Differential dynamics of Rab3A and Rab27A on secretory granules

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Summary

We have assessed the dynamics of the association of Rab3A and Rab27A with secretory granules at various stages of their life in PC12 cells. Endogenous Rab3A colocalised with the secretory granule marker secretogranin II (SGII) and expressed EGFP-Rab3A and ECFP-Rab27A colocalised with one another. The extent of colocalisation between EGFP-Rab3A or EGFP-Rab27 and SGII increased after longer times post transfection suggesting that these Rab proteins are preferentially recruited to newly synthesised granules. Following the release of immature secretory granules from the trans-Golgi network, Rab3A and Rab27A became associated with the immature granules after a lag period of around 20 minutes. Rab dynamics on granules were analysed in fluorescence recovery after photobleaching (FRAP) experiments. The recovery profile of EGFP-Rab27A was comparable to that of ppANF-EGFP, whereas the recovery profile of EGFP-Rab3A was significantly faster, indicating that Rab3A but not Rab27A might be rapidly exchanged between granules and cytosol. Inhibition of heat-shock protein 90 with 10 μM geldanamycin did not affect the exchange process or regulated exocytosis. Rab dynamics during stimulation with 300 μM ATP were analysed in live cells. Loss of granular ppANF-EGFP fluorescence was seen at the cell periphery after stimulation but only limited changes in EGFP-Rab3A and EGFP-Rab27A fluorescence was observed, indicating that the Rab proteins do not immediately dissociate or disperse on stimulation. The data suggest potentially distinct roles for Rab3A and Rab27A and we suggest that the finding that young secretory granules have a higher capacity for binding Rab3A and Rab27A is functionally important for preferential exocytosis from these granules.

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Key words: Rab proteins, GTPases, Exocytosis, Secretion, Secretory vesicle

Introduction

Rab proteins comprise the largest group within the Ras superfamily of monomeric GTPases, with 11 Rabs identified in yeast, and over 60 identified in mammals (Gurkan et al., 2005). Individual Rab subtypes characteristically associate with specific membrane compartments and interact with specific effector(s) to regulate trafficking between compartments (Pereira-Leal and Seabra, 2000). The expansion of the Rab gene family in higher eukaryotes is likely to reflect the increased complexity and regulation of membrane trafficking in these organisms and particularly that associated with the specialisation of membrane-trafficking pathways in specialised cell types (Bock et al., 2001). Regulated exocytosis is the release of vesicular or secretory granule contents in response to stimulation. A broad range of cell types including neuronal, exocrine, endocrine and immune cells are specialised for regulated exocytosis, and there are sometimes dramatic differences in the regulation of the process and in the morphology of the organelles involved (Burgoyne and Morgan, 2003). It is thought that the core machinery of exocytosis, and indeed that of membrane fusion in general, is conserved in the form of the soluble N-ethyl maleimide-sensitive attachment protein receptor (SNARE) proteins, whereas many other proteins, including the Rabs and their effectors, are involved in adapting the process according to its discrete cellular or physiological roles.

Two groups of Rab proteins localised to vesicles and secretory granules, the isoforms of Rab3 and Rab27, have been directly implicated in regulated exocytosis and much work has focused on the Rab3A and Rab27A isoforms. Rab3A overexpression is reported to be inhibitory (Holz et al., 1994; Johannes et al., 1998; Regazzi et al., 1996), although it might enhance constitutive exocytosis (Schluter et al., 2002). Rab27A overexpression is variously reported to inhibit or enhance regulated exocytosis (Desnos et al., 2003; Johnson et al., 2005; Menasche et al., 2003; Yi et al., 2002). Endogenous Rab3 and Rab27 isoforms, however, have positive roles in regulated exocytosis. Rab3A knockout mice show a moderate phenotype, with increased Ca^{2+}-triggered exocytosis leading to faster than normal ‘rundown’ of synaptic transmission in cultured hippocampal neurons (Geppert et al., 1994; Geppert et al., 1997) and reduced spontaneous neurotransmitter release at the neuromuscular synapse (Sons and Plomp, 2006). Mutation of the gene encoding Rab27A in humans results in Griscelli syndrome (Menasche et al., 2000) and loss of functional Rab27A in ashen mice is associated with defects in melanosome trafficking, defects in the exocytosis of lytic granules by cytotoxic T lymphocytes leading to

immunodeficiency and impaired release of insulin from pancreatic β-cells leading to glucose intolerance (Haddad et al., 2001; Kasai et al., 2005; Stinchcombe et al., 2004; Wilson et al., 2000). The importance of the Rab proteins in exocytosis might be even greater than the phenotypes of the mutant animals suggest if other Rab3 and Rab27 isoforms have roles that overlap with those of Rab3A and Rab27A, and partially compensate for their absence in the mutant animals. A quadruple knockout of Rab3A, Rab3B, Rab3C and Rab3D is postnatally lethal, and evoked exocytotic responses of cultured hippocampal neurons from these animals were reduced by ~30% compared with controls: a much greater effect than that seen when these isoforms were deleted singly (Schluter et al., 2004). A knock-in study in which mutant animals expressed EGFP-Rab27A on the native Rab27a promoter showed more widespread protein expression than expected in secretory endocrine and exocrine cells, and it is suggested that the most pronounced deficits in ashen mice are found in tissues that express only Rab27A (Tolmachova et al., 2004).

Like other small GTPases, Rabs are 'molecular switches', present in either GDP- or GTP-bound forms. When membrane associated, they may become GTP-bound through interaction with specific guanine nucleotide exchange factors (GEFs). It is generally the GTP-bound form of the Rab that is considered active, and which interacts with effectors, although the Rab27 effector granuphilin-a/Slp4-a can interact with the GDP-bound Rab (Fukuda, 2003). Rabs hydrolyse bound GTP to GDP following interaction with specific GTPase-activating proteins (GAPs). In the GDP-bound form, Rabs may be vulnerable to extraction to the cytosol: a process involving guanine nucleotide dissociation inhibitors (GDIs) (Luan et al., 1999). GDIs stabilise the GDP-bound conformation of Rabs and are required for their cytosolic localisation, because all cytosolic Rab protein is GDI bound. They are also thought to be important for correct targeting of Rabs back onto target membranes, a process also hypothesised to require a GDI displacement factor (GDF) (Pfeffer, 2005). Standard models of Rab dynamics suggest a continuous cycle of organelle association and disassociation (Grosshans et al., 2006).

The regulation and dynamics of Rab3A in neurons have been partially characterised but less is known about Rab27A. Candidates for the Rab3GEP and Rab3GAP have been described (Fukui et al., 1997; Nagano et al., 1998; Wada et al., 1996), and Rab3A is likely to associate with GDIα in preference to the other two GDI isoforms (Ishizaki et al., 2000). Rab3A on synaptic vesicles is largely GTP-bound, while GTP-hydrolysis has been linked to exocytosis (Stahl et al., 1994). Extraction of Rab3A from membranes is also suggested to be coupled to exocytosis as stimulation of neurons or synaptosomes was found to increase the proportion of the protein that was cytosolic (Fischer von Mollard et al., 1991; Sakisaka et al., 2002; Star et al., 2005). The extraction process is proposed to involve a GDIα-Hsp90 chaperone complex (GCC) comprising GDIα, the Hsp90/Hsc70 chaperone system and the cysteine-string protein (CSP) (Sakisaka et al., 2002) and inhibition of Hsp90 has been shown to inhibit dissociation of Rab3A and exocytosis in brain synaptosomes. Although it has been proposed that Rab3A cycles off membranes during or following exocytosis nothing is known about its constitutive recycling.

