Disruption of Rab3–calmodulin interaction, but not other effector interactions, prevents Rab3 inhibition of exocytosis

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Rab GTPases regulate membrane traffic between the cellular compartments of eukaryotic cells. Rab3 is associated with secretory vesicles of neuronal and endocrine cells and controls the Ca2+-triggered release of neurotransmitters and hormones. To clarify the mode of action of Rab3 we generated mutants of the GTPase that do not interact efficiently with its putative effectors Rabphilin and RIM. Surprisingly, these mutants transfected in PC12 cells were still capable of inhibiting Ca2+-evoked secretion. Rab3 was shown previously to bind to calmodulin in a Ca2+-dependent manner. By replacing two arginines conserved between Rab3 isoforms, we generated a mutant with a reduced affinity for calmodulin. This mutant retained the capacity to interact with the Rab3 regulatory proteins, Rabphilin, RIM, Mss4 and RabGDI, and was correctly targeted to dense-core secretory granules. However, replacement of the two arginines abolished the ability of the GTP-bound form of Rab3 to inhibit exocytosis of catecholamine- and insulin-secreting cells. We propose that a Rab3–calmodulin complex generated by elevated Ca2+ concentrations mediated at least some of the effects of the GTPase and limited the number of exocytotic events that occurred in response to secretory stimuli.

Keywords: GTP/insulin/neurotransmitter/secretion

Introduction

Rab proteins are Ras-like GTPases involved in the trafficking of secretory and endocytic vesicles of eukaryotic cells (Novick and Zerial, 1997; Martinez and Goud, 1998). Rab GTPases associate reversibly with the membranes of transport vesicles via polyisoprenoid groups post-translationally attached to the C-terminus of the proteins (Farnsworth et al., 1991). Like other GTPases, Rab proteins can switch between two conformational states, an active GTP-bound and an inactive GDP-bound form. Rab GTPases in their GDP-bound state are cytosolic and are associated with RabGDI-dissociation inhibitor (RabGDI) (Regazzi et al., 1992; Ullrich et al., 1993). RabGDI is capable of chaperoning the GTPases in the cytosol and delivering them to the appropriate membrane (Pfeffer et al., 1995). RabGDI controls the interaction of Rabs with phospholipids by binding in a guanine-nucleotide-dependent manner to their prenylated C-terminus (Burstein et al., 1993). Once Rabs have been delivered to the vesicle membrane, guanine nucleotide exchange proteins (GEPs) promote the exchange of bound GDP for GTP and the release of RabGDI. Some GEPS, such as Mss4, act on a wide range of Rabs, whereas others appear to be more specific (Burton et al., 1994; Wada et al., 1997). The signals elicited by Rabs are switched off thanks to the stimulation of their intrinsic GTPase activity by specific GTPase-activating proteins (GAP) (Burstein et al., 1993). Rab GTPases, again in the GDP-ligated state, re-form a complex with RabGDI, dissociate from the membranes and are, thereby, ready for another cycle.

Rab GTPases are localized within different cellular compartments and control specific transport steps (Novick and Zerial, 1997; Martinez and Goud, 1998). Members of the Rab3 family are associated with secretory vesicles of neuronal and endocrine cells, and regulate the final steps in the secretory pathway (Bean and Scheller, 1997; Geppert and Südhof, 1998). Several lines of evidence indicate that the GTP-bound form of Rab3 acts as a negative modulator of exocytosis. First, overexpression of GTPase-deficient mutants of Rab3 inhibits the release of neurotransmitters and hormones elicited by different secretagogues (Holz et al., 1994; Johannes et al., 1994; Regazzi et al., 1996; Iezzi et al., 1999). Secondly, overexpression of the catalytic domain of Rab3 GEP, which promotes the exchange of GDP for GTP on Rab3, reduces exocytosis (Oishi et al., 1998). Thirdly, microinjection of antisense oligonucleotides that decrease the expression of Rab3a augments the response to repetitive stimulations (Johannes et al., 1994). Finally, the number of fusion events occurring in response to a nerve impulse is increased in mice lacking Rab3a (Geppert et al., 1997).

