

GTP binds to Rab3A in a complex with Ca²⁺/calmodulin

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Ras-like small GTP-binding proteins of the Rab family regulate trafficking of the secretory or endocytic pathways. Rab3 proteins within the Rab family are expressed at high levels in neurons and endocrine cells, where they regulate release of dense-core granules and synaptic vesicles (SVs). Rab3A is present as either the soluble or the SV membrane-bound form in neurons that are dependent on the GDP- or GTP-bound states respectively. GDP dissociation inhibitor (GDI) is known to induce the dissociation of Rab3A from synaptic membranes when GTP is depleted. In an earlier study, Ca²⁺/calmodulin (CaM) was also shown to dissociate Rab3A from synaptic membranes by forming an equimolar complex with Rab3A *in vitro*. We have examined a possible role for Ca²⁺/CaM in modulating both the binding of guanine nucleotides to Rab3A and the GTPase activity of Rab3A.

The basal level of Rab3A GTPase activity was not affected by an association with Ca²⁺/CaM. Ca²⁺/CaM–Rab3A complex that was formed in synaptic membranes was able to bind guanine nucleotides, whereas the Rab3A–GDI complex could not. In addition, Ca²⁺/CaM led to the replacement of the GDP molecule in the Rab3A–GDI complex with GTP in Rab3A. Taken together, these results suggest that CaM may have a role in stimulating GTP binding to Rab3A that is complexed with GDI, which leads to the formation of an active GTP-bound form of the Rab3A–Ca²⁺/CaM complex.

Key words: GDP dissociation inhibitor (GDI), GTP binding, synaptic vesicle.

INTRODUCTION

In nerve terminals, neurotransmitters are stored in synaptic vesicles (SVs) and are subsequently released by the fusion of these vesicles with the pre-synaptic plasma membrane. Thus SVs are organelles that have a central role in neurotransmitter release [1]. SVs possess a diverse set of proteins comprising synapsins, synaptotagmin, synaptobrevin/vesicle-associated membrane protein ('VAMP'), synaptophysin, synaptophorin, secretory carrier membrane proteins ('SCAMPs'), neurotransmitter transporters, proton pumps, SV2 and Rab3A [2].

Rab3A belongs to the Ras superfamily of small GTP-binding proteins [3]. Mammalian cells contain at least 50 related small GTP-binding proteins that regulate processes as diverse as cell replication and differentiation, cytoskeletal organization, secretion and endocytosis [3]. Ras-related GTP-binding proteins alter their conformation in response to the phosphorylation state of bound guanine nucleotides. The binding of GTP switches the protein to an active form, in which it is able to interact with a downstream effector to trigger an appropriate response. GTP hydrolysis returns the active GTP-bound protein to an inactive GDP-bound protein form. The association of GDP and GTP with small GTP-binding proteins is controlled by regulatory proteins. Regeneration of the active GTP-bound state requires an exchange of the bound GDP with an exogenous GTP molecule, a reaction which is stimulated by specific guanine nucleotide exchange proteins (GEPs). Members of the Ras-related GTP-binding protein family are also able to catalyse the hydrolysis of GTP. GTPase-activating proteins (GAPs) have a role in enhancing further the intrinsic GTPase activity. GEPs and GAPs are likely to be as diverse in structure and function as are their cognate GTPases. In addition, members of the Rab and Rho families are known to interact with GDP dissociation inhibitors

(GDIs), which can bind to the GDP-bound form and prevent an exchange of guanine nucleotides [4].

