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The GTPase Rab3a negatively controls calcium-dependent exocytosis in neuroendocrine cells

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There is accumulating evidence that small GTPases of the *rab* family regulate intracellular vesicle traffic along biosynthetic and endocytotic pathways in eukaryotic cells. It has been suggested that Rab3a, which is associated with synaptic vesicles in neurons and with secretory granules in adrenal chromaffin cells, might regulate exocytosis. We report here that overexpression in PC12 cells of Rab3a mutant proteins defective in either GTP hydrolysis or in guanine nucleotide binding inhibited exocytosis, as measured by a double indirect immunofluorescence assay. Moreover, injection of the purified mutant proteins into bovine adrenal chromaffin cells also inhibited exocytosis, as monitored by membrane capacitance measurements. Finally, the electrophysiological approach showed that bovine chromaffin cells which were intracellularly injected with antisense oligonucleotides targeted to the *rab3a* messenger exhibited an increasing potential to respond to repetitive stimulations. In contrast, control cells showed a phenomenon of desensitization. These results provide clear evidence that Rab3a is involved in regulated exocytosis and suggest that Rab3a is a regulatory factor that prevents exocytosis from occurring unless secretion is triggered. Furthermore, it is proposed that Rab3a is involved in adaptive processes such as response habituation.

Key words: chromaffin cells/exocytosis/GTP-binding protein/membrane capacitance/Rab3a

Introduction

Small GTPases of the *ras* superfamily regulate many fundamental processes in eukaryotic cells such as growth, vesicle traffic and cytoskeletal organization (reviewed by Hall, 1990, 1993). As these proteins exist in either a GTP- or GDP-bound conformation, each form being associated with a particular activity, they have been described as molecular switches (reviewed by Bourne *et al.*, 1991). Within this superfamily, the *rab* proteins have been proposed to control membrane fusion events between specific intracellular compartments (reviewed by Pfeffer, 1992; Simons and Zerial, 1993). Nevertheless, it has not yet been elucidated how *rab* proteins relate to the membrane fusion machinery.

Rab3a is a member of the *rab* family which was originally cloned from rat brain (Touchot *et al.*, 1987), human pheochromocytoma cells (Zahraoui *et al.*, 1989) and bovine brain (Matsui *et al.*, 1988). Members of the *rab3* subfamily have also been found in organisms as diverse as *Drosophila melanogaster* (Johnston *et al.*, 1991) and the marine ray *Discopyge ommata* (Volknandt *et al.*, 1991). The specific association of Rab3a with synaptic vesicles (Fischer von Mollard *et al.*, 1990) or chromaffin granules in adrenal chromaffin cells (Darchen *et al.*, 1990) was taken as the first evidence favoring a role for Rab3a in regulated exocytosis. This hypothesis was supported further by the observation that Rab3a dissociates from synaptic vesicles upon stimulation of synaptosomes (Fischer von Mollard *et al.*, 1991) and by studies using peptides derived from the putative effector domain of Rab3a which stimulated secretion in several exocrine and endocrine cells (Oberhauser *et al.*, 1992; Padfield *et al.*, 1992; Senyshyn *et al.*, 1992). However, the specificity of these peptides has been challenged since the stimulatory effect persisted when the amino acid sequence was substantially modified (MacLean *et al.*, 1993). Moreover, a mastoparan-like effect of these peptides on heterotrimeric GTP-binding proteins has been reported (Law *et al.*, 1993). In addition, the most effective peptide carried mutations that, in Rab3a, abolish the interaction with all known target proteins and regulatory elements (McKiernan *et al.*, 1993). Thus, direct evidence for Rab3a being involved in regulated exocytosis is still missing.

The interconversion between GTP- and GDP-bound conformation of Rab3a is catalyzed by interaction partners. Two of these proteins have been described biochemically: Rab3a-GTPase activating protein (Rab3a-GAP) and Rab3a-GDP dissociation stimulator (Rab3a-GDS) (Burstein *et al.*, 1991; Burstein and Macara, 1992). The cDNAs of two other proteins, Rab3a-guanine dissociation inhibitor (Rab3a-GDI) and Rabphilin-3a, were cloned due to the ability of these proteins to interact specifically with Rab3a. Rab3a-GDI (Matsui *et al.*, 1990) stabilizes the binding of GDP to Rab3a (Sasaki *et al.*, 1990) and other *rab* proteins (Ullrich *et al.*, 1993), and inhibits the binding to and stimulates the dissociation from synaptosomal membranes of the GDP-bound form of Rab3a (Araki *et al.*, 1990). Rabphilin-3a forms a specific complex with Rab3a bound to a non-hydrolyzable analog of GTP but not with the GDP-bound form of Rab3a (Shirataki *et al.*, 1993). The C2 domains of this protein show sequence homology to those of synaptotagmin, a protein associated with synaptic vesicles (Shirataki *et al.*, 1993).

We have investigated the function of Rab3a in bovine adrenal chromaffin cells and PC12 cells by introducing into these cells mutant Rab3a proteins and antisense oligonucleotides directed to the *rab3a* messenger. Our results show that Rab3a is involved in regulated exocytosis, and suggest that its function is to control negatively the secretory activity. In addition, we propose that Rab3a might regulate the calcium sensitivity of the secretory response.

Results

Overexpression of mutant Rab3a proteins in PC12 cells with T7 RNA polymerase recombinant vaccinia virus system

A double indirect immunofluorescence assay was developed to analyze, at the single cell level, the effect of overexpression of wild type Rab3a and mutant Rab3a proteins on exocytosis. After the transfection of respective cDNAs under control of the T7 promoter, PC12 cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase (Fuerst *et al.*, 1986). After 4 h the cells were stimulated by KCl depolarization to secrete and processed for double indirect immunofluorescent staining. Cells overexpressing a particular construct were identified with specific primary antibodies and fluorescein-conjugated secondary antibodies (Figure 1c and d). The secretory activity was measured by counting, in the rhodamine channel, the number of fluorescent patches corresponding to dopamine β-hydroxylase (DBH) immunoreactivity at the cell surface (Figure 1e and f). DBH was used as a marker for vesicle fusion (Elferink *et al.*, 1993) since it is located inside secretory granules and exposed to the cell surface during exocytosis. The appearance of DBH on the plasma membrane is dependent on Ca^{2+} stimulation: in the presence of Ca^{2+}, 85% of the cells displayed >10 fluorescent patches, whereas in its absence only 16% of the cells were found in these categories, confirming the notion that DBH is a valid marker for vesicle fusion at the single cell level (Elferink *et al.*, 1993).

