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Barg, Sebastian

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Mechanisms of Exocytosis in Insulin-Secreting B-Cells and Glucagon-Secreting A-Cells

Sebastian Barg

Department of Physiological Sciences, Molecular and Cellular Physiology, Lund University, Sölvegatan 19, BMC F11,
S-221 84 Lund, Sweden

Abstract: In pancreatic B- and A-cells, metabolic stimuli regulate biochemical and electrical processes that culminate in Ca^{2+}-influx and release of insulin or glucagon, respectively. Like in other (neuro)endocrine cells, Ca^{2+}-influx triggers the rapid exocytosis of hormone-containing secretory granules. Only a small fraction of granules ($<1\%$ in insulin-secreting B-cells) can be released immediately, while the remainder requires translocation to the plasma membrane and further "priming" for release by several ATP- and Ca^{2+}-dependent reactions. Such functional organization may account for systemic features such as the biphasic time course of glucose-stimulated insulin secretion. Since this release pattern is altered in type-2 diabetes mellitus, it is conceivable that disturbances in the exocytotic machinery underlie the disease. Here I will review recent data from our laboratory relevant for the understanding of these processes in insulin-secreting B-cells and glucagon-secreting A-cells and for the identification of novel targets for antidiabetic drug action. Two aspects are discussed in detail: 1) The importance of a tight interaction between L-type Ca^{2+}-channels and the exocytotic machinery for efficient secretion; and 2) the role of intragranular acidification for the priming of secretory granules and its regulation by a granular 65-kDa sulfonylurea-binding protein.

The importance of insulin

The introduction of a novel medical treatment has rarely had such a dramatic impact on the patient's life as the introduction and use of the blood glucose-lowering hormone insulin. Within two years of its discovery in 1921, insulin was available in large quantities and used routinely in the treatment of diabetes mellitus. It took only a few weeks of insulin treatment to revitalize severely starved diabetic patients, illustrating the fundamental role the hormone plays in the metabolic regulation of the body. Diabetes mellitus is characterized by chronic hyperglycaemia, which results from a failure of the body to release adequate amounts of the blood glucose-lowering hormone insulin, the inability of the target organs to respond to insulin with increased uptake of glucose, or a combination of both. The two main classes, "insulin-dependent diabetes" (IDDM or type-1) and non-insulin-dependent diabetes" (NIDDM or type-2) are easily distinguished by the severity of insulin deficiency, which is more profound in IDDM (Pickup & Williams 1997). However, NIDDM is becoming an ever greater public health problem, currently affecting an estimated 150 million people worldwide (Amos *et al.* 1997). NIDDM is character-

ized by insufficient insulin secretion relative to the blood glucose level. Additionally, already at early stages of the disease the normal pattern of insulin release is disturbed so that the rapid but transient peak of secretion in response to a glucose challenge (1st phase) is absent, while a slow but sustained component remains (2nd phase; 1992). Because of the secretional defects associated with diabetes, it is important to understand the molecular mechanisms underlying normal and pathological insulin release.

Insulin secretion from pancreatic B-cells

Insulin is produced in the B-cells of the islets of Langerhans, where it is stored in ∼10000 secretory vesicles or "granules" waiting to be released into the blood stream. The latter occurs by fusion of the granule with the plasma membrane, a process referred to as exocytosis and used in most cells of the body to release substances or to insert newly synthesized proteins into the plasma membrane. Like in other endocrine cells and in neurones, exocytosis in B-cells is regulated and the amount of insulin circulating in the blood depends more on the rate of exocytosis than on the rate of insulin biosynthesis.

B-cells are electrically active and respond to elevated blood glucose by generating action potentials (fig. 1). Electrophysiological experiments during the past 15 years have thrown light on the way that release of insulin is coupled to stimulation. In this model, glucose is taken up by the cell

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Author for correspondence: Sebastian Barg, Department of Physiological Sciences, Molecular and Cellular Physiology, Lund University, Sölvegatan 19, BMC F11, S-221 84 Lund, Sweden (fax +46 222 7763, e-mail sebastian.barg@mphy.lu.se).
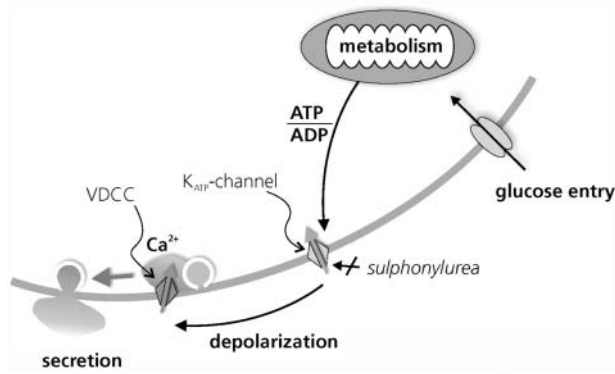
Fig. 1. Stimulus secretion coupling in pancreatic B-cells. Glucose enters the cell via GLUT-2 transporters, and is metabolised to produce ATP. The resulting increase in the cytosolic ATP/ADP-ratio closes K_{ATP}-channels in the plasma membrane, which leads to depolarization of the cell and influx of Ca^{2+} through voltage dependent L-type Ca^{2+}-channels (VDCC). The increase in Ca^{2+} at the granule then triggers exocytosis and thus insulin secretion. Sulfonylurea close the K_{ATP}-channel by binding to its SUR-subunits, and thereby cause membrane depolarization independent of the metabolic state of the cell.

and metabolized to ATP. The increase in the ATP/ADP ratio then leads to closure of ATP-sensitive K^+-channels (K_{ATP}-channels) in the plasma membrane (fig 1). Since these channels are responsible for maintaining a resting potential of about -70 mV, i.e., close to the K^+-equilibrium potential, this leads to a gradual depolarization of the cell. Eventually, voltage-dependent Ca^{2+}-channels become activated and initiate action potentials. Influx of Ca^{2+} through these channels and the resulting elevation of the cytosolic Ca^{2+}-concentration then triggers exocytosis of the insulin-containing granules (Ashcroft & Rorsman 1989).