In the present study, we describe our findings regarding the regulation and dynamics of Rab3A and Rab27A in an endocrine secretory cell type, the PC12 cell. PC12 cells are a commonly used model endocrine cell line used in the study of exocytosis (Burgoyne and Morgan, 2003). Importantly, recent work has established that Rab3A and Rab27A are likely to be the only two Rabs that function in regulated exocytosis in this cell type and that both contribute in a non-redundant manner to secretory granule exocytosis (Tsuboi and Fukuda, 2006). Secretory granules are distributed throughout the cytosol in PC12 cells and have an approximately ten times greater diameter than synaptic vesicles (Warashina, 1985; Willig et al., 2006). It is therefore possible to resolve labelled secretory granules by conventional confocal microscopy and it is similarly practical to carry out fluorescence recovery after photobleaching (FRAP) experiments to investigate Rab dynamics directly on secretory granules. Secretory granules can exist in differing states during their life cycle. After budding from the trans-Golgi network (TGN) immature granules move to the cell periphery where they are releasable but undergo maturation processes that result in an increase in their size, removal of specific components and their immobilisation in the cell cortex (Dittie et al., 1997; Rudolf et al., 2001; Tooze et al., 1991), Newly synthesised secretory granules (up to 16 hours old) undergo exocytosis in preference to older granules (Duncan et al., 2003). We have investigated the association of Rab3A and Rab27A with granules during various stages in their life cycle. We were able to demonstrate that Rab3A and Rab27A are recruited to immature granules shortly after budding and are associated preferentially with newly synthesised granules. In addition, Rab3A but not Rab27A is capable of exchanging rapidly between newly synthesised and older granules and cytosol but this exchange occurs in an Hsp90-independent manner. Finally, neither Rab3A nor Rab27A disperses immediately when secretory granules undergo exocytosis.

Results

Exogenously expressed Rab3A and Rab27A are preferentially recruited to newly-synthesised secretory granules

To study the localisation and dynamics of Rab3A and Rab27A, expression vectors for N-terminally fluorescently tagged versions of these proteins were generated. Coding sequences for the rat orthologues of the Rab proteins were amplified from an existing construct or rat PC12 cell cDNA respectively, and then subcloned into pEGFP-C1 or pECFP-C1 expression vectors. It was decided to use N-terminally tagged constructs to avoid any potential deleterious affects on the C-terminal geranylgeranyl site of the Rabs that is required for membrane association. N-terminal modification does not appear to affect Rab protein function, because chimeric Rab27A N-terminally tagged with GFP has the capacity to functionally replace the wild-type protein in a mouse 'knock-in' model (Tolmachova et al., 2004).

Endogenous Rab3A and Rab27A are reported to associate with secretory granules in PC12 cells (Tsuboi and Fukuda, 2006). We confirmed this localisation in PC12 cells by probing fixed cells with antibodies against Rab3A and the secretory granule marker protein secretogranin II (SGII) (Fig. 1A-C) (Steiner et al., 1989). It was not possible to carry out a similar experiment to determine the localisation of endogenous

Rab27A because antibodies were not available. However, when ECFP-Rab27A and EGFP-Rab3A were coexpressed (Fig. 1D-F), their localisation was punctate, closely resembled that of endogenous Rab3A, and the two tagged Rab proteins showed punctate colocalisation. To determine whether the localisation of the exogenous Rab proteins matched that of the endogenous protein, cells were transfected with each of the constructs, fixed 18 hours post transfection and then probed with the anti-SGII antibody. Surprisingly, the colocalisation between the exogenous Rabs and SGII was only partial (for an example, see the colocalisation between ECFP-Rab27A and SGII in Fig. 1G-I). Similar findings were made when cells were probed for cysteine string protein (CSP) (Fig. 1J-L), another marker of secretory granules (Chamberlain et al., 1996). One possibility is that exogenously expressed Rabs become localised with only part of the cellular compliment of granules because the protein synthesised post transfection was preferentially recruited to newly synthesised secretory granules. To test this hypothesis, a transfectable granule content marker, prepro-atrial natriuretic factor-EGFP (ppANF-EGFP), was used. ppANF-EGFP is packaged within the granule core, and so can only label granules formed post-transfection (Burke et al., 1997). When it was cotransfected with ECFP-Rab27A (Fig. 1M-O), ppANF-EGFP showed almost complete colocalisation, suggesting that the Rab protein is recruited to the granules synthesised post-transfection as they mature, but is present to a lesser extent on older pre-existing secretory granules.

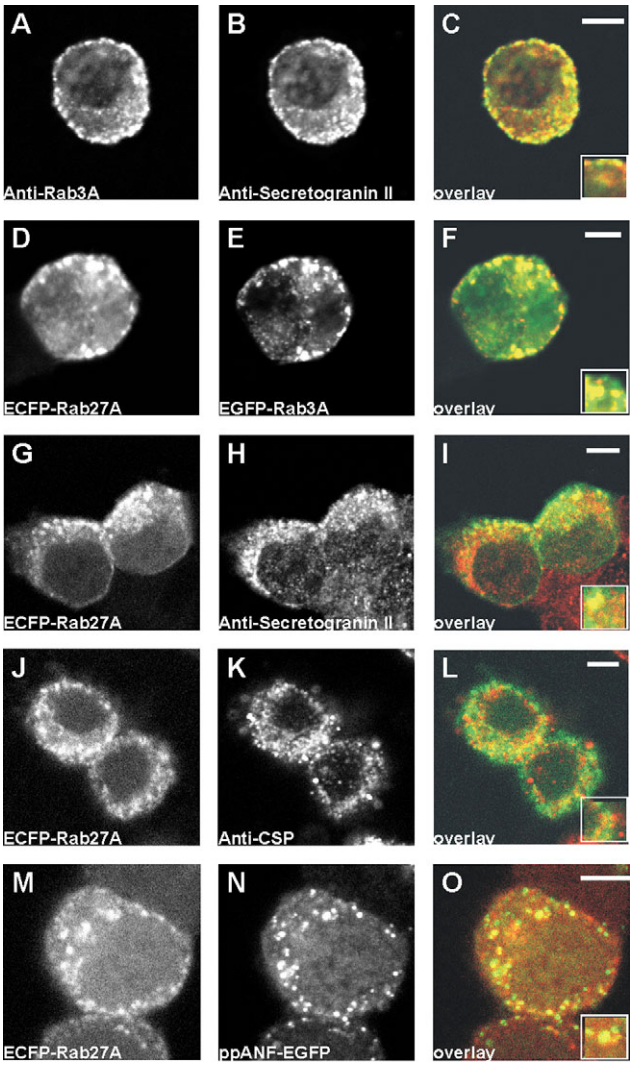

Fig. 1. Recruitment of EGFP-Rab3A and EGFP-Rab27A to newly synthesised granules in PC12 cells. The cells were examined 18 hours after transfection. (A-C) Cells after fixation co-immunostained with anti-Rab3A and anti-secretogranin II (anti-SGII). (D-F) Cells co-transfected with plasmids encoding ECFP-Rab27A and EGFP-Rab3A. (G-I) Cells transfected with a plasmid encoding ECFP-Rab27A, which after fixation, were immunostained with anti-SGII. (J-L) Cells cotransfected with plasmids encoding ECFP-Rab27A and prepro-atrial natriuretic factor-EGFP (ppANF-EGFP). Each overlay image on the right of the figure is a composite of green from the left image and red from the centre image. Areas of overlap appear in yellow. Magnified views of areas of colocalisation are shown at the bottom right. Bars, 4 μm.

If the exogenous Rab proteins are recruited to granules during granule maturation, their colocalisation with SGII should improve over time as granules synthesised post transfection make up a greater proportion of the total cellular compliment. To test this prediction, cells were transfected with EGFP-tagged Rab3A or Rab27A, or with ppANF-EGFP as a control, fixed at 18 hours and 30 hours post transfection, probed with anti-SGII, and compared. Colocalisation between each construct and SGII was partial after 18 hours but more extensive 30 hours after transfection (Fig. 2) and this was confirmed by quantitative analysis (Fig. 2S). It seems therefore that the tagged Rab proteins are preferentially recruited to newly synthesised secretory granules.

Association of Rab3A and Rab27A with immature secretory granules following their biogenesis.

