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G-protein βγ-binding domains regulate insulin exocytosis in clonal pancreatic β-cells

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Abstract We have tested the putative role of G-protein β-subunits in insulin exocytosis by transient expression of βγ-binding proteins targeted to the plasma membrane. The PH domain of the G-protein-linked receptor kinase 2 fused to the transmembrane domain of a cell surface receptor and the α-subunit of the retinal G-protein transducin inhibited stimulated insulin release from intact and permeabilised HIT-T15 cells. This effect cannot be imputed to an increase in free Gα, as the RGS protein RGS3 did not reverse this effect. Among the isoforms of Gβ examined, Gβ2 was detected on the plasma membrane by confocal immunomicroscopy. These observations suggest a role for G-protein βγ-subunits in insulin exocytosis.

Key words: Exocytosis; Calcium; Insulin; G-protein; Guanosine triphosphate

1. Introduction

The peptide hormone insulin is stored in large dense-core vesicles (LDCVs) in pancreatic β-cells. Following transport to the plasma membrane and ATP-dependent priming of the vesicle, the hormone is released by exocytosis, that is fusion between vesicle membranes and the plasma membrane [1,2]. Exocytosis constitutes the final step in the biosynthetic pathway from the endoplasmic reticulum to the cell surface [3,4]. This membrane fusion event is triggered in a late step by Ca^{2+} and stimulated in a Ca^{2+}-independent manner by GTP or its more stable analogue GTPγS [5–7]. Several GTPases including the heterotrimeric GTP-binding proteins are known to regulate transport throughout the biosynthetic pathway [8–11]. Heterotrimeric G-proteins are activated by receptors through a GDP/GTP exchange at the α-subunit [12] and this leads to dissociation of the trimer into Gα and the Gβγ dimer. Both the α-subunit and the βγ-dimer regulate downstream effectors like ion channels, enzymes or kinases [12,13]. Termination of G-protein signalling is controlled by the GTPase activity inherent in the α-subunit and GTPase activation factors, such as the recently characterised RGS proteins [14], leading to reassembly of Gα-GDP with Gβγ into the inactive trimer Gα-GDPβγ.

Activation of the pertussis toxin (PTX)-sensitive G-proteins G_i/G_o by plasma membrane receptors for several inhibitory hormones and neurotransmitters, such as galanin, somatostatin and epinephrine, inhibits insulin exocytosis [15]. This regulation can still be observed in permeabilised cells and therefore occurs without the involvement of soluble second messengers [16–20]. We have previously demonstrated that the direct inhibition of insulin exocytosis can be mediated by the α-subunit of $G_i1,2,3$ or G_o2 and that it occurs after ATP-dependent priming, that is at a late step in exocytosis [21]. We have now addressed the question whether the βγ-dimer also plays a role in the regulation of insulin exocytosis. To this end we transiently expressed proteins endowed with βγ subunit-binding domains, such as the α-subunit of the retinal G-protein transducin (TD) [12] or the PH domain of G-protein-coupled receptor kinase 2 (GRK2PH) [22,23]. As free Gβγ associates with these proteins, Gβγ-mediated signalling is impeded [24–29].

We observed a pronounced effect of βγ subunit-binding domains on stimulated insulin secretion in intact cells as well as on insulin exocytosis in permeabilised cells stimulated by Ca^{2+} or by GTPγS. These observations indicate an important role for Gβγ-dimers in the direct regulation of insulin exocytosis.

2. Materials and methods

2.1. Materials

Recombinant pore-forming toxins were kindly provided by Dr U. Weller (Mainz, Germany). The plasmids pcDNA-TM and pcDNA-TM-GRK2PH [26] were kindly donated by Dr S. Gutkind (NIH, Bethesda, MD, USA), pcDNA3-RGS3 [30] and pcDNA-TDα [31] were generously provided by Dr R. Fisher (University of Iowa, Iowa City, IA, USA) and Dr R.C. Tsu (Hong Kong University of Science and Technology, Kowloon, China).

2.2. Cell culture, transient cotransfection, C-peptide release, immunoblots and confocal immunofluorescence microscopy

Culture of primary islet cells or HIT T15 cells, transient cotransfections, release experiments from intact or permeabilised cells and determination of human insulin C-peptide were performed as published [21,32,33]. Western blots and confocal immunofluorescence microscopy were performed as described previously [32,34,35]. The primary antibodies were used at the following dilutions: anti-CD8 (Boehringer, Rotkreuz, Switzerland) 1:4000; anti-insulin (Sigma, Buchs, Switzerland) 1:800; and isoform-specific anti-Gβ antibodies (Santa Cruz, Glaser AG, Bale, Switzerland) at 1:300 (immunofluorescence) or 1:3000 (immunoblots).

