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Ral and Rab3a are major GTP-binding proteins of axonal rapid transport and synaptic vesicles and do not redistribute following depolarization stimulated synaptosomal exocytosis

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We have employed high-resolution SDS polyacrylamide gels to demonstrate that there are two major low-molecular-weight GTP-binding proteins associated with axonal membranes including synaptic vesicles, rapid transported membranes and clathrin-coated vesicles. We demonstrate that one of the major proteins is Ral and that the other is Rab3A. Following the depolarization of synaptosomes resulting in increased neurotransmitter release, we see no significant dissociation of either Ral or Rab3a from synaptic vesicle derived membranes in contrast to results reported previously.

Introduction

Recent evidence from a variety of systems implicates low-molecular-weight GTP-binding proteins (LMW-GBPs) as key regulators of membrane transport between organelles. Based on a genetic and biochemical analysis of Sec4, a yeast LMW-GBP involved in vesicle transport from the Golgi to the plasma membrane, it was postulated that a cycling of these proteins between membrane-bound and soluble states is required for regulating membrane fission and fusion events [1]. Another yeast LMW-GBP, Ypt1, appears to be involved in the control of vesicle movement between the endoplasmic reticulum and the Golgi [2]. A theoretical model has also been proposed utilizing this concept to explain the vectorial control of intracellular vesicle traffic [3]. Evidence has been obtained that several mammalian LMW-GBPs, including the *rab3A* gene product, a recently characterized LMW-GBP found on synaptic vesicles, exist in two forms, an integral membrane-bound and cytosolic form [4–6].

Synaptosomes, which consist of pinched-off nerve terminals, have provided an excellent system to study molecular events associated with Ca^{2+}-regulated exocytic membrane fusion and recycling events. Recently, it was reported that Rab3A rat cerebral cortical synaptosomes, slowly but almost quantitatively dissociate from the synaptic vesicle membrane during the course of Ca^{2+}-stimulated exocytosis and recycling [7]. This observation lends support for an association-dissociation cycle of LMW-GBPs during transport, fusion and recycling of the synaptic vesicle.

In this report we confirm the identity of Rab3A as a major LMW-GBP in both synaptic vesicle and axonal rapid transport vesicles. Finally we also identify a second, slightly larger LMW-GBP constituent of purified synaptic vesicles and rapid transport vesicles as Ral. We also present data indicating that there is no significant decrease in the amount of either Rab3A nor Ral associated with the synaptic vesicle membrane during exocytosis in synaptosomes, a finding at variance with reported results [7].

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Abbreviations: LMW-GBP, low-molecular-weight GTP-binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; PAS, protein A Sepharose; HBS, 142 mM NaCl, 2.4 mM KCl, 1.2 mM K_2HPO_4, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1 mM EGTA and 10 mM Hepes adjusted to pH 7.4 with Tris.

Materials and Methods

Materials

[^{3}H]Norepinephrine and [^{14}C]ACh were from Amersham. [α-^{32}P]GTP (approx. 3000 Ci/mmol), L-

[*methyl*-^{3}H]methionine (80 Ci/mmol), [^{125}I]goat-anti-rabbit F(ab)$_2$ fragments (5.3 μCi/μg) and nitrocellulose paper were from Dupont New England Nuclear. Trans ^{35}S-label methionine (> 1000 Ci/mmol) was from ICN Biomedicals. Rab1B was a generous gift from Channing Der (La Jolla, CA, USA). Recombinant H-Ras was from Oncogene Science. Protein A Sepharose, carboxypeptidase A and the rabbit anti-rat secondary antibody were from Sigma. Proteinase inhibitors were from Boehringer-Mannheim. Nitrocellulose filters were from Millipore. Centricon-10 Microconcentrators were from Amicon. The Y13-259 p21^{ras} antibody was kindly supplied by Dr. Richard Roth (Stanford, CA, USA). [α-^{32}P]GTP was converted to [α-^{32}P]GDP as previously described [8]. Monoclonal anti-synaptophysin antibody was from Accurate. Peroxidase-labelled antibodies were from Sigma and ECL detection reagents were from Amersham.

Production and characterization of recombinant Rab3A

The rat rab3A open reading frame was amplified by the polymerase chain reaction and subcloned into the pET3a expression vector as described previously [8]. Recombinant protein was purified to > 95% homogeneity and > 95% activity from *Escherichia coli* transformed with the pET-rab3A plasmid, as described [9].

Production of antiserum

Purified, active r-Rab3A (0.5 mg) was emulsified in Freund's complete adjuvant and injected subcutaneously at multiple sites into a New Zealand rabbit. After 3.5 weeks, another 0.4 mg of r-Rab3A emulsified in Freund's incomplete adjuvant was injected, and 3 weeks later serum was collected.

Immunoblotting

Proteins were separated by 14% or 18% SDS-PAGE, transferred to nitrocellulose paper and immunoblotted as described previously [5] using a 1:200 dilution of either anti-Rab3A antiserum or preimmune serum, or 1 μg/ml anti-synaptophysin. Detection of antigen/antibody complexes was accomplished by using either ^{125}I-labelled goat anti-rabbit F(ab)$_2$ fragments or the enhanced chemiluminescence (ECL) Western blotting detection system. For the ECL system, the nitrocellulose which had been probed with primary antibody was washed and incubated for 1 h with horseradish peroxidase-labelled goat anti-mouse IgG or anti-rabbit IgG (1:2000 dilution) in phosphate-buffered saline containing 0.1% Tween-20. After washing, the nitrocellulose was incubated for 1 min with the ECL detection reagents, then immediately exposed on film for 30 s to 3 min.

[^{32}P]GTP blotting

The GTP-binding capacity of axonal and other neuronal proteins was analyzed on nitrocellulose sheets. Proteins were first separated by SDS-PAGE, then transferred onto nitrocellulose as described above. The blots were incubated for 1 h at room temperature in blocking solution (10 mM Tris (pH 7.4), 0.15 m NaCl, 5% dry milk and 0.05% Tween-20), then incubated for 1 h at room temperature in binding buffer (50 mM Tris (pH 7.5), 0.3% Tween-20, 5 mM $MgCl_2$ and 1 mM EGTA) containing 2 μCi/ml of [α-^{32}P]GTP (3000 Ci/mmol). Blots were then washed three times for 20 min each in binding buffer. The blots were rubbed with gloved fingers after the second wash to decrease non-specific background. They were then dried and exposed to Kodak XAR-5 film with DuPont Cronex image intensifying screens at −70°C for varying times, depending on the amount of sample transferred.