Studies in PC12 cells have shown that immature secretory granules move within seconds to the cell periphery following their budding from the TGN, and after a further period of tens of minutes become immobilised in the cell cortex (Rudolf et al., 2001). During maturation secretory granules increase in size and specific components are removed from them with a half-time of around 45 minutes (Dittie et al., 1997; Tooze et al., 1991). During the maturation period the granules can undergo regulated exocytosis (Tooze et al., 1991). We aimed to examine when it is during their biogenesis that the two Rab proteins become associated with secretory granules. To do this we made use of the blockade of vesicle formation at the TGN that is produced when cells are incubated at 20°C. This has been used previously to follow immature granules in PC12 cells (Rudolf et al., 2001). Following culture for 18 hours at 20°C, cells expressing ppANF-EGFP showed accumulated fluorescence in a perinuclear region, as expected if it was trapped in the TGN. Switching of the cells to 37°C for 30 minutes resulted in the extensive appearance of labelled punctate structures throughout the cells, which are likely to be immature granules (Fig. 3A). By contrast, expressed EGFP-tagged Rab3A and Rab27A showed a more diffuse cytosolic distribution at 20°C, although punctate structures were visible indicating that a proportion of the Rabs can associate with some pre-existing granules. After 30 minutes at 37°C, EGFP-Rab3A and EGFP-Rab27A were almost completely punctate in localisation, suggesting that they had become associated with the immature granules. To follow the time course of their

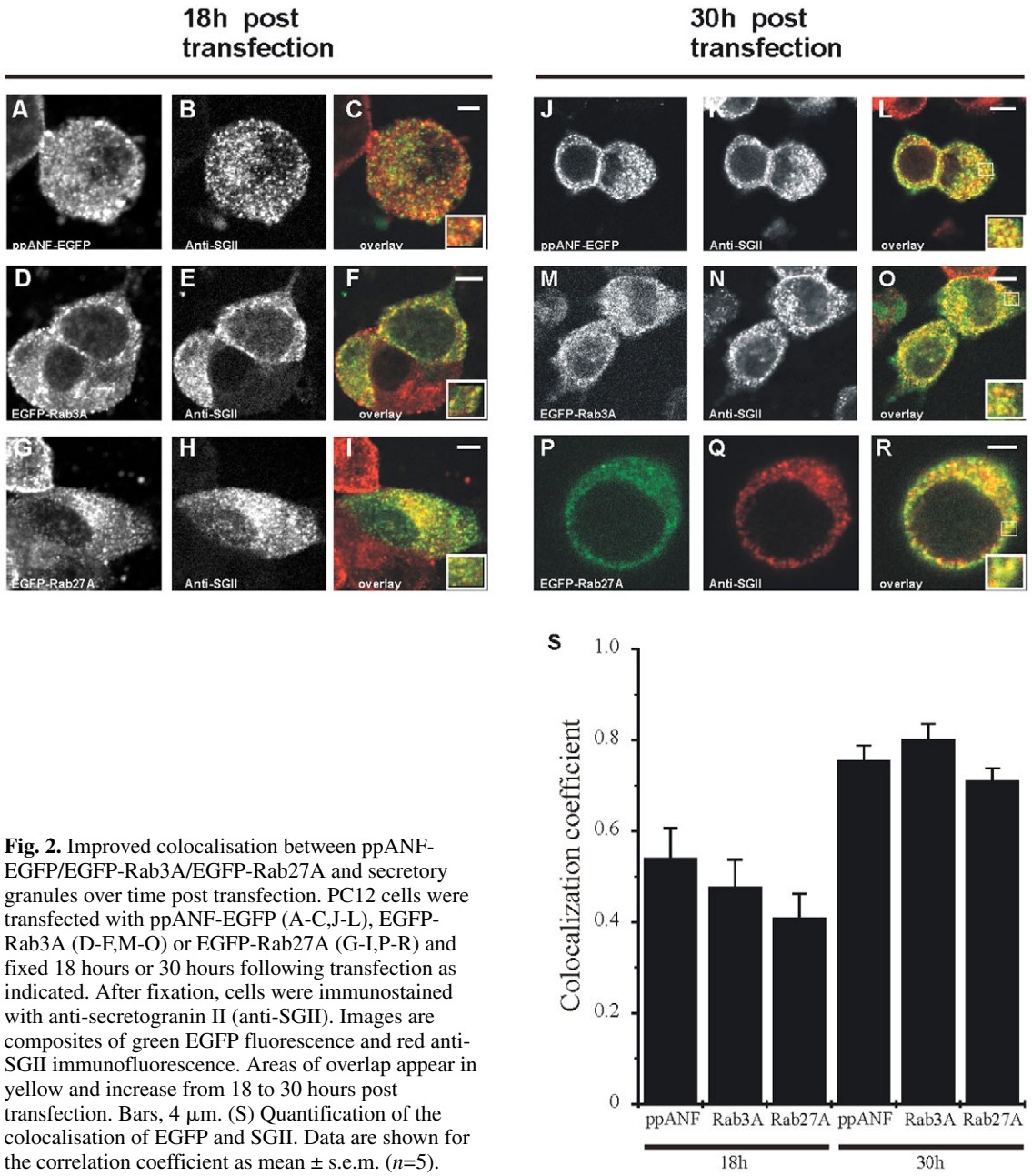

Fig. 2. Improved colocalisation between ppANF-EGFP/EGFP-Rab3A/EGFP-Rab27A and secretory granules over time post transfection. PC12 cells were transfected with ppANF-EGFP (A-C,J-L), EGFP-Rab3A (D-F,M-O) or EGFP-Rab27A (G-I,P-R) and fixed 18 hours or 30 hours following transfection as indicated. After fixation, cells were immunostained with anti-secretogranin II (anti-SGII). Images are composites of green EGFP fluorescence and red anti-SGII immunofluorescence. Areas of overlap appear in yellow and increase from 18 to 30 hours post transfection. Bars, 4 μm. (S) Quantification of the colocalisation of EGFP and SGII. Data are shown for the correlation coefficient as mean ± s.e.m. (n=5).

association with immature granules, cells were transfected to coexpress ppANF-EGFP and Rab3A or Rab27A tagged with monomeric red fluorescent protein (Fig. 3B). Immature secretory granules were visible throughout the cells after 5-10 minutes following incubation at 37°C. However, colocalisation of ppANFP-EGFP and mRFP-Rab3A or mRFP-Rab27A was not evident until the cells had been incubated at 37°C for 20 minutes or more indicating a lag period between release of immature secretory granules from the TGN and the association of the Rab proteins of up to 20 minutes.

Rab3A but not Rab27A is dynamically associated with secretory granules in PC12 cells

Recruitment of Rab3A and Rab27A to newly synthesised secretory granules is consistent with the suggestion made for synaptic vesicles that once the proteins associate with vesicles, they are only released during or following exocytosis (Fischer von Mollard et al., 1991; Star et al., 2005). To directly address this possibility, it was decided to use FRAP experiments. In a FRAP experiment, the fluorescent protein within a small region of interest (ROI) of a cell is bleached using a high-intensity laser, and the recovery of fluorescence within this area is subsequently monitored (Lippincott-Schwartz and Patterson, 2003). The recorded fluorescence recovery reflects the replacement of bleached protein in the ROI with unbleached protein from outside this region. To test whether the protocol that was to be used could successfully resolve exchange of fluorescent protein between cytosol and membrane in PC12 cells, bleaching experiments were carried out on ARF1-EGFP-transfected cells. ARF1 is a small GTPase shown to cycle