The mechanism by which Rab3 controls exocytosis remains to be established. To clarify the mode of action of Rab3 a major effort has been made to identify its effectors. Rabphilin is a neuron-specific protein isolated by chemical cross-linking of Rab3 with bovine brain extracts (Shirataki et al., 1993). Rabphilin binds to the surface of secretory vesicles where it interacts with the GTP-bound form of Rab3 (Shirataki et al., 1996; McKerrow et al., 1996; Stahl et al., 1996). Overexpression of Rabphilin in chromaffin cells or in cells that secrete insulin increases stimulated exocytosis (Chung et al., 1995; Arribas et al., 1997). This effect is possibly due to the ability of Rabphilin to interact with α-actinin and thereby regulate the organization of the actin cytoskeleton (Kato et al., 1996). Rabphilin has also been shown to bind to Rabaptin-5 and be involved in endocytosis (Ohya et al., 1992).
et al., 1998). RIM is another putative Rab3 effector identified in a yeast two-hybrid screen using Rab3 as bait (Wang et al., 1997). RIM is localized to presynaptic active zones in synapses and has been suggested to mediate at least some of the functions of Rab3, but the precise role of RIM in the secretory process remains to be established (Wang et al., 1997). The structural basis for the formation of the complex between Rab3a and these two effectors has recently been elucidated (Ostermeier and Brunger, 1999).

Noc2, a zinc-finger protein predominantly expressed in endocrine tissues, displays high similarity to the Rab3-binding domain of Rabphilin. However, despite these characteristics, Noc2 does not bind efficiently to Rab3 (Kotake et al., 1997; T.Coppola and R.Regazzi, unpublished observation).

In addition to these putative effectors, Rab3 interacts in a Ca\(^{2+}\)-dependent manner with calmodulin (CaM) (Park et al., 1997). The binding of Rab3 to Ca\(^{2+}/\)CaM occurs by a mechanism similar to that of RabGDI and requires the prenylation of the protein and the presence of bound guanine nucleotide (Park et al., 1997). In contrast to RabGDI, Ca\(^{2+}/\)CaM does not discriminate between the GDP- and GTP-bound forms of Rab3.

Here, using point mutants of Rab3 we investigated the involvement of the putative effectors of the GTPase in Ca\(^{2+}\)-triggered exocytosis of catecholamine- and insulin-secreting cells. Our data provide evidence that the inhibition of secretion caused by the GTP-bound form of Rab3 requires an interaction with Ca\(^{2+}/\)CaM. In contrast, interactions of Rab3 with Rabphilin and RIM are neither required nor sufficient to sustain the effect of Rab3 on exocytosis.

**Results**

Rabphilin and RIM have been suggested to mediate at least some of the effects of Rab3 on regulated secretion (Bean and Scheller, 1997; Geppert and Südhof, 1998). We tested the role of these two putative Rab3 effectors in exocytosis by modifying key amino acids within a GTPase-deficient mutant of Rab3a (Rab3a Q81L) (Brondyk et al., 1993). As shown in Figure 1A, replacement of Phe19 and Asp20 with Leu and Glu, respectively (F19L, D20E), reduced the ability of Rab3a to interact with RabGDI and requires clusters of basic amino acid residues (Rhoads and Friedberg, 1997). As shown in Figure 1A, replacement of Phe19 and Asp20 with Leu and Glu, respectively (F19L, D20E), reduced the ability of Rab3a to interact with RabGDI and Rabphilin. As expected from the structural analysis of the Rab3–Rabphilin complex (Ostermeier and Brunger, 1999), replacement of Val55 with Glu (V55E) had a more drastic effect. In this case, the binding of Rab3a to both RabGDI and Rabphilin was abolished almost completely (Figure 1A). Surprisingly, these mutants with an impaired capacity to bind to Rabphilin and RIM were still able to inhibit K\(^{+}\)-induced exocytosis of PC12 cells, which suggests that another, as yet unidentified, effector may mediate the effect of Rab3a (Figure 1B).