An amino acid substitution in Rab3a was made at position 135 (Asn to Ile). This position lies in a region of the protein which is thought to be implicated in guanine nucleotide binding, as predicted by comparison with the 3-D structure of the sequence-related *ras* p21 protein (Bourne *et al.*, 1991). In addition, a c-Myc epitope was added to the N-terminus of this mutant, yielding TR3aN135I for tagged Rab3aN135I. When this mutant protein was overexpressed in PC12 cells, a pronounced inhibition of the secretory response was observed (Figures 1a, c and e, and 2). More than 50% of the cells overexpressing TR3aN135I showed less than three DBH-fluorescent patches on their surface, whereas <5% of the control cells fell into this category (Figure 2). Similarly, significantly different results were found in all other categories. Overexpression of the epitope tagged wild type Rab3a, termed TR3a, also resulted in inhibition. However, in this case, the effect was less pronounced (Figure 2). The expression of Rab6 with an N → I mutation analogous to TR3aN135I (Figure 2) or of the unrelated MHC class II invariant chain (data not shown) did not inhibit secretion. Furthermore, TR3aV55E, carrying a mutation in the putative effector loop (Figures 1b, d and f, and 2) was equally inactive. An equivalent mutation at amino acid position 36 of v-H-ras corresponding to position 55 of Rab3a decreases the transforming activity of v-H-ras (McCormick, 1989). The absence of effect of TR3aV55E on exocytosis might therefore be due to the loss of interaction with an effector protein required for the function of Rab3a.

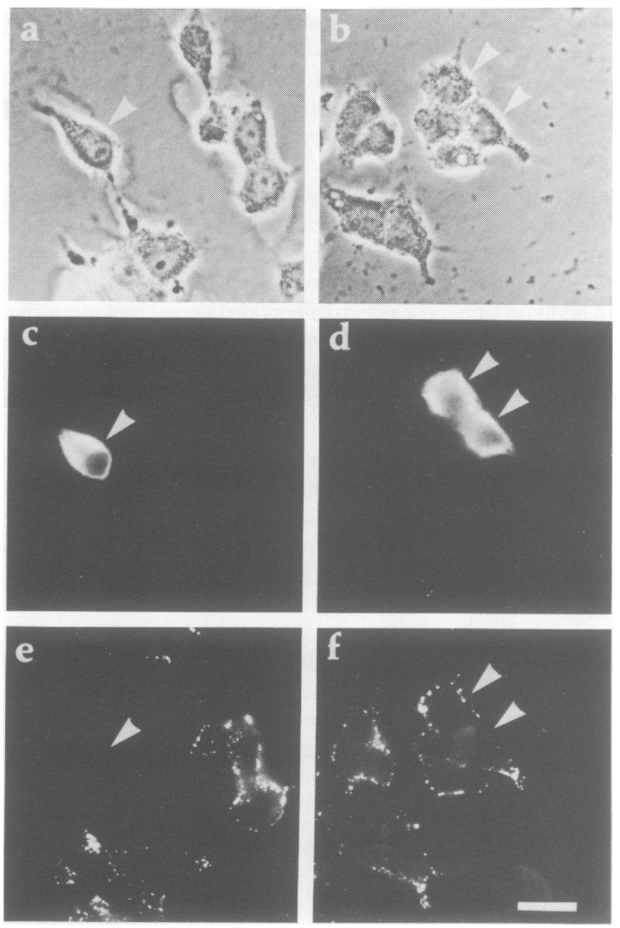

Fig. 1. Double immunofluorescence assay of exocytosis in PC12 cells expressing c-Myc tagged Rab3a mutants. Recombinant vaccinia virus expressing T7 RNA polymerase was used to overexpress the Rab3a mutated proteins TR3aN135I (**a**, **c** and **e**) or TR3aV55E (**b**, **d** and **f**). Cells expressing the mutants were identified by their c-Myc immunoreactivity (c and d). Secretion by these cells was revealed by immunodetection of the secretory granule membrane marker DBH at the cell surface (e and f). Arrows indicate a cell that does not secrete (a, c and e) or cells that respond to depolarization (b, d and f). Note the positive response of the non-transfected cells (e and f). Scale bar, 10 μm.

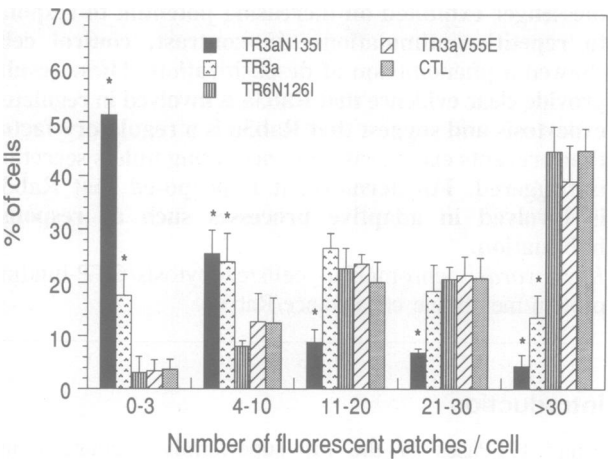

Fig. 2. Quantitation of the secretory response in cells overexpressing various constructs. Cells overexpressing a particular construct were grouped into categories according to the number of DBH-fluorescent patches on their surface, reflecting the magnitude of their secretory response. For each construct, results are given as percents of cells in the different categories. Non-transfected cells are figured as a control (CTL). Overexpression of a Rab6 mutant protein with an N → I mutation at position 126 (TR6N126I) and of TR3aV55E had little effect on secretion. At least 50 cells per experiment and per condition were analyzed. Data are shown as the means ± SE of five experiments, and are indicated with an asterisk (*) when significantly different ($P < 0.001$; chi square test) from control.

Two other mutants were designed in order to analyze more accurately the activity of Rab3a either blocked in the GTP- or the GDP-bound state. The mutant protein TR3aQ81L is deficient in GTP hydrolysis (Brondyk *et al.*, 1993) and is therefore thought to persist for a longer time in the GTP-bound conformation than the wild type protein. TR3aQ81L efficiently inhibited secretion (Figure 3). In contrast, TR3aT36N is deficient in GTP-binding and therefore thought to be predominantly in the GDP-bound conformation (Burstein *et al.*, 1992). TR3aT36N gave results that were not significantly different from those of control cells (Figure 3).