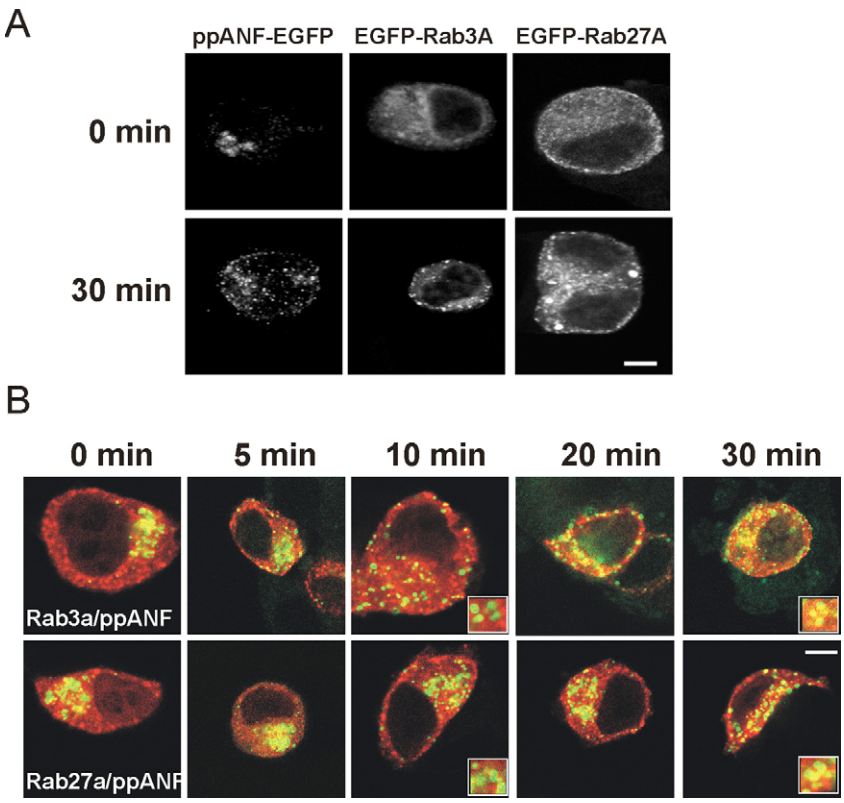

Fig. 3. Appearance of Rab3A and Rab27A on immature secretory granules during granule biogenesis and maturation. (A) PC12 cells were transfected with ppANF-EGFP, EGFP-Rab3A or EGFP-Rab27A and cultured for 18 hours at 20°C to block granule budding at the TGN and then fixed (time 0) or incubated for 30 minutes at 37°C to allow budding of immature granules. (B) PC12 cells were co-transfected with ppANF-EGFP and mRFP-Rab3A or mRFP-Rab27A, cultured for 18 hours at 20°C and then fixed at the indicated times after incubation at 37°C. The images show overlays of ppANF-EGFP fluorescence in green and mRFP fluorescence in red. Expanded inserts are shown for the 10- and 30-minute time points to show lack of colocalisation at 10 minutes and colocalisation at 30 minutes. Bars, 4 μm.

rapidly between cytosolic and Golgi-associated states in other cell types (Presley et al., 2002; Vasudevan et al., 1998). Expressed ARF-1-EGFP was partially cytosolic but also showed a clear localisation to a perinuclear region presumed to be the Golgi complex in PC12 cells (Fig. 4A). Following the bleaching of an area of the Golgi complex, fluorescence recovery of this ARF-1-EGFP occurred with a $t_{1/2}$ of 44 seconds and was almost complete (fractional recovery of 87.5%) within the recorded timeframe of the experiments (Fig. 4A,E). This behaviour was similar to that described for ARF1 previously and confirms the applicability of the FRAP protocol in PC12 cells.

Following bleaching of fluorescently tagged EGFP-Rab3A or EGFP-Rab27A, fluorescence recovery in the ROI could be attributed to three sources: the diffusion of unbleached cytosolic protein, exchange between bleached and unbleached protein on granule membranes, and the movement from other parts of the cell of granules carrying unbleached protein. Compared with the other components of recovery, diffusion of unbleached protein into the ROI was likely to be rapid (~1 second), whereas recovery as a result of granule movement was initially an unknown quantity. For the latter aspect, the recovery following bleaching of ppANF-EGFP was assessed (Fig. 4B,E,F). Since this protein is contained within granules, its recovery profile would be solely due to the effects of granule movement, and provides a reference for comparison with the other data. Imaging of live cells expressing ppANF-EGFP indicated that the labelled secretory granules had only limited mobility (see supplementary material Movie 1). For photobleaching experiments, a small area of the cell was chosen containing several labelled secretory granules. Following photobleaching of ppANF-EGFP (Fig. 4A,E,F) only a slow and partial recovery of fluorescence (fractional recovery of 23.7%) in the bleached region was observed with a $t_{1/2}$ of 58 seconds consistent with limited secretory granule mobility under resting conditions in these cells.

EGFP-Rab27A or EGFP-RAB3A transfected cells were used in the FRAP experiments 18 hours post-transfection to analyse Rab dynamics on newly synthesised granules and only cells expressing relatively low levels of the exogenous protein were selected. For Rab27A (Fig. 3C,E), as expected, it was found that proportionate fluorescence recovery at ~1.6 seconds post bleaching (i.e. the first post-bleaching data point) was greater than seen in the ppANF-EGFP-transfected cells, consistent with a greater cytosolic component of recovery. However, when the data were normalised with respect to this initial data point, the recovery profiles of these proteins were not significantly different, suggesting that the minimal EGFP-Rab27A recovery seen after photobleaching was largely or entirely due to granule movement rather than exchange of Rab27A on the granules (Fig. 4F). In the case of EGFP-Rab3A (fractional recovery 66.6%), the recovery profile (Fig. 4D,E,F) was strikingly different to that of EGFP-Rab27A (fractional recovery 37.8%). The recovery of EGFP-Rab3A was more similar to that of the ARF1-EGFP with extensive although not complete recovery with a $t_{1/2}$ of 32 seconds. These data suggest that like ARF1 but unlike Rab27A, Rab3A cycles continuously between the cytosol and membranes, and that in PC12 cells at least, this recycling is not exclusively coupled to the exocytotic event.

The apparent difference between newly synthesised and pre-existing secretory granules in terms of the amount of

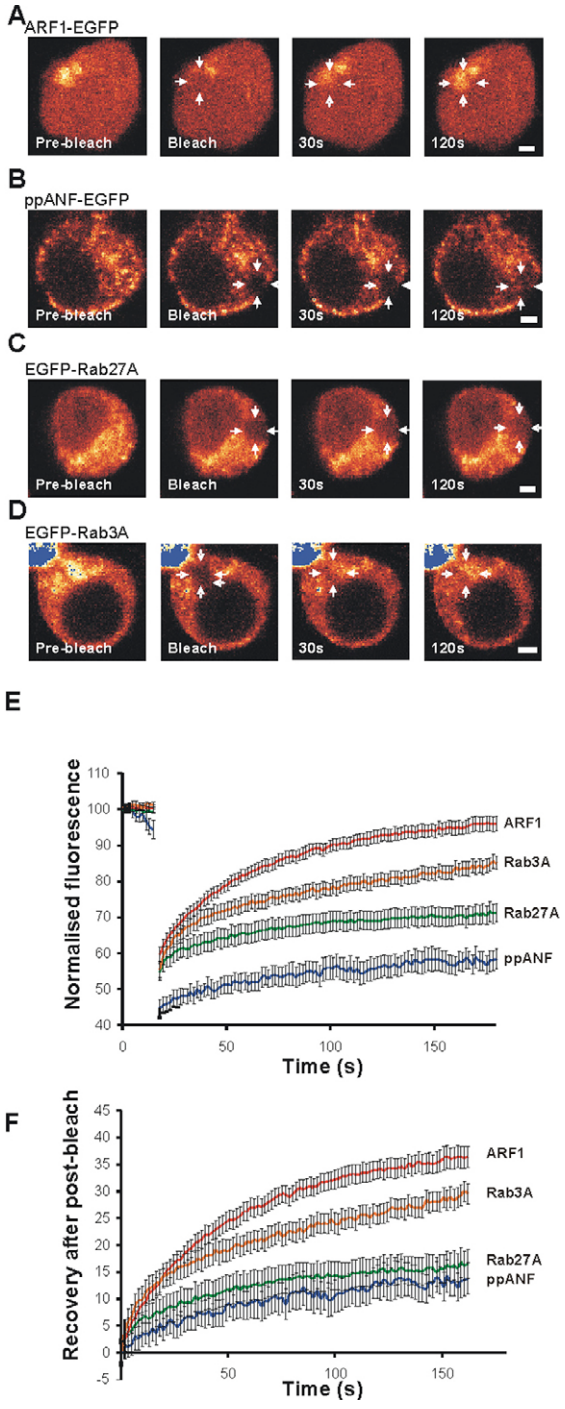

Fig. 4. Dynamics of EGFP-Rab3A and EGFP-Rab27A on newly synthesised secretory granules in PC12 cells. (A-D) PC12 cells were transfected to express the indicated EGFP-tagged protein and examined after 18 hours. Regions of interest (ROIs) in each cell were bleached with high intensity laser (arrows) and the fluorescence recovery of these areas was followed over time. (E) Fluorescence in the ROI was recorded over time during low-intensity imaging. The data are shown as mean ± s.e.m. of 14 ARF1-EGFP, 13 ppANF-EGFP, 21 EGFP-Rab27 and 21 EGFP-Rab3A expressing cells. The data were corrected for general photobleaching for each cell at each time point and normalised by setting the initial fluorescence value for each cell to 100. (F) The data were replotted with the initial post-bleach data point set to 0. Bars, 2 μm.

tagged Rab3A that becomes associated to give differing fluorescence intensities at 18 hours post transfection and more homogenous intensity after 30 hours post transfection could have two explanations. The first is that Rab3A on older granules is unable to cycle between the granules and cytosol. The second is that the capacity of old granules for recruitment of Rab3A is lower than that of newer granules. To test the possibility of a difference in cycling we carried out FRAP experiments on older EGFP-Rab3A-labelled granules in cells at 30 and 54 hours after transfection and compared these to granules in cells at 18 hours post transfection. EGFP-Rab3A recovered with similar kinetics onto granules in cells at 18, 30 and 54 hours but with a slightly higher fractional recovery for the older granules (Fig. 5). These data indicate that Rab3A can cycle on and off both newly synthesised and older granules.