Rab3a has been shown to bind to calmodulin in a Ca\(^{2+}\)-dependent manner (Parker et al., 1997). To investigate the functional relevance of this interaction we first analyzed the effect on exocytosis of a synthetic peptide corresponding to amino acids 62–85 of Rab3a (K62–R85). This peptide competes for the binding of Ca\(^{2+}/\)CaM to Rab3a (Parker et al., 1997). The K62–R85 peptide was introduced in streptolysin-O permeabilized HIT-T15 cells, an insulin-secreting cell line expressing different isoforms of Rab3 (Regazzi et al., 1996). At 50 \(\mu\)M, the peptide increased, by ~2-fold, the amount of hormone released at stimulatory Ca\(^{2+}\) concentrations (10 \(\mu\)M free Ca\(^{2+}\): 5.24 ± 0.36 ng versus 2.58 ± 0.51, \(n = 3\)). In contrast, basal insulin secretion was not affected significantly (0.88 ± 0.42 ng versus 0.85 ± 0.30 for the control, \(n = 3\)). This is reminiscent of the situation encountered in Rab3a knockout mice in which each nerve impulse leads to a higher-than-usual number of exocytotic events (Geppert et al., 1997). The K62–R85 peptide is also known to prevent the interaction of Rab3a with RabGDI (Parker et al., 1997). To clarify whether the effect of the peptide on exocytosis was due to the disruption of the Rab3–CaM complex we generated a mutant of Rab3a defective in Ca\(^{2+}/\)CaM binding but still capable of associating with RabGDI.

Ca\(^{2+}/\)CaM forms complexes with many different proteins. The mechanism underlying these interactions is not fully understood, but Ca\(^{2+}/\)CaM binding is known to require clusters of basic amino acid residues (Rhoads and Friedberg, 1997). The region of Rab3a involved in Ca\(^{2+}/\)CaM and RabGDI binding contains two positively charged amino acids, Arg66 and Arg70, not found in other members of the Rab family. These two basic amino acids are conserved in the other three mammalian isoforms of Rab3 and are also present in the Rab3 homologs of...
**Caenorhabditis elegans**, *Drosophila melanogaster* and *Aplysia californica*. Although this pair of arginines appears to be a distinctive feature of Rab3 proteins, its relevance for the function of the GTPase has never been studied. Most Rab GTPases, including Rab1 and Rab5, that are known to associate with RabGDI (Ullrich et al., 1993), but not with Ca²⁺/CaM (Park et al., 1997), have respectively a Leu and a Thr in the positions corresponding to Arg66 and Arg70 of Rab3a.

The K62–R85 peptide mimicking the sequence of wild-type Rab3a can be cross-linked to Ca²⁺/CaM (Park et al., 1997) with an affinity of ~20 μM (Figure 2A). However, the replacement of the amino acids corresponding to positions 66 and 70 with Leu and Thr caused a strong decrease (~6-fold) in the ability of the peptide to cross-link to Ca²⁺/CaM (Figure 2A). This indicates that the two arginines present in Rab3 isoforms are required for efficient binding to Ca²⁺/CaM. In the hope of generating a Rab3 mutant retaining the general properties of Rab GTPases, but with a decreased affinity for Ca²⁺/CaM, we exchanged the two arginines of Rab3a with Leu and Thr (Rab3a LT). To evaluate the impact of the mutations on the ability to interact with Ca²⁺/CaM, we expressed wild-type Rab3a and Rab3a LT in catecholamine-secreting PC12 cells or in insulin-secreting HIT-T15 cells. Membrane-associated wild-type Rab3a was able to associate in a Ca²⁺-dependent manner with CaM, forming a soluble complex (Figure 2B). Rab3a Q81L, which is locked in the GTP-bound form (Brondyk et al., 1993), was capable of interacting with Ca²⁺/CaM as well as wild-type Rab3a (not shown), confirming that Ca²⁺/CaM is able to complex to both the GDP- and the GTP-bound form of Rab3a (Park et al., 1997). As predicted, exchange of Arg66 and Arg70 with Leu and Thr decreased the binding of Rab3a LT to Ca²⁺/CaM (Figure 2B). Similar results were obtained after replacing Arg66 and Arg70 in the GTPase-deficient mutant of Rab3a (not shown). Taken together, these results confirm that Arg66 and Arg70 are required for efficient binding to Ca²⁺/CaM. In contrast, exchanging these two amino acids with Leu and Thr did not alter the ability of Rab3a to form a complex with RabGDI. As shown in Figure 2C, RabGDI was able to bind to both wild-type Rab3a and to Rab3a LT.