The exocytotic machinery

B-cells are fairly typical examples of peptide-secreting endocrine cells. Proinsulin, the precursor of insulin, is synthesized in the endoplasmic reticulum and undergoes a series of maturation steps, starting already in the Golgi. The product is then packaged into secretory granules that gradually acidify, allowing further processing to insulin (Hutton 1994). These granules are found throughout the cytosol and eventually translocated to the plasma membrane. The ultimate fusion of the granule with the plasma membrane is triggered by Ca^{2+} and controlled by a complex network of protein-protein and protein-lipid interactions that are similar in all cellular membrane fusion events, and largely conserved from yeast to man. A vast body of molecular and physiological data accumulated over the past years has led to a unifying model for membrane fusion (Calakos & Scheller 1996). It is therefore not surprising that many of the proteins involved in the regulation of neurotransmitter release have also been identified in the pancreatic B-cell and demonstrated to participate in insulin secretion (Lang 1999). These proteins include the SNAREs (soluble *N*-ethylmaleimide-sensitive fusion protein [NSF] attachment protein [SNAP] receptors) synaptobrevin-2, syntaxin-1A, and SNAP-25 (25-kDa synaptosomal-associatedprotein). It is now clear that a ternary complex consisting of these proteins plays a central role in exocytosis (fig 4; Söllner *et al.* 1993), by tethering the granules to the plasma membrane (Sutton *et al.* 1998). Synaptotagmin is the most likely candidate for the Ca^{2+}-sensor of the exocytotic machinery (Fernandez-Chacon *et al.* 2001), and essential for Ca^{2+}-dependent neurotransmission (Littleton *et al.* 1993) and exocytosis of insulin-containing granules (Lang *et al.* 1997). It is a protein of 65 kDa that spans the vesicle membrane once and contains a large cytoplasmic domain with two Ca^{2+}-binding C2 domains (protein kinase C-homology domains), C2A and C2B (Sutton *et al.* 1995). In addition to binding Ca^{2+}, the C2 domains are involved in the association of syt with phospholipids, SNAREs, and a variety of other proteins. Several isoforms of the protein have been identified, of which synaptotagminIII and synaptotagminVII are present in B-cells (Lang *et al.* 1997; Gao *et al.* 2000).

Docking, priming, ATP-dependence, and kinetics of exocytosis

Fusion occurs rapidly upon Ca^{2+}-influx but obviously requires the granule to be situated in the immediate vicinity of the plasma membrane. Electron microscopy on various neuronal and endocrine cells (von Gersdorff *et al.* 1996; Plattner *et al.* 1997), including B-cells (Olofsson *et al.* 2002), has revealed a subset of secretory granules that is "docked" beneath the plasma membrane. These findings correlate with data obtained with capacitance measurements (fig. 2) demonstrating a burst of exocytosis in response to a step elevation of Ca^{2+}, followed by a slow but sustained phase (fig. 3). In B-cells, the size of the burst that can be released immediately upon Ca^{2+}-influx corresponds to less than 1% of the total number of granules (Barg *et al.* 2001a, 2002a & b). Refilling of this functionally defined "readily releasable pool" is ATP-, Ca^{2+}- and temperature-dependent (Bittner & Holz 1992; Parsons *et al.* 1995, Renström *et al.* 1996a & b, Eliasson *et al.* 1997; Gromada *et al.* 1999), and commonly referred to as "priming" (Hay & Martin 1992). In the B-cells, the "readily releasable pool" can be depleted in less than a second (Barg *et al.* 2001a & 2002a), whereas its refilling takes up to a minute (Gromada *et al.* 1999; Barg *et al.* 2002a). Under conditions of prolonged stimulation, the priming of vesicles for exocytosis therefore becomes rate-limiting, which may account for the biphasic time course in glucose-stimulated insulin secretion. In this scenario, the early rapid component (1st phase secretion) corresponds to the Ca^{2+}-dependent release of granules already belonging to the "readily releasable pool", while the sustained component (2nd phase) reflects the relatively slow priming of granules and to some extent probably their translocation to the release site (docking) (Rorsman *et al.* 2000; Barg *et al.* 2002a). Interestingly, priming occurs after
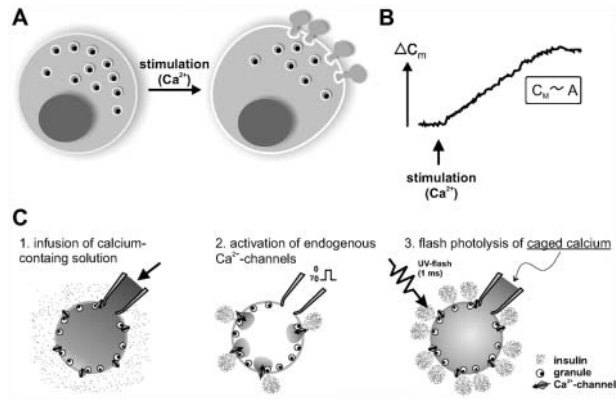
Fig. 2. Exocytosis measured as capacitance increase. In this method, a high resistance seal is formed between a patch of the cell surface membrane and a recording electrode made of glass. Electrical contact with the cytosol is then established by rupturing the membrane within the patch. Capacitance can be measured by circuit analysis, and since the cell capacitance is proportional to its surface area (10 $fF/\mu m^2$), any membrane that is added by exocytosis is detected as capacitance increase. Exocytosis can be measured in single cells at millisecond time resolution, making the study of release kinetics feasible. A. Schematic of a cell undergoing exocytosis; granule membrane is added to the plasma membrane and thereby increases the cell's capacitance. B. Typical capacitance recording of a cell stimulated with Ca^{2+} in the recording electrode. C. Means of eliciting exocytosis in patch-clamped cells; 1. infusion of an EGTA/Ca^{2+}-mixture leads to slow, sustained exocytosis (see B); 2. voltage-clamp depolarization evokes the opening of voltage-gated Ca^{2+}-channels (VDCC), Ca^{2+}-influx, and triggering of exocytosis in a near physiological manner and results in steep spatial Ca^{2+}-gradients; 3. flash photolysis of "caged" Ca^{2+}; Ca^{2+} is chelated by the NP-EGTA cage and introduced into the cell via the recording electrode. A flash (<1 msec.) is then directed at the cell to photolyse the case, increasing Ca^{2+} uniformly within a few hundreds of microseconds. Note that in all cases access to the cytosol is conveniently provided through the recording electrode, and test substances can be applied intracellularly simply by including them in the electrode solution.

