

## REVIEW ARTICLE

# Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine secretion

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Secretion of the peptide hormone insulin from pancreatic  $\beta$  cells constitutes an important step in the regulation of body homeostasis. Insulin is stored in large dense core vesicles and released by exocytosis, a multistage process involving transport of vesicles to the plasma membrane, their docking, priming and finally their fusion with the plasma membrane. Some of the protein components necessary for this process have been identified in  $\beta$  cells. The export of potent and potentially harmful substances has to be tightly controlled. The secretory response in pancreatic  $\beta$  cells requires the concerted action of nutrients together with enteric hormones and neurotransmitters acting on G-protein coupled receptors. It is well established that glucose and other metabolizable nutrients depolarize the  $\beta$ -cell membrane and the ensuing  $\text{Ca}^{2+}$  influx through voltage-dependent channels constitutes a main stimulus for insulin exocytosis. Theoretical considerations and recent observations suggest in addition an organizing role for the  $\text{Ca}^{2+}$  channel similar to neurotransmission. A second regulatory control on exocytosis is exerted by monomeric and heterotrimeric G-proteins. The monomeric GTPase Rab3A controls insulin secretion through cycling between a guanosine triphosphate liganded vesicle-bound form and a guanosine diphosphate liganded, cytosolic form. The effect of neurohormones is transduced by the heterotrimeric GTPases. Whereas pertussis-toxin sensitive  $\alpha$ -subunits exert direct inhibition at the level of exocytosis, the  $\text{G}\beta\gamma$ -subunits are required for stimulation. It is possible that these GTPases exert immediate regulation, while protein kinases and phosphatases may modulate long-term adaptation at the exocytotic machinery itself. The molecular nature of their activators and effectors still await identification. Insights into the progression of the exocytotic vesicle from docking to fusion and how these processes are precisely regulated by proteins and second messengers may provide the basis for new therapeutic principles.

**Keywords:** calcium; exocytosis; G-proteins; hormones; insulin; membrane fusion; neurotransmission; SNARE proteins; vesicle trafficking;  $\beta$  cells.

The process of regulated secretion forms the basis for a broad variety of physiological processes such as intracellular communication in the nervous system, acute defence against bacterial invasion and the regulation of body homeostasis by hormones. Consequently, secretory systems can be classified as myeloid, neuronal, neuroendocrine and endocrine. The best characterized endocrine system is represented by the pancreatic  $\beta$  cell in which the molecular basis of insulin release and its regulation has been extensively studied. The control of insulin secretion from the pancreatic  $\beta$  cells and the biological actions of this peptide hormone on its target tissues ensure the vital function of

glucose homeostasis [1]. Insulin secretion is inadequate in various forms of diabetes, in particular in pre-type I (insulin-dependent diabetes mellitus) and in many forms of the more common type II non-insulin-dependent diabetes (NIDDM) [2,3].

Insulin is transported in pancreatic  $\beta$  cells in large dense core vesicles (LDCV), the secretory granules. Insulin is released from the  $\beta$  cells by exocytosis. This process is mediated by the action of specific proteins, the exocytotic machinery, and proceeds through distinct steps. Exocytosis finally results in the fusion of the secretory granule membrane with the plasma membrane followed by release of the granule content, insulin, into the extracellular space to reach the capillary blood flow. The secretory granules serve not only as a transport vesicle, but also as a storage pool of insulin. Only a small proportion of insulin is released even under maximal stimulatory conditions. The concentration of circulating insulin therefore depends mainly on the regulation of secretion, rather than on the rate of biosynthesis of the hormone itself. The pancreatic  $\beta$  cell responds by sensing the levels of nutrients such as glucose and certain amino acids. Moreover,  $\beta$  cells have to integrate the physiological demands which are signalled through neurotransmitters and hormones acting on G-protein coupled receptors. This regulation provides the correct levels of insulin corresponding to changing requirements; e.g. increased secretion after meals and reduced output during physical stress. The comprehension of insulin secretion from pancreatic  $\beta$  cells at the molecular level requires the understanding of both, the exocytotic machinery and its regulatory features.

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**Abbreviations:** BoNT, botulinum neurotoxins; CoA, coenzyme A; Csps, cysteine string proteins; GAP, GTPase-activating protein; GDI, GTP-dissociation inhibitor; GDP, guanosine diphosphate; GEP, GDP/GTP-exchange protein; GIP, glucose-dependent insulinotropic polypeptide; GPCRs, G-protein coupled receptors; GTP, guanosine triphosphate; IGF, insulin-like growth factor; LDCVs, large dense core vesicles; NIDDM, non-insulin-dependent diabetes; PACAP, pituitary adenylate cyclase activating protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SLMVs, synaptic-like microvesicles; SVs, synaptic vesicles; VDCC, voltage-dependent calcium channels

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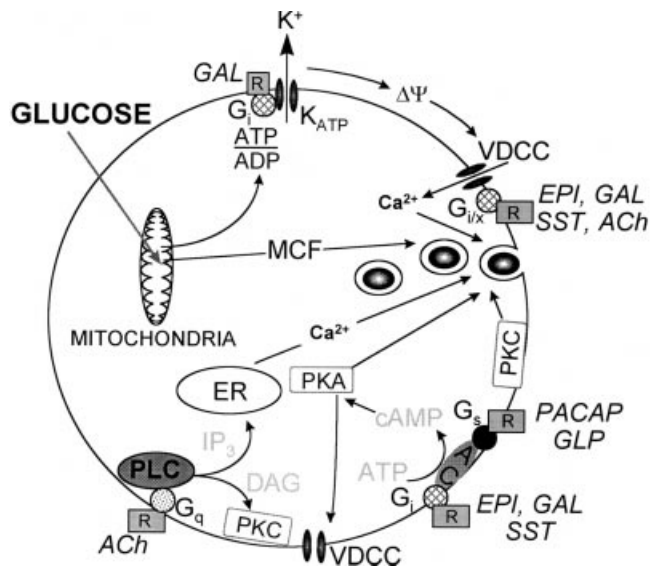
## STIMULUS-SECRETION COUPLING IN INSULIN SECRETION

The secretory response in pancreatic  $\beta$  cells results from the concerted action of nutrients (glucose and amino acids) together with the effect of hormones and of neurotransmitters acting on G-protein coupled receptors (see Fig. 1). Their site of action can be divided into proximal steps regulating the levels of second messengers and into distal steps. The latter term implies effects beyond the control of second messengers, that is at the exocytotic machinery itself.

### Proximal regulatory steps

The generation of metabolic coupling factors through sugar metabolism in the  $\beta$  cell is central to glucose-induced insulin secretion [4]. The currently best-characterized metabolic coupling event is a change in the adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio, which promotes closure of ATP-sensitive  $K^+$ -channel  $K_{ATP}$ . This leads to membrane depolarization and opening of voltage-dependent calcium channels (VDCC). The subsequent increase in cytosolic free  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ) plays an important part in the stimulation of insulin secretion [5].

