabilised cells [78] and may thus directly regulate the ATPase activity of kinesin by binding to the enzyme's active site. The loss of an inhibitory phosphorylation event, catalysed by AMPK [53], is another potential mechanism. Supporting the latter hypothesis, activation of endogenous AMPK or overexpression of the activated enzyme markedly decrease vesicle movements, not only in intact cells (where the effects are largely attributable to the suppression of glucose metabolism, see above) and in permeabilised cells where ATP concentration could be altered at will. In future it will be important to determine whether either of kinesin's subunits can be directly phosphorylated by AMPK (Fig. 3c). Another intriguing but untested possibility is whether inhibition of AMPK is involved in the second, sustained phase of glucose-stimulated insulin secretion, or in the "amplification" of insulin secretion by glucose when intracellular [Ca^{2+}] is clamped [72].

Recent data suggest that, whilst microfilaments probably play no role in mediating long-range movement of vesicles (see above), they are probably important as vesicles approach the cell surface. In beta, as in other neuroendocrine cells [84], the majority of cellular actin is located immediately beneath the plasma membrane under basal conditions, forming a "cortical actin network" that must be breached for vesicles to reach the plasmalemma [53, 85]. Correspondingly, stabilisation of the network with jasplakinolide causes a near complete inhibition of exocytosis, whilst its disruption with latrunculin B increases vesicle mobility and the number of release events [53]. Expression

of a dominant–negative form of the actin-dependent motor myosin Va (myoVa), which is believed to be involved in the transport of vesicles in other systems [86], or its inactivation by RNA silencing (A. Varadi, G.A. Rutter, unpublished results), decreases the number of vesicles beneath the membrane and causes a profound inhibition of stimulated exocytosis. Similarly, antibodies against myosin light chain kinase inhibit insulin secretion in permeabilised cells [87]. Moreover, a small proportion of the cellular vesicle complement can be shown to be localised just beneath the plasma membrane (within ~100 nm) at low glucose concentrations and in apparent association with actin filaments (A. Varadi, G.A. Rutter, unpublished results). These interactions may play a dual role: (i) to deliver newly arrived vesicles to "holding" sites prior to release; (ii) to tether them prior to the arrival of a stimulatory signal (usually Ca^{2+}). Interestingly, reversible interactions between melanosomes and myoVa have been demonstrated [88] and shown to be regulated by Ca^{2+}-dependent phosphorylation mediated by calmodulin kinase II [45]. In beta cells, the Ca^{2+} trigger may lead to the dissociation of myoVa from its tethering site and the collapse of the actin network, thus liberating vesicles for fusion (Fig. 5). In addition to influx across the plasma membrane, Ca^{2+} released from the vesicle itself [89], as a result of the generation [90] of nicotinic acid adenine dinucleotide phosphate [91], may establish a local "microdomain" of high Ca^{2+} concentration [92] to activate both processes (Fig. 5a).

Might changes in vesicle motility or recruitment play a role in the pathology of type 2 diabetes? We [93, 94] and others [95, 96] have recently shown that elevations of beta cell triglyceride content caused by overexpression of the lipogenic transcription factor sterol regulatory element binding protein (SREBP1c) leads to a substantial accumulation of intracellular lipid and the near-complete elimination of both phases of glucose-stimulated insulin secretion from beta cells and islets, consistent with reports of inhibitory effects on insulin secretion of lipid infusion in vivo [97] and the culture of islets with fatty acids in vitro [98]. The effects of SREBP1c overexpression are associated with decreased glucose-induced increases in cytosolic [ATP], vesicle motility at both low and high glucose concentrations, and a reduction in the number of glucose-stimulated release events [99] (Fig. 5b). Whereas the effects of SREBP1c on glucose-stimulated vesicle excursions are probably due to the lowering of ATP concentrations in these conditions [94], the blockade of vesicle motility at low glucose concentrations, where there is no difference in ATP between control and SREBP-infected cells, may be due to the physical effects of numerous lipid droplets in the cell cytosol, as well as to changes in the expression of elements of the fusion machinery including Rim1 [100]. Interestingly, depolarisation-stimulated release is unaffected