docking of the granule at the release site and the ATP-dependence is therefore not due to the energy required for translocation. Several sequential steps are necessary to render the granule release-competent, and numerous ideas have been put forward to explain the ATP-dependence of priming. These include the ATP-dependent synthesis of phosphoinositides and protein kinase-mediated protein phosphorylation (Klenchin & Martin 2000). More recently, the presynaptic proteins munc-13 and Rim (Rab3-interacting molecule) have also been implicated in priming, mainly because they act after docking but before fusion (Ashery *et al.* 2000; Koushika *et al.* 2001).

Coupling of Ca^{2+}-influx and exocytosis

Despite many similarities of the exocytotic machinery in (neuro-) endocrine cells and that in neurotransmitter-releasing fast synapses, there are important differences: 1) synaptic vesicles are small (diameter <30 nm; von Gersdorff 1996) compared with peptide containing vesicles (hundreds of nm; Plattner *et al.* 1997; Olofsson *et al.* 2002); 2) synaptic exocytosis occurs without delay after the influx of Ca^{2+} and is at least one order-of magnitude faster than peptide secretion (Ämmälä *et al.* 1993; Mennerick & Matthews 1996); 3) synaptic vesicles may stay largely intact during exocytosis and are refilled with new transmitter molecules immediately after endocytosis, whereas peptide-containing granules are assembled and filled with secretory peptides in the Golgi apparatus (Betz & Bewick 1992; Hutton 1994); 4) synaptic exocytosis generally requires higher concentrations of Ca^{2+} (Ämmälä *et al.* 1993; Mennerick & Matthews 1996, Chow *et al.* 1996; Bollmann *et al.* 2000); and 5) although most secretory cells contain more than one type of voltage-gated Ca^{2+}-channel, synaptic transmitter exocytosis depends on the faster N- or P-type (Hirning *et al.* 1988; Uchitel *et al.* 1992), whereas endocrine cells preferentially use the L-type (Lopez *et al.* 1994; Barg *et al.* 2001a). Taken together, the findings in neurones suggest that the site of Ca^{2+}-influx is within minimal distance of the Ca^{2+}-sensor at the synaptic vesicle and functionally associated within release sites. This "coupling" appears to be crucial for fast synapses in order to avoid the diffusional delay between Ca^{2+}-influx and exocytosis. Notably, the high concentrations of Ca^{2+} required for exocytosis in certain neurons (~100 µM; Heidelberger *et al.* 1994) are not observed throughout the cytosol, and can in fact be expected only at the pore of the Ca^{2+}-channel. Such an organization can easily be envisaged to be energetically advantageous as it restricts the Ca^{2+}-signal to a small part of the cell where it can quickly trigger exocytosis, because less Ca^{2+} has to be cleared from the cytosol after the stimulation. In addition, Ca^{2+} may still be used for other signal transduction purposes like the control of gene transcription or synaptic plasticity (Berridge 1998).

In endocrine cells such functional coupling between Ca^{2+}-channel and exocytotic machinery seems less critical. In chromaffin cells for example, exocytosis persists even after Ca^{2+}-entry has stopped, due to the relatively low Ca^{2+}-dependence of exocytosis (Augustine & Neher 1992) and the time required to remove Ca^{2+} from the cytosol (Chow *et al.* 1996). However, recent estimates of the K_D
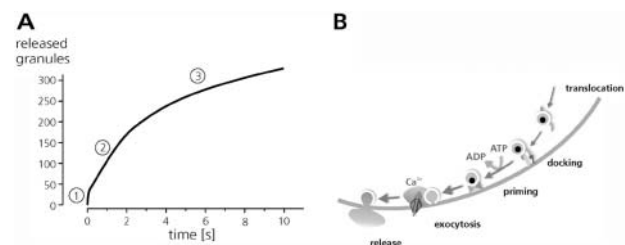
Fig. 3. Kinetics of exocytosis. A. Three distinct phases of exocytosis are readily distinguished in mouse pancreatic B-cells: (1) exocytosis of readily releasable pool (2–3) priming/retrieval of new granules; data combined from Barg *et al.* (2001). B, schematic of regulated exocytosis. Granules are translocated to the plasma membrane, where they dock and are primed for release by an ATP-dependent reaction. Ca^{2+}-influx triggers release of the primed granules.

of Ca^{2+}-dependent exocytosis in B-cells are around 20 μM (Takahashi *et al.* 1997; Barg *et al.* 2001a), and in fact similar to that of some neuronal terminals (Bollmann *et al.* 2000; Beutner *et al.* 2001). This value is surprisingly high because the average cytosolic (global) Ca^{2+} in the B-cell does not exceed ~2 μM during stimulation (Ämmälä *et al.* 1993; Bokvist *et al.* 1995; Dryselius *et al.* 1999). Obviously, steep spatial Ca^{2+}-gradients must exist and exocytosis is probably triggered in these zones where $[Ca^{2+}]_i$ may be 10 times higher than in the remainder of the cell (Bokvist *et al.* 1995; Qian & Kennedy 2001).