Some of the receptor agonists that potentiate insulin secretion,



**Fig. 1. Proximal events in insulin secretion.** Glucose metabolism in the mitochondria alters the ATP/ADP ratio, which leads to the closure of the  $K_{ATP}^+$  channel. The ensuing membrane depolarization ( $\Delta\Psi$ ) opens voltage-dependent calcium channels (VDCC) and subsequent  $Ca^{2+}$  influx leads to insulin secretion. In addition to alterations in ATP/ADP ratios, glucose metabolism generates other, still poorly characterized, metabolic coupling factors (MCF) acting on secretion. Insulin secretion is further regulated by several hormones and neurotransmitters such as epinephrine (or noradrenaline) (EPI), galanin (GAL), somatostatin (SST), acetylcholine (ACh), pituitary adenylate cyclase-activating peptide (PACAP) or glucagon-like peptide (GLP) which bind to their cognate receptors. Coupling to heterotrimeric G-proteins ( $G_s$ ,  $G_i$ ,  $G_x$ ) activates adenylate cyclase (AC), controls VDCC,  $K_{ATP}$  or activates phospholipase C (PLC). Activation of PLC generates  $IP_3$  with subsequent liberation of  $Ca^{2+}$  from intracellular stores (ER, endoplasmic reticulum) and diacylglycerol (DAG), an activator of protein kinase C (PKC). Stimulation of protein kinase A (PKA) or PKC alters second messenger generating enzymes, ion channels and also has a more direct influence on secretion.

such as acetylcholine and cholecystokinin, promote phosphoinositide breakdown with a consequent mobilization of  $Ca^{2+}$  from intracellular stores,  $Ca^{2+}$  influx across the cell membrane and activation of protein kinase C (PKC) [6–8]. Other potentiators, including GLP-1, glucose-dependent insulinotropic polypeptide (GIP) and pituitary adenylate cyclase activating protein (PACAP) raise cyclic AMP (cAMP) levels and activate cAMP-dependent protein kinase (PKA) [9–11]. PKC and PKA exert some of their actions at early steps involving the generation of second messengers [11,12]. Inhibition of insulin secretion as encountered in stress is induced by neurotransmitters and hormones such as noradrenaline, galanin and somatostatin. They bind to receptors that are coupled to pertussis toxin-sensitive heterotrimeric GTPases (G-proteins) and thereby inhibit adenylate cyclase and modify  $Ca^{2+}$  and  $K^+$  channel gating [13].

### Distal regulatory steps

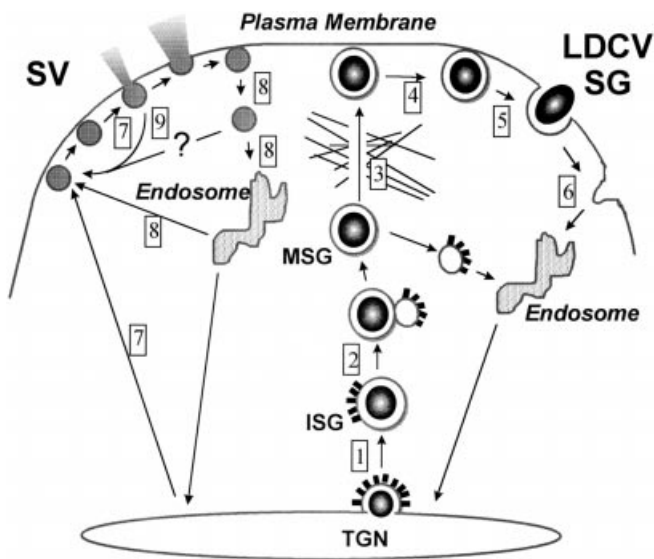
The above mentioned events do not sufficiently account for the physiological regulation of insulin release and important regulatory steps occur distal to changes in second messengers such as  $[Ca^{2+}]_i$  or cAMP. Indeed, hormone release can be induced by glucose through a mechanism distinct from closure of  $K_{ATP}$  channels, alterations in  $[Ca^{2+}]_c$  or activation of protein kinases A or C [14]. Such a mechanism may imply metabolic coupling factors other than ATP [15]. Although these effects of glucose occur only at a significant level in the presence of  $Ca^{2+}$ , it is yet unclear whether  $Ca^{2+}$  is required for glucose metabolism or at the site of exocytosis. Malonyl-coenzyme A (CoA) and long chain fatty acid CoA have been proposed as alternative metabolic coupling factors in  $\beta$  cells linking fuel metabolism to insulin secretion [16]. At least fatty acyl-CoA is required for budding of coated transport vesicles from Golgi cisternae [17] and may thus also be involved in other transport steps. Insulin exocytosis can also be induced independently from  $Ca^{2+}$  by intracellular application of guanine nucleotides such as GTP, GppNHp and  $GTP\gamma S$  [18,19]. In addition, PKA and PKC exert their actions at least in part on late steps controlling the exocytotic process itself [20,21]. Finally, several inhibitory hormones and neurotransmitters alter levels of second messengers only transiently or partially, but completely inhibit secretion [13]. All these observations point towards a more direct mechanism of regulation at the level of exocytosis itself.

## EXOCYTOSIS, A SEQUENCE OF EVENTS

### Characteristics of secretory granules

Neurotransmitters and hormones are packaged in distinct vesicles, mainly synaptic vesicles (SV) and secretory granules or LDCVs, respectively. Whereas SV are capable of undergoing rapid cycles of exocytosis, endocytosis, refilling with transmitters and a subsequent round of fusion, LDCVs have to bud from the trans-Golgi network to ensure their filling with the appropriate biosynthetic cargo (see Fig. 2). LDCVs have to process their peptide hormones and shed their coat proteins to achieve maturation [22]. Subsequently, exocytosis proceeds in sequential steps: vesicle recruitment and docking to the plasma membrane, vesicle priming and, finally, fusion between vesicle and plasma membranes by the generation of a fusion pore (see Fig. 2).

Certain features have to be taken into account in the study of  $\beta$ -cell exocytosis. Similar to SVs, exocytosis of LDCVs in chromaffin or  $\beta$  cells is linked to endocytosis, which occurs



**Fig. 2. Schematic presentation of exocytosis.** LDCVs such as secretory granules (LDCV/SG) but from the trans Golgi network (TGN, 1) yielding immature secretory granules (ISG) which shed their coat proteins (2) and become mature secretory granules (MSG). They have to traverse the subcortical actin network (3) and dock to the plasma membrane (4). Subsequently, LDCVs become primed in an ATP-dependent reaction (4) and their membranes fuse with the plasma membrane (5). The ensuing endocytosis retrieves the membrane material, which is redirected to the TGN through the endocytotic pathway (6). Exocytosis of synaptic vesicles SV (7) also implies vesicle recruitment, docking, priming and fusion. However, in contradistinction to LDCV/SGs, SV may undergo several cycles of exocytosis either by recycling only through the endosomal compartment (8) or eventually by only partial fusion with transient opening of a fusion pore (kiss and run, 9).

rapidly if massive stimulation has been applied [23,24]. As several proteins implicated in exocytosis may also have a role in endocytosis [25,26], this close link may complicate their analysis under certain experimental conditions. Moreover, pancreatic endocrine cells express many gene products characteristic of neurons, although they are probably of endodermal origin [27]. It is therefore not surprising that peptide-secreting endocrine cells such as the  $\beta$  cell contain a class of microvesicles, termed synaptic-like microvesicles (SLMVs), in addition to LDCVs. SLMVs resemble SV in membrane composition, biogenesis and life cycle [28]. They have been suggested to store and secrete neurotransmitters, which may act as paracrine signalling molecules. Global assays may not always permit the distinction between events linked to SLMVs vs. phenomena proper to LDCVs.

### Vesicle recruitment and docking

Vesicle recruitment requires the myosin–actin system and ATP, which may serve as an energy donor for molecular motors such as myosin and protein kinases including the myosin light chain kinase [29]. A requirement for actin network disassembly has been suggested for primary  $\beta$  cells by the use of *Clostridium botulinum* C2 toxin [30]. Continuous vesicle recruitment prevents the depletion of releasable vesicles. In nerve terminals this process is supported by the liberation of  $\text{Ca}^{2+}$  from intracellular stores [31]. Such a mechanism may explain the enhancement of insulin exocytosis by the neurotransmitter acetylcholine in  $\beta$  cells and probably involves several kinases [32,33]. The concept

of vesicle docking stems from ultrastructural visualization in neuronal and neuroendocrine cells of vesicles in close proximity to the plasma membrane. In general, manipulation of protein function leading to the accumulation of vesicles at the plasma membrane has been interpreted as an interference with the subsequent fusion step by the altered proteins [34]. Recently developed optical methods should permit dynamic investigation of docked vesicles and increase our understanding of this important step in exocytosis [35,36].