Inhibition of HSP90 does not affect Rab3A dynamics or regulated exocytosis in PC12 cells

Recent work has suggested that Rab protein extraction from membranes involves the heat-shock protein Hsp90 (Chen and Balch, 2006; Sakisaka et al., 2002). More specifically, it was previously found that extraction of Rab3A from synaptosome membranes was regulated by a chaperone complex containing GDIα, Hsp90, Hsc70 and CSP (Sakisaka et al., 2002) and also that inhibition of Hsp90 inhibited Ca^{2+}-triggered exocytosis in synaptosomes. While the authors suggest that Hsp90-dependent Rab3A extraction is coupled to exocytosis, it was possible that the continuous cycling of EGFP-Rab3A between membrane and cytosol that we observed was also mediated via this complex. To investigate this possibility, parallel bleaching experiments were carried out on control EGFP-Rab3A-transfected cells and cells incubated with 10 μM geldanamycin for 1 hour before bleaching. Geldanamycin is a potent and specific inhibitor of Hsp90, and was used here at the

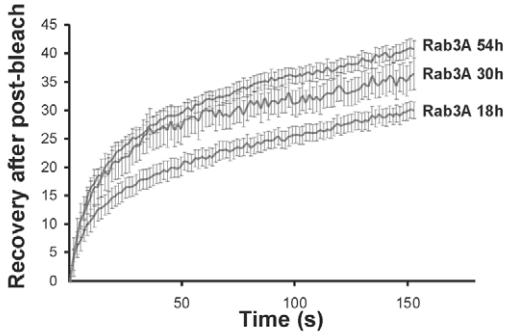

Fig. 5. Dynamics of EGFP-Rab3A on newly synthesised and old secretory granules in PC12 cells. PC12 cells were transfected to express the indicated EGFP-tagged protein and examined 18, 30 or 54 hours post transfection. Regions of interest (ROIs) in each cell were bleached with high intensity laser and the fluorescence recovery of these areas was followed over time. Fluorescence in the ROI was recorded over time during low-intensity imaging. The data are shown as mean ± s.e.m. for 32 EGFP-Rab3A expressing cells at 18 hours, 10 at 30 hours and 27 at 54 hours. The data were corrected for general photobleaching for each cell at each time point and normalised by setting the initial fluorescence value for each cell to 100 and with the initial post-bleach data point set to 0.

concentration found to virtually abolish extraction of Rab3A from synaptosomal membranes (Sakisaka et al., 2002) following stimulation with Ca^{2+}. The recovery profile for EGFP-Rab3A following photobleaching in control cells was closely similar to that in the earlier experiments (compare Fig. 4 with Fig. 6). It was found that recovery profiles for control and treated cells were virtually identical (Fig. 6A) with $t_{1/2}$ of 29 and 28.6 seconds for control and geldanamycin-treated cells, respectively, suggesting that the observed EGFP-Rab3A cycling occurs independently of Hsp90. It was also reported that geldanamycin could block neurotransmitter release from synaptosomes. To see whether there was a similar effect on the release of secretory granule contents from PC12 cells, we assayed ATP-stimulated release of exogenously-expressed human growth hormone (hGH) from PC12 cells pre-incubated for 1 hour before stimulation with varying concentrations of the inhibitor (see Fig. 6B). The release of hGH was assayed as this would allow examination of release from newly synthesised granules in transfected cells (i.e. those that exogenous Rabs become associated with). As shown, geldanamycin had no significant effect on hGH release within the range of concentrations used suggesting that secretory granule exocytosis occurs in an Hsp90-independent manner in this cell type.

It was possible that the negative results described above were caused because the geldanamycin used was inactive, or because PC12 cells were in some way resistant to its effects. To rule out these possibilities, the effects of the inhibitor on the well characterised Hsp90-dependent nuclear translocation of glucocorticoid receptor (GCR) were determined. It has been found that 10 μM geldanamycin can block dexamethasone-induced nuclear translocation of GCR-EGFP in NIH-3T3 cells (Galigniana et al., 1998), and so this protocol was applied to PC12 cells. GCR-EGFP-transfected cells were serum starved, chilled on ice, exposed to 1 μM dexamethasone, and then either vehicle or 10 μM geldanamycin. Following a brief period of incubation at 37°C and fixation, 100 cells per treatment expressing moderate amounts of this protein were scored according to its localisation. In individual cells in the cultures the GCR-EGFP was either predominantly in the cytosol or nucleus (Fig. 6C). As expected, the presence of geldanamycin increased the proportion of cells in which the