Ca^{2+}-channels are part of the fusion machinery

The N-type Ca^{2+}-channel binds to the synaptic core complex via syntaxin, SNAP-25, and the C2B domain of synaptotagminI (fig. 4), suggesting that in neurones the Ca^{2+}-channel is an integral part of the exocytotic machinery (Mochida *et al.* 1996). The interacting site of the channel is located between transmembrane regions II and III of its pore-forming α1 subunit (Sheng *et al.* 1996; Wiser *et al.* 1997; Atlas 2001), and is often referred to as the "synprint"-region. Recombinant peptides containing the synprint region dissociate the channel from the exocytotic complex and, when injected into presynaptic neurones, reversibly inhibit synaptic transmission without affecting Ca^{2+}-influx (Mochida *et al.* 1996). Syntaxin appears to act as a scaffold for the exocytotic machinery, keeping the proteins of the vesicle docking site in place (Wu *et al.* 1999). Interestingly, syntaxin alone (i.e., in the absence of other synaptic proteins) binds to the N-type channel and *decreases* its activity (Bezprozvanny *et al.* 1995). This could serve to prevent Ca^{2+}-influx at sites that are not occupied with docked secretory vesicles. Once the site is occupied with a vesicle, it would bind to the Ca^{2+}-channels in the vicinity with its synaptotagmin, and thus promote Ca^{2+}-channel activity.
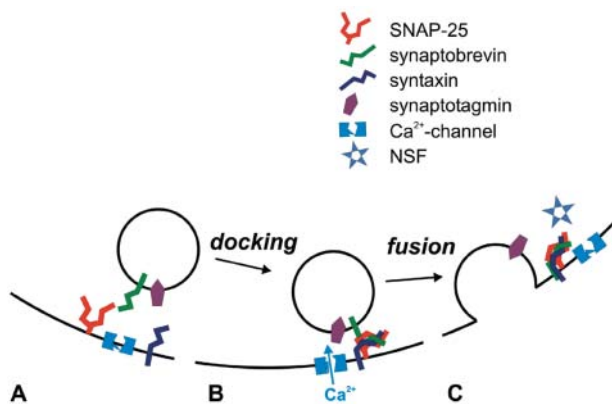

Fig. 4. Proteins involved in exocytosis. A, an insulin-containing granule that approaches the plasma membrane. B, a docked vesicle in the moment just before fusion. The SNARE proteins have formed a parallel four-helix bundle, which pulls the granule down the plasma membrane C, after fusion, the SNARE protein-complex is disassembled by the ATPase NSF.

Syntaxin, SNAP-25, and synaptotagmin also bind to the synprint site of the L-type Ca^{2+}-channel, which mediates Ca^{2+}-influx in endocrine cells (Wiser *et al.* 1997 & 1999; Atlas 2001). Like in neurones, interfering with the interaction between the Ca^{2+}-channel and the core complex by introducing the corresponding L-type synprint peptide ($Lc_{753-893}$) completely abolishes depolarization-evoked insulin exocytosis (Wiser *et al.* 1999; Barg *et al.* 2001a). Importantly, neither Ca^{2+}-dependent exocytosis itself nor Ca^{2+}-currents are affected, suggesting that the exogenous synprint peptide dissociates Ca^{2+}-channel/SNARE/synaptotagmin complexes, thereby increasing the average distance between channels and docked granules. This interpretation is supported by the finding that synprint-blocked exocytosis can be rescued by strong, prolonged stimulation, which increases the total Ca^{2+}-influx and leaves more time for its diffusion away from the channel pore (Barg *et al.* 2001a). The latter should increase the Ca^{2+}-concentration sufficiently to trigger exocytosis even at sites that are not in the immediate vicinity of Ca^{2+}-influx.

How many channels are needed to make a granule fuse?

In B-cells, the magnitude of the whole-cell Ca^{2+}-current is very small, and non-stationary fluctuation analysis shows that mouse B-cells are equipped with fewer than 500 α1C L-type Ca^{2+}-channels (Barg *et al.* 2001a). This corresponds to a Ca^{2+}-channel density of ~0.9 channel per μm^2 or only 5–10% of that reported for other endocrine cells (9–20/μm^2; Fenwick *et al.* 1982). Nevertheless, B-cells are capable of fast exocytosis. Analysis of the kinetics of exocytosis in these cells during voltage-clamp depolarizations reveals an early, very rapid component of about 60 granules. Exocytosis of this pool takes place at a peak rate of ~700 granules per second (Barg *et al.* 2001a), suggesting very high levels of Ca^{2+} (probably >100 μM) at the site of exocytosis. Assuming that the 60 granules that can be released at maximum rate are associated with about 500 Ca^{2+}-channels, it seems likely that more than one channel binds to each exocytotic site. Indeed, cell-attached patches on B-cells (~1% of the cell surface) predominantly contain either no channels at all or three active Ca^{2+}-channels, indicating non-random distribution of the channels on the plasma membrane (Barg *et al.* 2001a). Further support for Ca^{2+}-channel clustering comes from experiments with the L-type Ca^{2+}-channel agonist BayK8644, which acts by increasing the open lifetimes. Prolonged openings of single Ca^{2+}-channel should make exocytosis more likely, because of the increased probability that the Ca^{2+}-concentration around the granule lasts sufficiently long to trigger exocytosis. However, the efficiency of exocytosis was not altered in presence of BayK8644, despite 6 times longer Ca^{2+}-channel open-times (Barg *et al.* 2001a). The reason might be that the openings of several clustered Ca^{2+}-channels overlap in time and thereby achieve a similar effect as BayK8644 would if the channels were not clustered.