More than 30 Rab proteins have been described, which are thought to regulate membrane traffic among different subcellular compartments in eukaryotic cells [5]. Rab3A is found most abundantly in the brain, where it is enriched with SVs. Rab3A has two cysteine residues (Cys²¹⁸ and Cys²²⁰) that are geranylgeranylated [6], and the protein may be bound to the membrane via these geranylgeranyl groups [7]. It has been proposed that two key players in SV fusion are Rab3A, which serves to select and limit the number of SVs released at a central synapse, and synaptotagmin, the putative Ca²⁺ sensor that drives SV fusion [8]. Overexpression of a Rab3A mutant protein defective in either GTP hydrolysis or guanine nucleotide binding inhibits exocytosis in PC12 cells [9]. On the other hand, micro-injection of antisense oligonucleotides to Rab3A mRNA increases the secretory activity at a low concentration of Ca²⁺ (approx. 0.2–4 μM; [10]), suggesting that Rab3A inhibits the sensitivity of the exocytotic process that is associated with the internal concentration of Ca²⁺. Recently, it has been reported that Noc2, a putative Rab3A effector in dense-core granule exocytosis, has a direct inhibitory effect on Ca²⁺-triggered exocytosis in permeabilized PC12 cells [11]. Rab3A was found to dissociate in a quantitative manner from the vesicle membrane after Ca²⁺-dependent exocytosis. This dissociation is partly reversible during recovery after stimulation [12]. A considerable portion of Rab3A in the brain is cytosolic and complexed with GDI, a protein capable both of enveloping the geranylgeranyl groups of Rab proteins and of dissociating them from membranes [13].

However, it has not yet been elucidated how an influx of Ca²⁺ signals the dissociation of Rab3A. There are many Ca²⁺-sensing proteins in nerve terminals. These include rabphilin [14], isoforms of protein kinase C [15], dynamin [16], synaptotagmin [17],

Abbreviations used: BS³, bis(sulphosuccinimidyl) suberate; CaM, calmodulin; 2D, two-dimensional; DMPC, dimyristoyl phosphatidylcholine; DTT, dithiothreitol; GAP, GTPase-activating protein; GDI, GDP dissociation inhibitor; GEP, guanine nucleotide exchange protein; GST, glutathione S-transferase; [γ -³⁵S]GTP, guanosine 5'-[γ -³⁵S]thio]triphosphate; IPTG, isopropyl β -D-thiogalactoside; LP2, crude synaptic vesicle membrane fraction; SV, synaptic vesicle; TX-100, Triton X-100.

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Doc2, Munc13 [18] and calmodulin (CaM) [19], which forms a complex with calcineurin [20] and CaM-dependent protein kinase II [19].

In 1997, Park et al. [21] discovered that $\text{Ca}^{2+}/\text{CaM}$ could also dissociate Rab3A from Rab3A-enriched membranes of the monkey brain. Like Rab-GDI, $\text{Ca}^{2+}/\text{CaM}$ forms a complex with dissociated Rab3A with a stoichiometry of 1:1. Indeed, it has been proposed that the interaction of CaM with Rab3A is important for exocytosis *in vivo*: a mutation in Rab3A that reduced the affinity of the protein for CaM led to the abolition of the ability of the GTP-bound form of Rab3A to inhibit exocytosis in PC12 cells [22]. Furthermore, CaM has been identified as a major target effector protein in pancreatic β -cells, and the formation of the Rab3A-CaM complex has been confirmed by co-immunoprecipitation analysis [23]. The regulatory function of $\text{Ca}^{2+}/\text{CaM}$ with Rab3A, however, has not been fully elucidated. Thus in the present study we have examined a possible role for $\text{Ca}^{2+}/\text{CaM}$ in the regulation of guanine nucleotide binding to Rab3A, and in the GTPase activity of Rab3A *in vitro*.

MATERIALS AND METHODS

Materials

CaM and GDI were purified from bovine brain. Supra-pure grade CaCl_2 was from EM Science (Gibbstown, NJ, U.S.A.). Antibodies raised against Rab1, Rab3A, Rab5 and Cdc42, and horseradish-peroxidase-conjugated anti-IgG antibody were purchased from Santa-Cruz Antibody (Santa Cruz, CA, U.S.A.). GDP and guanosine 5'-[γ -thio]triphosphate (GTP[S]) were purchased from Calbiochem (La Jolla, CA, U.S.A.). [^3H]GDP was from NEN Research Products (Boston, MA, U.S.A.). [α - ^{32}P]GTP, [γ - ^{32}P]GTP and guanosine 5'-[γ - ^{35}S]thio]triphosphate ([γ - ^{35}S]GTP) were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). Glycine, SDS, Tris base and Tween 20 (electrophoresis grade) were purchased from Bio-Rad (Hercules, CA, U.S.A.). PVDF membranes were from Millipore (Bedford, MA, U.S.A.). DEAE-Sephacel, phenyl-Sepharose and glutathione-Sepharose were purchased from Pharmacia (Uppsala, Sweden). BA85 and NC45 membrane filters (25 mm) were purchased from Schleicher & Schuell (Keene, NH, U.S.A.). Triton X-100 (TX-100), BSA, PMSF, isopropyl β -D-thiogalactoside (IPTG), glutathione, dimyristoyl phosphatidylcholine (DMPC) and other reagents were purchased from Sigma (St Louis, MO, U.S.A.). QIAEX II DNA isolation beads were from Qiagen (Hilden, Germany). Bis(sulphosuccinimidyl) suberate (BS^3) was purchased from Pierce (Rockford, IL, U.S.A.).