2.3. Statistical analysis

Results are presented as mean ± S.D. from experiments performed independently on at least three different cell preparations. Statistical analysis was performed by Student's two-tailed *t*-test for unpaired data (2*P*).

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Abbreviations: GRK2, G-protein-linked receptor kinase 2; PH domain, pleckstrin homology domain; RGS, regulator protein of G-protein signalling; TD, transducin; TDα, the α-subunit of the retinal G-protein transducin; TM, transmembrane domain of CD8; TM-GRK2PH, the PH domain of the G-protein-linked receptor kinase 2 fused to the transmembrane domain of CD8

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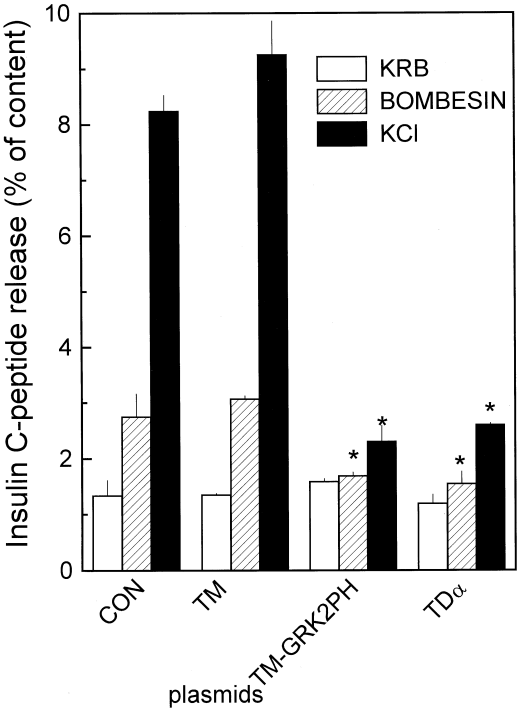

Fig. 1. Transient expression of βγ-binding domains inhibits insulin secretion from intact HIT-T15 cells. HIT-T15 cells were transiently cotransfected with a plasmid encoding human preproinsulin (pHINS) and either pcDNA3 (CON), the transmembrane domain of the surface receptor CD8 (TM), the PH domain of G-protein-coupled receptor kinase 2 fused to TM (TM-GRK2PH) or the α-subunit of the retinal G-protein transducin (TDα). 48 h later cells were washed and subsequently exposed for 8 min to Krebs-Ringer buffer alone (KRB) or KRB containing 50 mM KCl (KCl) or 10 μM of the receptor agonist bombesin (BBS). The secretion of the reporter gene human insulin C-peptide was measured by ELISA and values are expressed as percent of cellular content. $N = 8$–20 for each condition from four different experiments; *$2P < 0.01$ as compared to corresponding incubation in CON.

3. Results and discussion

3.1. Effects of Gβγ-binding proteins on insulin secretion from intact cells

We first examined the effect of Gβγ-binding domains on insulin release from intact HIT-T15 cells, a clonal pancreatic β-cell line. Recent reports suggest that heterotrimeric G-proteins influence membrane fusion or vesicle transport in the biosynthetic pathway prior to exocytosis [8–11]. To avoid interference with these earlier transport steps, we employed constructs targeted to the plasma membrane. The PH domain of GRK2 fused to the transmembrane domain of the cell surface receptor CD8, termed TM-GRK2PH, and TDα, the α-subunit of the retinal G-protein, have been shown to be expressed at the plasma membrane ([12,26]; see also below). To test the effect of their transient expression, cells were cotransfected with a plasmid encoding human preproinsulin (phINS) and the constructs to be studied. Release of human insulin C-peptide serves as reporter gene for exocytosis in the hamster insulin-secreting cell line HIT-T15 and permits the study of hormone release exclusively from cotransfected cells [21,32,33]. None of the constructs studied here altered the content (data not shown) or basal release (see Figs. 1–3) of the reporter gene product thus excluding an effect on early steps in the biosynthetic pathway prior to exocytosis.