### Vesicle priming and fusion

Biochemical assays established the absolute necessity of ATP at a stage prior to the release of vesicle contents, a step termed ‘priming’, in all cellular systems studied [18,37,38]. This process requires submicromolar  $\text{Ca}^{2+}$  [39] and involves alterations in membrane lipids by phosphatidylinositol transfer protein and, by type I phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) [40]. PI4P5K has also recently been cloned from clonal  $\beta$  cells and may play a part in insulin exocytosis [41]. Glucose metabolism in  $\beta$  cells has been proposed to influence the priming state of insulin containing LDCVs by virtue of altering the levels of intracellular ATP [42]. However, such a mechanism is likely to be of minor importance as  $\text{Ca}^{2+}$ -induced insulin secretion is still preserved in clonal  $\rho$ -mutant  $\beta$  cells, which are devoid of metabolically competent mitochondria [43]. We still have only a faint notion about the final fusion pore itself. Although this structure has the initial size of a small ion channel [44], the relative contribution of proteins vs. lipids is still unresolved [45].

Electrophysiological investigations lead to the distinction between a small readily releasable pool of granules from a larger pool of slowly released granules in different systems [42,46]. Primed and readily releasable vesicles may represent the same vesicle pool. However, the terms docking, priming and readily releasable pool are operational definitions derived from different observation methods and we do not yet know the precise correlation between electrophysiological, biochemical and morphological descriptions.

## THE SNARE HYPOTHESIS

### The SNARE complex

A unifying hypothesis has been proposed to account in molecular terms for membrane docking and/or fusion of transport vesicles throughout the secretory pathway from the endoplasmic reticulum to the plasma membrane [47]. This process is thought to be induced by the pairing of specific proteins on the vesicle membrane, termed v-SNAREs, with cognate proteins on the target membrane, the t-SNAREs. These proteins mainly interact via coiled–coiled regions present in distinct helices and are capable of forming extremely stable complexes arranged in ring-like structures [48]. On addition of the cytosolic components NSF (*N*-ethylmaleimide-sensitive factor) and  $\alpha/\beta$ -SNAPs, this complex is converted into a multisubunit aggregate, the 20S particle. This complex contains ATP and is disassembled when NSF hydrolyzes the nucleotide. The use of particular v- and t-SNAREs may confer spatial specificity as suggested by the observation of multiple isoforms with distinct subcellular localizations and defined interactions [49]. The exocytotic machinery is not only defined by the SNARE proteins but also by additional proteins, such as munc18/sec1 and munc13 [50,51], which regulate their function. Moreover, the machinery is thought to encompass a large number of additional gene

products which intervene at different steps of exocytosis. Indeed, studies in yeast and in mammalian cells already indicated the presence of a large multimeric protein complex termed the exocyst [52,53].

The functional importance of the v-SNAREs VAMP/synaptobrevin/cellubrevin and the t-SNAREs syntaxin and SNAP-25 have been confirmed in exocytosis by the correlation between the blockade of exocytosis by clostridial neurotoxins and their selective cleavage [54–56]. In addition, v- and t-SNAREs reconstituted into phospholipid vesicles have been reported to produce complete lipid mixing between distinct vesicles [57]. However, the observed efficiency and kinetics differ largely from those in biological systems and several assumptions of the initial SNARE hypothesis remain a matter of debate. At least in *Drosophila*, spontaneous exocytosis persists in the absence of synaptobrevin [58,59]. Moreover, their spatial organization, the temporal sequence of events and their precise function in priming, docking and/or fusion are still to be resolved (reviewed in [60,61]).

### SNARE proteins in $\beta$ cells

The SNARE proteins were initially regarded to be neuron-specific. The immunochemical identification of the v-SNAREs and t-SNAREs synaptobrevin, syntaxin and SNAP-25 in primary and clonal  $\beta$  cells, along with NSF and  $\alpha$ -SNAP suggested their general role in exocytosis [62,63]. As insulin-secreting cells are impermeable to tetanus (TeNT) or botulinum neurotoxins (BoNT), functional studies have been conducted either in streptolysin-O permeabilized cells, which allow the dialysis of macromolecules and subsequent stimulation of insulin exocytosis by  $\text{Ca}^{2+}$  [64], or after transient expression of BoNT light chains [65]. These approaches demonstrated the requirement for synaptobrevin, SNAP-25 and syntaxin in  $\text{Ca}^{2+}$ -evoked exocytosis of insulin [66–68]. Similar results have been obtained for syntaxin using a functional antibody [69], whereas dialysis of cytosol and the use of recombinant proteins demonstrated a role for  $\alpha$ -SNAP [63]. Insulin-secreting cells are endowed with several isoforms of SNARE proteins as synaptobrevin/VAMP can be replaced by the isoform cellubrevin and functional knock-out of SNAP-25 can be recovered by SNAP-23 [70,71]. Interestingly, certain forms of tetanus toxin-insensitive exocytosis were found in several systems including insulin exocytosis in clonal  $\beta$  cells [66,72,73]. As a toxin-insensitive isoform of VAMP, TI-VAMP, has recently been described [74], these observations may suggest specificity for SNARE isoforms in defined circumstances. This is corroborated by studies on syntaxin, which suggested a difference between syntaxin 1a and syntaxin 1b [75].

### CONSTITUTIVE AND REGULATED EXOCYTOSIS

In contrast to constitutive exocytosis, neurotransmission and release of potent hormones have to be tightly controlled. The capacity of the  $\beta$  cell to respond to stimulation is influenced by a balance between protein phosphorylation and dephosphorylation [21]. Putative targets for such a regulatory mechanism at the level of the docking and fusion machinery have been suggested from *in vitro* experiments [76–79]. The physiological relevance of these post-translational alterations is currently unclear except for VAMP, where phosphorylation is seemingly not required for  $\text{Ca}^{2+}$ -stimulated insulin exocytosis in clonal  $\beta$  cells [70]. As these SNARE proteins have counterparts in yeast, a model of constitutive exocytosis, they may form targets for important

long-term adaptations, but are less likely to confer regulatory features for the acute control of exocytosis. In contrast, regulated exocytosis is strongly dependent on  $\text{Ca}^{2+}$  and is modulated by guanine nucleotides. The control of cytosolic  $\text{Ca}^{2+}$ , the action of its effectors and the guanine nucleotide-dependent systems are therefore central to the regulation of exocytosis.

### $\text{Ca}^{2+}$ ACTION IN EXOCYTOSIS

#### $\text{Ca}^{2+}$ concentrations and exocytosis

Calcium constitutes the major stimulus for exocytosis. Exocytotic vesicles undergo exocytosis at different concentrations of  $\text{Ca}^{2+}$  depending on their functional state and the cellular system.  $\text{Ca}^{2+}$  regulates several steps in exocytosis, such as the size of vesicle pools, the fusion event and the size of the fusion pore, and may act on distinct protein targets [80–82]. The replenishment of a releasable vesicle pool occurs at submicromolar  $\text{Ca}^{2+}$  and implies a phorbol-ester-sensitive site such as PKC [83]. In contrast, the apparent  $\text{EC}_{50}$  of  $\text{Ca}^{2+}$  in the fusion event ranges from an  $\text{EC}_{50}$  of 190  $\mu\text{M}$  as in bipolar retinal neurons releasing vesicles at a maximal rate of 2000–3000 per second [82] to low micromolar levels in dorsal root neurons [84] or neuroendocrine cells [85]. The observed differences in  $\text{Ca}^{2+}$ -sensitivity and release rates may be vesicle-specific rather than cell-specific [86]. Moreover, they may result from the organization of the exocytotic site [87] and the number of vesicles in close proximity to the point of  $\text{Ca}^{2+}$  influx.