Cortical explant cultures

Cerebral cortex explant cultures were prepared as described elsewhere [10]. In brief, 1-day-old rat pups were decapitated and the brains removed and placed in pre-warmed media. For all labelling experiments, one-half of the cerebral cortex was used per 35-mm plate containing 1 ml of labelling media. For [^{3}H]-methionine labelling, minced tissue was labelled in methionine-free MEM supplemented with 1 mM glutamine, 10% horse serum and 400 μCi/ml L-[*methyl*-^{3}H]methionine.

Immunoprecipitation and gel electrophoresis

Anti-Rab3A antiserum or pre-immune serum was coupled to an equal volume of resuspended Protein A Sepharose (PAS) in buffer A for 1 h, washed twice in Buffer A and brought back up to the original volume of the 1:1 suspension with Buffer A. Labelled brain extracts were then added (100 μl of this 1:1 suspension will quantitatively immunoprecipitate 200 ng Rab3A; data not shown) and incubated at 4°C with gentle rocking for 2 h. This mixture was then layered on a 700 μl sucrose cushion (Buffer A with 1 M sucrose), the PAS beads were pelleted through the cushion at 10000 × g for 2 min, and the unpelleted material aspirated off to the top of the sucrose cushion. The area above the cushion was washed with 1 ml of 2 M urea, then the urea and cushion removed. The pellet was washed twice with Buffer A plus 500 mM NaCl and 10 mM $MgCl_2$, then solubilized in Laemmli sample buffer and heated for 7 min before being loaded and run on 12% SDS-PAGE gels.

Chromatography

The Mono-Q column has a 1-ml bed volume. The column was eluted at a flow rate of 1 ml/min and 0.65 ml fractions collected. Buffers used were 10 mM Tris

(pH 7.0 at room temperature), 0.5% CHAPS and 1 mM $MgCl_2$. Buffer A contained no NaCl, while buffer B contained 1 M NaCl. Fresh dithiothreitol (1–2 mM) was added to buffers before using the column. Protein was pre-loaded with [α^{32}P]GDP before injection onto the column and the fractions assayed by filter binding as previously described [8].

Isolation of axonal transport vesicles

Subcellular fractionation was done using a modification of the method of Lorenz and Willard [11]. Within 10 min of killing by lethal injection, optic nerves and tracts were rapidly dissected and placed in 7 ml of ice-cold homogenization buffer (1 mM triethanolamine, 320 mM sucrose (pH 7.4), containing 30 μg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin and 0.5 μg/ml aprotinin). Homogenization consisted of three strokes in a motor-driven Teflon/glass homogenizer in a volume of 7 ml, by three strokes in a glass Dounce homogenizer in a volume of 10 ml. Homogenates were diluted to 40 ml with homogenization buffer and spun at $1200 \times g$ for 7 min. The resulting supernatant was then spun at $100\,000 \times g$ for 60 min. The resulting pellet was resuspended in 2 ml of homogenization buffer and loaded onto a discontinuous gradient consisting of 20, 26, 31, 37 and 45% sucrose (w/w). These gradients were spun at $150\,000 \times g$ in a SW 40 rotor (Beckman) for 16 h. The region of the gradient (26–31% sucrose interphase) containing the rapidly transported membranes having highest specific activity in labelled optic nerve [12] was collected. Electron microscopy and agarose gel electrophoresis, as described previously, demonstrated that these membranes consisted almost entirely of small (approx. 100 nm) enclosed vesicles and were devoid of myelin [12].

Isolation of synaptic vesicles and clathrin-coated vesicles

Synaptic vesicles were isolated from bovine brains according to the method of Huttner et al. [13] with minor modifications. Briefly, cerebral cortices were homogenized with a motor driven Teflon pestle in buffered sucrose (320 ml sucrose, 4 mM Hepes-NaOH (pH 7.3)). The homogenate was subjected to three steps of differential centrifugation, yielding a crude synaptosomal fraction. The synaptosomes were lysed by hypoosmotic shock and subjected to two more rounds of differential centrifugation. The resulting pellet was resuspended in 40 mM sucrose and layered on top of a discontinuous sucrose gradient. After equilibrium centrifugation, The gradient revealed a band of high turbidity in the 200–400 mM sucrose interphase. This material was collected as synaptic vesicles. Coated vesicles were isolated from rabbit brain according to the method of Blitz et al. [14]. Electron microscopy demonstrated that they were at least 95% pure.

Preparation of synaptosomes

Synaptosomes were prepared according to a published procedure [15]. Cerebral cortices of two rats were homogenized in 15 ml ice-cold sucrose (320 mM). The homogenate was brought to a volume of 25 ml and cleared of debris by centrifugation at $2600 \times g$ for 2 min. The supernatant was centrifuged at $14\,500 \times g$ for 12 min, and the pellet was washed once in 25 ml 320 mM sucrose. Before discarding the supernatant, the tube was given a vigorous twist to suspend the upper, white layer of the pellet. The remaining pellet was suspended in 10 ml Hepes-buffered saline (HBS; 142 mM NaCl, 2.4 mM KCl, 1.2 mM K_2HPO_4, 1 mM $MgCl_2$, 5 mM D-glucose, 0.1 mM EGTA and 10 mM Hepes, adjusted to pH 7.4 with Tris base), with the addition of 0.5 mM ascorbic acid and 20 μM pargyline. This material was centrifuged at $12\,000 \times g$ for 5 min, and the resulting pellet was stored on ice before use.

Assay of neurotransmitter release

Synaptosomes were prepared as described above and loaded with neurotransmitter using a published procedure [15]. Synaptosomes were resuspended at 2 mg protein/ml in oxygenated HBS/ascorbic acid/pargyline (as above). They were then incubated with 0.05 μM [^{3}H]norepinephrine and 4 μM [^{14}C]choline for 10 min at 37°C with agitation. Labelled synaptosomes were washed 3 times by microcentrifuging for 10 s with HBS/0.5 mM ascorbic acid/20 μM pargyline and resuspended at 2 mg/ml in the same buffer. The synaptosomes maintained a high negative membrane potential as monitored by the fluorescent dye, 3,3-dipropylthiadicarbocyanine. The potential was rapidly reduced upon addition of K^+.