As demonstrated in Figure 3, Rab3a LT was able to interact with the other putative effector proteins, Rabphilin and RIM, and with the guanine nucleotide-exchange factor Mss4. In fact, in vitro translated Mss4, RIM and Rabphilin were bound with equal efficiency to glutathione S-transferase (GST) affinity columns of wild-type Rab3a and Rab3a LT. This indicates that Arg66 and Arg70 are not directly involved in the interaction with these Rab3 effector proteins. The ability of Rabphilin to bind the Rab3a LT mutant was also tested using a mammalian two-hybrid system. As shown in Figure 4, in transfected HIT-T15 cells the GTPase-deficient forms of Rab3a and of Rab3a LT were capable of binding equally well to Rabphi-
Fig. 4. Interaction between Rabphilin and different Rab3a mutants in intact cells. HIT-T15 cells were cotransfected with a fusion protein of VP16 with Rabphilin, a fusion protein of GAL4 with the indicated mutants of Rab3a and a plasmid containing five binding sites for GAL4 upstream of the firefly luciferase gene. The interaction between Rabphilin and the Rab3a mutants was quantitated by measuring firefly luciferase activity. The luciferase activity produced by the association of VP16–Rabphilin with GAL4–Rab3a Q81L was set to 100%. The figure shows the mean ± SD of 2–4 independent experiments performed in duplicate.

Fig. 5. Rab3a LT is targeted to secretory granules. PC12 cells transfected with myc-tagged wild-type Rab3a or Rab3a LT were immunostained with an antibody against the myc epitope and an antibody against chromogranin A (as a marker for secretory granules). The cells were then analyzed using confocal microscopy. Identical results were obtained with a GTPase-deficient mutant of Rab3a in which Arg66 and Arg70 were replaced with Leu and Thr.

Fig. 6. Interaction of Rab3a with Ca²⁺/CaM is required for inhibition of exocytosis. PC12 cells (A) or HIT-T15 cells (B) were cotransfected with hGH and with constructs of Rab3a or of Rab3a Q81L (Rab3a GTP). The constructs contained at position 66 and 70 either two Arg (RR) or Leu and Thr (LT). Exocytosis from transfected cells was assessed by measuring the amount of hGH released into the medium. The figure shows the mean ± SD of four independent experiments. In cells transfected with vector alone K⁺ depolarization increased the release of hGH by ~4-fold.

lin. In contrast, Rabphilin was unable to interact with the GTPase-deficient form of Rab3a V55E and with a mutant of Rab3a that is preferentially in the GDP-bound conformation (Rab3a T36N). These findings confirm the results obtained in Figures 1 and 3. In addition, they provide evidence that the GTPase-deficient form of Rab3a LT is able to acquire an activated GTP-bound conformation in transfected HIT-T15 cells.

Next, we investigated the subcellular distribution of Rab3a LT. Rab GTPases are found in two pools: a membrane-bound pool and a soluble pool in which the protein is associated with RabGDI (Regazzi et al., 1992; Ullrich et al., 1993). Fractionation of transfected PC12 and HIT-T15 cells indicated that the ratio between the two pools was identical for wild-type Rab3a and Rab3a LT (not shown). The precise mechanism underlying the targeting of Rab3 proteins to secretory granules has not been elucidated. To determine more precisely the subcellular distribution of the protein, PC12 cells transfected with wild-type Rab3a or the LT mutant were analyzed using confocal microscopy. Both proteins were targeted to dense-core chromogranin-containing secretory granules (Figure 5), and similar results were obtained with transfected HIT-T15 cells (not shown).