$\text{Ca}^{2+}$  imaging in single rat  $\beta$  cells demonstrated that glucose elicited an initial rise at the periphery of the cell with subsequent spreading throughout the cytosol and an average  $[\text{Ca}^{2+}]_i$  in the range of 300–600 nM [7]. Owing to the strong buffering capacity of the cytosol, reliable measurements of  $[\text{Ca}^{2+}]_i$  at the cytosolic mouth of the  $\text{Ca}^{2+}$  channels have not been performed in  $\beta$  cells.  $\text{Ca}^{2+}$  concentrations could reach values of 100  $\mu\text{M}$  or more as observed in smooth muscle cells using  $\text{Ca}^{2+}$ -sensitive fusion proteins situated close to the  $\text{Ca}^{2+}$  channel itself [88]. Hence the true  $\text{Ca}^{2+}$  concentration required for insulin secretion is not known. Experimental approaches that allow the equilibration of free  $\text{Ca}^{2+}$  over the whole cytoplasm determined the overall macroscopic  $\text{Ca}^{2+}$  dependency of insulin secretion regardless of granule subsets and distinct functional states. Fluorescent methods coupled to capacitance measurements or biochemical approaches in permeabilized cells revealed maximal secretion at 7–10  $\mu\text{M}$  of free  $\text{Ca}^{2+}$  and an  $\text{EC}_{50}$  of 1.6  $\mu\text{M}$  free  $\text{Ca}^{2+}$  [18,89]. It should, however, be noted that higher  $\text{EC}_{50}$  values have been reported ( $K_d$  20  $\mu\text{M}$ ), which resemble more closely values found in neuroendocrine cells [90,91]. The latter may apply for subpopulations of exocytotic vesicles in  $\beta$  cells, such as readily releasable LDCVs, whereas the lower values may represent contribution of various steps prior to membrane fusion.

#### $\text{Ca}^{2+}$ channels and exocytosis

Subsequent to  $\text{Ca}^{2+}$  influx, synaptic vesicle exocytosis occurs in less than a millisecond suggesting a physical link between  $\text{Ca}^{2+}$  channels and docked vesicles [92]. Indeed, physical interactions between SNARE proteins and neuronal P/Q or N-type  $\text{Ca}^{2+}$  channels, but not L-type  $\text{Ca}^{2+}$  channels, occur at a restricted region, namely the intracellular loop connecting domains II and III of the  $\text{Ca}^{2+}$ -channel  $\alpha_1$ -subunit ( $L_{II-III}$ ) [93–95]. Such interactions may be altered by protein phosphorylations [96] and eventually provide regulation of  $\text{Ca}^{2+}$  influx pending on SNARE protein status [93,97]. However, several electrophysiological

investigations clearly suggest that these protein interactions organize exocytosis at the site of maximal presynaptic  $\text{Ca}^{2+}$  influx, although the exocytotic machinery on the plasma membrane occurs not only in close vicinity to  $\text{Ca}^{2+}$  channels [98].

While pancreatic  $\beta$  cells express N-, P/Q- and L-type  $\text{Ca}^{2+}$  channels [99], the major contribution to  $\text{Ca}^{2+}$ -influx eliciting insulin exocytosis is contributed by L-type channels [100]. A spatial relationship between L-type  $\text{Ca}^{2+}$ -channels and the site of insulin exocytosis was suggested by the observation that depolarization-induced  $\text{Ca}^{2+}$  influx has a more pronounced effect on exocytosis than intracellular  $\text{Ca}^{2+}$ -mobilization [23]. Moreover,  $\text{Ca}^{2+}$ -influx sites have been found to be polarized at the part of the cell containing the secretory granules [89]. This macroscopic arrangement may serve to restrict maximal  $\text{Ca}^{2+}$  levels to the exocytotic pole of the cell. Although SNARE proteins did not bind to recombinant L<sub>II-III</sub> of L-type channel  $\alpha_1$ -subunits [93,95,101], a functional interaction has been reported upon their coexpression in *Xenopus* oocytes [102] and peptide injection in primary  $\beta$  cells [103]. The question whether such a mechanism mediates polarized insulin exocytosis will only be answered after demonstration of physical and functional interactions in endocrine cells *in situ*.

Exocytotic vesicles and  $\text{Ca}^{2+}$  channels may be co-ordinated by chaperones such as the cysteine string proteins (Csps). Csps were first discovered as presynaptic vesicle proteins in *Drosophila* and a densely clustered string of 12–15 cysteine residues gave rise to their name [104]. Deletion of the Csp gene in *Drosophila* causes a temperature-sensitive block of synaptic transmission, followed by paralysis and premature death [105,106]. This deficiency involves only exocytosis elicited by  $\text{Ca}^{2+}$  influx through presynaptic  $\text{Ca}^{2+}$  channels [107]. Interestingly, it has recently been shown by Leveque *et al.* [108] that Csp1 interacts *in-vitro* with presynaptic  $\text{Ca}^{2+}$  channels and the SNARE protein VAMP. This led to the proposal that Csp acts as a chaperone during the assembly, rearrangement or disassembly of protein complexes at this specific site of exocytosis. Such a function of Csp may provide a molecular basis for its earlier suggested role as a link between SV and presynaptic  $\text{Ca}^{2+}$  channels [109]. In pancreatic  $\beta$  cells Csps are expressed on insulin-containing vesicles [110,111] and suppression of Csp expression inhibits insulin secretion and exocytosis [110]. Chaperone activity, but not the regulation of ion channels by Csp is required for its function in  $\beta$  cells [110–112]. These observations may further indicate that  $\text{Ca}^{2+}$  channels may provide a spatial organization of insulin exocytosis as discussed above.

## TARGETS OF $\text{Ca}^{2+}$ IN EXOCYTOSIS

### $\text{Ca}^{2+}$ -binding proteins

$\text{Ca}^{2+}$  acts at several defined steps during exocytosis and distinct protein targets for its effect on exocytosis have been characterized [39,80–82]. Candidate proteins include calmodulin, CaM Kinase II ( $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase II), annexin and CAPS ( $\text{Ca}^{2+}$ -dependent activator protein for secretion). Moreover, a number of proteins such as synaptotagmins, unc13, doc2 and rabphilin contain C<sub>2</sub> domains, which mediate  $\text{Ca}^{2+}$ -induced attachment to lipid membrane proteins [113]. Work on CamKinase II and one of its substrates, synapsin, suggested their role in exocytosis [114]. CaM kinase II  $\delta_2$ , the predominant subtype in insulin-secreting cells, is associated with insulin secretory granules [115]. However, at least in neurons, CamKinase II and synapsin are not indispensable for exocytosis to occur [116] and their role may be confined to early

steps in exocytosis, such as vesicle recruitment to the plasma membrane. Similarly, diminished levels of rabphilin produced only subtle changes of the phenotype [117]. Most intriguingly unc13 contains C<sub>2</sub> and C<sub>1</sub> domains. It binds diacylglycerol and phorbol esters via its C<sub>1</sub> domain and subsequently interacts with Doc2 [118]. This may provide the molecular basis for the activation of insulin exocytosis by diacylglycerol [18]. CAPS exhibits  $\text{Ca}^{2+}$ -dependent binding to phospholipids and aggregates liposomes [119]. It may thereby appose vesicle and plasma membrane bilayers in the preparation of exocytotic fusion. The phenotype in loss-of-function mutants of its *Caenorhabditis elegans* analogue, *unc-31*, suggests its major role in neurosecretion. CAPS is expressed in neurons, neuroendocrine and insulin-secreting cells and is specifically involved in the exocytosis of LDCVs, but not of SVs in synaptosomes [120].