Exocytosis was stimulated according to the basic procedure of Fisher von Mollard et al. [7]. Either $CaCl_2$ (1 mM) or EGTA (0.5 mM) was added with KCl (50 mM) to 40 μg of the synaptosomes, which were then incubated at 37°C for either 2, 5, or 10 min. Control samples had no addition of the stimulants. After the required incubation, exocytosis was stopped by placing the tubes on ice. Synaptosomes were pelleted by microcentrifuging for 10 s, and the supernatants were counted in an LKB Rackbeta counter in the presence of 4 ml of scintillant.

Assay of the distribution of GTP-binding proteins during synaptic vesicle exocytosis

Synaptosomes were prepared as described above and resuspended at 2 mg/ml in HBS. The procedure of Fischer von Mollard et al. [7] was then followed, with minor modifications. Either $CaCl_2$ (1 mM) or EGTA (0.5 mM) was added with KCl (50 mM) to 40 μg of the synaptosomes, which were then incubated at 37°C for either 2, 5 or 10 min. Control samples had no addition of the stimulants. At the end of the incuba-

tion, samples were diluted with 6 ml ice-cold HBS and centrifuged for 10 min at $12000 \times g$. All subsequent steps were at 4°C. Pellets resuspended in 0.3 ml HBS were lysed by addition of 2.7 ml H_2O, followed by rapid homogenization (6 strokes in a Teflon/glass homogenizer). Samples were centrifuged for 10 min at $12000 \times g$. A crude synaptic vesicle fraction was isolated from the supernatant by centrifugation for 2 h at $150000 \times g$. Pellets were analyzed by SDS-PAGE and immuno- or ^{32}P-blotting.

Results

Antiserum characterization

There are probably over 30 small GTP-binding proteins in rat brain, all of similar size and with significant homology to one another and to Rab3A [16,17]. It was therefore imperative to determine the specificity of the anti Rab3A antiserum prior to its use in detecting endogenous Rab3A. Initially, recombinant Ha-Ras, Rab1B and Rab2 proteins were screened alongside Rab3A using the antiserum for both immunoprecipitation (Fig. 1A) and Western blotting (Fig. 1B). These proteins show 28%, 45% and 36% amino-acid identity with Rab3A, respectively, which is within the same range as almost all of the other small GTP-binding proteins for which sequence data are available. Only Rab3A was recognized by the antiserum in these assays. Autoradiography of *methyl*-^{3}H-methionine-labelled explant cultures revealed the specific immunoprecipitation by immune serum of a single 25-kDa band (Fig. 1C). Another stringent test of specificity took advantage of the fact that although both liver and brain membranes contain over 10 distinct small GDP-binding proteins that can be resolved by Mono-Q anion-exchange chromatography (Fig. 2A,B), many of which are common to both tissues [16], Rab3A is expressed specifically in brain [5,18]. Immunoprecipitations were therefore performed on cytosolic and RIPA-solubilized membrane fractions from rat brain and liver, and quantitated by [α-^{32}P]GDP binding (Fig. 2C). No significant binding activity was immunoprecipitated from liver, while activity was detected in both the membrane and soluble fractions from brain. When the peaks of GDP-binding activity from brain which had been separated by Mono-Q chromatography were immunoprecipitated with the anti Rab3A antiserum and analyzed for GDP binding, only a single peak showed detectable activity (peak 4, Fig. 2A). In other studies, Western blots with an anti-peptide antiserum directed against a peptide unique to the C-terminal region of Rab3A specifically recognized a 25-kDa band from a similar peak in the Mono-Q profile from brain membranes. While the Mono-Q column does not resolve every small GTPase present in cells, it can resolve at least 10 [8]. The fact that only a single peak cross-reacted with the antiserum, therefore, provides strong support for its specificity. We have shown previously, as have other laboratories [5,17,19,20], that Rab3A is only partially membrane-bound. Therefore, chromatographic characteristics, tissue distribution and subcellular localization all support the conclusion that the antiserum is specific for Rab3A.

Two major blottable LMW-GBPs are found in rabbit and bovine rapid transport and synaptic vesicles

Having previously revealed the presence of low-molecular-weight GTP-binding proteins in various neuronal vesicles [4], we sought to identify these proteins as known or unknown GTP-binding proteins. To aid in our analysis, we used 18% polyacrylamide gels for protein separation, instead of the 12% gels that were used earlier. We purified bovine cerebral cortical clathrin-coated and synaptic vesicles, bovine and rabbit optic nerve rapid transport vesicles as described previously [4]. As shown in the [^{32}P]GTP blot of Fig. 3, the use of 18% gels results in good resolution of the GTP-binding proteins. Two major bands are visible, including an upper band of 24 kDa and a lower band of approx. 23.5 kDa, together with a few minor bands. These two major bands are common in bovine synaptic vesicles, rabbit and bovine rapid transport vesicles, and bovine brain coated vesicles, although the ratio of the two proteins appears to vary.

We also employed Mono-Q ion-exchange chromatography exactly as described previously [8] to analyse the LMW-GBP composition of bovine optic nerve rapid transport vesicles and cerebral cortical vesicles. As can be seen in Fig. 4, both vesicles contain two major GDP-binding proteins which are eluted late in the gradient. One of these proteins elutes in the some position as Rab3A, a blottable GTP-binding protein previously identified as a synaptic vesicle component [6].

While both vesicle types contained these two major proteins, the optic nerve rapid transport vesicles contained a much larger amount of other GDP-binding proteins. This diversity is expected because of these vesicles which contain precursors to both synaptic vesicles and plasma membrane components, as well as to yet unidentified membranes [12].

Identification of one major LMW-GBP as Rab3A

We employed a monospecific antibody to recombinant Rab3A to definitively identify Rab3A in synaptic vesicles. Bovine synaptic vesicles were solubilized with SDS and Triton X-100, and the extract was incubated with either anti-Rab3A or control antibody (normal rabbit serum). Protein A-Sepharose beads were used to precipitate the antibody complexes, which were then electrophoresed and blotted with [^{32}P]GTP. Fig. 5 shows that anti-Rab3A precipitates a protein (lane 2)

which corresponds to the lower of the two major bands in synaptic vesicles (lane 1). An identical result was obtained with synaptic vesicles subjected to further purification by electrophoresis through agarose or by immunoabsorption with antisynaptophysin, as previously described [4]. We therefore conclude that the smaller of the two major GTP-binding proteins in synaptic vesicles is Rab3A. A similar result was obtained with rabbit optic nerve rapid transport vesicles (data not shown).