Similar results were found when secretion was triggered by increasing the intracellular free calcium concentration with ionomycin, bypassing membrane receptors and ionic channels (data not shown). Therefore, it is unlikely that an effect on calcium entry into PC12 cells can account for the inhibition of secretion by TR3aN135I, TR3aQ81L and TR3a.

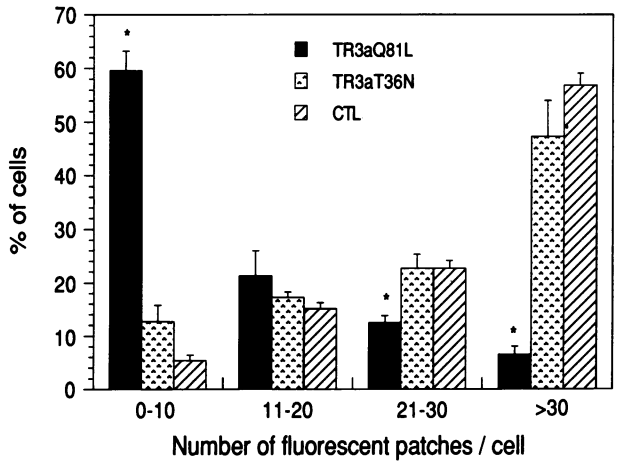

Fig. 3. Quantitation of the secretory response in cells overexpressing various constructs, as described in Figure 2. Non-transfected cells are figured as a control (CTL). Overexpression of TR3aT36N had little effect on secretion while TR3aQ81L was inhibitory. At least 50 cells per experiment and per condition were analyzed. Data are shown as the means ± SE of four experiments and are indicated with an asterisk (*) when significantly different ($P < 0.01$; chi square test) from control.

Injection of mutant Rab3a proteins into chromaffin cells

In a second set of experiments, the cDNAs coding for TR3a, TR3aT36N, TR3aQ81L and TR3aN135I were fused to that of glutathione *S*-transferase (GST). The purified fusion proteins, GST−TR3a, GST−TR3aT36N, GST−TR3aQ81L, GST−TR3aN135I, and GST alone were injected into bovine adrenal chromaffin cells. Exocytosis was subsequently analyzed by monitoring changes in cell membrane capacitance (C_m) induced by a depolarizing voltage step, with the whole-cell patch-clamp technique (Neher and Marty, 1982; Neher and Zucker, 1993). Consistent with the results obtained on PC12 cells, GST−TR3aQ81L and GST−TR3aN135I inhibited the secretory response of chromaffin cells (Figures 4 and 5). This inhibitory effect was dose-dependent since it was overcome by progressively diluting GST−TR3aN135I (data not shown). The same result was obtained when the GST moiety was released from GST−TR3aN135I by digestion with thrombin (Figure 5). In this test, GST, GST−TR3a, GST−TR3aT36N (Figures 4 and 5) and wild type Rab3a (data not shown) were not inhibitory. The discrepancy between the results obtained for TR3a either overexpressed in PC12 cells or injected into chromaffin cells might be attributed to different intracellular levels of recombinant proteins or to differences due to cell type.

Effects on exocytosis of antisense oligonucleotides targeted to the rab3a messenger

Antisense oligonucleotides complementary to different regions of the *rab3a* messenger and control oligonucleotides were injected into cultured bovine adrenal chromaffin cells. Exocytosis was subsequently analyzed by monitoring changes in C_m induced by successive depolarizing voltage steps. In non-injected cells and in cells injected with the con-

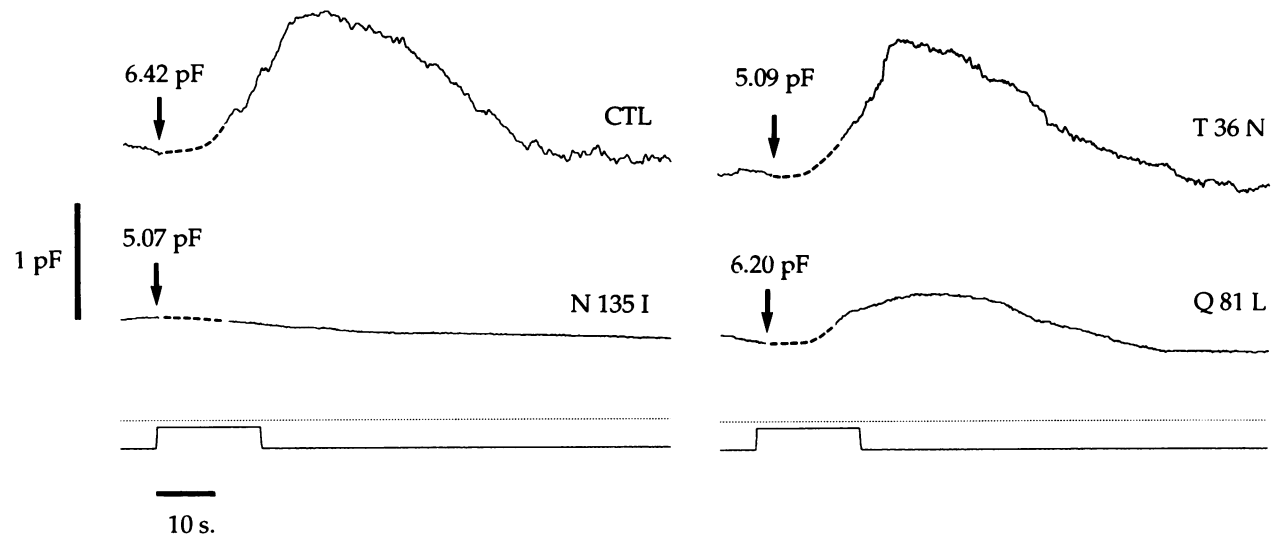

Fig. 4. Inhibitory effect of TR3aQ81L and TR3aN135I on the secretory response of chromaffin cells. C_m changes, measured with the patch clamp technique (Neher and Marty, 1982) result from exocytosis and/or endocytosis. The secretory responses were induced by a voltage depolarizing step from −80 to −20 mV during 20 s, indicated below the C_m traces. Numbers adjacent to C_m traces represent C_m values before the voltage step. Dotted line corresponds to voltage zero level. Representative traces are shown. Measurements were made 3−24 h after microinjection of the mutant Rab3a proteins GST−TR3aT36N (T36N), GST−TR3aQ81L (Q81L) or GST−TR3aN135I (N135I). A non-injected cell is shown as control (CTL).

trol oligonucleotides ASR3b, S5 (Figures 6A and 7) and an antisense oligonucleotide directed to rat *rab3b* messenger (data not shown), the secretory response (ΔC_m) decreased rapidly with successive stimulations. In contrast, cells injected with antisense oligonucleotide AS5 overlapping the translation initiation codon of *rab3a* mRNA showed, 5 days after microinjection, a sustained secretory response (Figure 7). The amplitude of ΔC_m even increased significantly with repetitive stimulations (Figure 7). Similar results were obtained with antisense oligonucleotide ONT3, corresponding to residues 209–230 of *rab3a* mRNA (Figures 6B and 7). Furthermore, the rate of secretion ($\Delta C_m/\Delta t$) was found to increase with repetitive stimulation in cells injected with either AS5 or ONT3 (Figure 6B and Table I).