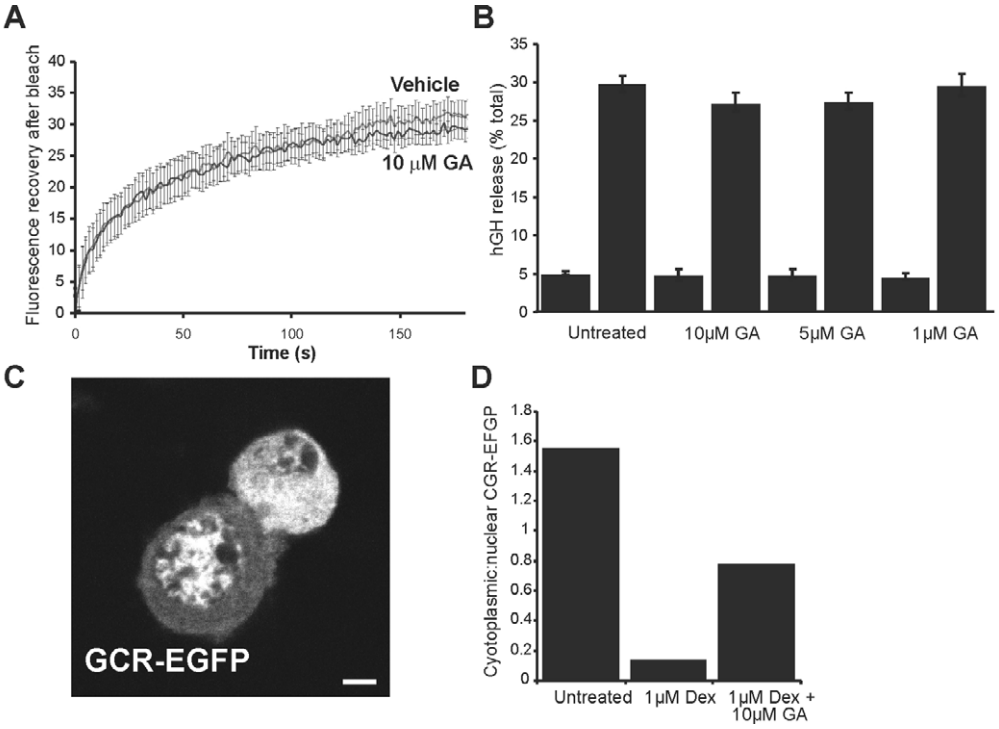

Fig. 6. Inhibition of Hsp90 does not affect EGFP-Rab3A dynamics or exocytosis in PC12 cells. (A) Cells were transfected with 0.5 μg EGFP-Rab3A and at 18 hours post transfection small regions of interest (ROIs) in each cell were bleached with high intensity laser. Fluorescence recovery was recorded over time and corrected to account for bleaching sustained during low-intensity imaging, summed and normalised to the first data point post-bleach. Data are shown from control cells and cells recorded in parallel that had been treated with 10 μM geldanamycin (GA) for 1 hour before bleaching (n=11 for each condition). (B) Secretion of exogenous human growth hormone (hGH) from cells treated with varying concentrations of GA for 1 hour before stimulation was assayed. Stimulated cells were exposed to 300 μM ATP, hGH release over 15 minutes was assayed and expressed as a percentage of total hGH. (C) Representative image of control cells expressing glucocorticoid receptor-EGFP (GCR-EGFP) 18 hours after transfection with 0.5 μg GCR-EGFP showing two adjacent cells in which the GCR-EGFP is either predominantly cytosolic or alternatively nuclear. Bar, 2 μm. (D) Effect of geldanamycin on GCR-EGFP translocation to the nucleus. After transfection with GCR-EGFP, treated cells were exposed to 1 μM dexamethasone on ice for 1 hour or 1 μM dexamethasone on ice for 1 hour with the addition of 10 μM geldanamycin for the final 30 minutes. Following a 20-minute incubation at 37°C and fixation, 100 cells were scored for each condition according to the cytosolic or nuclear localisation of GCR-EGFP Data are expressed as cytoplasmic:nuclear ratios and are representative of two independent experiments.

CGR-EGFP was cytosolic (Fig. 6D), indicating that it was indeed active in PC12 cells.

Exocytosis of secretory granules in PC12 cells does not lead to immediate dispersal of Rab3A and Rab27A to the cytosol

It has previously been reported that activation of exocytosis leads to Rab3A extraction from synaptic membranes (Fischer von Mollard et al., 1991; Star et al., 2005). Indeed, Star et al. have directly imaged the reversible dispersal of EGFP-Rab3A away from presynaptic terminals and into neighbouring axonal regions during periods of synaptic activity. It is unknown, however, whether dissociation and dispersal occurs for Rabs located on dense core secretory granules. In addition, experiments on synaptic Rabs requires use of either biochemical approaches or imaging of whole synaptic terminals whereas study of PC12 cells allows imaging of events on single secretory granules in live cells. Therefore, it was decided to image EGFP-Rab3A and EGFP-Rab27A-transfected cells during stimulation with ATP to raise intracellular calcium and activate exocytosis in PC12 cells. As it has been reported that overexpression of Rab3A or Rab27A in PC12 cells inhibits secretion (Chung et al., 1999; Desnos et al., 2003; Schluter et al., 2002), it was first necessary to make sure that transfected cells were capable of exocytosis. Cells were co-transfected with a range of concentrations of each Rab expression vector and an expression vector encoding human growth hormone. At 48 hours post-transfection, cells were stimulated with 300 μM ATP and secreted hGH was assayed. Transfection with high levels of EGFP-Rab3A or EGFP-Rab27A plasmids substantially inhibited evoked exocytosis of hGH and plasmids encoding untagged Rabs had a similar effect. However, exocytosis was only partially reduced at lower levels of plasmid (0.05 or 0.1 μg) (data not shown), indicating that the imaging experiments could be carried out.

Live cell imaging experiments were continued using low plasmid concentrations. In addition, cells transfected with 0.1 μg of each plasmid were imaged at only 18 hours post transfection so that expression levels of the Rabs would be less than in the 48-hour hGH experiments and only those cells expressing low levels of fluorescence were monitored. Cells were loaded with the Ca^{2+} indicator X-Rhod for measurement of changes in intracellular Ca^{2+} concentration resulting from stimulation to confirm that the cells responded to 300 μM ATP. An increase in Ca^{2+} concentration was found to occur in all cells analysed and the mean change in X-Rhod fluorescence over the time course of experiments is shown in Fig. 7A. Images were taken using settings to image within a thick confocal section to ensure that granules were not lost because of movement out of the confocal section and cells were rejected if any focus drift as a result of cell movement was detected. Initially, ppANF-EGFP-transfected cells were observed to see if the cells underwent exocytosis in response to 300 μM ATP

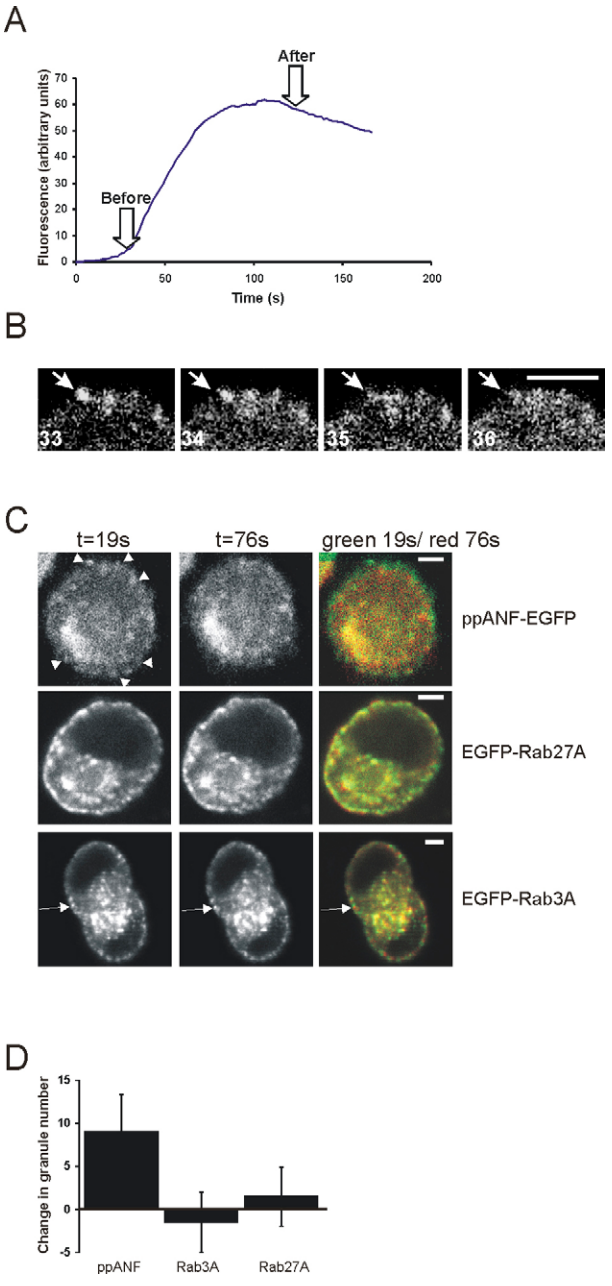

Fig. 7. Effect of activation of exocytosis on secretory granules labelled with EGFP-ppANF, EGFP-Rab3A or EGFP-Rab27A in live cells. Cells were transfected to express EGFP-ppANF, EGFP-Rab3A or EGFP-Rab27A and imaged 18 hours after transfection. Cells showing low levels of fusion protein expression were selected for observation and stimulated by perfusion with 300 μM ATP. (A) Example of the time course of Ca^{2+} changes from monitoring of X-Rhod fluorescence following ATP stimulation (n=11 cells). (B) Disappearance of a ppANP-EGFP-labelled granule during stimulation. The images shown are sequential frames in which the sudden disappearance of a granule (arrow) can be observed. (C) Effect of stimulation with 300 μM ATP on secretory granules labelled with EGFP-ppANF, EGFP-Rab3A or EGFP-Rab27A as indicated. Images were taken at 19 seconds and 76 seconds (after the elevation in cytosolic Ca^{2+}). Overlay images are shown with t=19 seconds in green and t=76 seconds in red so that granules that disappear between the images appear green. Granules that disappeared in the ppANF-EGFP cell are indicated with arrowheads. Some granules did not disappear but moved during the recording and so appear green in the overlay; an example is indicated by the arrow in the EGFP-Rab3A-transacted cell. Bars, 4 μm. (D) The number of granules that disappeared between the before and after images was identified by the comparison of images at 19 and 76 seconds, quantified and shown as mean ± s.e.m. for eight cells expressing each construct.