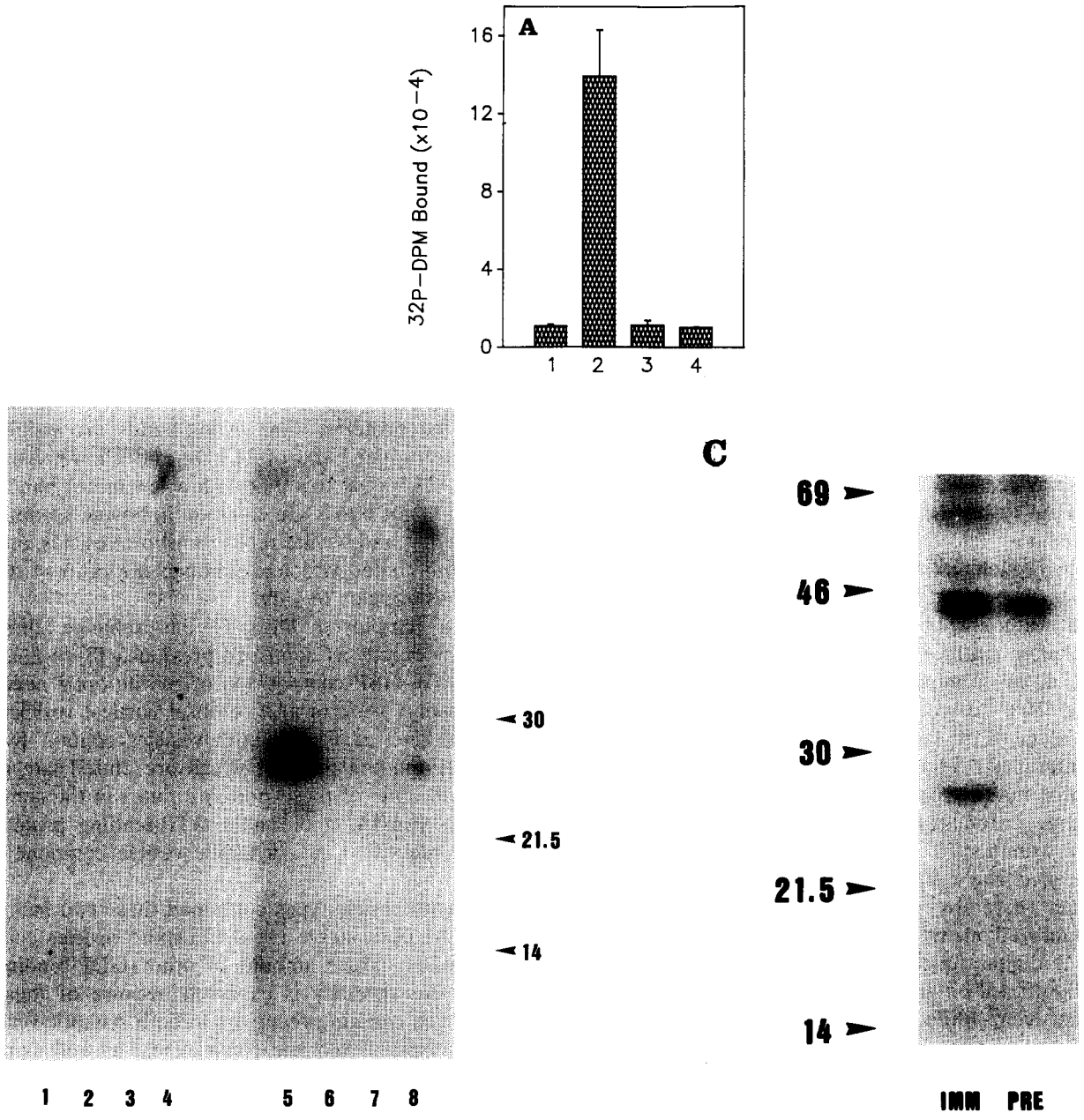

Fig. 1. Characterization of anti-Rab3A antiserum. (A) Equal amounts (500 ng each) of purified Rab3A (lane 2), Rab2 (lane 3) and Ras (lane 4) in 100 μl washing buffer (50 mM Hepes, 100 mM KCl, 5 mM EDTA (pH 7.6)) were incubated with 2 μl of immune serum for 1 h at 4°C. 20 μl of resuspended PAS was then added to each reaction and the incubation continued for an additional 30 min. The pellets were immunoprecipitated as described in Materials and Methods. Pellets were washed once with washing buffer then incubated in the same buffer plus 5 nCi/μl [α-^{32}P]GDP at 30°C for 10 min. This reaction was terminated by washing the pellets three times in buffer containing 10 mM Mg^{2+}, then counting the pellets for ^{32}P. Rab3A was also immunoprecipitated with 2 μl of pre-immune serum as a negative control (lane 1). Values shown are the average of duplicate experiments. (B) Equal amounts (500 ng each) of purified Rab3A (lanes 1 and 5), ras (lanes 2 and 6) or rab1B (lanes 3 and 7) were run on SDS-PAGE, transferred to nitrocellulose paper and immunoblotted. Hippocampal whole cell lysate (lanes 4 and 8) was run as a positive control. Lanes 1–4 were probed with pre-immune serum (1:200 dilution); lanes 5–8 were probed with immune serum (1:200 dilution). The numbers on the sides of this and subsequent figuress refer to the molecular mass in kilodaltons of the marker polypeptides. (C) Rat cerebral cortex extracts were labelled with L-[*methyl*-^{3}H]methionine as described in Materials and Methods. Immunoprecipitations were from 400 μg labelled whole cell lysate. Dried gel lanes were exposed to film for 4 weeks.