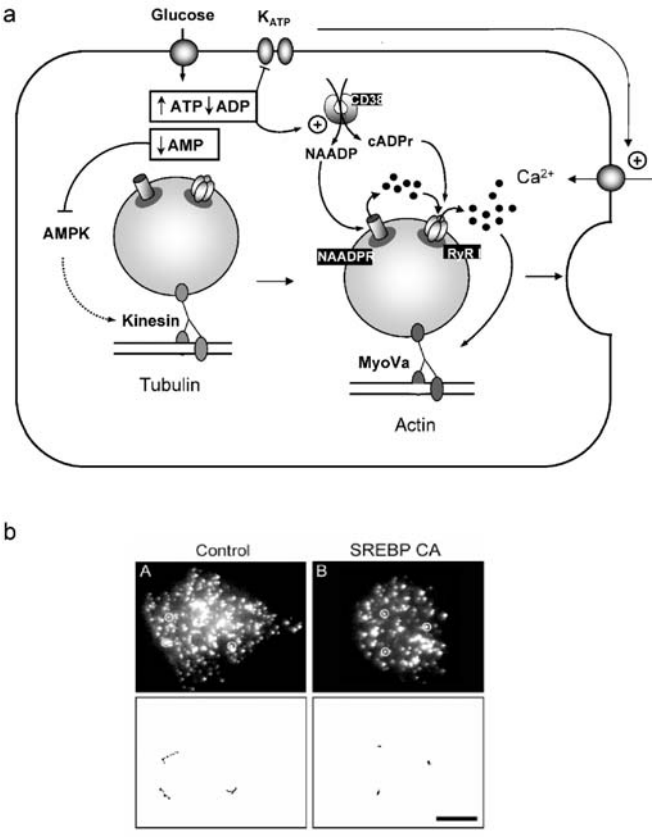

Fig. 5. Kiss and run or "cavicapture" exocytosis of vesicles. **a.** Vesicles close to the plasma membrane and initially tethered to the cortical actin network may be released during glucose stimulation as a result of Ca^{2+} influx and potentially of the mobilisation of Ca^{2+} held within the vesicle (cADPr, cyclicADPribose; CD38, cADPr synthase; RyR1, ryanodine receptor-1). Dephosphorylation of kinesin resulting from a fall in intracellular AMP concentration and a decrease in AMPK activity may lead to activated movement of vesicles more remote from the plasma membrane and their translocation towards site of exocytosis along microtubules. **b.** Vesicle movement, measured at 30 mmol/l glucose using confocal analysis of expressed phogrin.EGFP, is impeded in clonal beta cells overexpressing SREBP1c [99]. Tracks show the trajectories of three vesicles analysed at random. Scale bar: 5 μm

by SREBP1c overexpression, despite a decrease in the number of "morphologically docked" vesicles (i.e. those within ~100 nm and so detectable within an evanescent field). This finding is consistent with the view that these short, Ca^{2+}-dependent final movements of the vesicle towards the plasma membrane are independent of microtubules/kinesin, but rely instead on myoVa/actin interactions.

Mechanisms of vesicle release at the cell surface: full fusion or "kiss and run"?

What is the fate of the secretory granule membrane once it finally arrives at the plasma membrane? It is generally accepted that the molecular machinery of