Under experimental conditions with 1 μM free Ca^{2+} in the pipette, the increase in C_m was followed by a decrease in all control cells (Figure 6A and Table I), consistent with net membrane retrieval. In contrast, in cells injected with antisense oligonucleotides, C_m did not decrease after the initial rise due to step depolarization and sometimes even continued to increase (Figure 6B and Table I). However, when the intracellular calcium concentration was reduced by removing Ca^{2+} from the pipette (Figure 6D), the secretory responses of cells loaded with AS5 (phase I) were followed by a decrease in C_m (phase II and Table I), thus indicating that antisense oligonucleotide AS5 was unlikely to affect endocytosis. It should also be noted that the rate of spontaneous secretion in the absence of Ca^{2+} did not seem to be impaired in these cells (Figure 6D, phase III).

To demonstrate further the specificity of the antisense approach, a rescue experiment was designed. If the observed effects of antisense oligonucleotides were due to suppression of *rab3a* gene expression, reintroduction of purified Rab3a should restore the original phenotype. The results of such an experiment are illustrated in Figures 6C and 8. Cells which were first injected with antisense oligonucleotides, and 5 days later with purified Rab3a protein, exhibited a secretory response similar to that measured in control cells (Figures 6C and 8). In contrast, injection of another *rab* protein, Rab1

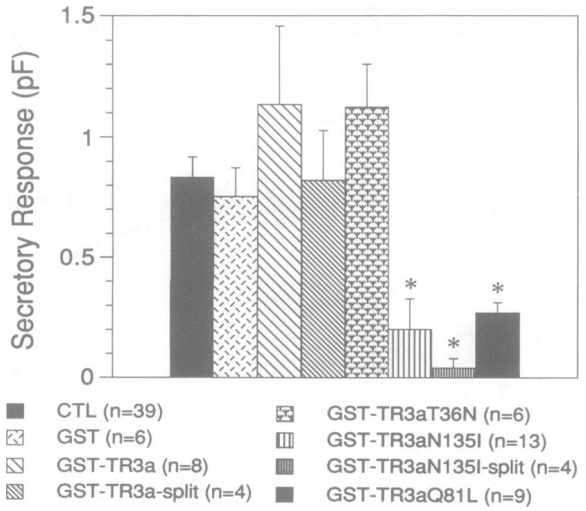

Fig. 5. Quantitative analysis of the secretory response of chromaffin cells after microinjection of recombinant proteins. The response to the first depolarization is shown. Data are given as difference between the maximum value of C_m after depolarization and the C_m value before depolarization (see traces in Figure 4). The cleavage of fusion proteins with thrombin (GST–TR3aN135I-split and GST–TR3a-split) did not change the result. Numbers of tested cells are indicated. Data are shown as mean ± SE and an asterisk (*) indicates $P < 0.001$ versus CTL (Mann–Whitney test).

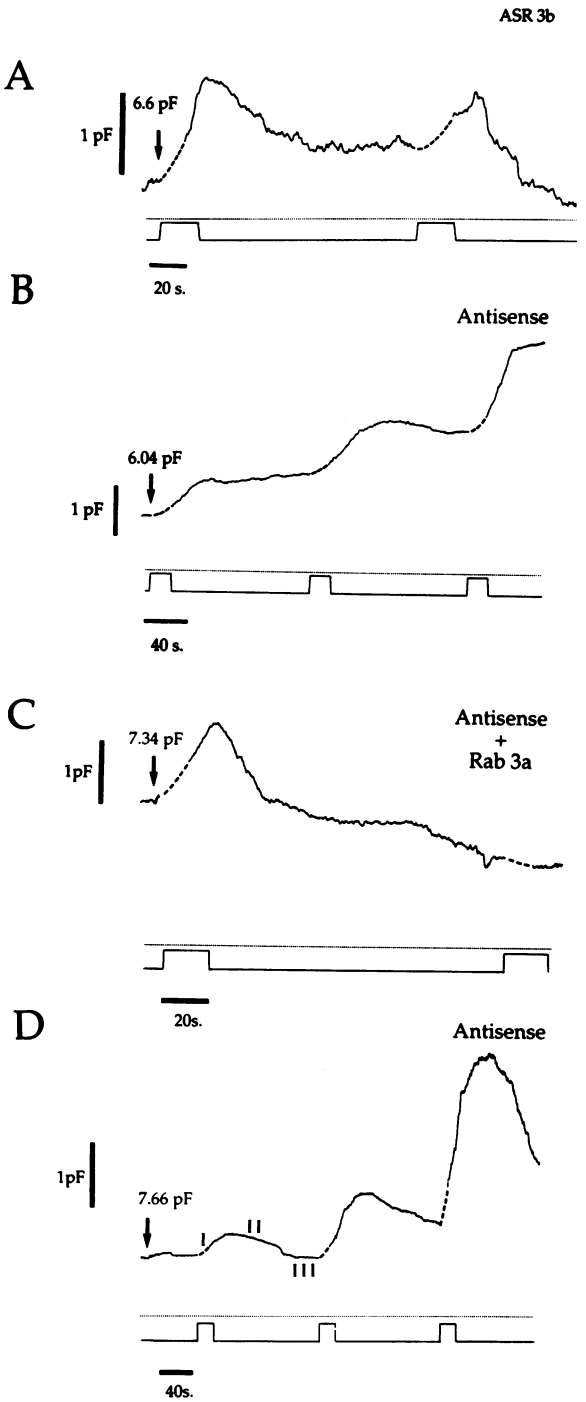

Fig. 6. Effects of antisense oligonucleotides directed to *rab3a* mRNA on exocytosis in chromaffin cells. Membrane capacitance changes were measured as described in Figure 4. Representative traces are shown. Cells were injected with antisense oligonucleotide ASR3b directed to bovine *rab3b* (**A**) as control and with antisense oligonucleotides ONT3 (**B**) and AS5 (**C** and **D**) to bovine *rab3a* 5 days before patch clamp recording. In (C) the cell was sequentially loaded with AS5 for 5 days and with recombinant Rab3a protein 4 h before recording. Injection of Rab3a protein completely reversed the AS5 effect. In (D) calcium was omitted from the pipette solution. Phase I, II and III are described in the text.