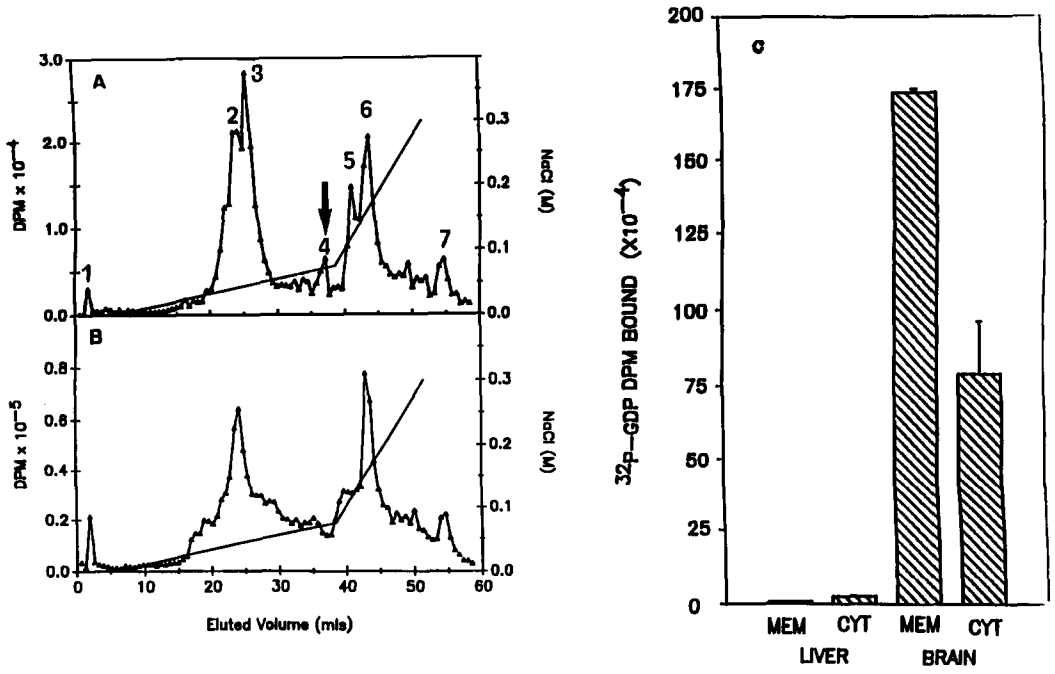

Fig. 2. Specificity of Rab3A antiserum. (A) 100 μg of Haps-solubilized brain membranes were chromatographed on a Mono-Q column as described in Materials and Methods and the fractions assayed by filter-binding for the presence of GDP-binding proteins. Peak fractions were pooled and concentrated in Centricon 10 microconcentrators, then used in immunoprecipitations with 40 μl of the pre-coupled anti-Rab3A antibody. Pellets were GDP-bound as described in the legend to Fig. 1; [α-^{32}P]GDP bound to the pellets was as follows (in dpm): peak 1, 2650; peak 2, 7095; peak 3, 5889; peak 4, 73926; peak 5, 4968; peak 6, 3374; peak 7, 5516. Arrow indicates presence of Rab3A. (B) As in (A), except 100 μg of Haps-solubilized liver membranes was used. (C) Equal amounts of rat brain or liver cytosol, RIPA-solubilized rat brain or liver membranes (100 μg each) were incubated with 40 μl of anti-Rab3A antiserum pre-coupled to PAS or 40 μl pre-coupled pre-immune serum for 2 h at 4°C. Immunoprecipitation was conducted as described in Materials and Methods and the pellets washed once with washing buffer. [α-^{32}P]GDP at 50 nCi/μl. Values shown were calculated as follows: (mean of duplicate immune serum precipitations)−(mean of duplicate pre-immune serum precipitations).

The larger of the two major LMW-GBPs found in bovine synaptic vesicles is Ral

We next set about to determine whether the other major protein species, localized on GTP blots as an upper band, might be a novel GTP-binding protein, or one that has previously been identified. Identification of this protein was accomplished by separation of the upper band by gel electrophoresis followed by amino-acid sequence analysis. First, in order to maximize the amount of GTP-binding proteins loaded on the gel, bovine synaptic vesicles were solubilized in approx. 3% cholate for 1 h on ice. The cholate concentration was then adjusted to 2%, followed by centrifugation at 100000 × g for 1 h. The cholate extract (supernatant) was subjected to SDS-PAGE by loading a total of 1.8 mg protein in three lanes of a 14% SDS acrylamide gel. In addition, adjacent lanes were loaded with lesser amounts of protein.

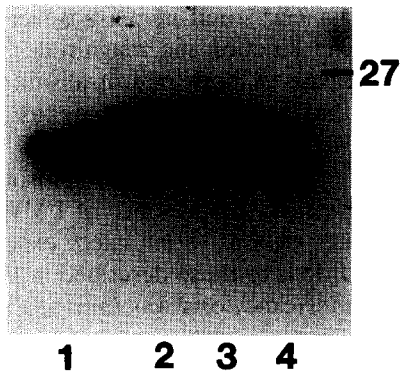

Fig. 3. Two major GTP-binding proteins are found in various neuronal vesicular compartments. Neuronal vesicles of various types were isolated as described in Materials and Methods. Vesicle proteins (10 μg/lane) were separated by SDS-PAGE on 18% polyacrylamide gels, electroblotted onto nitrocellulose and incubated with [^{32}P]GTP. Lane 1, bovine synaptic vesicles; lane 2, rabbit rapid transport vesicles; lane 3, bovine rapid transport vesicles; lane 4, bovine brain coated vesicles.

The strip of nitrocellulose, containing an estimated 3.6 μg protein, was given to the Harvard University Department of Microchemistry for amino-acid sequence analysis. The method for this analysis included digestion of the protein with trypsin followed by separation of the resulting peptide fragments by HPLC [21]. One of these fragments was sequenced in a gas-phase sequenator, yielding the amino-acid sequence shown in Fig. 6. This sequence was found to correspond exactly to amino acids 31–55 of the amino-acid sequence predicted from simian RalA and human RalA and RalB

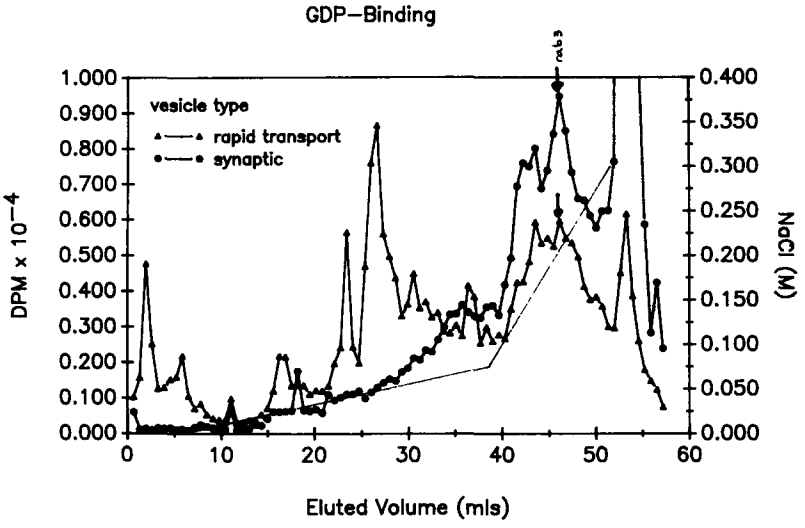

Fig. 4. GDP-binding protein profiles of bovine brain synaptic vesicles and bovine optic nerve rapid transport vesicles. 100-μg aliquots of CHAPS-solubilized synaptic vesicles (●) or rapid transport vesicles (▲) were prebound with [^{32}P]GDP and subjected to chromatography on a Mono-Q column as described in Materials and Methods.

cDNAs [22,23]. We confirmed the identity of this protein as Ral by use of a polyclonal monospecific antibody against Ral (Kind gift of Dr. Larry Feig). As can be seen in Fig. 7 the antibody reacted exclusively with the higher-molecular-weight LMW-GBP, on a highly-purified synaptic vesicle preparation.