Effects of Ca²⁺ on the recruitment of granules into the readily releasable pool

In addition to triggering exocytosis, Ca^{2+} stimulates the recruitment of release-competent granules (Ämmälä *et al.* 1993; Heidelberger *et al.* 1994). Thus, the size of readily releasable pool depends on the concentration of cytosolic Ca^{2+} that is present during the minute or so *before* the initiation of exocytosis (Påhlman & Barg, unpublished results). The previous finding that exogenous Ca^{2+}-chelators inhibit exocytosis in endocrine cells (Ämmälä *et al.* 1993; Chow *et al.* 1996) may therefore be due to reduced rate of supply of granules ready for release due to artificially low basal $[Ca^{2+}]_i$. However, even at elevated $[Ca^{2+}]_i$ recruitment of granules in the B-cell is a slow process (~5 granules/sec., compared with the exocytotic burst of 700 granules/sec.). During sustained stimulation the rate of supply of new granules quickly becomes rate-limiting for the process of exocytosis. A number of Ca^{2+}-binding proteins are known to affect exocytosis, and may underlie the Ca^{2+}-dependence of granule recruitment. These include calmodulin (Chen *et al.* 1999), the Ca^{2+}-dependent phosphatase calcineurin (Renström *et al.* 1996b), CAPS (Ca^{2+}-dependent activator protein for secretion; Ann *et al.* 1997), and Ca^{2+}/calmodulin kinase II (Ämmälä *et al.* 1993; Gromada *et al.* 1999). Blockage of the latter inhibited the slow phase, and suggested a myosin-actin-dependent step in the granule recruitment (Bi *et al.* 1997). The basal concentration of cytosolic Ca^{2+} depends to a great extent on intracellular Ca^{2+}-handling, and there is evidence for Ca^{2+}-release from the ER (Tengholm *et al.* 1998; Holz *et al.* 1999), for Ca^{2+}-uptake by the mitochondria (Rutter *et al.* 1993), and for accumulation of Ca^{2+} in the granules (Scheenen *et al.* 1998; Mitchell *et al.* 2001).

Comparison between insulin-secreting B-cells and glucagon-secreting A-cells

Glucagon is a major catabolic and hyperglycaemic 29 a.a. hormone, which is secreted from the A-cells. Its main biological effect is to enhance synthesis and mobilization of glucose in the liver. Secretion of glucagon is stimulated by low blood glucose (Gorus *et al.* 1984), amino acids (Pipeleers *et al.* 1985), and a variety of hormones and neurotransmitters, such as adrenaline, glucose-dependent insulinotropic polypeptide (GIP), and glucagon-like peptide-1 (Ding *et al.* 1997; Gromada *et al.* 1997). Insulin, somatostatin, and fatty acids reduce glucagon secretion (Gerich *et al.* 1976; Ding *et al.* 1997). Whereas insulin levels are inadequately low in hyperglycaemic diabetic patients, glucagon levels are elevated, and this aggravates the disease (Unger 1971). The reason for this abnormality of glucagon secretion is not known. Electrical activity has been observed in A-cells (Rorsman & Hellman 1988; Barg *et al.* 2000; Göpel *et al.* 2000) and is, at least in part, attributable to voltage-gated Ca^{2+}-channels. A- and B-cells in the mouse can be distinguished electrophysiologically because A-cells 1) are small in size (capacitance of 3 pF compared with 6–7 pF in B-cells, corresponding to diameters of 10 and 15 μm; Barg *et al.* 2000), 2) are equipped with a large tetrodotoxin-sensitive and transient Na^+-current (Barg *et al.* 2000; Göpel *et al.* 2000), 3) have almost no K_{ATP}-conductance (Barg *et al.* 2000; Göpel *et al.* 2000), and 4) are, contrary to B-cells, electrically active at low glucose levels (Barg *et al.* 2000; Göpel *et al.* 1999 & 2000). The latter observation is in agreement with the tentative physiological role of A-cells and has been explained by a combination of a small (hyperpolarizing) K_{ATP}-current and the fact that regenerative electrical activity depends on Na^+, rather than Ca^{2+} in these cells (Göpel *et al.* 2000). In this scenario, elevation of glucose results in an increased cytoplasmic ATP/ADP-ratio, which closes the few K_{ATP}-channels that are present in A-cells (Bokvist *et al.* 1999; Barg *et al.* 2000; Göpel *et al.* 2000). This is associated with membrane depolarization, which in turn prevents reactivation of the Na^+-channels after an action potential.