(Figure 8) and of an unrelated protein, GST (data not shown), was ineffective in restoring the original phenotype.

Pre-hybridization of AS5 to an oligonucleotide corresponding in length to AS5, but with the sequence of the opposite strand (S5), resulted in neutralization of the AS5 effect (Figure 7), indicating that AS5 has to be single-stranded to exert its function. In addition, it can also be excluded that any impurity of the oligonucleotide preparation is responsible for the observed changes in the secretory response. The same conclusion is suggested by the fact that AS5 from different commercial sources gave the same result (data not shown). The specificity of the antisense oligonucleotides was further supported by the fact that oligonucleotide AS5 inhibited the *in vitro* translation of *rab3a* mRNA, but not that of *rab3b* mRNA (data not shown). To demonstrate that the *in vivo* effect of AS5 and ONT3 was not due to a non-specific interference with *rab3b* gene activity, an antisense oligonucleotide (ASR3b) specifically directed to the bovine *rab3b* messenger was taken to replace AS5 and ONT3 in equivalent experiments. ASR3b did not have any effect on secretion (Figure 7 and Table I).

Finally, the effects of antisense oligonucleotides directed to the *rab3a* messenger were found to be reversible. They developed after a 2 day lag and were maximal 5 days after loading. After 9 days, the secretory response was similar to that of non-injected cells, thus arguing against a toxic effect of the oligonucleotides (data not shown).

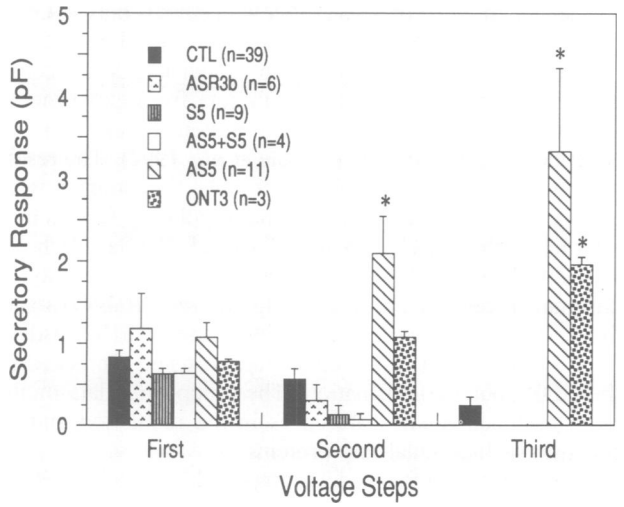

Fig. 7. Quantitation of the effect of different oligonucleotides on exocytosis. Different oligonucleotides were injected into chromaffin cells: antisense directed to *rab3a* (AS5 and ONT3), sense to *rab3a* (S5) or antisense to bovine *rab3b* (ASR3b). Open columns indicate cells that were injected with 10 μg/ml AS5 pre-hybridized to a 3-fold molar excess of S5. Non-injected cells are abbreviated as CTL. Numbers of tested cells are indicated. Data were determined as described in Figure 5. Bars represent standard error and an asterisk (*) indicates $P < 0.01$ versus CTL (Mann–Whitney test). The secretory response increased significantly from the first voltage step to the third one in ONT3 and AS5 treated cells ($P < 0.03$; Mann–Whitney test) and decreased significantly in control cells ($P < 0.001$; Mann–Whitney test).

Discussion

The recent finding that the *N*-ethylmaleimide-sensitive fusion protein (NSF), the soluble NSF attachment proteins (SNAPs), syntaxin A and B, SNAP25 and VAMP/synaptobrevin-2 assemble in a tight multimolecular complex (Söllner *et al.*, 1993) has shed some light on the molecular events underlying the process of exocytosis. Since VAMP/synaptobrevin-2 (Südhof *et al.*, 1989) and syntaxin A and B (Bennett *et al.*, 1993) are associated with, respectively, synaptic vesicles and plasma membrane, and since both proteins belong to protein families that have been implicated in defined transport steps in yeast, it has been suggested that these proteins provide a molecular basis for docking of secretory vesicles at release sites (reviewed by Warren, 1993). Rab3a, which is specifically associated with secretory vesicles in neurons (Fischer von Mollard *et al.*, 1990) and neuroendocrine cells (Darchen *et al.*, 1990), has recently been proposed to be part of this complex (Horikawa *et al.*, 1993), further suggesting a role for Rab3a in regulated exocytosis. The experiments presented in this paper confirm this hypothesis.

Overexpression in PC12 cells of an epitope tagged Rab3a protein mutated at position 135 (Asn to Ile), termed TR3aN135I, resulted in a pronounced inhibition of the secretory response. The same conclusion was reached after injection of the purified mutant protein into chromaffin cells. The corresponding mutations in *rab1a, rab1b, rab2* (Tisdale *et al.*, 1992), *rab5* (Bucci *et al.*, 1992) and *SEC4* (Walworth *et al.*, 1989) result in each case in a mutant protein that specifically inhibits a particular vesicle transport step in a *trans*-dominant manner.