Distribution of LMW-GBPs during Ca^{2+}-stimulated neurotransmitter release

In view of our finding that Ral, as well as Rab3A, was present on synaptic vesicles, we were very interested in The report that Rab3A was almost quantitatively dissociated from the synaptic vesicle membrane

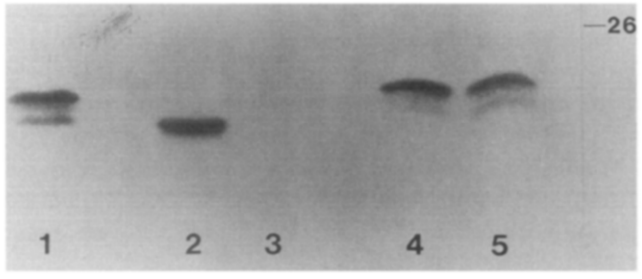

Fig. 5. One of the two major synaptic vesicle GTP-binding proteins is immunoprecipitated by antibody against Rab3A. A solubilized extract of bovine synaptic vesicles was incubated with either a polyclonal antibody to Rab3A or normal rabbit serum, followed by the addition of protein A-Sepharose beads as described in Materials and Methods. The beads, containing the antibody complexes, were separated from supernatant (1/20 of total volume) and subjected to SDS-PAGE on 18% polyacrylamide gels followed by GTP blotting. Lane 1, bovine synaptic vesicles (12 μg); lane 2, beads resulting from immunoprecipitation with anti-Rab3A; lane 3, beads resulting from immunoprecipitation with normal rabbit serum; lane 4, supernatant resulting from immunoprecipitation with anti-Rab3A; lane 5, supernatant resulting from immunoprecipitation with normal rabbit serum.

after Ca^{2+}-dependent exocytosis in synaptosome preparations [7]. To determine whether Ral and possibly other LMW-GBPs behaved in a similar manner, we carried out similar experiments employing rat cortical synaptosomes.

Crude synaptosomes were isolated and preloaded with [^{3}H]norepinephrine and [^{14}C]choline as described in Materials and Methods. The preloaded synaptosome preparation was divided into several aliquots. To one set of tubes was added KCl (50 mM) and either $CaCl_2$ (1 mM) or EGTA (0.5 mM). The tubes were then incubated at 37°C for either 2, 5 or 10 min followed by cooling on ice and microcentrifugation. Fig. 8 shows the amounts of [^{14}C]ACh and [^{3}H]NE released during this time interval. As can be seen, the secretory response of the synaptosomal preparation was dependent on extracellular Ca^{2+} and increased over the time of incubation. The kinetics and magnitude of the Ca^{2+}-stimulated release of both neurotransmitter were approximately that seen for glutamate as reported by Fischer von Mollard et al. [7].

The remaining portion of the synaptosomal preparation was assayed for the presence of LMW-GBPs during the course of Ca^{2+}-stimulated exocytosis using identical conditions to those described in Fig. 8. At 2, 5 and 10 min after exocytosis was initiated, the synapto-

(N-terminus)

S A L T L Q F M Y D E F

V E D Y E P T K A D S Y (C-terminus)

Fig. 6. The amino-acid sequence of a 25-residue peptide generated by trypsin digestion of the larger of the two major GTP-binding proteins in bovine synaptic vesicles.

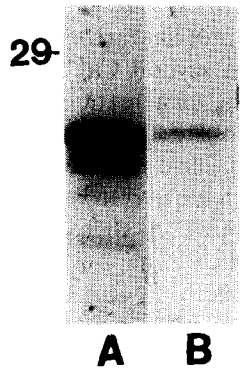

Fig. 7. The higher-molecular-weight GTP-binding protein in synaptic vesicles is Ral. A 2% cholate extract of bovine brain synaptic vesicles was divided into two portions (20 μg) and subjected to SDS-PAGE and transferred to nitrocellulose. One aliquot (A) was blotted with [^{32}P]GTP as described in Materials and Methods. The other aliquot (B) was Western blotted with 1 μg of an affinity purified polyclonal antibody specific for Ral. (Kind gift of Dr. Larry Feig).

somes were quickly chilled on ice and a crude synaptic vesicle fraction was prepared following the procedure of Fischer Von Mollard et al. [7]. Electron microscopy after homogenization of the synaptosomes demonstrated an almost total disruption of the plasma membranes without a significant amount of fragmentation of plasma membrane. Small vesicles appeared to be intact (data not shown). Also, over 95% of the LMW-GBPs and synaptophysin were in the supernatant after the low-speed spin following lysis (data not shown).

The crude synaptic vesicles were subjected to SDS-PAGE and GTP blotting as described in Materials and Methods. As the GTP blot in Fig. 9a shows, there were only small apparent differences in the amounts of GTP binding to The LMW-GBPs in all of the experimental conditions employed. An identical aliquot of each sample shown in Fig. 9a was subjected to Western blotting using a monoclonal antibody against synaptophysin and our specific, polyclonal antiserum against Rab3A (Fig. 9b,c) Again, it is evident by visual inspection that there is no obvious decrease in either Rab3A or synaptophysin during the course of stimulation. The data of this experiment were combined with data obtained from an identical experiment and the results normalized to the amount of synaptophysin present in each sample (Fig. 10). As can be seen, there is a small and not statistically significant decrease in Rab3A amount and in the amount of the other major blottable LMW-GBP, Ral, bound to synaptic vesicle-derived membranes during the Ca^{2+}-dependent exocytic release of ACh and NE, two transmitters contained in synaptic vesicles. Also, whereas Fischer Von Mollard et al. [7] report an almost quantitative removal of Rab3A from the synaptic vesicle membrane after 10 min of stimulated exocytosis, we see a small increase in both Rab3A and Ral binding to the synaptic vesicle membrane at this time.