### Synaptotagmin as a $\text{Ca}^{2+}$ sensor

The  $\text{Ca}^{2+}$ - and phospholipid-binding protein synaptotagmin has been extensively studied [113]. The deletion mutation of one of its isoforms, i.e. synaptotagmin I in transgenic mice or of *Drosophila* synaptotagmin, leads to a major impairment of the fast component of  $\text{Ca}^{2+}$ -induced neuroexocytosis and changes in  $\text{Ca}^{2+}$  cooperativity have been reported [121–124]. This change in cooperativity is currently the best indication for a role as a specific  $\text{Ca}^{2+}$  sensor in exocytosis. Moreover, biochemical data indicate that synaptotagmin acts after ATP on primed vesicles, as would be expected for a  $\text{Ca}^{2+}$  sensor [125]. Synaptotagmin is an integral membrane protein of SV and of neurohormone containing secretory vesicles. It is composed of a short intravesicular N-terminal sequence, a single transmembrane region and a large cytosolic portion [113]. The main features of the cytosolic part are two  $\text{Ca}^{2+}$ -binding repeats, the C<sub>2</sub> domains, to which several functions have been assigned. The first C<sub>2</sub> domain (C<sub>2</sub>A) binds to phospholipids at low micromolar free  $\text{Ca}^{2+}$  and to the plasma membrane protein syntaxin 1 at several hundred micromolar  $\text{Ca}^{2+}$  [126]. The latter  $\text{Ca}^{2+}$  requirement parallels that necessary for neuroexocytosis [82]. In addition to  $\text{Ca}^{2+}$ -sensitive actions, the second C<sub>2</sub> domain (C<sub>2</sub>B) binds independently from  $\text{Ca}^{2+}$  inositol-1,3,4,5-tetrakisphosphate (IP<sub>4</sub>), inositol-1,2,3,4,5-pentakisphosphate (IP<sub>5</sub>) and inositol-1,2,3,4,5,6-hexakisphosphate (IP<sub>6</sub>) [127]. These events may mimic binding to membrane phospholipids as the injection of IP<sub>4</sub> into squid giant synapse preterminal inhibits neuroexocytosis specifically at the C<sub>2</sub>B domain [128]. The IP<sub>4</sub>-binding site in the C<sub>2</sub>B domain may therefore mediate attachment to lipid membranes and this function might be controlled by cellular levels of IP<sub>4</sub>, IP<sub>5</sub> or IP<sub>6</sub>.

Synaptotagmin is, however, probably not the only  $\text{Ca}^{2+}$  sensor, as slow components of stimulated neurotransmission persist in its absence [121–123]. Interpretation of *in-vivo* data on synaptotagmin function is not always straightforward. Observed effects suggest both a stimulatory role as well as an inhibitory (clamp) function in exocytosis, diminishing noise and enhancing transmission fidelity [121,129]. These two facets may be explained by different functions of the two C<sub>2</sub> domains of synaptotagmin. Moreover, the protein may also play a part in endocytosis [25] and numerous isoforms and putative homologues of synaptotagmin have been described, which exhibit distinct *in-vitro* properties and cellular distribution [113,126,130–132]. It is therefore not surprising that the precise physiological role of synaptotagmin is still a matter of debate.

### Synaptotagmins in $\beta$ cells

The presence of several synaptotagmin isoforms in non-neuronal tissues suggested their role in endocrine exocytosis

as well [62,130,132]. Synaptotagmin I and II are expressed in clonal pancreatic  $\beta$  cells and are specifically required for  $\text{Ca}^{2+}$ -evoked hormone exocytosis [133]. Their activity requires epitopes of the first  $\text{C}_2$  domains known to bind phospholipids. As this effect occurs at low micromolar  $\text{Ca}^{2+}$ , it may represent the physiological basis for the function of synaptotagmin I and II in endocrine exocytosis. In contrast to neuroexocytosis,  $\text{IP}_4$ ,  $\text{IP}_5$  and  $\text{IP}_6$  stimulate insulin exocytosis [134]. Their action may, however, be mediated by effectors different from the  $\text{C}_2\text{B}$  domain of synaptotagmin such as protein phosphatases known to be involved in insulin exocytosis [21,135]. This interpretation is also suggested by the observation that mutations in the  $\text{IP}_4$ -binding site of synaptotagmin II did not alter insulin exocytosis [133]. Primary  $\beta$  cells express synaptotagmin III, which has been advocated as a candidate in endocrine cells due to its expression pattern and high  $\text{Ca}^{2+}$  affinity [126,130]. Insulin exocytosis is indeed inhibited by an isoform-specific IgG in one clonal cell line [136]. However, synaptotagmin III is also expressed in neurons, which argues against a simple cell-type specificity [132] and the true  $\text{Ca}^{2+}$  affinity of insulin exocytosis is unknown (see above). In fact, specific patterns may arise by combinations of isoforms expressed within the same cell on the same vesicle as initially suggested by Hilbush and Morgan [137]. Synaptotagmin IV is expressed as an embryonal and as an adult form. Pancreatic  $\beta$  cells in culture contain almost exclusively the embryonal form, which is not located on exocytotic vesicles (J. Lang, unpublished observations). Its function has still to be determined.

## EFFECTS OF GUANINE NUCLEOTIDES ON EXOCYTOSIS

Exocytosis can be induced by intracellular application of GTP or its slowly hydrolyzed analogue  $\text{GTP}\gamma\text{S}$  independently of  $\text{Ca}^{2+}$  in a variety of systems including chromaffin cells and neutrophils, as well as in clonal and primary  $\beta$  cells [18,138–140]. The pharmacological effect of GTP gave rise to the hypothesis that a G-protein, termed  $\text{G}_E$  for exocytosis, directly regulates this final step in secretion [141]. The term  $\text{G}_E$  implies a regulatory G-protein acting on exocytosis without the involvement of soluble second messengers but does not determine its precise nature, subcellular location or final outcome of its action, i.e. inhibitory or stimulatory. It is obvious that GTP or its analogues will interfere with a number of different GTPases including the heterotrimeric G-proteins, the ras-like monomeric G-proteins and dynamin. Several pharmacological tools such as aluminium fluoride or mastoparan have been employed to distinguish between monomeric and heterotrimeric G-proteins. Unfortunately, the specificity of these tools is questionable [142,143].

$\text{GTP}\gamma\text{S}$ -induced insulin exocytosis exhibits several distinct features (Table 1). This mode of stimulation is characterized by slow but persistent hormone release [18,19,140] and does not require the activation of protein kinases A and C or phospholipase C [18]. The regulation by guanine nucleotides is preserved in streptolysin-O permeabilized cells, which permit the dialysis even of macromolecules [61,144]. This stimulation is therefore independent of diffusible second messengers or ion fluxes. As both  $\text{Ca}^{2+}$ -induced and  $\text{GTP}\gamma\text{S}$ -induced exocytosis require ATP [18,19,66], their site(s) of action should be located after vesicle priming. Glucose metabolism alters the cytosolic levels not only of ATP but also of GTP [145]. It has been proposed that glucose metabolism may thereby be linked to the regulation of exocytosis [146]. However, as G-proteins are enzymes tightly co-ordinated by proteins controlling GTPase activation and nucleotide exchange (see below), a regulation of exocytosis solely by changes of the levels of endogenous GTP seems unlikely.

It is of interest that certain SNARE proteins and cytosolic factors are not required for  $\text{GTP}\gamma\text{S}$ -induced vesicular hormone release as it is insensitive to tetanus toxin, which cleaves VAMP, to syntaxin peptides, to the dialysis of cytosol and to mutations in the first  $\text{C}_2$  domain of synaptotagmin [63,133,147]. The basis for the differential behaviour between  $\text{Ca}^{2+}$ - and  $\text{GTP}\gamma\text{S}$ -induced exocytosis may be the following: (a) the two stimuli act on distinct vesicle pools endowed with different sets/isoforms of SNARE proteins or other components of the exocytotic docking/fusion machinery, and (b) alternatively,  $\text{GTP}\gamma\text{S}$  and  $\text{Ca}^{2+}$  act at distinct steps of the fusion process of the same vesicle and  $\text{GTP}\gamma\text{S}$  may drive exocytotic vesicles into a slow release pathway that is no longer sensitive to  $\text{Ca}^{2+}$ . The latter hypothesis is suggested by kinetic studies in permeabilized cells as the addition of  $\text{GTP}\gamma\text{S}$  prior to  $\text{Ca}^{2+}$  inhibits the cation-induced hormone release whereas concomitant addition of  $\text{GTP}\gamma\text{S}$  and  $\text{Ca}^{2+}$  slightly potentiates the effect of the cation [140]. Moreover, the inhibition of  $\text{Ca}^{2+}$ -stimulated release by  $\text{GDP}\beta\text{S}$ , which locks G-proteins in their inactive conformation [20,21], suggests that a GTP-binding protein acts in parallel to  $\text{Ca}^{2+}$ .