Preparation of glutathione S-transferase (GST)-Rab3A fusion protein

Two primers (5'-CCGGAATTCATGGCATCGGCCACAGACTCGCG-3' and 5'-GCCGTCGACTCAGCAGGCGCAGCTCTGGTGC-3') were synthesized from Universal DNA, Inc. (Tigard, OR, U.S.A.). These primers were mixed with a human brain cDNA library, and PCR was performed to synthesize Rab3A cDNA containing *EcoRI* and *SalI* sites at the 5' and 3' ends respectively. PCR products were separated by agarose gel electrophoresis, and then isolated using the QIAEX II gel isolation kit (Qiagen). The cDNA product was blunt-ended, and the fragment was then introduced into the blunt-ended *EcoRV* site of pBlueScript. Ligated pBlueScript vector was transformed and amplified in *Escherichia coli* DH5 α cells, and the purified plasmid was subsequently digested with *SalI* and *EcoRI*. The digested DNA fragment was ligated into the *EcoRI* and *SalI*

restriction sites of pGEX4T-1, and amplified in *E. coli* DH5 α cells. For the induction of GST-Rab3A fusion protein, 0.1 mM IPTG was added to the cultured *E. coli* cells. Cells were then harvested and disrupted in lysis buffer [50 mM Hepes (pH 7.4)/50 mM NaCl/5 mM MgCl_2 /1 mM DTT containing 1 $\mu\text{g}/\text{ml}$ aprotinin, 1 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM PMSF] by sonication using a sonic dismembrator (Fisher model 300; Farmingdale, NY, U.S.A.) with a microtip at 30% maximal power for 1 min three times on ice. The GST-Rab3A fusion protein was then purified by passage through glutathione-Sepharose beads (Pharmacia). GST-Rab3A that was bound to the beads was washed with the lysis buffer seven times, and was released from them by washing with lysis buffer containing 5 mM glutathione, before the released GST-Rab3A solution was dialysed against lysis buffer lacking MgCl_2 . SDS/PAGE revealed the presence of the GST-Rab3A fusion protein as a single protein band, and Western blot analysis was used to confirm the specific interaction of anti-Rab3A antibody with this fusion protein.

Preparation of crude synaptic vesicle membrane fraction (LP2) from the rat brain

Synaptosomes were prepared from rat brain by Ficoll gradient centrifugation [12,21]. LP2 containing Rab3A-enriched membranes was prepared by the lysis of the synaptosomes in hypotonic solution [21,24].

Purification of CaM

CaM was purified from bovine brain as described previously [25,26], but with a minor modification. Bovine brain tissue (350 g) that was suspended in 350 ml of 30 mM Hepes buffer, pH 7.4, containing 2 mM EDTA, 2 mM EGTA, 1 mM CaCl_2 , 1 mM dithiothreitol (DTT), 1 mM PMSF, 4 $\mu\text{g}/\text{ml}$ aprotinin and 2 $\mu\text{g}/\text{ml}$ leupeptin was homogenized in two steps, first using a Warring blender and then using a Potter-Elvehjem-type homogenizer. The homogenized sample was purified by centrifugation for 30 min at 66000 g at 4 °C. The supernatant solution was added to CaCl_2 (final concentration of 5 mM), heated at 85 °C for 3 min, and then cooled on ice. Heat-denatured material was removed by centrifugation for 30 min at 12000 g at 4 °C. The supernatant was loaded on to a phenyl-Sepharose column (50 ml bed-volume) pre-equilibrated in buffer I [30 mM Hepes (pH 7.4)/1 mM CaCl_2 /0.1 mM DTT], and then washed with 10 column vol. of buffer I and 5 column vol. of buffer II [30 mM Hepes (pH 7.4)/200 mM NaCl/1 mM CaCl_2 /0.1 mM DTT]. Bound CaM was eluted with buffer III [30 mM Hepes (pH 7.4)/2 mM EGTA/0.1 mM DTT]. The pooled CaM containing fractions were concentrated to 1.5 ml using ultrafiltration with a PM10 membrane (Amicon, Beverly, MA, U.S.A.), equilibrated with 5% (v/v) glycerol and stored at -70 °C. The purified CaM was revealed as a single band on SDS/PAGE analysis.