Discussion

We have employed high-resolution SDS-PAGE and highly-specific antisera directed against Rab3A and Ral to demonstrate that these two proteins are the major LMW-GBPs associated with a variety of membranes known to be transported into CNS axons and to function there. Rab3A was previously reported to be exclusively associated with synaptic vesicles [8]. However, we demonstrate in this study that it is also associated with several classes of rapid transport vesicles and clathrin-coated vesicles in addition to synaptic vesicles. We find that Ral, previously found associated with platelet membranes [23,24], is also a major constituent

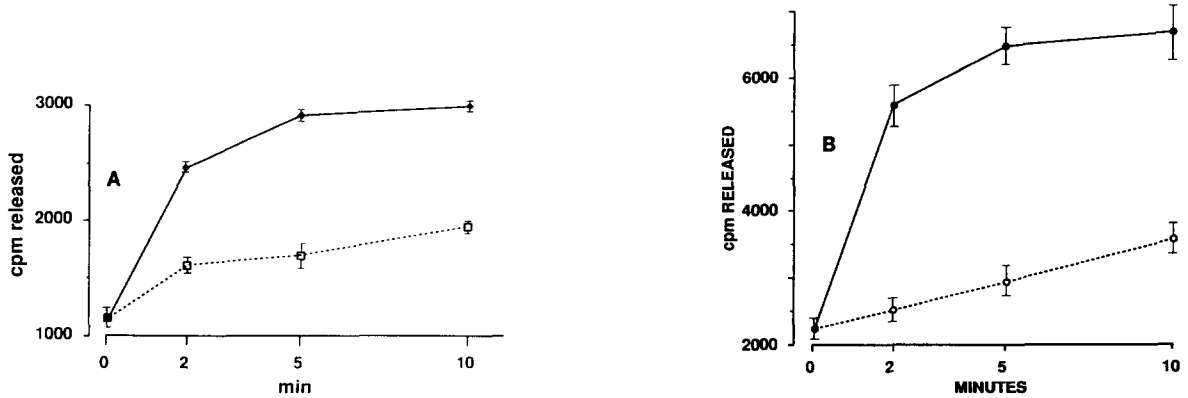

Fig. 8. Ca^{2+}-dependent stimulation of acetylcholine and norepinephrine release by K^+-depolarization of isolated synaptosomes. Synaptosomes were prepared and loaded with [^{3}H]norepinephrine and [^{14}C]choline as described in Materials and Methods. They were then incubated at 37°C for varying lengths of time in a buffer containing 50 mM K^+ and either 1 mM Ca^{2+} or 0.5 mM EGTA. Control samples (time 0) contained neither K^+ nor Ca^{2+}. At the end of the incubation, samples were placed on ice, centrifuged and the amount of radiolabelled neurotransmitter released into the supernatant was measured in a scintillation counter. (A) The cpm released of [^{14}C]acetylcholine; the results are representative of two independent experiments. (B) Results for [^{3}H]norepinephrine release. Values are means of duplicate samples (bar indicates range).

of synaptic vesicles as well as rapid transport and clathrin-coated vesicles.

While Rab3A and Ral are the major GTP-binding proteins on synaptic vesicles, there are a number of other LMW-GDPs associated with rapid transport vesicles (Fig. 4). These other proteins may play functional roles in a variety of axonal sorting and fusion events, as well as possibly serving other functions, including the regulation of the cytoskeletal and signal transduction. We are presently developing techniques to identify these proteins and to assay their possible functions.

There is now strong evidence that functional Rab3A or other related proteins are critical for Ca^{2+}-stimulated exocytosis in several Ca^{2+}-regulated systems including mast cells, pancreatic acini and chromaffin cells [25–27]. Consistent with this evidence, Fischer Von Mollard et al. have reported that Rab3A actually is quantitatively removed from the synaptic vesicle membrane during Ca^{2+}-mediated glutamic acid release from cerebral cortical synaptosomes [7]. Using a well-characterized antibody to Rab3A antibody as well as [^{32}P]GTP blotting, we find no evidence that the