Kinetics of glucagon exocytosis

Exocytosis of glucagon (A-cells) is Ca^{2+}-dependent and slightly faster than exocytosis of insulin (B-cells; fig. 5). The difference reflects the number of granules belonging to the RRP (~120 granules in A-cells and ~60 granules in B-cells), and the rate of refilling after depletion of the readily releasable pool (~20 granules/s in A-cells and 5–10 granules/sec. in B-cells). In addition, there is a strong secondary acceleration of the exocytotic rate in A-cells after depletion of the readily releasable pool (fig. 5), which may reflect fast Ca^{2+}-dependent translocation of granules from the interior of the cell towards the release sites. Interestingly, the *maximum* rate of 750 granules/s during a depolarization is achieved within about 20 msec. and fairly similar to that observed in B-cells (~700 granules/sec. after 15 msec.). Taken together, the data therefore suggest a fairly similar organization of Ca^{2+}-dependent exocytosis in both cell types, but a higher number of release sites in A-cells. It seems further likely that the large Ca^{2+}-currents of the relatively small A-cells, leading to larger and less localized Ca^{2+} transients, may explain part of the differences,
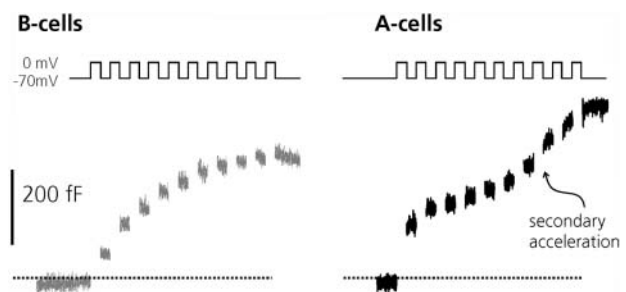

Fig. 5. Comparison of exocytosis in B- and A-cells. A single B-cell (left) or A-cell (right) was stimulated with a train of depolarizations (upper trace). While the exocytotic responses in the B-cell become progressively smaller throughout the train, there is a secondary acceleration in A-cells.

e.g., faster Ca^{2+}-dependent recruitment of granules and the secondary acceleration. It can be speculated that the these features enable the A-cell to respond with a bout of glucagon release during short periods of glucose demand, as required during intense physical activity and in certain life-threatening situations such as shock (van der Meer *et al.* 1981) and trauma (Efendic *et al.* 1974).

Sulfonylureas stimulate insulin release

Sulfonylureas have been used in the treatment of NIDDM for almost 45 years, but it is only recently that the molecular mechanisms of their action have been elucidated. The principal effect of the sulfonylureas is to stimulate insulin secretion from the B-cells (Ashcroft & Ashcroft 1992). This is a consequence of their ability to selectively inhibit the K_{ATP}-channels in the B-cell plasma membrane (Trube *et al.* 1986) by binding to its sulfonylurea receptor (Aguilar-Bryan *et al.* 1995) subunit. This short-circuits the normal physiological regulation of the channel by the metabolic state of the cell (via the ATP/ADP-ratio) and results in membrane depolarization, Ca^{2+}-influx and insulin secretion (Ashcroft & Rorsman 1989).

In addition to the stimulation induced by the inhibition of the K_{ATP}-channel, therapeutic concentrations of sulfonylureas potentiate insulin secretion by a mechanism that is exerted downstream of the depolarization, i.e., *directly* on the exocytotic machinery (Eliasson *et al.* 1996; Tian *et al.* 1998; Barg *et al.* 1999 & 2001b). Thus, sulfonylureas remain stimulatory in conditions that preclude the effects of sulfonylureas on the membrane potential, i.e. after permeabilization (Tian *et al.* 1998) or voltage-clamping of the cells (Eliasson *et al.* 1996; Barg *et al.* 1999 & 2001b). However, since sulfonylureas normally depolarize the B-cell and thereby indirectly stimulate insulin release, any *direct* stimulation of the exocytotic machinery may easily be overlooked. Our laboratory has used patch-clamp capacitance measurements to elucidate the mechanisms underlying sulfonylurea-dependent stimulation of exocytosis (Barg *et al.* 1999 & 2001b). In these experiments, sulfonylureas (tolbutamide) failed to stimulate exocytosis in the absence of Ca^{2+} and stimulated at concentrations that were only slightly higher than resting $[Ca^{2+}]_i$. This suggests that sulfonylureas do not induce, but rather stimulate (Ca^{2+}-dependent) exocytosis (Barg *et al.* 1999). Similar effects of sulfonylureas have been reported in pancreatic A-cells (Høy *et al.* 2000) and clonal neuroendocrine PC12-cells (Taylor *et al.* 1999), suggesting that the underlying mechanism is not restricted to B-cells and insulin secretion.

Regulation of exocytosis by a granular 65-kDa sulfonylurea-binding protein

Pharmacologically, the mechanism that mediates the *direct* stimulation of the exocytotic machinery is very similar to the B-cell variety of the K_{ATP}-channel, which consists of an octameric complex of four type 1 sulphonylurea receptors (SUR1) and four K^+-channel (Kir6.2) subunits. Thus, exocytosis is inhibited by intracellular application of diazoxide, an activator of B-cell K_{ATP}-channels, while pinacidil, an activator of vascular K_{ATP}-channels that contain SUR2A (Shindo *et al.* 1998), has no effect (Barg *et al.* 1999). K_{ATP}-channels are physiologically activated with reduced ATP/ADP ratio, and a similar regulation leads to inhibition of the exocytotic machinery. Interestingly, this inhibition of Ca^{2+}-dependent exocytosis is fully rescued by addition of tolbutamide (Barg *et al.* 2001b). The latter suggests that a direct stimulation of the exocytotic machinery could contribute to the insulinotropic action of sulfonylureas in diabetic patients, where the ATP/ADP ratio may be reduced as a consequence of impaired glucose metabolism.