Purification of GDI

GDI was purified from bovine brain as described previously [27], but with a minor modification, using DEAE-ion-exchange chromatography, ammonium sulphate precipitation and Mono-Q ion-exchange chromatography. All buffers used after the ammonium sulphate precipitation step contained 10% (v/v) glycerol, 0.25 mM PMSF, 2 $\mu\text{g}/\text{ml}$ aprotinin, 2 $\mu\text{g}/\text{ml}$ leupeptin and 1 $\mu\text{g}/\text{ml}$ pepstatin A. Aliquots of purified GDI solution were stored at -70 °C [20].

Dissociation of Rab3A from LP2

LP2 (60 μg of protein) was incubated for 30 min at 30 °C with CaM or GDI in 50 μl of the reaction buffer [50 mM Hepes (pH 7.4)/0.5 μM MgCl₂/1 mM DTT/2 $\mu\text{g}/\text{ml}$ aprotinin/2 $\mu\text{g}/\text{ml}$ leupeptin/0.1 mM CaCl₂]. The reacted mixture was centrifuged for 30 min at 100000 g in a Beckman TLA45 rotor. The supernatant was saved, and the pellet was then suspended in 50 μl of the same reaction buffer before homogenization by mild sonication. Rab3A released into the soluble fraction was analysed by Western blotting using the anti-Rab3A antibody, and quantified by scanning of the Western blot with a densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.) [21].

Two-dimensional (2D)-gel electrophoresis and transfer on to PVDF membranes, and overlay with [α -³²P]GTP

The protein sample for the 2D-gel electrophoresis was prepared by suspending the sample in 80% (v/v) acetone for 30 min at –20 °C. The precipitates were collected by centrifugation (12000 g for 20 min) at 4 °C, and then dried in air. Samples dissolved in 9.5 M urea solution containing 2% (w/v) TX-100, 5% (v/v) 2-mercaptoethanol, 1.6% (w/v) ampholyte (pH 5–7) and 0.4% (w/v) ampholyte (pH 3–10) were electrofocused on isoelectric focusing gels (diameter 1 mm \times length 7 cm) for 7 h at 700 V, following the instructions of Bio-Rad, and then transferred on to SDS/14% polyacrylamide gels before 2D-gel electrophoresis. The proteins separated by SDS/PAGE were transferred on to PVDF membranes and overlaid with GTP as described by Huber and co-workers [28], but with a minor modification. The polyacrylamide gel was washed twice for 15 min in 50 mM Tris/HCl, pH 7.5, containing 20% (v/v) glycerol, before the proteins were electrophoretically transferred on to PVDF membranes in 10 mM Na₂CO₃ buffer, pH 9.8. The transfer blot was rinsed for 30 min in GTP-binding buffer [50 mM Na₂CO₃ (pH 7.5)/10 μM MgCl₂/2 mM DTT/0.2% (v/v) Tween 20/4 μM ATP], and then incubated with [α -³²P]GTP (1 $\mu\text{Ci}/\text{ml}$) for 2 h. The blot was rinsed ten times (6 min for each wash) with GTP-binding buffer containing 25 mM MgCl₂ instead of 10 μM MgCl₂, and then dried in air. [α -³²P]GTP binding spots were visualized by autoradiography using Kodak Xomat film exposed for 12–24 h at –70 °C.