Taken together, these findings indicate that the LT mutant retains the known properties of Rab3 GTPases, except for the ability to interact with Ca²⁺/CaM. We then tested whether the LT mutant was able to control Ca²⁺-induced exocytosis. For this purpose, different Rab3a constructs were expressed transiently in PC12 cells (Figure 6A) or in HIT-T15 cells (Figure 6B) together with human growth hormone (hGH). Since hGH is targeted to dense-core secretory granules of transfected cells (Schweitzer and Kelly, 1985), hGH release can be used to monitor exocytosis from the cells producing the Rab3a constructs (Holz et al., 1994; Wang et al., 1997; Iezzi et al., 1999). Overexpression of wild-type Rab3a did not significantly modify K⁺-evoked hGH secretion and exchange of the two Arg with Leu and Thr on wild-type Rab3a was without effect (Figure 6). Overexpression of the GTPase-deficient mutant of Rab3a locked in the GTP-bound form decreased hGH exocytosis, in agreement with results published previously (Holz et al., 1994; Johannes et al., 1994; Regazzi et al., 1996; Iezzi et al., 1999). Introduction of Leu and Thr in positions 66 and 70 of the GTPase-deficient mutant reduced the protein’s affinity for Ca²⁺/CaM and caused a complete loss of the inhibitory action of the GTPase (Figure 6). Thus, our results strongly
suggest that activated Rab3a requires interaction with Ca\(^{2+}/\)
CaM for the control of exocytosis.

Ca\(^{2+}/\)CaM is thought to play a positive role in exocytosis (Chamberlain et al., 1995). Thus, a straightforward interpretation of our results might be that overexpression of the GTP-bound form of Rab3a inhibits Ca\(^{2+}\)-induced exocytosis by sequestering Ca\(^{2+}/\)CaM. To test this hypothesis, we analyzed whether co-expression of Ca\(^{2+}/\)CaM with Rab3a could prevent the effect of the GTPase on secretion (Figure 7). Calmodulin transfected alone in PC12 cells had no effect on exocytosis whereas, as expected, the GTPase-deficient mutant of Rab3a decreased K\(^{+}\)-evoked secretion. When the two proteins were co-expressed at similar levels in the same cells, Rab3a was still capable of decreasing hGH release, indicating that the GTPase did not act by sequestering Ca\(^{2+}/\)CaM.

**Discussion**

In eukaryotes, intracellular membrane fusion is a fundamental process for the maintenance of cell integrity and secretion. All fusions appear to rely on a similar mechanism, involving evolutionarily conserved components (Ferro-Novick and Jahn, 1994; Rothman, 1994) and an increase in cytosolic Ca\(^{2+}\) concentration (Peters and Mayer, 1998). However, in contrast to other intracellular transport steps in which, after ensuring the correct targeting of the vesicle, the highest efficiency of fusion is desired, the number of exocytotic events has to be tightly controlled in regulated secretion. Rab3a regulates a late step in exocytosis and limits the extent of Ca\(^{2+}\)-triggered vesicle fusion (Bean and Scheller, 1997; Geppert and Südhof, 1998). Thus, in mice lacking Rab3a, the arrival of a nerve impulse causes an unusually high number of exocytotic events (Geppert et al., 1997). Conversely, overexpression of constitutively active mutants of Rab3a or of the other Rab3 isoforms decreases secretion from endocrine and neuroendocrine cells (Holz et al., 1994; Johannes et al., 1994; Regazzi et al., 1996; Iezzi et al., 1999). So far, the effect of Rab3 isoforms on exocytosis has been attributed to Rabphilin and/or RIM, two proteins that interact selectively with the GTP-bound form of the GTPase (Bean and Scheller, 1997; Geppert and Südhof, 1998). However, Rabphilin has a positive effect on secretion (Chung et al., 1995; Arribas et al., 1997) and the exact role of RIM remains to be determined (Wang et al., 1997). Here we provide strong evidence that Rabphilin and RIM are not the effectors responsible for Rab3-mediated inhibition of exocytosis. First, point mutations that decreased the binding of Rab3 to Rabphilin and RIM did not interfere with the ability of the GTPase to control exocytosis. Secondly, the association of Rab3 with Rabphilin and RIM was not sufficient to control exocytosis. Two papers published by others during the revision of this manuscript strongly support our findings. Thus, the phenotype of Rabphilin knockout mice indicates that Rabphilin is not required for the regulatory function of Rab3a in neurotransmitter release (Schlüter et al., 1999). In addition, Holz and collaborators reported that different mutations in the effector domain of Rab3a that strongly impair the interaction of the GTPase with Rabphilin (and RIM, our unpublished observation) do not alter the ability of Rab3a to inhibit exocytosis (Chung et al., 1999). All these observations suggest that the inhibitory action of Rab3a on exocytosis may not be mediated by a protein that is interacting with the effector domain of the GTPase.