As shown in Fig. 1 (CON), secretion of the reporter gene product human insulin C-peptide can be induced by KCl-induced membrane depolarisation and subsequent Ca^{2+} influx through voltage-dependent Ca^{2+} channels [1] or by the receptor agonist bombesin through activation of phospholipase C and liberation of Ca^{2+} from intracellular stores [36]. Expression of the transmembrane domain of CD8 alone (TM) did not alter secretion (Fig. 1, TM). However, human insulin C-peptide release evoked by either KCl or bombesin was largely reduced by expression of the Gβγ-binding PH domain of GRK2 linked to the transmembrane domain of CD8 (TM-GRK2PH). In addition, the transient expression of the Gβγ-binding protein TDα produced a comparable effect (see Fig. 1). These results suggest that binding of Gβγ and blocking of its signalling function inhibits insulin secretion.

Binding of Gβγ may increase the amount of free active Gα (Gα-GTP) and the observed effects might reflect the action of Gα-GTP. Indeed, we have shown previously that inhibition of insulin exocytosis can be mediated by the α-subunits of the PTX-sensitive G-proteins G_i/G_o [21]. Although such a mechanism seems unlikely [24–29], we tested this assumption directly. To this end we employed an isoform of the recently identified regulators of G-protein signalling, the RGS proteins

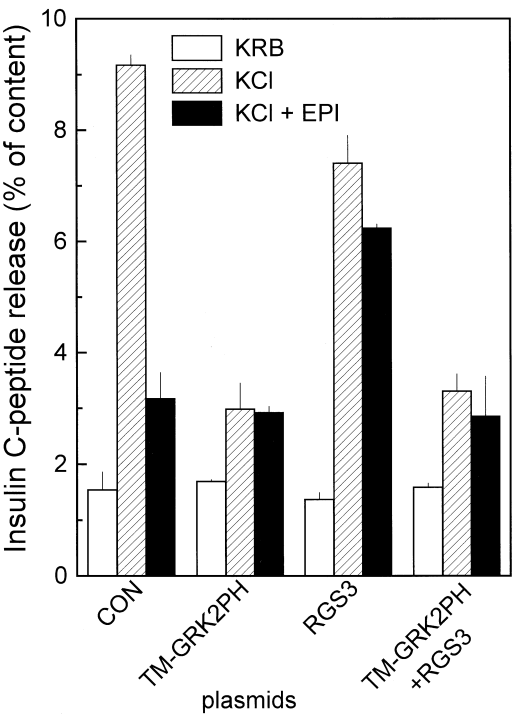

Fig. 2. Regulator of G-protein signalling (RGS3) reverts epinephrine-induced inhibition of secretion, but not the effect of TM-GRKPH. HIT-T15 cells were cotransfected with phINS (2.5 μg/well) and either pcDNA3 (CON, 1.25 μg/dish), TM-GRK2PH (TM-GRK2PH; 1.25 μg/well), RGS3 (RGS3, 1.25 μg/well) or RGS3 and TM-GRK2PH (1.25 μg of each/well). The total amount of cDNA was filled up to 5 μg/well with pcDNA3. 48 h later cells were washed and subsequently exposed for 8 min to KRB alone (CON), KRB containing 50 mM KCl (KCl) or containing 50 mM KCl and 10 μM epinephrine (KCl+EPI). Secretion of the reporter gene product was determined and values are expressed as in Fig. 1. $N = 8$ for each condition from four different experiments; *$2P < 0.01$ as compared to corresponding incubation in CON.