(Fig. 7B,C). As noted above, there is little movement or loss of granules from the imaged field under resting conditions. During stimulation, ppANF-labelled granules in the cell periphery but not those in the cell interior were seen to disappear abruptly often between successive images (Fig. 7B), indicating exocytosis of these granules. The number of granules lost per cell was variable but on average close to 20% of labelled granules disappeared (nine per cell, Fig. 7C). This can be seen in the example shown in Fig. 7C in which images before (green) and after (red) stimulation have been overlaid so that granules that remained appear yellow. By contrast, when EGFP-Rab3A or EGFP-Rab27A-transfected cells were imaged during stimulation, although some short-range granule movement was noted, there was no overall loss of fluorescent granules at the cell periphery or in the cell interior (see Fig. 7C). These results suggest that dissociation of Rab3A and Rab27A from secretory granules does not occur following stimulation nor do the Rabs immediately disperse from the sites of exocytosis at the plasma membrane in PC12 cells.

Discussion

We have examined various aspects of the dynamics and lifecycle of Rab3A and Rab27A on secretory granules in PC12 cells. Four lines of evidence from the data presented here point to the suggestion that on exogenous expression Rabs 3A and 27A are recruited preferentially to newly synthesised granules in PC12 cells: (1) the initially partial colocalisation of the exogenous Rab proteins with SGII; (2) their stronger colocalisation with ppANF-EGFP; (3) their stronger colocalisation with SGII over time post transfection; and (4) their more diffuse cytosolic localisation in cells in which budding of new granules from the TGN has been blocked. Under conditions where formation of new granules is blocked, more of the Rab3A and Rab27A were cytosolic, but some was associated with pre-existing granules indicating that the exogenously expressed Rabs can associate to a lesser extent with older granules. The 20°C block experiments also demonstrate that Rab3A and Rab27A only become associated with immature secretory granules after a lag period of 10-20 minutes following budding from the TGN. At this time, immature secretory granules in PC12 cells can undergo regulated exocytosis (Tooze et al., 1991).

The data from FRAP analysis showed that Rab3A, but not Rab27A can cycle rapidly between the granule membrane and the cytosol. The reproducibility of the Rab3A cycling data was shown by the independent series of experiments carried out, including those in the presence or absence of gelanamycin. The findings for Rab3A are not unexpected because other small GTPases (Mochizuki et al., 2001; Presley et al., 2002; Vasudevan et al., 1998), and indeed Rab5 and Rab7, have shown similar properties. FRAP experiments on EGFP-Rab5 exogenously expressed in CHO cells showed that the bleached protein on phagosomes is rapidly replaced with a $t_{1/2}$ of ~5 seconds (Vieira et al., 2003). EGFP-Rab7 expressed in Mel JuSo cells is replaced on endosomes and lysosomes with a $t_{1/2}$ of ~52 seconds, whereas 31% of the protein was found to be in an immobile fraction (Jordens et al., 2001). The contrasting finding that Rab27A does not cycle once it becomes granule associated is surprising and differs from other Rabs that have been examined, but is consistent with other recent data showing that in unstimulated platelets Rab27A is predominantly in a GTP-bound and membrane-associated form (Kondo et al., 2006) whereas in the case of Rab3A in synaptosomes there is a significant cytosolic GDP-bound pool (Stahl et al., 1994).

How can the preferential recruitment of Rab3A to newly synthesised granules be explained? One possibility is that this is due to differences in the exchangeability of Rab3A between new and old granules and that although Rab3A can initially cycle between these and the cytosol, over time, it becomes progressively more tightly associated and unexchangable as the granules age. Perhaps Rab3A becomes segregated to a stable multimolecular ‘rab domain’ such as that reported for Rab5 and Rab9 (Ganley et al., 2004; Zerial and McBride, 2001), and this domain is resistant to disassembly because of positive feedback activation of Rab GEF. Alternatively, it could be suggested that sites for tight binding between the Rab protein and granule membrane are saturable and that the capacity of older granules for binding Rab3 is lower than younger granules. The finding that EGFP-Rab3A could cycle between the cytosol and newly synthesised (18-hour) or older (30- and 54-hour) granules in FRAP experiments provides an important clue that supports the latter explanation.

What is the explanation for the striking difference between Rab3A and Rab27A dynamics? Both Rab3A and Rab27A are likely to be targeted to saturable binding sites on newly synthesised granules. What is the mechanistic basis for the observed difference between Rab3A and Rab27A? A simple explanation for this difference might be that on granules, Rab3A has a higher intrinsic GTP hydrolysis rate than Rab27A, with cycling dependent on the presence of releasable GDP-bound Rab protein. Another possibility would be differential regulation by Rab-specific GAPs. Rab3GAP is present on synaptic vesicles, and may be active here and also on secretory granules (Sakane et al., 2006) and a distinct candidate Rab27 GAP has been described recently (Itoh and Fukuda, 2006). Another possibility is that GTP-bound Rab27A is unable to dissociate from membranes once it is bound. This would be consistent with data showing that Rab27A has a low intrinsic GTPase activity and also that the GDP-bound form of Rab27A in platelets remains membrane associated in stimulated cells (Kondo et al., 2006). The differential dynamics of Rab3A and Rab27A on secretory granules suggests that these two Rabs might have distinct functional roles in exocytosis and this would be consistent with differences in effectors preferred by these Rabs (Fukuda, 2005) and the finding that they appear to act in concert on granule docking (Tsuboi and Fukuda, 2006).

One already identified mechanism of rab3A extraction into the cytosol is that involving an Hsp90-containing complex (Sakisaka et al., 2002). However, we found that Hsp90 inhibition by geldanamycin had no effect on Rab3A cycling as identified by FRAP, nor did it affect exocytosis. The concentration of the inhibitor used was sufficient to inhibit dexamethasone-induced nuclear translocation of GCR-EGFP. One interpretation of the data is that the mechanism for membrane-extraction of Rab3A differs between PC12 cells and neurons. However, Chen and Balch (Chen and Balch, 2006) reported that an Hsp90-dependent mechanism of Rab extraction operates on other Rab subtypes and in various cell types, making this interpretation unlikely. Another interpretation is that separate mechanisms exist for Rab

extraction during rapid cycling and where a donor membrane compartment meets its target membrane. Under this interpretation the Hsp90-dependent mechanism would control only Rab extraction at the target membrane. Removal of the Rab during cycling may involve the removal of the Rab alone, whereas removal of the Rab at the target membrane is likely to require the disassembly of Rab-containing complexes. However, it should be noted that unlike the situation in synaptosomes (Sakisaka et al., 2002), geldanamycin had no detectable effect on exocytosis in PC12 cells.

Previous studies on Rab3A have identified neuronal Rab3A dissociation and dispersal coupled to exocytosis (Fischer von Mollard et al., 1991; Sakisaka et al., 2002; Star et al., 2005). By contrast, one study on Rab3A in synaptosomes observed that GDP-loaded Rab3A remained membrane associated after stimulation (Stahl et al., 1994). We found that in stimulated PC12 cells, secretory-granule-associated Rab3A and Rab27A did not dissociate from granules nor disperse when cells were stimulated. Evidence has been provided for very rapid retrieval of dense-core granules (Graham et al., 2002; Holroyd et al., 2002). It is possible therefore, that Rab3A and Rab27A in PC12 cells remain clustered at the plasma membrane following exocytosis in a manner similar to synaptotagmin in neurons (Willig et al., 2006). In PC12 cells, the functional requirement for the Rab, or the scale of the dispersal process, might differ in this regard to that in neurons. Nevertheless, the findings reported here suggest that the dissociation of Rab3A and Rab27A from membranes is not an integral aspect of the exocytotic process. In addition, the stable association of Rab3A and Rab27A with secretory granules would be consistent with a role for these Rabs in maintaining the fusion competency of secretory vesicles and perhaps the differing dynamics of these two Rabs may underlie the dual requirement for them in dense-core granule exocytosis (Tsuboi and Fukuda, 2006).