ATP- and sulfonylurea-sensitive K^+ and Cl^--conductances have been found in isolated zymogen granules of the exocrine pancreas (Thevenod *et al.* 1992 & 1994). In these granules, a 65-kDa sulfonylurea-binding protein is involved in the modulation of the activity of the granular ion channels. This protein cross-reacts with antibodies originally raised against a SUR-related protein, the 170-kDa mdr1 (multi-drug-resistance P-glycoprotein; Thevenod *et al.* 1994; Braun *et al.* 1997). Interestingly, the majority (90%) of the high-affinity sulfonylurea binding sites in the B-cell are intracellular and associated with the insulin-containing secretory granules (Ozanne *et al.* 1995). It seems therefore possible that such channels are also present in B-cell granules, and there are several arguments in favor of this idea. First, two different antibodies (JSB-1 and C219) originally raised against mdr1 detect a protein of 65 kDa in B-cell granules. Second, intracellular application of one of these antibodies (JSB-1) abolishes the stimulatory action of sulfonylureas on exocytosis, yet does not interfere with their capacity to block the K_{ATP}-channels (Barg *et al.* 1999). Third, the sulfonylurea glibenclamide binds to a 65 kDa protein in insulin-containing granules (Renström *et al.* 2002). Finally, both SUR and mdr1 belong to the family of ATP-binding cassette (ABC)-proteins (Schwiebert *et al.* 1999), and tamoxifen, a blocker of ABC-protein function in a variety of contexts, prevents the stimulation of exocytosis by tolbutamide (Barg *et al.* 1999). Collectively, the data argue that sulfonylureas bind to a mdr1-related granular protein of 65 kDa, similar to the protein found in the zymogen granules of the exocrine pancreas. The binding of sulfonylureas to the 65 kDa protein lead to an augmentation of the exocytotic machinery, possibly via activation of ion channels in the granule membrane.

Granular Cl^--flux through ClC-3 channels is required for insulin exocytosis

By analogy with other ABC-proteins, especially SUR, it is conceivable that the 65-kDa granular sulfonylurea binding protein acts as a regulatory subunit of a K^+-channel. This may indeed be the case in A-cells, where intracellular application of certain K^+-channel blockers interferes with exo-

cytosis (Høy *et al.* 2000). However, exocytosis in the B-cell is unaffected by replacing intracellular K^+ with Cs^+. It is instead strongly inhibited by the Cl^--channel blocker DIDS (4,4′-di-isothiocyanato-stilbene-2,2′-disulfonic acid) or by replacing intracellular Cl^- with glutamate (Barg *et al.* 2001b). This suggests that intracellular Cl^--, rather than K^+-fluxes, participate in the control of exocytosis in the B-cell. Indeed, antibodies against the Cl^--channel ClC-3 label B-cell granules and detect a protein with a molecular weight of ~90 kDa in B-cell homogenates and in a granule-enriched subcellular fraction of clonal insulin-containing Ins-1 cells. One of the antibodies, antiClC-3-C, selectively blocks ClC-3 channel function, which can be exploited to test the role of this channel in the process of exocytosis. Notably, the antibody abolishes exocytosis when it is dialyzed into the cell via the patch pipette, while the non-functional antibody antiClC-3-N has no effect (Barg *et al.* 2001b). These data raise the interesting possibility that granular Cl^--flux carried by ClC-3 channels is required for Ca^{2+}-dependent exocytosis in the B-cell. This phenomenon does not seem to be limited to the B-cell, as Cl^- is required for hormone release in a variety of other cells, including pancreatic acinar cells (Fuller *et al.* 1989) and pituitary melanotrophs (Rupnik & Zorec 1992).

Chloride influx enables granule acidification

How does influx of Cl^- into the granule stimulate exocytosis? One consequence of Cl^--influx would be net accumulation of electrolytes, water uptake, and increased hydrostatic pressure inside the granules. The latter has been suggested to promote membrane fusion (Woodbury 1995), but there is evidence to the contrary (Breckenridge & Almers 1987). Osmotic effects are unlikely to be involved in the B-cell because reversing osmotic gradients with strongly hyperosmotic intracellular solutions has no effect on exocytosis (Barg *et al.* 2001b). Alternatively, electrogenic H^+-pumping, as required for granule acidification, might require a shunt-conductance to prevent the development of a large electrical potential (positive inside). Indeed, regulation of vesicle acidification by chloride counter-ion flux seems to be a common phenomenon (al Awqati *et al.* 1992). For example, ClC-5 was found on endocytic vesicles of renal cells (Günther *et al.* 1998), and disruption of this channel impaired endocytosis and vesicle acidification (Piwon *et al.* 2000).

In the B-cell, interfering with either the granular v-type H^+-ATPase with bafilomycin, or with intracellular H^+-gradients by introducing the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) suppresses exocytosis (Barg *et al.* 2001b). This implicates granule acidification as an important step during the preparation of granules for exocytosis. H^+-pumping itself may be controlled by sulfonylurea-sensitive mechanism, as direct measurements of granular pH with the fluorescent probe LysoSensor show that tolbutamide acidifies granules. Importantly, CCCP-induced equilibration of the granule pH can be prevented by

blocking ClC-3 channel function with DIDS or antiClC-3-C. Why is this so? Apparently, protons leave the granule only when the Cl^--channel is open, because the (electrogenic) proton efflux requires charge neutralization. The electrical potential (negative inside) across the granule membrane would otherwise balance the H^+-gradient and prevent equilibration. This feature can be used to study the pharmacology of the granular Cl^--channel. It turns out that the CCCP-dependent H^+-leak is prevented by the very same maneuvers that also block exocytosis. ADP or diazoxide inhibit the leak, while tolbutamide enhances it (Barg *et al.* 2001b). Thus, exocytosis in the B-cell depends, like CCCP-dependent proton leakage from the granule, on open ClC-3 channels.