The general concept of G-protein control in intracellular vesicle trafficking and exocytosis is appealing as G-proteins are well designed molecular switches [149,150]. Their active life-span is tightly associated with the inherent GTPase activity, which is in turn co-ordinated by a number of proteins including GDP/GTP exchange factors (receptors, GEF) and GTPase-activating proteins (GAP) (effectors, GAP, RGS). As not only G-proteins, but also their regulators constitute large families of differentially distributed isoforms, sequence-specific interactions are guaranteed. This design ensures a precise temporal and spatial organization, an important feature required for the delivery and fusion of vesicles at the right place and time.

**Table 1. Characteristics of  $\text{Ca}^{2+}$ -evoked and of  $\text{GTP}\gamma\text{S}$ -evoked insulin exocytosis in  $\beta$  cells**

	$\text{Ca}^{2+}$ -stimulated exocytosis	$\text{GTP}\gamma\text{S}$ -stimulated exocytosis
Rate ( $\text{granules}\cdot\text{s}^{-1}$ )	1–60 <sup>a</sup>	5 <sup>a</sup>
Maximal secretion (granules)	4000/2 min <sup>a</sup>	1000/10 min <sup>a</sup>
$\text{Ca}^{2+}$	0.5–10 $\mu\text{M}^{\text{a-c}}$	Not required <sup>a,b</sup>
Effect of GDP or $\text{GDP}\beta\text{S}$	Inhibition <sup>a,b</sup>	Inhibition <sup>a,b</sup>
ATP	Required <sup>a,b</sup>	Required <sup>a,b</sup>
Cytosol	Required <sup>d</sup>	Not required <sup>d</sup>
$\alpha$ -SNAP	Required <sup>d</sup>	Not required <sup>d</sup>
BoNT/TeNT sensitive	Fully sensitive <sup>e</sup>	Not always sensitive <sup>e,f</sup>
Synaptotagmin	Required <sup>e</sup>	Not required <sup>e</sup>
$\text{G}\beta\gamma$	Required <sup>h</sup>	Required <sup>h</sup>

<sup>a</sup>[19], <sup>b</sup>[18]; <sup>c</sup>[140]; <sup>d</sup>[63]; <sup>e</sup>[64–68]; <sup>f</sup>Lang and Niemann, unpublished observations; <sup>g</sup>[133]; <sup>h</sup>[208].

## MONOMERIC G-PROTEINS REGULATING EXOCYTOSIS

### The Rac, Rho and ARF subfamilies

The superfamily of small GTP-binding proteins consists of more than 50 members from yeast to mammal and large numbers of them are implicated in intracellular vesicle trafficking, including exocytosis [151]. The effect of clostridial exotoxins in pancreatic  $\beta$  cells suggests that only Rac but not Rho is needed for stimulated insulin secretion [152]. The ADP-ribosylation factors ARFs function at multiple vesicle trafficking events. The isoform ARF1 has been shown to regulate budding of secretory vesicles from the trans-Golgi network by controlling phospholipase D [153]. As ARF interacts with heterotrimeric G-proteins, this may provide a link between heterotrimeric and monomeric GTPases [154]. Indeed ARF6 is associated with secretory chromaffin granules and may be part of a complex with  $\beta\gamma$ -subunits of heterotrimeric G-proteins [155]. Interestingly, ARF can be redistributed in clonal  $\beta$  cells upon incubation with GTP $\gamma$ S and may therefore be involved in guanine-nucleotide-induced insulin exocytosis [156].

### The Rab subfamily

This group of proteins contains more than 40 members, which localize to distinct membrane compartments [150]. They exert functions in different trafficking steps on the secretory and endocytic pathways. Cycling of Rab proteins between the GDP-bound inactive and GTP-bound active forms and between the cytosol and membrane fractions is essential for their action in vesicle trafficking. The recruitment of the inactive GDP-bound form of Rab from the cytosol to the correct membrane compartment is initiated by the dissociation of Rab from its cytosolic carrier protein, GTP-dissociation inhibitor (GDI). At least one isoform of RabGDI is expressed in insulin-secreting cells and forms a cytosolic complex with several Rab proteins [157]. A functional role for RabGDI in the  $\beta$  cells is inferred from the observation that overexpression of this protein inhibits stimulated insulin secretion [158].

Subsequent to its dissociation from RabGDI, GDP-bound Rab is converted to the GTP-bound form by the action of proteins promoting GDP/GTP exchange such as Rab GDP/GTP-exchange protein (GEP) [159]. The association of GTP-bound Rab with the vesicle membrane depends on the presence of GEP and is thought to assist docking and eventually fusion of transport vesicles through the control of SNARE protein assembly [160–162]. Such a role is compatible with the phenotype of Rab3A-negative transgenic mice which do not exhibit any obvious change in synaptic transmission except during repetitive stimulation when synaptic vesicle recruitment to the presynaptic membrane becomes rate-limiting [117,163]. During or after membrane fusion the intrinsic GTPase activity of the Rabs is stimulated by a Rab-specific GAP. Once the GDP-bound form is produced, it is complexed with Rab GDI and returns to the cytosol fraction.

Rab3A associates with the secretory granules characteristic of pancreatic  $\beta$  cells [164]. Initial evidence for a functional role of Rab3A in insulin exocytosis relied on the introduction of synthetic peptides corresponding to the putative Rab3 effector domain in permeabilized cells [144,165]. Similarly, nutrient induced insulin exocytosis was reduced by overexpression of the Rab3A mutants that cannot cycle between the GDP and the GTP state [164]. Rab3A binds in a GTP-dependent manner to the putative downstream effector rabphilin, a C<sub>2</sub> domain containing soluble protein [117,166,167]. Overexpression of full-length

rabphilin, but not of deletion mutants lacking the C<sub>2</sub> domains, resulted in an increase in catecholamine secretion in chromaffin cells [168]. However, the Rab3A-binding sites of rabphilin are not required for correct targeting or for the stimulatory function in chromaffin and insulin-secreting cells [169,170]. Therefore, the interaction between Rab3A and rabphilin may either be of minor physiological importance in exocytosis or the complex between the two proteins has to be dissociated in order for exocytosis to occur. Interestingly, not only rabphilin, but also the Ca<sup>2+</sup>-binding protein calmodulin interacts with Rab3A and dissociates the G-protein from synaptic membranes *in vitro* in a Ca<sup>2+</sup>-dependent fashion [171]. Although the functional significance *in vivo* of these effects remains to be determined, they may provide links between Ca<sup>2+</sup>- and GTP-dependent regulation of exocytosis.