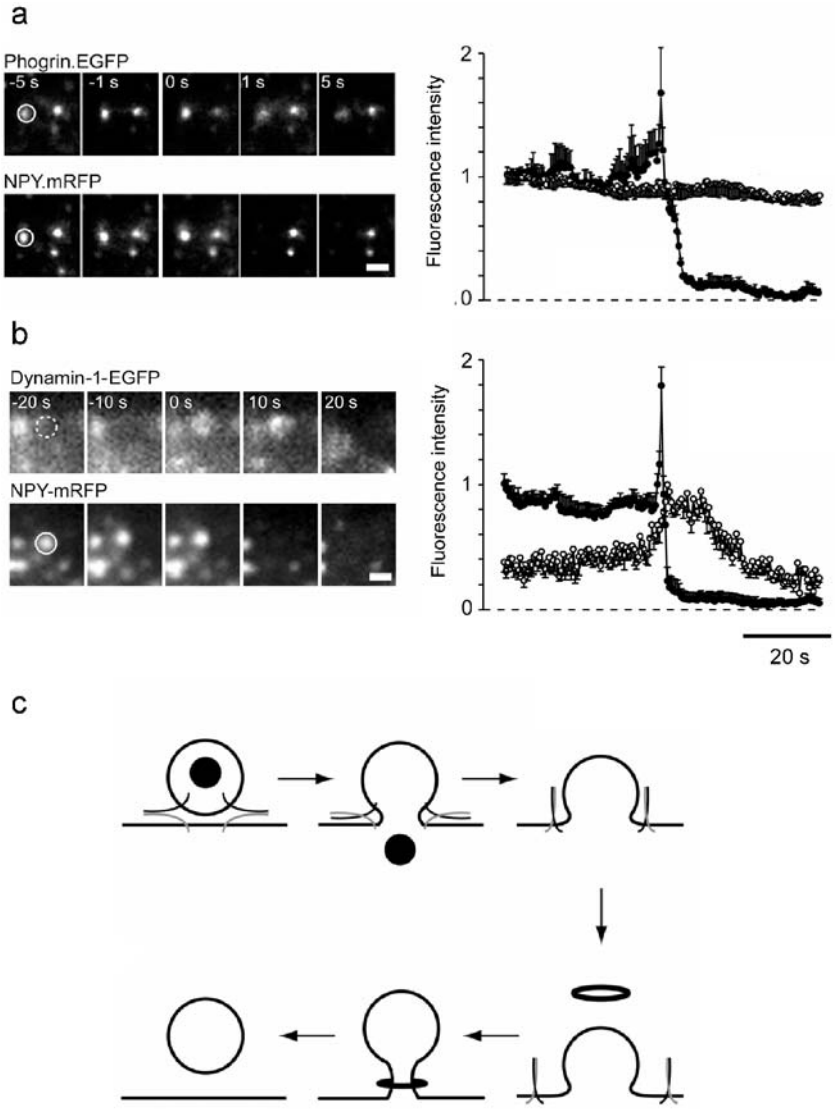

Fig. 6. Control of vesicle fusion. Five-sequential dual colour TIRF images showing the behaviour of the same individual vesicles bearing (**a**) phogrin.EGFP (time course: open circles) and NPY.mRFP (closed circles) or (**b**) dynamin-1 (open circles) plus NPY.mRFP (closed circles) after applying 50 mmol/l KCl to the cell. The vesicle position before exocytosis is outlined by a circle. The scale bar represents 1 μm. The graphs (on right) show averaged fluorescence intensity traces (**a**: 36 events, 8 cells), (**b**: 34 events, 8 cells). Values are presented as the mean ± SEM of the normalised fluorescence intensity. **c.** Model demonstrating the proposed role of dynamin-1 in vesicle recapture. The closed circle represents insulin, and v- (black) and t- (grey) N-ethyl-maleimide-sensitive fusion protein attachment receptors are shown initially tethering the vesicle and plasma membranes before catalysing the fusion of the two. Dynamin-1 is represented as an ellipse, which is recruited to the neck of the fusion pore to catalyse pore closure. Modified from [120]

vesicle fusion involves the interaction between soluble N-ethyl-maleimide-sensitive fusion protein attachment receptors that are present on the vesicle and the plasma membrane [101], and such mechanisms appear to be operative in the beta cell [21].

For many years it was assumed that the release of insulin required the complete fusion of the secretory and plasma membranes, as was also believed to be the case during synaptic vesicle fusion [102]. Moreover, the detection by electron microscopy of the vesicle core apparently leaving the open mouth of a fused vesicle en masse suggested a complete-collapse model [103]. On the other hand, the uptake of relatively high molecular mass markers into dense core secretory vesicles has been reported both in insulin-secreting [104] and chromaffin cell-derived PC12 cells [105], suggesting that the fusion pore must subsequently close, recapturing extracellular material. As in the nerve terminal [106], it now seems likely that such transient events are predominant at physiological levels of stimulation, where the rate of exocytosis does not exceed the cell's capacity for endocytosis [107]. Indeed, when the fate of the insulin-containing vesicle is imaged simultaneously in living beta cells, using either the low-molecular-mass dye acridine orange [82] or the vesicle cargo protein neuropeptide Y (NPY) fused to monomeric red fluorescent protein (mRFP) or