Table I. Membrane capacitance changes in chromaffin cells injected with various oligonucleotides

Condition	Number of cells	$\Sigma\ \Delta C_m$ (pF)[a]	$\Delta C_m/\Delta t$ (fF/s)[b] during depolarization		$\Delta C_m/\Delta t$ (fF/s)[b] after depolarization
			step 1	step 2	
CTL	39	1.38 ± 1.36	40.2 ± 32.3	ND	−14.0 ± 9.9
S5	9	0.84 ± 0.61	28.2 ± 16.7	ND	−11.9 ± 22.5
ASR3b	6	1.72 ± 1.87	33.8 ± 52.8	ND	−23.1 ± 20.9
AS5	11	5.10 ± 3.65[c]	43.7 ± 27.1	104.9 ± 78.6[e]	+2.9 ± 4.7[c]
ONT3	3	3.16 ± 1.07[d]	20.7 ± 1.9	41.4 ± 21.4	+3.2 ± 2.9[c]
AS5 ($-Ca^{2+}$)	5	5.27 ± 4.4[c]	34.1 ± 24.4	55.6 ± 24.4	−11.7 ± 7.6

[a]$\Sigma\ \Delta C_m$ was obtained by cumulating the response to successive depolarizations.
[b]The rate of capacitance changes was measured during the initial rise in C_m, and after depolarization (corresponding to phases I and II, respectively, in Figure 6D). Data are shown as mean ± standard deviation.
[c]$P < 0.01$ (Mann–Whitney test) versus non-injected cells (CTL).
[d]$P < 0.04$ (Mann–Whitney test) versus non-injected cells (CTL).
[e]$P < 0.05$ (Mann–Whitney test) versus step 1.
ND = not determined.

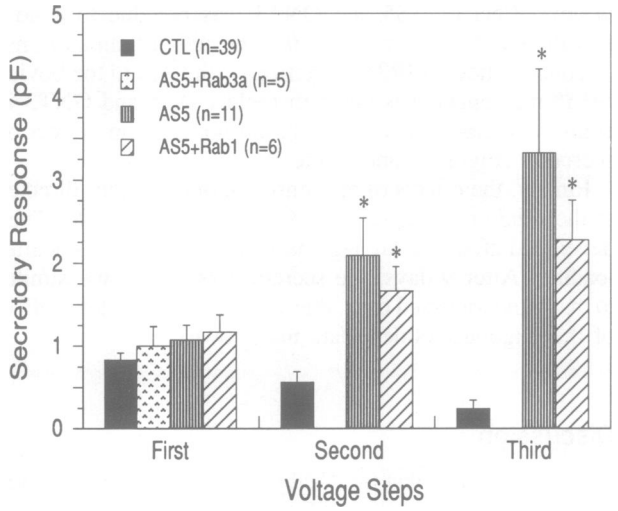

Fig. 8. Rescue experiment. Bovine chromaffin cells were sequentially loaded with antisense oligonucleotide AS5 directed to the *rab3a* messenger for 5 days and with recombinant Rab3a or Rab1 protein 4 h before recording. Injection of Rab3a completely reversed the AS5 effect while injection of Rab1 did not have any significant effect. Data are determined as described in Figure 5. Bars represent standard error and an asterisk (*) indicates $P < 0.01$ versus CTL (Mann−Whitney test).

That Rab3a is involved in regulated exocytosis was further supported by the effect of antisense oligonucleotides directed to the *rab3a* messenger. Whereas in control cells the amplitude of successive secretory responses diminished, the opposite was found in antisense DNA loaded cells which showed an increase in rate and amplitude of their responses. This effect appears to be specific since: (i) microinjection of purified Rab3a into antisense-injected cells reversed the antisense effect, whereas injection of another *rab* protein, Rab1, and of an unrelated protein, GST, was inefficient. (ii) Two oligonucleotides complementary to different regions of the *rab3a* mRNA had the same effect on secretion. (iii) Control oligonucleotides did not modify the secretory response. (iv) Pre-hybridization of the antisense oligonucleotide AS5 to a complementary oligonucleotide neutralized the effect of AS5. (v) The oligonucleotide AS5 inhibited the *in vitro* translation of *rab3a* mRNA, but not of *rab3b* mRNA.

Rab3a does not seem to be necessary for secretory granules to fuse with the plasma membrane since cells in which *rab3a* gene expression was inhibited still responded to membrane depolarization. What might be the function of Rab3a? We propose that Rab3a is a negative regulator of exocytosis. It might exert its inhibitory function by interacting, in its GTP-bound form, with components of the molecular complex proposed by Söllner *et al.* (1993), thus acting as a barrier to fusion. When secretion is triggered, GTP hydrolysis might be stimulated leading to the removal of the proposed barrier. The fact that cells which were injected with antisense oligonucleotides exhibited a higher ability to respond to successive stimulations supports the proposal of Rab3a being an inhibitor. Furthermore, a prediction from this model would be that a Rab3a mutant protein which is preferentially bound to GTP will inhibit secretion. The intrinsic GTP hydrolysis rate constant of Rab3a mutated at position 81 is strongly reduced (Brondyk *et al.*, 1993). This mutant protein, Rab3aQ81L, is still sensitive to Rab3a-GAP activity, but even in the presence of Rab3a-GAP the rate constant of GTP hydrolysis remains 8-fold lower than that of wild type Rab3a (Brondyk *et al.*, 1993). Rab3aQ81L is therefore thought to be predominantly in the GTP-bound conformation. Consistent with the proposed model we find that this mutant protein inhibits exocytosis, as measured by different techniques in different, though related cellular systems. The inhibitory effect of Rab3a mutated at position 135 is also consistent with the proposed model. This mutant protein has a greatly reduced affinity for guanine nucleotides (Brondyk *et al.*, 1993). Considering the dissociation rate constants for GDP and GTP and the intracellular GTP:GDP ratio, it was suggested that Rab3aN135I is preferentially associated with GTP *in vivo* (Brondyk *et al.*, 1993). However, the conformational state of the latter mutant has not yet been clearly established.

The lack of effect of TR3aT36N which binds only to GDP (Burstein *et al.*, 1992) indicates that the GDP-bound form of Rab3a does not inhibit secretion in neuroendocrine cells. This observation is consistent with data from experiments with the equivalent Rab2 mutant protein blocked in the GDP-bound conformation (Tisdale *et al.*, 1992). The result indicates that Rab3aT36N is not able to compete with endogenous Rab3a protein, and that regulatory factors that interact with the GDP-bound form of Rab3a, such as Rab3a-GRF or GDI, are not limiting for Rab3a activity in chromaffin cells or PC12 cells. In contrast, Rab5 (Li and Stahl, 1993; Stenmark *et al.*, 1994) and Rab1b (Tisdale *et al.*, 1992) inhibit a particular transport step if blocked in the GDP-bound conformation. These opposing data might point to fundamental differences in the regulation and/or function of individual *rab* proteins.