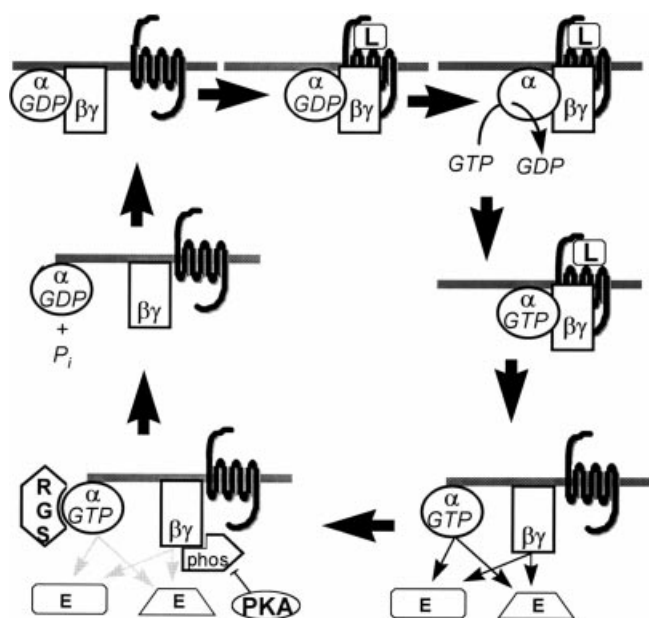
## HETEROTRIMERIC G-PROTEINS AS REGULATORS OF EXOCYTOSIS

Emerging evidence from a number of systems indicates that not only monomeric GTPases, but also heterotrimeric G-proteins are involved in vesicle transport steps throughout the secretory pathway. The heterotrimeric GTPases consist of an  $\alpha$ -subunit and a  $\beta\gamma$  dimer [149,172]. Activation of the  $\alpha$ -subunit by a receptor induces the exchange of GTP for GDP on the  $\alpha$ -subunit leading to the active conformation, that is a dissociation of the heterotrimer into  $\alpha_{\text{GTP}}$  and  $\beta\gamma$ . Both the  $\alpha_{\text{GTP}}$  and the  $\beta\gamma$ -subunits influence a number of intracellular effectors such as ion channels, adenylate cyclases, phospholipases and kinases (see Fig. 3). Moreover, G-protein  $\beta\gamma$ -subunits may provide scaffolds to organize protein-protein interactions as shown for the membrane translocation of G-protein coupled receptor-kinases during receptor desensitization [173]. The activity of heterotrimeric GTPases is terminated by the GTPase-activity intrinsic to the  $\alpha$ -subunit. In addition to receptors other regulatory proteins have been described. RGS (*Regulator of G-protein Signalling*) proteins stimulate the GTPase activity of  $\alpha$ -subunits thereby shortening signal transduction by G $\alpha$  [174]. The phosphoprotein phospho-ducin constitutes a ubiquitous regulator of G-protein function by its interaction mainly with G $\beta\gamma$  and blockade of G $\beta\gamma$ -stimulated cellular pathways [175,176]. Interestingly, phospho-ducin function is regulated by PKA, which provides an integration point for different signalling pathways.

A large number of isoforms of all three G-protein subunits have been identified [177]. The heterogeneity of isoforms offers a large combinatorial force although not every possible combination occurs. A series of elegant experiments using an antisense oligonucleotide approach revealed that the actual composition of the heterotrimer in terms of  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunit determines its specific activation by a receptor and its interaction with a distinct effector [178]. As, in addition, a single effector molecule such as adenylate cyclase is capable of integrating signals from distinct subunits [179], an extremely complex but finely tuned regulation results.

### Heterotrimeric G-proteins regulate vesicle transport throughout the biosynthetic pathway

The refined analysis of the subcellular localization of heterotrimeric G-proteins has demonstrated their presence on several endomembranes including SV and endocrine or exocrine granules [180–183]. Whereas the  $\alpha$ -subunit of G<sub>i3</sub> retards the constitutive transport through the Golgi in LLC-PK1 renal cells [184] the heterotrimer has been shown to stimulate histamine release from mast cells [185]. Moreover, G $\alpha_s$  and  $\beta\gamma$ -subunits



**Fig. 3. The GTPase cycle of heterotrimeric G-proteins.** The heterotrimeric GTPases consist of an  $\alpha$ -subunit and a  $\beta\gamma$ -dimer. Activation of the  $\alpha$ -subunit by a ligand-bound receptor induces the exchange of GTP for GDP on the  $\alpha$ -subunit leading to active conformation, that is a dissociation of the heterotrimer into  $\alpha_{GTP}$  and  $\beta\gamma$ . Both, the  $\alpha_{GTP}$  and the  $\beta\gamma$ -subunits regulate intracellular effectors. The activity of heterotrimeric GTPases is terminated by the GTPase-activity intrinsic to the  $\alpha$ -subunit. Subsequently,  $\alpha_{GDP}$  and  $\beta\gamma$  reassociate. In addition to receptors, other regulatory proteins have been described. RGS (Regulator of G-protein Signalling) proteins stimulate the GTPase activity of  $\alpha$ -subunits thereby shortening signal transduction by  $G\alpha$ . The phosphoprotein phosphoducin (phos) constitutes a ubiquitous regulator of G-protein function by its interaction with  $G\beta\gamma$  and blockade of  $G\beta\gamma$ -stimulated cellular pathways. Interestingly, the function of phosphoducin is inactivated by protein kinase A (PKA).

have been reported to stimulate transcytosis [186] while inhibiting endosome fusion [187]. In addition, the  $\beta\gamma$ -dimer of heterotrimeric G-proteins is involved in the organization of the Golgi apparatus [188,189] and G-proteins play a part in the vesicle biogenesis at the Golgi [190]. Moreover, G-proteins control the loading of SV with neurotransmitters and may thereby regulate quantal release in neurotransmission [191].

Extensive studies in chromaffin cells suggested a sequential involvement of distinct heterotrimeric G-proteins in the regulation of exocytosis.  $G_o$  is located on secretory granules [183] and inhibits exocytosis by lowering the affinity of the exocytotic machinery for  $Ca^{2+}$  [192,193] at an ATP-dependent ('priming') step, which is linked to rho-dependent disassembly of cortical actin filaments and phosphatidylinositol 4-kinase [194]. The growth-cone associated protein GAP43, a known stimulator of  $G_o$ , may provide an upstream signal [195], although the activator of GAP43 has still to be identified. The ATP-independent ('postpriming') step of chromaffin exocytosis has been reported to be positively regulated by  $G_i3$  through the activation of a phospholipase [196]. A comparable effect of  $G_o$  and GAP43 is probably not present in  $\beta$  cells as both are not localized to secretory granules (J. Lang, unpublished observations). However, other isoforms of heterotrimeric GTPases have been found in subcellular fractions enriched in insulin-containing granules or by electronmicroscopy on secretory granules [181,197]. Their activation by mastoparan coincides with a

stimulation of exocytosis and they may represent a target for  $GTP\gamma S$  in the stimulation of exocytosis.

### Inhibitory G-proteins in insulin exocytosis

To ensure adequate insulin release, the pancreatic  $\beta$  cell integrates messages from neurotransmitters and hormones acting on G-protein coupled receptors (GPCRs) and from intracellular signals generated by glucose. Pancreatic  $\beta$  cells express a wide variety of heterotrimeric G-proteins [198] and the role of the plasma membrane receptor activated heterotrimeric GTPases in the general regulation of insulin secretion has been fairly well investigated [9–13]. However, regulation of levels of second messengers such as  $Ca^{2+}$  or cAMP cannot solely explain the observed effect of GPCR activation on secretion (for example see [199–203]). A direct control of secretion at the level of exocytosis by plasma membrane activated G-proteins would offer several advantages to the cell. First, this mode of signal transmission is expected to be rapid and rather specific as compared with signal transmission via second messengers. The high combinatorial force offered by the assembly of distinct  $G\alpha\beta\gamma$  isoforms into heterotrimers probably provides sufficient selectivity for interactions with specific receptors and effectors. Second, prolonged elevations of second messenger concentrations such as  $Ca^{2+}$  are toxic for the cells and their obligatory dissipation constitutes an energy demanding process.

Controlled permeabilization of the cellular plasma membrane to gain access to the cytosol permits to maintain the concentrations of intracellular second messengers such as  $Ca^{2+}$  or cAMP at constant levels [64]. The persistence of GPCR-mediated inhibition of exocytosis under these circumstances indicates a general role for heterotrimeric GTPases in the direct control of secretion at a late step of exocytosis in pancreatic  $\beta$  cells and in the neuroendocrine clonal cell lines AtT-20 and PC12 [199–206]. The refined analysis of the inhibitory effect in streptolysin-O permeabilized clonal and primary  $\beta$  cells ruled out any participation of diffusible molecules including large cytosolic proteins and revealed that all  $\alpha_i$  isoforms and  $\alpha_o2$ , but not  $\alpha_o1$ , are efficient mediators of the inhibitory  $G_i/G_o$  effect [207]. Not only the  $G\alpha$ -subunit, but also the  $G\beta\gamma$ -dimer plays an important part in exocytosis. Indeed, inactivation of free  $G\beta\gamma$  at the level of the plasma membrane completely abolishes  $Ca^{2+}$ -evoked and  $GTP\gamma S$ -evoked insulin release in clonal  $\beta$  cells [208]. This situation is reminiscent of neutrophil exocytosis and of endocytosis, where a pool of free  $G\beta\gamma$  is required [209,210]. This defines  $G\beta\gamma$  as a crucial component in vesicle trafficking at the plasma membrane. Its upstream activator may be a GPCR or a component of the fusion machinery itself.