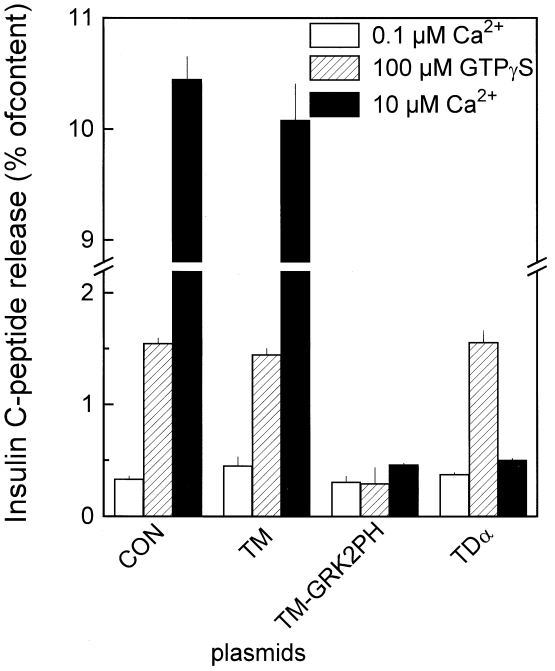

Fig. 3. Insulin exocytosis in permeabilised HIT-T15 cells is inhibited by βγ-binding domains. HIT-T15 cells were transiently cotransfected as in Fig. 1, permeabilised with α-toxin from *S. aureus* and subsequently exposed to 0.1 μM Ca^{2+}, 0.1 μM Ca^{2+} and 100 μM GTPγS or 10 μM Ca^{2+}. The exocytotic release of the reporter gene product was determined and values are expressed as in Fig. 1. $N = 10–13$ from five different experiments; $*2P < 0.05$ as compared to control cotransfections.

[37]. Similar to the effect of GAP on monomeric G-proteins [38], RGS proteins interact with Gα-subunits and enhance their GTPase activity thereby driving them into the inactive GDP-bound state [39]. If the effect of Gβγ-binding domains is mediated by the increased amount of active Gα (Gα-GTP), it should be antagonised by coexpression of RGS. One member of the RGS family, RGS3 [37], has been shown to specifically antagonise the action of PTX-sensitive G-proteins in intact cells [30]. To test whether RGS3 interacts also with $G\alpha_i$/$G\alpha_o$ in HIT-T15 cells, we investigated its effect on epinephrine-evoked inhibition of insulin secretion, which is known to be mediated by α_2-adrenergic receptors activating the α-subunit of a PTX-sensitive G-protein [21]. As shown in Fig. 2, epinephrine decreased KCl-induced secretion by 75%. Note that RGS3 by itself also slightly reduced stimulated secretion, an observation that was not unexpected: if RGS3 decreases the amount of Gα-GTP, it will favour the formation of inactive Gαβγ heterotrimers and thereby reduce the amount of free Gβγ. More importantly, expression of RGS3 completely reversed the effect of epinephrine (Fig. 2) demonstrating its capacity to antagonise the activation of $G\alpha_i$/$G\alpha_o$. However, RGS3 expression did not antagonise the inhibition of human insulin C-peptide release caused by the concomitant expression of TM-GRK2PH. This clearly demonstrates that the effect of Gβγ-binding domains cannot be explained in terms of increased amounts of active Gα.

3.2. Effects of Gβγ-binding proteins on exocytosis

The effect of Gβγ-binding domains on insulin secretion may be mediated by a number of mechanisms in intact cells. Gβγ dimers regulate several effectors such as adenylate cyclase, phospholipase C, Ca^{2+} channels and K^+ channels [12], which all participate in the expression of the secretory response [1]. To ascertain an effect on exocytosis, the final step in secretion, it was therefore necessary to exclude changes in soluble second messengers generated by these effectors. Permeabilisation of cells by the α-toxin from *Staphylococcus aureus* creates small pores and permits effective dialysis of soluble cytosolic second messengers [40] while preserving vigorous exocytosis [34,41,42]. In such permeabilised cells, exocytosis can be induced either by Ca^{2+} or in a Ca^{2+}-independent fashion by GTP and its stable analogue GTPγS [34,41,42]. As shown in Fig. 3, GTPγS and Ca^{2+} increased exocytosis of the reporter gene product human insulin C-peptide 4.5- and 30-fold, respectively. Again the expression of the transmembrane domain of CD8 (TM) did not alter the response. Similar to the observations in intact cells, the Ca^{2+}-evoked exocytosis was abolished by both βγ-binding proteins, that is TM-GRK2PH and TDα (see Fig. 3). In contrast, GTPγS-evoked exocytosis was only inhibited by TM-GRK2PH but not by TDα (see Fig. 3). Indeed, GTPγS added to the permeabilised cells will bind to transiently expressed TDα and activated TDα-GTPγS will be unable to scavenge free Gβγ-dimers.