Cross-linking studies

LP2 (60 μg of protein) was incubated for 30 min at 30 °C with 40 μg of CaM or 2 μg of GDI in 50 μl of the reaction buffer. The reaction mixtures were centrifuged for 30 min at 100000 g , and the supernatants were pre-incubated with various concentrations of TX-100, before treatment with freshly prepared 1 mM BS³ for 30 min at 30 °C. The reactions were quenched with Tris buffer to a final concentration of 50 mM. Cross-linking products of Rab3A were analysed by Western blot analysis using the anti-Rab3A antibody [21,29].

Binding of GDP or GTP, and GTPase activity

For the binding of GDP or GTP, Rab3A was incubated with 0.1 μM [³H]GDP or [γ -³⁵S]GTP or [γ -³²P]GTP in 50 μl of the GTP-binding buffer [10 mM Hepes (pH 7.4)/0.5 μM MgCl₂/1 mM DTT/1 mM DMPC] for 10 min at 30 °C. For the GTPase assay, 50 μl of the GTPase buffer (10 mM Hepes, pH 7.4, containing 1 mM DTT, 5 mM MgCl₂ and 1 mM GTP) was added to the [γ -³²P]GTP-bound Rab3A solution (50 μl) and incubated for various time periods at 30 °C [30]. The binding of GDP or GTP and GTPase activity assays were terminated by adding 1 ml of ice-cold Stop buffer [20 mM Hepes (pH 7.4)/100 mM NaCl/

25 mM MgCl₂]. The reaction mixtures were filtered immediately on a BA85 membrane (Schleicher & Schuell), and the membranes were then washed 5 times with 1 ml of cold Stop buffer. The radioactivity of dried membranes in 5 ml of cocktail (Ready Safe; Beckman) was measured using a liquid-scintillation counter (Beckman LS5000TD).

RESULTS

Complex formation between Ca²⁺/CaM and Rab3A, and disruption of the complex by TX-100

In order to ascertain the specific activity of Ca²⁺/CaM inducing dissociation of membrane-bound Rab3A, the freshly prepared

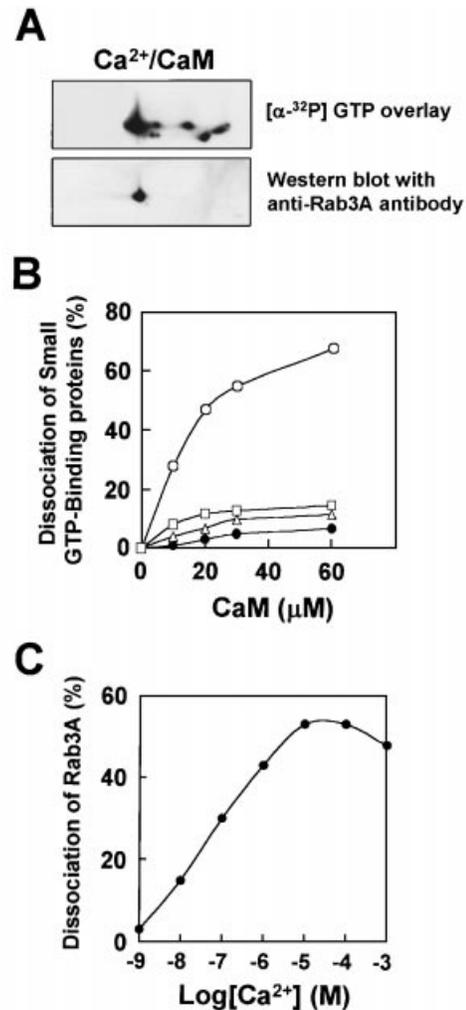


Figure 1 Dissociation of Rab3A by CaM

LP2 membranes (60 μg of protein) were incubated with 40 μg of CaM in 50 μl of the reaction buffer (see the Materials and methods section) for 30 min at 30 °C. Reaction mixtures were centrifuged at 100000 g for 30 min at 4 °C. GTP-binding proteins that were dissociated from LP2 membranes by Ca²⁺/CaM were separated by 2D gel electrophoresis and assayed by overlaying with [α -³²P]GTP on the transferred PVDF membrane (see the Materials and methods section), and Rab3A dissociated by Ca²⁺/CaM was identified using anti-Rab3A antibody (A). Small GTP-binding proteins dissociated from LP2 membranes by Ca²⁺/CaM were determined by Western blotting with anti-Rab1 (●), -Rab3A (○), -Rab5 (□) and -Cdc42 (△) antibodies. The intensity of the bands on the Western blot was determined by densitometry (B). CaM (30 μM) was incubated with LP2 membranes in various concentrations of Ca²⁺, and the dissociation of Rab3A was analysed by Western blotting using anti-Rab3A antibody (C).