The effectors of heterotrimeric G-proteins in the receptor-induced direct control of insulin exocytosis are still matters of debate. On the basis of capacitance studies using inhibitors of the phosphatase calcineurin, this enzyme was proposed as the target of GPCR-activated  $G_i/G_o$  in  $\beta$ -cell exocytosis [199]. This observation is in line with earlier studies demonstrating the importance of phosphatases in insulin exocytosis [21]. However, inhibitors of calcineurin were without effect on the control of insulin exocytosis by GPCR as measured by insulin radioimmunoassay (Nasrebi, Wollheim and Lang, unpublished observations). The basis for the discrepancy may reside in the approach used to monitor exocytosis. Capacitance measurements were conducted to determine mainly the contribution of readily releasable vesicles, whereas biochemical assays assess the overall rate of exocytosis. Additional effectors of heterotrimeric G-proteins in the GPCR-induced direct control of insulin exocytosis have therefore to be taken into account. One

might speculate that heterotrimeric G-proteins interact with phospholipases, which prepare the plasma membrane for fusion or regulate the organization of the cytoskeleton as had been proposed for neuroendocrine cells [194,211]. Alternatively, they may control the final fusion pore, which has the property of a small channel [44], in analogy to their known effects on ion channels. Indeed, interactions between SNARE proteins and heterotrimeric G-proteins have been demonstrated in neuroexocytosis [97].

### Stimulation of exocytosis by G-proteins

Comparison of exocytosis as effector to more classical effector systems would predict the existence of GPCRs, which directly stimulate exocytosis. Direct stimulation was indeed demonstrated by the work on the insulin-like growth factor II (IGFII) receptor in ob/ob mice [212] and on the recently cloned receptor for the black-widow spider toxin  $\alpha$ -latrotoxin, latrophilin/Ca<sup>2+</sup>-independent receptor of latrotoxin (Latrophilin/CIRL) in insulin-secreting and in chromaffin cells [213,214].  $\alpha$ -latrotoxin is known to induce exocytosis by opening cation permeable pores and by a calcium-independent mechanism [215]. Purification of  $\alpha$ -LTX-binding sites in brain led to the discovery of neurexins, a highly polymorphic family of neuronal cell-surface proteins [216]. Neurexin I $\alpha$  constitutes a specific high-affinity binding site for  $\alpha$ -LTX [217] and physically interacts with the calcium-sensor protein synaptotagmin I [218]. These findings suggested that  $\alpha$ -LTX might cause neuroexocytosis by direct activation of synaptotagmin I via neurexin, thus bypassing the requirement of Ca<sup>2+</sup> to activate synaptotagmin. However, in pancreatic  $\beta$  cells sensitivity to the toxin correlates strictly with the expression of Latrophilin/CIRL, but not with the expression of neurexin I $\alpha$  or synaptotagmin I [213]. Note that neurexin I $\alpha$  contributes to the neuronal effects of  $\alpha$ -LTX [219]. Latrophilin/CIRL displays several intriguing features [220–222]. Its sequence contains the signature of GPCRs and is the longest GPCR currently known. It belongs to the class II of GPCRs, which encompasses the secretin/calcitonin receptor family known to bind biologically active peptides and to participate in various secretion processes. Although its effects probably imply the activation of a heterotrimeric G-protein, the identity of the latter has yet to be determined. Downstream interaction with a phospholipase has been proposed as a mechanism of its Ca<sup>2+</sup>-dependent effect on exocytosis in synaptosomes, thereby lowering the apparent Ca<sup>2+</sup> sensitivity of the fusion machinery [223]. Moreover, binding of Latrophilin/CIRL to the t-SNARE protein syntaxin has been described [221] and may play a part in the regulation of exocytosis. It is noteworthy that a functional interaction involving G-proteins and syntaxin has already been found in the regulation of presynaptic calcium channels [97], a known target for heterotrimeric GTPases and a presumed docking site for SV in neurons [92].

### CONCLUSIONS

During recent years observation of such diverse fields as yeast genetics, biophysics and neurosciences produced considerable progress in our understanding of the molecular mechanisms of exocytosis. This puzzling process requires a large number of different gene products and relies on the interactions between proteins and between lipids and proteins. For each component of the exocytotic machinery the precise role and its involvement in the distinct steps has now to be clarified. Pancreatic  $\beta$  cells express many of these components initially described in neurons suggesting a high degree of conservation in the secretory

**Table 2. Comparison of exocytotic proteins functionally identified in  $\beta$  cells and neurons**

	$\beta$ cells	Neurons
<b>SNARE proteins</b>		
SNAP-25	+ [67]	+
$\alpha$ -SNAP	+ [63]	+
NSF	n.d.	+
Syntaxin	+ [65,69]	+
Synaptobrevin	+ [66,68]	+
Latrophilin/CIRL	+ [213]	+
<b>Ca<sup>2+</sup>-binding proteins</b>		
CAPS	n.d.	-/+
Rabphilin	n.d.	+
Synaptotagmin	+ [133,136]	+
<b>Ca<sup>2+</sup>-channels</b>		
L-type	+ [5]	-
P/Q-type	n.d.	-+
N-type	+ [99,100]	+
<b>G-proteins</b>		
Rab3	+ [164]	+
G $\alpha_o$	+ [207]	+
G $\alpha_i$	+ [181,207]	n.d.
G $\beta\gamma$	+ [208]	n.d.
<b>Others</b>		
CSP	+ [110–112]	+

n.d., not determined.

process (see Table 2). Moreover, neuron-specific proteins such as neurexins and latrophilin are well expressed in  $\beta$  cells [213] and certain protein tyrosine phosphatases representing important pancreatic autoantigens in diabetes are found apart from  $\beta$  cells only in brain [224].

Insulin exocytosis is under the control of metabolic coupling factors generated by glucose and of hormones and neurotransmitters. Only for ATP/ADP the immediate consequences have been identified, i.e. a rise in intracellular calcium as an important trigger for exocytosis. The action of Ca<sup>2+</sup> is mediated by different Ca<sup>2+</sup>-sensing proteins including synaptotagmins and exocytosis probably occurs in close vicinity to voltage-dependent Ca<sup>2+</sup> channels. Exocytosis requires the function of the SNARE complex and its chaperones, such as NSF and CSP, although their precise action has still to be characterized. Moreover, insulin exocytosis is sensitive to GTP and major insights have been obtained in the organization of its putative targets, the monomeric and heterotrimeric GTPases. It is now clear that both regulate exocytosis directly. Whereas the rab proteins may control proper assembly of the exocytotic machinery, heterotrimeric G-proteins directly regulate a late step in exocytosis at still unknown effectors and thereby permit integration of neuronal and hormonal signals. In addition, liberation of Ca<sup>2+</sup> from intracellular stores as evoked by neurotransmitters, supports vesicle supply to the pool of releasable granules. This process involves various protein kinases, which may also participate in long-term adaptation through phosphorylation of exocytotic proteins.

The study of insulin exocytosis will continue to provide an interesting and useful model to elucidate the molecular basis of stimulation-secretion coupling. Moreover, therapeutic principles promoting hormone secretion have basically not changed during the last decades. The knowledge obtained on insulin exocytosis and its regulation now suggests new targets. To this purpose the chemical nature and the effectors of putative metabolic coupling factors as well as G-proteins and their activators should be

identified. In addition, the expression of  $\beta$ -cell isoforms of components of the exocytotic machinery has to be investigated to determine specific targets. Finally, pathological states such as NIDDM are accompanied by secretory defects and the study of exocytosis may in the long term provide insight into mechanisms of this disease.

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