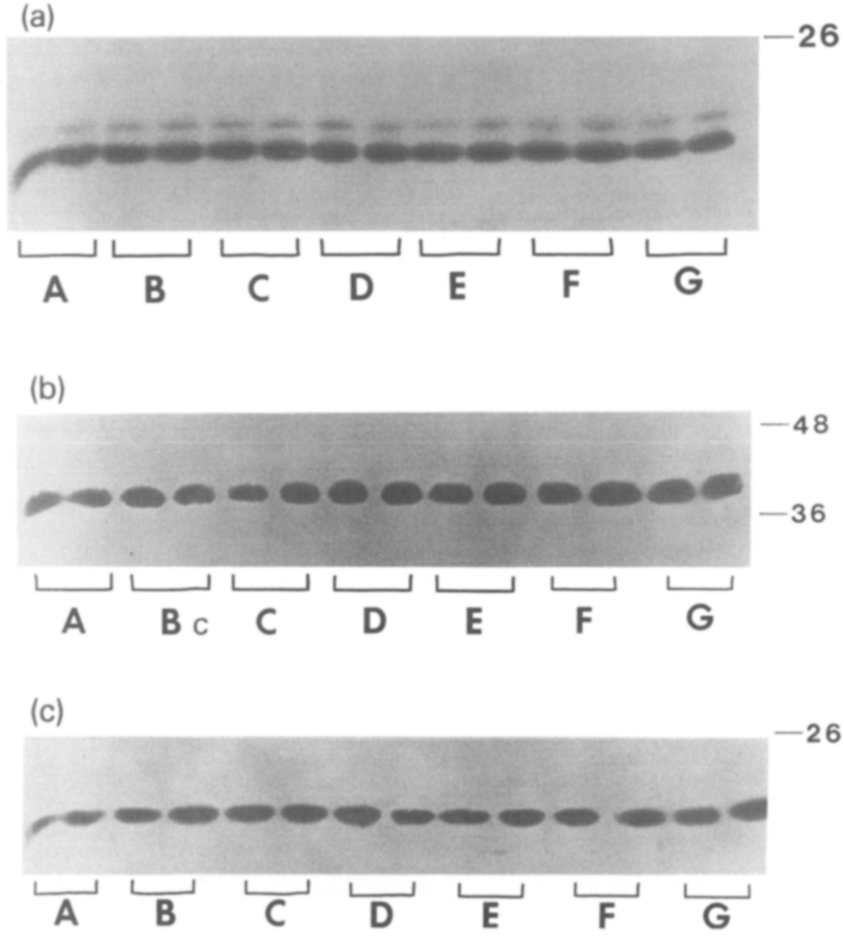

Fig. 9. (a) [^{32}P]GTP blot showing the profile of GTP-binding proteins in synaptic vesicles during exocytosis. Synaptosomes were prepared and incubated in depolarizing conditions for various times with or without Ca^{2+}, as described in the legend to Fig. 3. Control samples (time 0) contained synaptosomes to which no depolarizing stimulants were added. At the end of the incubation, samples were placed on ice and a crude preparation of synaptic vesicles was prepared according to the procedure described in Materials and Methods. The vesicles were electrophoresed on 18% SDS-polyacrylamide gels, electroblotted onto nitrocellulose, and the lower portion of the blot including proteins < 26 kDa was incubated with [^{32}P]GTP. Results shown are representative of two independent experiments. Brackets point to pairs of duplicate samples. A, time 0; B, +EGTA, 2 min; C, +EGTA, 5 min; D, +EGTA, 10 min; E, +Ca^{2+}, 2 min; F, +Ca^{2+}, 5 min; G, +Ca^{2+}, 10 min. (b) Immunoblot showing the content of synaptophysin in synaptic vesicles derived membrane during exocytosis. The upper portion of The nitrocellulose blot described in the legend to Fig. 9a was immunoblotted with an antibody against synaptophysin and detected with the ECL system. Results shown are representative of two independent experiments. Brackets point to pairs of duplicate samples. A, time 0; B, +EGTA, 2 min; C, +EGTA, 5 min; D, +EGTA, 10 min; E, +Ca^{2+}, 2 min; F, +Ca^{2+}, 5 min; G, +Ca^{2+}, 10 min. (c) Immunoblot showing the content of Rab3A in synaptic vesicles during exocytosis. The [^{32}P]GTP blot shown in Fig. 9a was stripped of [^{32}P]GTP by incubation in 0.1 M glycine (pH 2.0) for 30 min, followed by washing in phosphate-buffered saline for 1 h with two changes. It was then immunoblotted with an antibody against Rab3A and detected with the ECL system. Results shown are representative of two independent experiments. Brackets point to pairs of duplicate samples. A, time 0; B, +EGTA, 2 min; C, +EGTA, 5 min; D, +EGTA, 10 min; E, +Ca^{2+}, 5 min; G, +Ca^{2+}, 10 min.

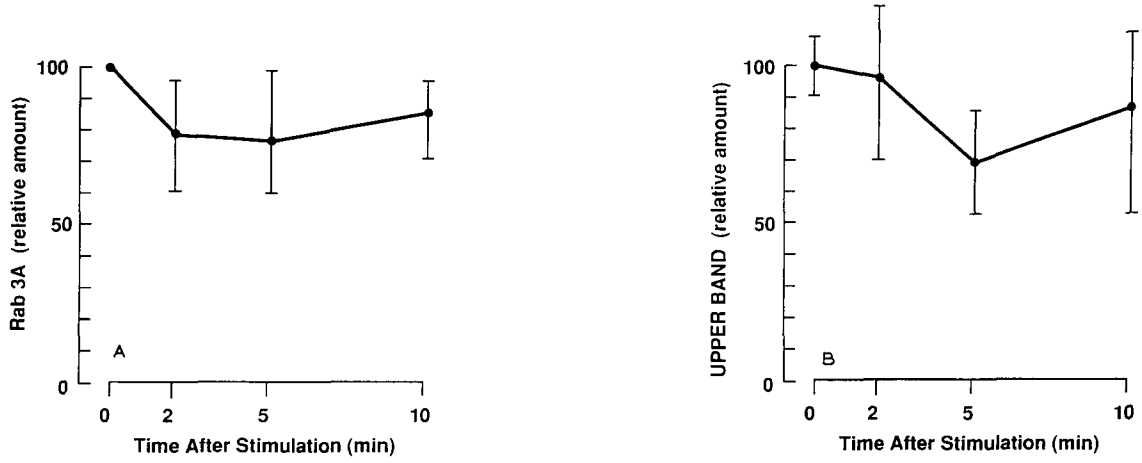

Fig. 10. (A) Quantitative determination of the content of Rab3A in synaptic vesicles during K^+-stimulated exocytosis in the presence of Ca^{2+}. Results shown are the relative amounts normalized to the amount of the vesicle marker synaptophysin, with the 0-min value represented by 100. All values are the means of four samples (duplicates from two independent experiments): bar indicates range. Quantitation was accomplished by scanning autoradiograms such as that shown in Fig. 9 with an LKB Ultrascan XL laser densitometer. (B) Quantitative determination of Ral visualized as the upper band in the profile of the [^{32}P]GTP blot of synaptic vesicles during K^+-induced exocytosis in the presence of Ca^{2+}. Results shown are the relative amounts normalized to the amount of the vesicle marker synaptophysin, with the 0-min value represented by 100. All values are the means of four samples (duplicates from two independent experiments); bar indicates range. Quantitation was accomplished by scanning the autoradiograms such as shown in Fig. 9a with an LKB Ultroscan XL laser densitometer.

steady-state level of Rab3A or of Ral significantly decreases during Ca^{2+}-stimulated exocytosis in cortical synaptosomes. The reason why these results are in disagreement with those of Fischer von Mollard et al. are not apparent. Since these investigators used an antibody against partially purified Rab3A in these studies, while we used both a very well-characterized monospecific antibody against Rab3A and GTP-blotting systems, we feel that our results are valid. It is, however, possible that the antibody used by Fischer Von Mollard et al. is in fact recognizing another protein which is dissociating from the vesicle membrane during the course of exocytosis and/or recycling. Whatever the identity of the protein, the rate of the release is too slow to be causally related to neurotransmitter release.

While our results provide no evidence that either Rab3A, Ral or any other blottable LMW-GBP dissociates from the membrane during the course of exocytosis and recycling, they do not rule out this possibility. It is known from several studies that a sizable proportion of LMW-GBPs are in the cytosol [4–6]. Hence it is possible that the membrane-bound LMW-GBPs do, in fact, dissociate from the vesicle membrane and are rapidly replaced by other molecules, thus maintaining the steady-state amounts bound to the synaptic (or recycling) membrane. To address this question, a permeabilized system which maintains Ca^{2+}-stimulated exocytosis and recycling must be employed, so that reagents which will block reassociation of LMW-GBPs with the membrane can be utilized. Using such a system, direct evidence has been obtained that a LMW-GBP, ARF, does associate and dissociate with membrane elements during vesicle transport through the Golgi [28].

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