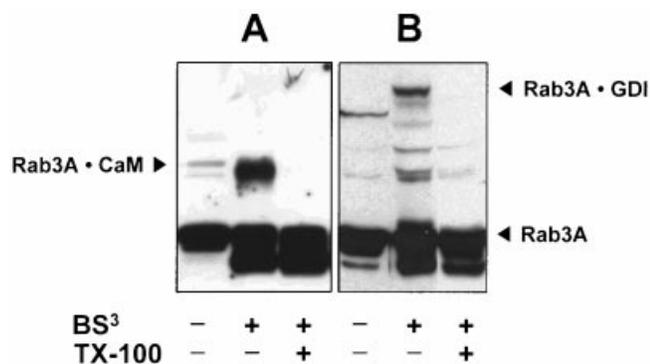


Figure 2 Cross-linking of CaM-Rab3A and GDI-Rab3A

LP2 (60 μ g of protein) was incubated for 30 min at 30 °C with 40 μ g of CaM (A) or with 2 μ g of GDI (B) in 50 μ l of the reaction buffer. The reaction mixtures were centrifuged for 30 min at 100 000 *g*, and the supernatants were pre-incubated with or without 0.1% (w/v) TX-100 for 20 min at 30 °C, and then treated with freshly prepared 1 mM BS³ for 30 min at 30 °C. The reactions were quenched with 1 M Tris buffer to the final concentration of 50 mM. The reacted proteins were separated by SDS/PAGE, and Rab3A and the cross-linked proteins of the Rab3A complex were analysed with Western blotting by using an anti-Rab3A antibody.

LP2 membranes were used, and solubilized Rab3A was measured by Western blot analysis with the Rab3A antibody. Ca²⁺/CaM readily induced the dissociation of Rab3A, along with that of other minor small GTP-binding proteins, from LP2 membranes, as reported previously [21]. GTP-binding proteins dissociated from LP2 membranes by Ca²⁺/CaM were easily separated by 2D-gel electrophoresis, and were assayed by overlaying with [α -³²P]GTP on the transferred PVDF membrane. The major GTP-binding spot was found to coincide with the Rab3A antibody-reactive protein spot, although other GTP-binding proteins of relatively minor importance were also present on the gel (Figure 1A). Of the small GTP-binding proteins tested, Rab3A was released preferentially from the LP2 membranes by Ca²⁺/CaM (Figure 1B). The dissociation of Rab3A induced by CaM was almost linearly dependent on Ca²⁺ concentration, and reached a plateau at 10–100 μ M Ca²⁺ (Figure 1C).

In addition to Ca²⁺/CaM, GDI also caused dissociation of Rab3A from LP2 membranes by binding to Rab3A [21,27]. Interaction of Rab3A with either Ca²⁺/CaM or GDI was confirmed by a combination of techniques: chemical cross-linking analysis of the proteins with BS³, SDS/PAGE analysis and identification with the Rab3A antibody, as shown in Figure 2. Three immunoreactive bands of p28, p43 and p84 correspond to Rab3A, Rab3A-CaM and Rab3A-GDI respectively. The control experiment (non-specific cross-linking by BS³) was performed by pre-treating the supernatant solution from the reaction mixture of LP2 membranes containing Rab3A and either Ca²⁺/CaM or GDI proteins with 0.1% TX-100 to disrupt complex formation. In this case, there was no evidence of cross-linking by BS³ (Figure 2).