Our results indicate that a conserved region of Rab3 proteins (K62–R85 in Rab3a), which does not appear to participate in interactions between Rab3 proteins and Rabphilin and RIM (Figures 3 and 4), is required for the Rab3-dependent inhibition of Ca\(^{2+}\)-induced exocytosis. Thus, when a GTPase-deficient form of Rab3a was modified in this region by replacement of Arg66 with Leu and Arg70 with Thr, and then overexpressed in PC12 cells or HIT-T15 cells, it was found to have no inhibitory effect.

This same region of wild-type Rab3a has been shown to act with the prenylated C-terminus of this protein to promote both the formation of a soluble complex between Ca\(^{2+}/\)CaM and Rab3a and the dissociation of Rab3a from synaptic vesicle membranes (Park et al., 1997). Furthermore, in the current study, we show that mutations in this region greatly attenuate the ability of Ca\(^{2+}/\)CaM to dissociate Rab3a from the membranes. In neurons, Ca\(^{2+}\)-free CaM is bound to a specific protein called neuromodulin, GAP43, or B-50, which is attached to the cytosolic surface of the synaptic plasma membrane (Van Lookeren-Campagne et al., 1989). When calcium levels rise within nerve terminals, Ca\(^{2+}/\)CaM is released from its binding site and this causes a local increase in its concentration (Hens et al., 1996). Ca\(^{2+}/\)CaM may then interact not only with high-affinity targets, e.g. CaM kinase II, but also with Rab3a and other low-affinity targets (Peresichini and Cronk, 1999).

An increase in intracellular Ca\(^{2+}\) concentration is the main trigger for exocytosis in most secretory systems, and the formation of complexes between Ca\(^{2+}/\)CaM and specific intracellular proteins is clearly involved. We suggest that Ca\(^{2+}/\)CaM also may form a complex with Rab3a and activate an inhibitory pathway, which contributes to the fine-tuning of exocytosis. The number of secretory vesicles fusing with the cell membrane in response to stimuli may be determined by the balance between the two opposing effects of Ca\(^{2+}\). Thus, as recently proposed for Ca\(^{2+}\) channel modu-
Provision of the mammalian expression vector pcDNA3 (Invitrogen) that the Research Institute, Seattle, WA). Calmodulin was subcloned in a modified calmodulin cDNA was obtained from Dr. C. Rhodes (Pacific Northwest 1996). The antibody against human chromogranin A was purchased from human myc proteins were kindly provided by Dr. T. Sudhof (University of Texas) and the Rabphilin and Mss4 cDNAs were obtained from Dr. P. De Camilli.

**Materials and methods**

**Materials**

The Rabphilin and Mss4 cDNAs were obtained from Dr. P. De Camilli (Yale University). The plasmids encoding RIM and Rabphilin GST fusion proteins were kindly provided by Dr. T. C. Sudhof (University of Texas) and Dr. I. G. Macara (University of Virginia), respectively. The generation of human myc-tagged wild-type Rab3a and of the mutant at position 81 (Q81L) has been described previously (Regazzi et al., 1996). Human calmodulin cDNA was obtained from Dr. C. Rhodes (Pacific Northwest Research Institute, Seattle, WA). Calmodulin was subcloned in a modified version of the mammalian expression vector pcDNA3 (Invitrogen) that provides a myc-epitope tag at the N-terminus of the protein (Regazzi et al., 1996). The antibody against human chromogranin A was purchased from Dako; the antibody against insulin was obtained from Linco Research Inc. Rab GDI was purified as described (Park et al., 1997).

**Site-directed mutagenesis**

Site-directed mutagenesis was performed using the QuickChange kit (Stratagene) and was verified by sequencing the inserts of the plasmids.