Recent work by Schluter et al. has improved the understanding of the function of Rab3A in synaptic exocytosis and suggested that Rab3A boosts the release probability of a subset of the vesicles of the readily releasable pool (Schluter et al., 2004). This finding presents the question as to how Rab3A, which is present on all vesicles, can regulate the properties of only some. It might be relevant that in chromaffin cells, one factor regulating the release probability of secretory granules is granule age, with newly synthesised granules reportedly released in preference to older granules (Duncan et al., 2003). The molecular basis for this difference between old and young granules has been unknown. Since we describe data suggesting that Rab3A and Rab27A associate preferentially with young granules, it is an intriguing possibility that this is a functionally important determinant of this phenomenon.

Materials and Methods

Plasmids

The rat Rab3A sequence was amplified from an existing vector (Haynes et al., 2001) by PCR and inserted into the pEGFP-C1 vector (Clontech, Basingstoke, UK) and the pmRFP-C1 vector (kind gift of R. Y. Tsien, Department of Pharmacology, University of California, San Diego, CA) to generate mammalian expression constructs for Rab3A, N-terminally tagged with EGFP and monomeric red fluorescent protein (mRFP) (Campbell et al., 2002). Primers contained restriction endonuclease sites (underlined) to facilitate cloning. The sense primer used was 5′-AGCAGAAGCTTTAAATATGGCATCTGCCACAGACGCT-3′; *Hin*dIII, and the antisense primer was 5′-CAAGTATGGATCCGCTCAGCAGGCGCAGTCCTGATGCGG-3′; *Bam*HI. PC12 cell cDNA was prepared from total PC12 cell RNA extracted using Trizol Reagent (Invitrogen) followed by first strand cDNA synthesis with oligo dT(15) primers and Improm reverse transcriptase (Promega). The rat Rab27A sequence was amplified from the PC12 cDNA by PCR and then inserted in frame into the pEGFP-C1, pECFP-C1 and pmRFP-C1 vectors to generate mammalian expression constructs for Rab27A, N-terminally tagged with EGFP, ECFP and mRFP. Primers contained restriction endonuclease sites (underlined) to facilitate subcloning. The sense primer used was 5′-AGCAGAAGCTTTAAATATGTCGGATGGAGATTATGAC-3′; *Hin*dIII, and the antisense primer was 5′-CAAGTATGGATCCGCTCAACAGCCGCATAACCCCTTCTC-3′; *Bam*HI. Sequences contained in the recombinant plasmids were verified by automated sequencing (DBS Genomics, Durham, UK) and comparison with published sequence information. The ppANF-EGFP (Burke et al., 1997), ARF1-EGFP (Haynes et al., 2005) and glucocorticoid receptor-EGFP (Galigniana et al., 1998) fusion constructs were as described previously. Human growth hormone (hGH) encoding plasmid (pXGH5) was obtained from Nichols Institute Diagnostics, San Clemente, CA.

Cell culture and transfection

PC12 cells were maintained in suspension in 75-cm^2 culture flasks at 37°C in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 10% horse serum, 5% foetal calf serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin. Transfection of PC12 cells was carried out as follows; 24-well plates were seeded with ~4×10^5 cells per well on glass coverslips and cells allowed to adhere overnight. Prior to transfection, the growth medium was replaced with 400 μl/well Optimem 1 media (Gibco). Transfection mixes consisting of 3 μl Lipofectamine 2000 reagent (Invitrogen, Paisley, UK) per μg plasmid in 100 μl Optimem 1 media (Gibco) were prepared and incubated at room temperature for 20 minutes before being added drop-wise to the cells. Five hours following their addition, transfection mixes were removed and replaced with normal growth medium.

Immunocytochemistry

Transfected cells grown on coverslips were washed in PBS and then fixed in 0.5 ml 4% (v/v) paraformaldehyde for 30 minutes at room temperature. Coverslips were then washed twice in PBS, and exposed to primary antibody in PBS containing 0.3% BSA and 0.1% Triton X-100 (PBT) for 1 hour. Primary antibodies, mouse anti-Rab3A (Transduction Labs, Cowley, UK), rabbit anti-secretogranin II (Abcam, Cambridge, UK) and rabbit anti-CSP (Chamberlain et al., 1996) were used at 1:400, 1:100 or 1:400 dilutions respectively. Unbound antibody was removed by washing three times with PBT, and coverslips were exposed to appropriate secondary antibodies at a 1:100 dilution in PBT for 60 minutes. Either Alexa Fluor 488-conjugated, Alexa Fluor 594-conjugated (Molecular Probes, Paisley, UK) or biotinylated (Amersham Biosciences, Buckinghamshire, UK) secondary antibodies were used. After an additional round of washing, coverslips previously exposed to biotinylated antibody were exposed to a 1:50 dilution of streptavidin-conjugated Texas Red (Amersham Biosciences) in PBT for 30 minutes. After a final washing step, coverslips were air dried before mounting on glass slides using Prolong antifade reagent (Molecular Probes).

Confocal microscopy

Confocal microscopy following immunocytochemistry was carried out on a Leica TCS-SP microscope (Leica Microsystems, Heidelberg, Germany) using a 63× oil-immersion objective with a 1.4 numerical aperture and pinhole set to airy1. ECFP-containing constructs were excited using a 405 nm laser, and emitted light was collected between 450-500 nm. EGFP-containing constructs were excited using a 488 nm laser and light collected between 500-550 nm. Texas Red, Alexa Fluor 594 and monomeric red fluorescent protein were excited using a 594 nm laser and light collected between 625-675 nm. For the quantification of colocalisation, images were analysed using the Manders Coefficient plug-in of ImageJ to generate values for the Pearson colocalisation coefficient

Live-cell confocal microscopy in bleaching and perfusion experiments was carried out on a Leica TCS-SP-MP microscope (Leica Microsystems) with images captured every 1.64 seconds. A 63× water-immersion objective with a 1.2 numerical aperture was used and the pinhole set to airy 2.06 (for photobleaching) or airy 3 for imaging before and after stimulation. For bleaching experiments, cells transfected with 0.5 μg of each EGFP-containing construct were excited with a 488 nm laser and light collected between 500-550 nm. Bleaching was carried out by selecting circular regions of interest of diameter 2.5 μm, using 75% of laser power. To determine the rate of fluorescence recovery, fluorescence in these regions was measured over time, and then normalised with respect to corresponding total cellular fluorescence at each individual time point to correct for bleaching during low power laser excitation. For perfusion experiments, cells were transfected with 0.1 μg of each EGFP-containing construct. Transmitted light images were taken during experiments to ensure that changes in fluorescence were not a result of focal drift. All imaging of live cells and was carried out on cells at room temperature in Krebs Ringer buffer (145 mM NaCl, 20 mM HEPES, 10 mM Glucose, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4). Where the effects of geldanamycin (GA) were analysed, control cells were incubated in Krebs, and treated cells incubated in Krebs containing 10 μM GA, for 1 hour before bleaching. For analysis of changes in intracellular Ca^{2+} concentration, cells were loaded with 5 μM X-Rhod (Molecular Probes, Paisley) in 3 mM Ca^{2+}-Krebs for 20 minutes and then in X-Rhod-free 3 mM Ca^{2+}-Krebs for a further 20 minutes

before imaging. Where cells were stimulated, 300 μM ATP in 3 mM Ca^{2+}-Krebs was applied by perfusion.

Assay of growth hormone secretion

Assay of exocytosis from transfected cells made use of heterologous expression of hGH expression (Wick et al., 1993) as described previously (Graham et al., 2000). In these experiments, 24-well poly-D-lysine-coated plates (BD Biosciences, San Jose, CA) were seeded with ~4×10^5 cells per well, and cells were transfected as described. Each well was transfected with 0.5 μg of the pXGH5 hGH-encoding vector either alone, or combined with other vectors as detailed in the text. 48 hours post transfection, cells were assayed for hGH release (Graham et al., 2000). Cells were washed once in 3 mM Ca^{2+}-Krebs and then exposed to 200 μl of 3 mM Ca^{2+}-Krebs (unstimulated) or 200 μl of 3 mM Ca^{2+}-Krebs and 300 μM ATP, (stimulated) for 15 minutes. hGH release (supernatant) was quantified using an enzyme-linked immunosorbent assay (ELISA) kit (Roche, East Sussex, UK) according to the manufacturer's instructions. Levels of remaining cellular hGH were also quantified, to determine levels of total cellular hGH. The data for hGH secretion were calculated as a percentage of total hGH for each well and are presented as mean ± s.e.m.

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