Overexpression of tagged wild type Rab3a protein in PC12 cells resulted in some inhibition of secretion, in agreement with the proposed model. In chromaffin cells we could not detect any effect of injection of tagged wild type protein TR3a, nor of the untagged wild type protein. We currently do not have an explanation for this difference between the two cell types. It is nevertheless tempting to speculate that in chromaffin cells in primary culture, the interplay between all regulatory factors is still well adapted to ensure a tight regulation of secretion. In contrast, the PC12 cell line is no more subjected to the selective pressure of a physiological background and changes in the regulatory apparatus may have occurred that might explain why the negative regulation by Rab3a can be improved by the introduction of additional recombinant protein.

Finally, the inhibition of exocytosis by non-hydrolyzable GTP analogs in permeabilized chromaffin cells (Ahnert-Hilger *et al.*, 1992; Sontag *et al.*, 1992) and in neurons (Hess *et al.*, 1993) is also in agreement with the proposed inhibitory role of the GTP-bound form of Rab3a.

Synaptotagmin has been proposed to serve as an inhibitor of regulated exocytosis (Popov and Poo, 1993). In this context, it is interesting to note that Rabphilin-3a, one of the interacting partners of GTP-bound Rab3a, shows sequence homologies to the C2 domains of synaptotagmin. It has been suggested that synaptotagmin and Rabphilin-3a might act in concert to control neurotransmitter release (DeBello *et al.*, 1993), on the basis that C2 domain peptides completely block secretion (Bommert *et al.*, 1993) whereas synaptotagmin mutations only produce a reduction in transmitter release (Littleton *et al.*, 1993).

One aspect of the physiological function of Rab3a could be to reduce the probability of spontaneous fusion events in resting cells. This is supported by the fact that injection of *rab3a* specific antisense oligonucleotides leads to a higher calcium sensitivity of the release mechanism, since in antisense oligonucleotide injected cells, endocytosis following stimulation was compensated by a sustained exocytosis when the pipette solution contained 1 μM free calcium (see Figure 6B). The C2 domains of Rabphilin-3a, which are known to confer Ca^{2+}-dependent phospholipid binding activity to protein kinase C and synaptotagmin, might provide a link between Rab3a and calcium signaling.

Another possible physiological function of Rab3a could be to adapt the secretory response to a given stimulus. A reduction in amplitude of the secretory response after successive stimulations has been reported for PC12 cells, termed response habituation (McFadden and Koshland, 1990), and for chromaffin cells (Von Rüden and Neher, 1993). Our results indicate that Rab3a might be responsible for this desensitization, since cells in which *rab3a* gene expression was inhibited did not show this phenomenon, thus suggesting that modification of Rab3a activity might be associated with adaptive processes. Such modifications could include phosphorylation as described for other *rab* proteins (Bailly *et al.*, 1991; Karniguian *et al.*, 1993). Since it is thought that presynaptic modifications leading to an increase in neurotransmitter release play a role in long-term potentiation (Bliss and Collingridge, 1993), these findings are in agreement with the possibility that Rab3a might also be implicated in this process.

Finally, it should be noted that the closely related protein Rab3b apparently acts in a different manner since exocytosis is inhibited in pituitary cells loaded with antisense oligonucleotides directed to *rab3b* mRNA (Lledo *et al.*, 1993). The fact that Rab3a and Rab3b are coexpressed in some cells (F.Nothias, personal communication) suggests that they might regulate different steps in calcium-induced secretion.

Materials and methods

Cell culture

Bovine chromaffin cells were isolated and cultured as previously described (Darchen *et al.*, 1990) and used 3–10 days after dissociation. PC12 cells were grown on poly-L-lysine coverslips in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (D.Dutscher, France), and differentiated for 48 h with 50 ng/ml NGF (Sigma, USA) before being stimulated for secretion.

Electrophysiology

Cells were voltage-clamped at a holding potential of around −80 mV. C_m was recorded using a dual phase lock-in amplifier (1600 Hz, 1 mV peak-to-peak) incorporated into a SWAM (Ljubljana, Slovenia) patch clamp amplifier (Zorec *et al.*, 1991), as previously described (Lledo *et al.*, 1993). The pipette solution contained (in mM): CsCl 150; $MgCl_2$ 2; HEPES 10; EGTA 10.25; $CaCl_2$ 9; cAMP 0.1; Na_2ATP 2 and GTP 0.4; pH 7.25 (with KOH). The calcium activity of this solution was found to be close to 1 μM (Lledo *et al.*, 1993). Where indicated, $CaCl_2$ was omitted. The recording medium contained (in mM): NaCl 127; KCl 5; $MgCl_2$ 2; $CaCl_2$ 5; NaH_2PO_4 0.5; $NaHCO_3$ 5; HEPES 10 and D-glucose 10; pH 7.25 (with NaOH).

Sequences: AS5 (5′-TGTGGCAGATGCCATCTTGTT-3′); S5 (5′-AACAAGATGGCATCTGCCACA-3′); ONT3 (5′-ACTCCCAGATCTGCAGCTTGATCC-3′); ASR3b (5′-GGTCACTGAAGCCATCTGGGA-3′). Searching the GenBank and EMBL databases did not detect any sequence that matched with AS5 and ONT3 except *rab3a*.

Microinjection

Antisense or control oligonucleotides (10 μg/ml) and proteins (0.5 mg/ml) were injected in buffer containing (in mM): sodium glutamate 135; NaCl 20; $MgCl_2$ 4; EGTA 0.5; GDP 0.05; HEPES 10, pH 7.2 and 1 mg/ml FITC–dextran (Sigma). Just before injection, samples were cleared by centrifugation at 140 000 *g* for 15 min. Injections were performed with an Eppendorf microinjector at constant pressure. The immunofluorescent signal of coinjected FITC–dextran allowed unambiguous identification of loaded cells. Membrane capacitance measurements were made for 2–9 days (for oligonucleotides) and 3–24 h (for proteins) after injection. For the rescue experiment, cells were first injected with antisense oligonucleotides and, 5 days later, with 0.5 mg/ml Rab3a, Rab1 or GST in buffer containing RITC–dextran instead of FITC–dextran. Cells injected with antisense oligonucleotides and subsequently with protein were identified positively by their fluorescence in the fluorescein and rhodamine channels. These cells showed normal ionic currents after stimulation, indicating that the integrity of their membranes was preserved (data not shown).