Venus [81] (where Venus is a highly fluorescent and relatively pH-insensitive derivative or GFP) [108], genuine peptide release events occur without the concomitant release of the vesicle membrane protein phogrin.EGFP into the plasma membrane. This is perhaps the most compelling evidence that complete fusion of the vesicle membrane and the plasmalemma do not occur during insulin release. Very similar findings have also been made in PC12 cells [109], and suggest a conserved role for kiss-and-run or "cavicapture" exocytosis in neurosecretion.

By the combined use of a range of fluorescent reporters differing in molecular mass and targeted either to the vesicle membrane or lumen, it was possible to demonstrate the existence of multiple forms of cavicapture exocytosis, termed "transient", "mixed" and (quasi) "full" events [81]. The proportions of these varied according to the strength of cellular stimulation [81]. Transient events, which were detected as changes in vesicular pH, probably involved the formation of a small (≤2 nm) fusion pore which is open for only a few seconds and allows the selective release of low-molecular-mass molecules (e.g. ATP) [110]. "Mixed" events allow the release of selected vesicle membrane proteins (e.g. VAMP2) [81], with K_{ATP} channel subunits [111] possibly providing a mechanism for delivering proteins ultimately destined to function at the plasma membrane. Finally, (quasi) "full" events permit the release of soluble vesicle cargoes including NPY.Venus (which has approximately the same molecular dimensions as insulin), but do not permit the release of larger proteins such as tissue plasminogen activator (tPA, 70 M_r) or phogrin (Fig. 6a). Importantly, this distinction between "full" but reversible events versus complete merger is difficult, if not impossible to detect, unless the fates of membrane and cargo markers are imaged simultaneously [81, 109]. This limitation may explain why recordings relying solely on soluble probes [112, 113] tend to invoke complete vesicle merger as the principal mechanism of insulin release.

The fate of the transiently fused vesicle: nanomechanics of insulin vesicle recapture

By what mechanisms is the recapture of the vesicle membrane achieved without full fusion? Dynamin is a 100 M_r GTP-driven mechanochemical enzyme related to mammalian mx-proteins, the *yeast vps 1* gene product and *Drosophila melanogaster shibire* [114]. First identified as a microtubule-associated motor protein-like activity [115], dynamin is implicated in the recapture of intact secretory vesicles in PC12 cells [105, 116]. Dynamin-1, the principal isoform in neuronal and neuroendocrine cells, possesses GTPase, pleckstrin homology and C-terminal proline-rich domains, and is proposed to act either as a mechanochemical enzyme that cleaves the neck of an endocytosing vesicle ("pinchase") [117], or as a molecular spring ("poppase") that extends and eventually ruptures the tubule linking the vesicle and donor membrane [118]. Dynamin-1 is required for "rapid endocytosis" detectable by capacitance measurements after mild (physiological) stimulation of chromaffin cells [119]. Imaged by TIRF microscopy, dynamin-1.EGFP is recruited, in the absence of other components of the classical endocytic pathway (e.g. clathrin and epsin), to sites of NPY.Venus release in MIN6 beta cells (Fig. 6b). Furthermore, overexpression of mutant dynamin-1 bearing a defective pleckstrin homology domain permits the release of large peptide cargoes including phogrin.EGFP and tPA [120]. Together, these findings indicate that dynamin-1 plays a key role in the endocytic mechanism by which the semi-fused secretory vesicle is recovered from the plasma membrane, essentially intact, following cargo release.

Conclusions

Imaging techniques have provided previously unsuspected information on the complex molecular machinery of insulin release, and demonstrated new layers of regulation. By identifying the important role of particular gene products (kinesin, myoVa, dynamin etc.), these approaches provide potential new therapeutic targets, as well as identifying potentially new diabetes genes that may be useful predictors of this disease.

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