Effect of Ca²⁺/CaM on the Rab3A GTPase activity

The possible influence of Ca²⁺/CaM on the intrinsic GTPase activity of Rab3A, examined by the direct hydrolysis of [γ -³²P]GTP, revealed that GTPase activity was very similar, regardless of whether Rab3A existed in a complex or was dissociated from Ca²⁺/CaM (Figure 3A). The dissociation of the complex mediated by 0.1% TX-100 (Figure 2) did not alter the rate of GTP hydrolysis. To corroborate this result,

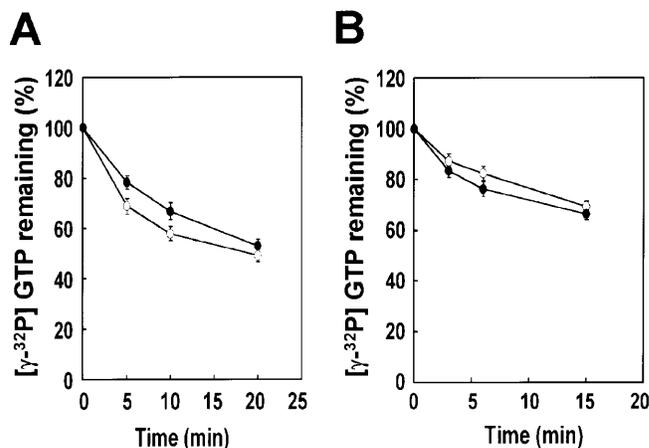


Figure 3 Effect of Ca²⁺/CaM on GTPase of Rab3A

Rab3A-Ca²⁺/CaM complex was prepared by incubation of LP2 membranes (80 μ g of protein) and 20 μ g of CaM in 50 μ l of the reaction buffer at 30 °C for 30 min, and then 10 μ l of Rab3A-Ca²⁺/CaM complex (containing 4 μ g of CaM) were pre-incubated in the presence (●) or absence (○) of 0.1% Triton X-100 in 50 μ l of the GTP-binding buffer (see the Materials and methods section). 0.1 μ M [γ -³²P]GTP was then added and incubated for 10 min at 30 °C (A). GST-Rab3A (50 ng) was pre-incubated with 35 μ g of BSA (○) or CaM (●) in 50 μ l of the GTP-binding buffer with 0.1 μ M [γ -³²P]GTP for 10 min at 30 °C (B). The GTPase reaction was started by adding 50 μ l of GTPase buffer (see the Materials and methods section) at 30 °C. GTPase reaction was stopped by the addition of 1 ml ice-cold Stop buffer. The values are expressed as means \pm S.E.M. (*n* = 3).

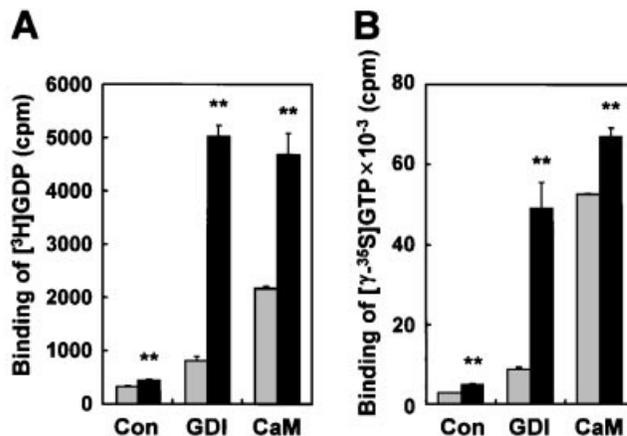


Figure 4 GTP- and GDP-binding to Rab3A dissociated from LP2 membranes by GDI or CaM

Of Rab3A dissociated by GDI (0.25 μ g) or CaM (4 μ g), 10 μ l was pre-incubated in the absence (grey bars) or presence (black bars) of 0.1% TX-100 and then 0.1 μ M [³H]GDP (A) or [γ -³⁵S]GTP (B) was added. After 10 min of incubation in 50 μ l of the GTP-binding buffer at 30 °C, the reaction was stopped by addition of 1 ml of ice-cold Stop buffer. The values are expressed as means \pm S.E.M. (*n* = 3); those marked with a double asterisk are significantly different (*P* < 0.01).

recombinant GST-Rab3A, which was shown to possess full intrinsic GTPase activity but was unable to form a complex with Ca²⁺/CaM [21], was utilized under similar experimental conditions. Ca²⁺/CaM neither affected the binding of GDP and GTP to recombinant GST-Rab3A (results not shown) nor altered the intrinsic GTPase activity of the recombinant GST-Rab3A protein (Figure 3B). Thus the results from both experiments suggest that the catalytic domain of GTPase in the Rab3A