Priming for exocytosis by granular acidification and Cl^--fluxes

Which step during the life of the insulin granule is modulated by granular ion fluxes, tolbutamide, and the ATP/ADP-ratio? Although capacitance measurements monitor only membrane fusion, kinetic analysis of the capacitance
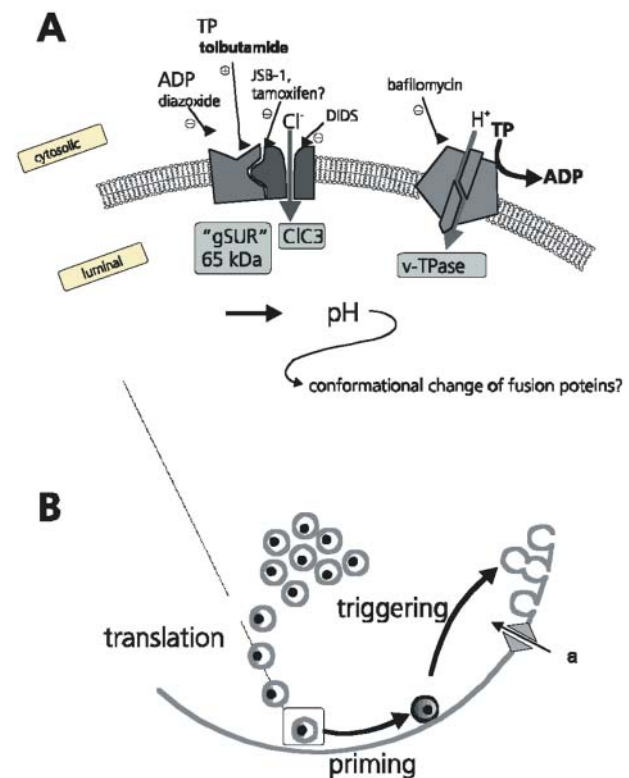
Fig. 6. Model for the regulation of granular pH and exocytosis. A. Metabolically regulated Cl^--influx into the granule through an ion channel complex comprised of ClC-3 and an mdr1-like 65-kDa protein, determines the rate of granular acidification by the V-type H^+-ATPase. Tolbutamide, diazoxide and ADP are likely to interact with the regulatory 65-kDa protein, whereas DIDS and anti-hClC-3-C bind to and inhibit ClC-3 directly. B. Replenishment of readily releasable pool depends on intragranular acidification. See text for details.

data allows limited predictions of processes that precede exocytosis, e.g., the rate at which granules become readily releasable (Parsons *et al.* 1995). During trains of depolarizations, exocytosis proceeds throughout the train, with the fastest rate during the first depolarization and progressively smaller responses during subsequent depolarizations (fig. 5). This is usually interpreted to mean that the first depolarizations rapidly deplete the readily releasable pool, and that the remainder of the train is associated with the slow recruitment of new granules. Such experiments show that ADP selectively blocks the slower second phase, and that the effect of ADP is counteracted by tolbutamide (Barg *et al.* 2001b). Similarly, intracellular application of the Cl^--channel blocker DIDS or the antibody anti-hClC-3-C remove the slow phase, but leave the initial burst intact. These data agree with a model in which ADP, tolbutamide, and granular Cl^--flux control the refilling of the readily releasable pool, without affecting exocytosis of granules that have already progressed into this pool. This is in contrast to the effects of the protonophore CCCP, which abolishes not only the late component of the release, but eliminates the readily releasable pool as well. The latter may indicate that low intragranular pH is required not only for the priming of the granules but also for maintaining their release competence. Importantly, granule acidification depends on a bafilomycin-sensitive ATP-driven protonpump. It is therefore conceivable, that acidification of the insulin-granule is not required merely for processing of insulin (Hutton 1994), but also represents one of the ATP-dependent priming reactions.

A possible model that accounts for the available data is presented in fig 6. Secretory granules become release-competent by intragranular acidification, which depends on the simultaneous activity of the v-type H^+-ATPase and the ClC-3 Cl^--channel. By analogy to the regulation of the K_{ATP}-channel in the plasma membrane (Gribble *et al.* 1997), it seems possible that ClC-3 together with a regulatory mdr1-like protein of 65-kDa (Barg *et al.* 1999; Renström *et al.* 2002) form an ion channel complex in the granular membrane. In this scenario, ATP enhances granular Cl^- uptake, acidification and priming by a direct interaction with ClC-3, whereas ADP attenuates the same chain of events by binding to the regulatory 65-kDa protein. Tolbutamide interacts with the 65-kDa protein and prevents ADP binding, thereby counteracting the inhibitory effects of ADP on priming. How does granular acidification affect the exocytotic machinery? It can only be speculated that low intragranular pH may induce conformational changes in some of the proteins constituting the exocytotic machinery, thereby perhaps rendering them more fusogenic (Sutton *et al.* 1998). Indeed, vacuole acidification has recently been demonstrated to be required for the pairing of the SNARE-proteins in yeast (Ungermann *et al.* 1999).

Finally, the model implies that the priming of granules for release is controlled by the metabolic state of the cell, which may be of significance for the coupling between the level of blood glucose and insulin release. Data from B-cells

of diabetic individuals are scarce, but it appears possible that slow priming would produce secretional defects similar to those seen in NIDDM. Since the granular sulfonylurea receptor is involved in the modulation of priming, it may represent an exciting target for the development of new antidiabetic drugs.

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