Site-directed mutagenesis

H-rab3a (Zahraoui *et al.*, 1989) was subcloned into pGEM4Z (Promega, USA), yielding pGEMRab3a. A c-Myc epitope of 10 amino acids (Evan *et al.*, 1985) was added to the N-terminus of wild type Rab3a using a PCR-based strategy (Johannes *et al.*, 1993), yielding pGEMTR3a. Briefly, primers ONT1 (5′-TATCAGAAGCTTGGCAAGATGGCATCGGAGCAGAAGCTAATCTCGGAGG-3′), ONT2 (5′-GTCTGTGGCCGATGCGGATCCTAGGTCCTCCTCCGAGATTAGCTTCTGC-3′) and ONT3 were used to generate a *Hin*dIII–*Pst*I fragment which was inserted into the corresponding sites of pGEMRab3a. Mutants pGEMTR3aN135I, pGEMTR3aV55E and pGEMTR3aQ81L were obtained by inserting, respectively, a *Pst*I–*Nhe*I PCR fragment (mutated at position 404), a *Hin*dIII–*Pst*I fragment (mutated at position 164) or a *Pst*I–*Nhe*I fragment (mutated at position 242) in pGEMTR3a (numbering according to Zahraoui *et al.*, 1989). The PCR primers were ONT1, ONT5 (5′-TTGGTCAGCTAGCTGCCGGCCACGTTCTGATGACACCACCCGCTCATCCTCCATGTCACACTTGATTCCTACCAGC-3′), V55E6 (5′-ACTCCCAGATCTGCAGCTTGATCCTCTTGTCGTTGCGATAGATGGTCTTGACCTTGAAGTCGATGCCCTCGGTGCTGACGAA-3′) and Q81L (5′-TCAAGCTGCAGATCTGGGACACAGCAGGGCTAGAGCGGTACCGGAC-3′). Mutation pGEMTR3aT36N was generated by a two step PCR method as described (Higuchi *et al.*, 1988; Ho *et al.*, 1989). The PCR primers were ONT1, ONT3, T36N8 (5′-CGGAAGAGGAAGGAATTCTTGCCCACGCT-3′) and T36N7 (5′-AGCGTGGGCAAGAATTCCTTCCTCTTCCG-3′). The final *Hin*dIII–*Pst*I fragment (mutated at positions 107 and 108) was inserted into pGEMTR3a. The constructs were verified by dideoxy sequencing (US Biochemicals).

Infection, transfection and secretion assay

PC12 cells were transfected using Transfectam (Sepracor, France) for 90 min at 37°C with various constructs under control of the T7 promoter, incubated overnight in serum-containing medium, and then infected with recombinant vaccinia virus expressing T7 RNA polymerase (Fuerst *et al.*, 1986). Infection was carried out at 37°C for 30 min in serum-free medium. More than 90% of PC12 cells were infected as indicated by the expression of the 14 kDa late phase vaccinia marker (Rodriguez *et al.*, 1985). Secretion was induced, 4 h later, by incubating the cells for 10 min at 37°C in Locke's medium (Sontag *et al.*, 1992) supplemented with 50 mM KCl. In some experiments, cells were depolarized with stimulation buffer devoid of $CaCl_2$, but supplemented with 2.2 mM $MgCl_2$ and 5 mM EGTA. Subsequently, cell-surface DBH was stained by incubating unfixed cells for 40 min at 4°C with anti-rat DBH antibody and then with rhodamine-conjugated secondary antibody (Biosys, France). The final steps were fixation with 4% paraformaldehyde/PBS, quenching in 50 mM NH_4Cl/PBS, permeabilization in 0.2% BSA/0.05% saponin/PBS, staining with the monoclonal anti-c-Myc 9E10 (Evan *et al.*, 1985) or IN1 antibodies and appropriate fluorescein-conjugated secondary antibodies (Amersham, UK). After mounting in Mowiol, the cells were viewed under a Zeiss Axiophot microscope.

Purification of fusion proteins

H-Rab3a was expressed in *Escherichia coli* and purified as described (Zahraoui *et al.*, 1989). For expression as GST fusion proteins, new *Eco*RI and *Hin*dIII restriction sites were added by PCR to the TR3a open reading frame using the primers EcoRI3 (5′-GAACTGAATTCCGAGCAGAAGCTAATCTC-3′) and 3Hind14 (5′-AGTACAAGCTTTGACATCTCCTAAGG-3′). The corresponding fragment was subcloned in frame into pGEX-2T (Pharmacia, USA). Subsequently, the *Bam*HI–*Sst*II fragment of this construct was exchanged for the corresponding fragments of

pGEMTR3a, pGEMTR3aT36N, pGEMTR3aV55E and pGEMTR3aN135I to yield pGEXTR3a, pGEXTR3aT36N, pGEXTR3aV55E and pGEX-TR3aN135I, respectively. Sequences derived by PCR were verified.

Fifty ml of an overnight culture of *E.coli* strain DH5α harboring respective pGEX-2T expression plasmids in LB medium with 50 μg/ml ampicillin were inoculated in 1000 ml of the same medium. After 90 min at 37°C the culture was induced with 0.5 mM IPTG for 5 h. The cells were harvested by centrifugation, resuspended in ice-cold 50 mM Tris−Cl, pH 8.0, 25% sucrose and lysed in the presence of 10 μM GDP and protease inhibitors by sequential addition of 3 mg/ml lysozyme (Sigma), 33 mM EDTA and 1% Triton X-100. Cell debris was removed by centrifugation for 45 min at 25 000 *g* at 4°C. The supernatant was incubated with 1.5 ml 50% suspension of washed glutathione−Sepharose 4B (Pharmacia) for 15 min at room temperature. The beads were washed three times at 4°C with PBS containing 1 mM $MgCl_2$, 50 μM GDP, three times with 50 mM Tris−Cl, pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 50 μM GDP, and once with 50 mM Tris−Cl, pH 7.5, 1 mM $MgCl_2$, 50 μM GDP. Proteins were eluted in the last wash buffer containing 10 mM reduced glutathione, dialyzed against 10 mM Tris−Cl, pH 7.5, 0.5 mM $MgCl_2$, 250 μM GDP and frozen in aliquots for storage at −70°C. SDS−PAGE analysis showed that the respective proteins were purified to near homogeneity and migrated as expected (data not shown). When indicated, GTP fusion proteins were split with thrombin (Sigma) in the elution buffer at a molar ratio of 80. After digestion, thrombin was inhibited by addition of 1 mM PMSF. Samples were dialyzed and stored as described.

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Note added

During completion of this work, we learnt that another group has also established a role for Rab3a in Ca^{2+}-dependent exocytosis in chromaffin cells, using the same mutant proteins (R.Holz, W.H.Brondyk, R.A.Senter, L.Kuzion and I.G.Macara, *J. Biol. Chem.*, in press.