To exclude the putative participation of cytosolic proteins, transiently cotransfected HIT-T15 cells were also permeabilised with streptolysin-O (SL-O). This bacterial haemolysin creates large pores in the plasma membrane, resulting in the complete dialysis of the cytosol including macromolecules [21,32,40]. In SL-O-permeabilised cells TM-GRK2PH again reduced Ca^{2+}-evoked exocytosis by 81 ± 14% as compared to control transfections ($n = 6$ for each condition, $2P < 0.01$) and TM alone did not produce any significant changes. The observations in permeabilised cells permit several conclusions. First, the effect of Gβγ-binding proteins takes place at the level of exocytosis and does not depend on the generation of second messengers or soluble proteins. Second, the observed effects are not due to cellular adaptations during the expression of these constructs, but are of acute nature. Indeed, if the observed changes were only of adaptive nature, we would expect changes also in the content or basal release of the reporter gene product. More importantly, addition of GTPγS to the permeabilised cells abolished the effect of transiently expressed TDα clearly demonstrating its acute reversibility.

3.3. Expression of Gβ and TM-GRK2-PH

We also investigated the endogenous expression of the targets of βγ-binding domains, namely the G-protein β-subunits, in insulin-secreting cells. Among the isoforms described [12], we restricted our analysis to Gβ1, Gβ2, Gβ3 and Gβ4. According to Western blot analysis (Fig. 4A) these isoforms are expressed to varying degrees in HIT-T15 cells, except for Gβ3, which was undetectable. Immunocytochemistry was performed to study their subcellular localisation. Costaining for insulin permitted analysis also in primary islet cells, a preparation always containing non-β-cells. As shown in Fig. 4B, Gβ2 was localised at the plasma membrane in HIT-T15 and primary β-cells. Transient expression of TM-GRK2PH in HIT-T15 cells led to its expression at the plasma membrane (Fig. 4B) and colocalisation with endogenous Gβ2. Gβ1 and Gβ4 were only found at intracellular sites distinct from the plasma membrane or secretory granules in HIT-15 and primary β-cells (data not shown).