**Interaction of Rab3a mutants with Rabphilin, RIM and Mss4**

The Rab3-binding domains of RIM (amino acids 1–398) or Rabphilin (amino acids 1–206) were produced as fusion proteins with GST and immobilized on glutathione–agarose beads. The beads were resuspended in 20 mM HEPES pH 7.5, 150 mM KCl, 1 mM dithiothreitol (DTT), 5% glycerol, 0.05% Tween and 1 mM/ml bovine serum albumin (BSA). They were then incubated with the 35S-labeled Rab3a mutants produced by in vitro translation (Promega). Proteins that remained associated with the affinity columns after extensive washing were analyzed using SDS–PAGE and revealed by autoradiography. In the experiments shown in Figure 3 wild-type Rab3a and the LT mutant were produced as GST-fusion proteins and were incubated with 35S-labeled Mss4, RIM and Rabphilin produced by in vitro translation under the same conditions described above. The interaction between different Rab3 mutants and Rabphilin was also tested using a mammalian two-hybrid system (CheckMate™, Promega). For this purpose the Rab3 mutants were subcloned in-frame with GAL4 in the expression vector pHIND, whereas full-length Rabphilin was subcloned in-frame with VP16 in the expression vector pACT. The GAL4 and VP16 fusion proteins were cotransfected in HIT-T15 cells along with a third plasmid (pG5 luc) encoding five GAL4-binding sites upstream from the firefly luciferase gene. Three days after transfection the cells were lysed and the amount of firefly luciferase was quantitated using the Dual-Luciferase™ Reporter Assay System (Promega).

**Secretion**

PC12 and HIT-T15 cells were transiently transfected with a plasmid encoding hGH and with the Rab3a constructs (Iezzi et al., 1999). Three days later, the cells were pre-incubated for 30 min in 20 mM HEPES pH 7.4, 128 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2.7 mM CaCl2 and 10 mM glucose. The medium was then aspirated and the cells were stimulated for 10 min with the same buffer but containing 53 mM NaCl and 80 mM KCl. Exocytosis from transfected cells was assessed by measuring hGH release (Iezzi et al., 1999).

**Cross-linking of peptides to CaM**

The K62–R85 peptide (KTIYRNDKRIKLQIWDTAGQERYR) and the LT peptide (KTYLNDKTIKLQWDTCGQYR) were cross-linked with 1 mM bis(sulfosuccinimidyl) suberate to 12 μM CaM (Park et al., 1997). The samples were then analyzed using SDS–PAGE and the Coomassie Brilliant Blue-stained bands were measured using densitometric scanning.

**Interaction of Rab3a LT with CaM and RabGDI**

HIT-T15 or PC12 cells were transiently transfected by electroporation with 10 μg of DNA/106 cells. After 2–3 days culture the cells were homogenized by sonication in buffer A (20 mM Tris–HCl pH 7.5, 1 mM EDTA, 6 mM MgCl2, 1 mM DTT, 10 μg/ml leupeptin and 2 μg/ml aprotinin). The homogenate was loaded on a gradient consisting of 1 ml of 2 M sucrose and 2.5 ml of 0.5 M sucrose. After 90 min centrifugation at 65 000 g, 100 μg of proteins collected at the interface between 0.5 and 2 M sucrose for 45 min at 30°C with 100 μM GDP or GTP followed by an additional 15 min period in the presence of Ca2+/CaM (60 μM) or EGTA (5 mM). The mixture was then centrifuged for 1 h at 70 000 g. Aliquots of the supernatant and of the pellet were analyzed by Western blotting using a monoclonal antibody (mAb) against the myc epitope (clone 9E10) present at the N-terminus of the Rab3 constructs. The same procedure was used to assess the binding of Rab3 proteins to RabGDI (1 μM).

**Targeting of Rab3a LT to dense-core secretory granules**

Colocalization of the myc-tagged Rab3a constructs with dense-core granules was determined using confocal microscopy as described previously (Iezzi et al., 1999). Briefly, 2 days after transfection the cells were fixed and simultaneously incubated with a mAb against the myc epitope (clone 9E10) and with a polyclonal antibody directed against human chromogranin A (PC12 cells) or with a polyclonal antibody against porcine insulin (HIT-T15). The localization of the myc-tagged Rab3a constructs was revealed using a rhodamine-conjugated antibody. The position of dense-core secretory granules was assessed using fluorescein-conjugated antibodies.
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