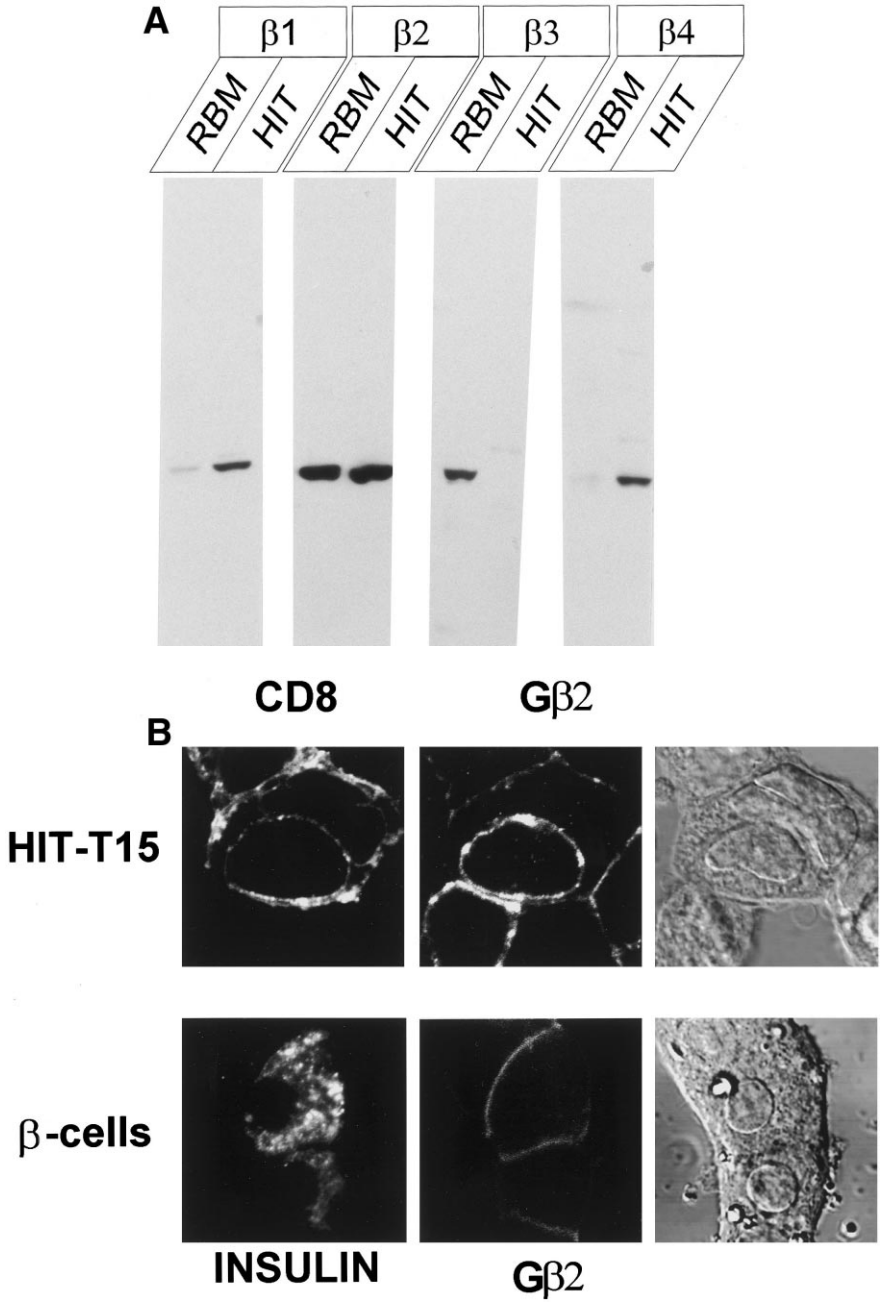

Fig. 4. Endogenous Gβ2 and transiently expressed TM-GRK2PH are located at the plasma membrane. A: Western blot of Gβ isoforms. Crude membranes from rat brain (RBM, 20 μg/lane) or HIT-T15 cells (HIT, 40 μg/lane) were separated by SDS-PAGE, electroblotted and incubated with Gβ-isoform-specific antibodies (anti-β1, -β2, -β3 and -β4). B: Confocal immunocytochemistry. Upper panel: HIT-T15 cells transiently expressing TM-GRK2PH were fixed, permeabilised and double stained with monoclonal anti-CD8 (CD8) antibody recognising the transmembrane domain of TM-GRK2PH and with a polyclonal anti-Gβ2 antibody (Gβ2). Lower panel: Primary islet cells were double-stained with a monoclonal anti-insulin antibody (INS) and with a polyclonal anti-Gβ2 antibody (Gβ2). Phase contrast images are given on the right.

3.4. Conclusions

Our observations demonstrate a pronounced effect of different βγ-binding domains on insulin exocytosis. The PH domain of GRK2 binds not only Gβγ, but also phospatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) [43]. PtdIns(4,5)P_2 plays an important role in vesicle fusion [44,45] and its binding to TM-GRK2PH might alter the exocytotic response. However, as exocytosis was also inhibited by TDα, another Gβγ-binding protein, the observed effects cannot be solely explained in terms of binding of PtdIns(4,5)P_2. Therefore, our data indicate a requirement for free Gβγ-dimers in the process of exocytosis.

We have not identified the molecular nature of the Gβγ in question. However, among the four isoforms of Gβ examined, only Gβ2 was found to be expressed at the plasma membrane whereas Gβ4 is clearly located to an intracellular organelle. Moreover, the PH domain of GRK2 binds Gβ1 and Gβ2, but not Gβ3 [29], excluding the latter isoform as a putative target. Gβ2 may thus be involved in exocytosis although we can currently not exclude a role for other isoforms such as Gβ5

[46]. It will obviously be an important future topic to identify the activator of Gβ implicated in exocytosis. We have recently demonstrated that receptor-mediated activation of heterotrimeric G-proteins can produce not only direct inhibition of exocytosis [15] mediated by PTX-sensitive Gα [21], but also direct stimulation of exocytosis in endocrine and neuroendocrine cells as in the case of the G-protein-coupled receptor latrophilin/CIRL [47,48]. Receptor-mediated direct stimulation of exocytosis by heterotrimeric G-proteins is therefore conceivable, although other modes of activation must exist [9].

Interestingly, the βγ-binding domain of GRK2 interfered with both stimuli for exocytosis, Ca^{2+} and GTPγS. Several differences between the two stimuli suggest the presence of two pathways. Whereas both require ATP-dependent priming of vesicles [6], an early step in exocytosis [2,4], GTPγS-induced exocytosis proceeds independently of the presence of cytosolic proteins [42] and the calcium-sensing vesicle protein synaptotagmin [32]. Moreover, electrophysiological studies have clearly indicated the presence of a GTP-dependent step during Ca^{2+} stimulation of exocytosis [49]. Thus the GTP-dependent step occurs most likely in parallel to or after the Ca^{2+}-dependent step in exocytosis. As the βγ-binding domains affect hormone release evoked by both stimuli, they probably interfere with a rather late step in exocytosis. It is tempting to speculate that Gβγ may be a mediator of the GTP effect. Future research should provide an answer to this question.

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