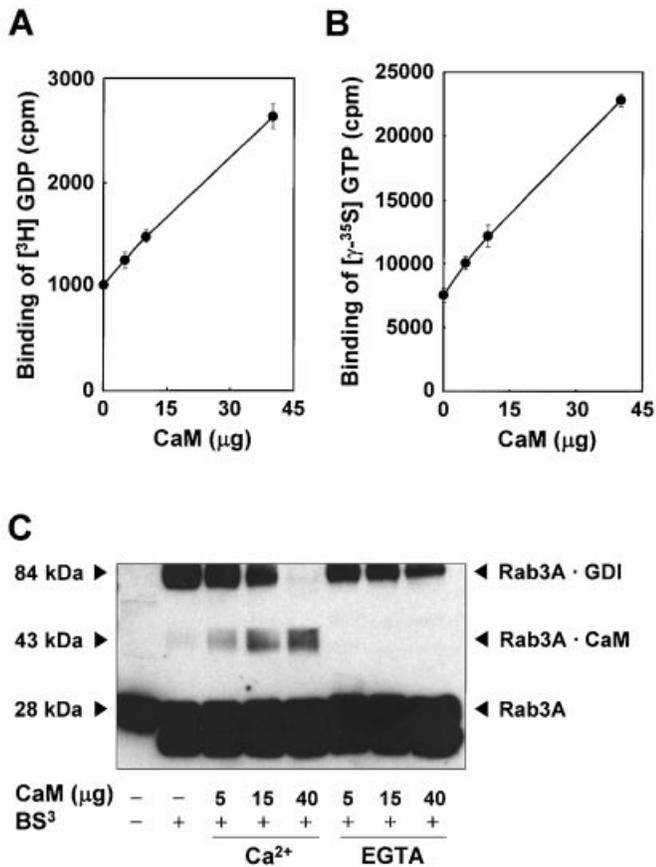


Figure 5 Stimulation of GDP and GTP binding to Rab3A–GDI complex by Ca²⁺/CaM

LP2 membranes (60 μg of protein) were incubated with 1.25 μg of GDI in 50 μl of the reaction buffer for 30 min at 30 $^{\circ}\text{C}$ to obtain Rab3A–GDI complex. The supernatant (0.25 μg of GDI/10 μl) was incubated with various concentrations of CaM in 50 μl of the GTP-binding buffer for 30 min at 30 $^{\circ}\text{C}$, and then 0.1 μM [³H]GDP (A) or [γ -³⁵S]GTP (B) was added and allowed to react for 10 min at 30 $^{\circ}\text{C}$. The reaction of guanine nucleotide binding was stopped by the addition of 1 ml of ice-cold Stop buffer. The values are expressed as means \pm S.E.M. ($n = 3$). The supernatant (0.25 μg of GDI/10 μl) was incubated with various concentrations of CaM in the presence of 0.1 mM Ca²⁺ or 10 mM EGTA, and then the samples were treated with BS³ for cross-linking and analysed by Western blotting using the anti-Rab3A antibody (C).

molecule appears to be independent from the Ca²⁺/CaM-binding domain, and furthermore was not affected by the association.

GDP and GTP binding to the Ca²⁺/CaM–Rab3A complex

The resultant Ca²⁺/CaM–Rab3A (or GDI–Rab3A) complex was incubated with isotopically labelled GDP or GTP to measure guanine nucleotide binding. The guanine nucleotides binding to Rab3A reached a plateau within 10 min. Rab3A in the GDI–Rab3A complex demonstrated a rather poor binding of GDP or GTP, whereas the TX-100-treated GDI–Rab3A complex was able to bind GDP or GTP up to the maximum level (Figure 4), suggesting that GDI blocks the binding of GDP or GTP to Rab3A. In the case of the Ca²⁺/CaM–Rab3A complex, however, GDP or GTP could bind to Rab3A at an appreciable level, even in the absence of TX-100 (Figure 4). Furthermore, there was a definitive preference shown by the Ca²⁺/CaM–Rab3A complex for GTP than for GDP. These results suggest that Ca²⁺/CaM does not inhibit the binding of GTP to Rab3A, even in the form of the Ca²⁺/CaM–Rab3A complex.

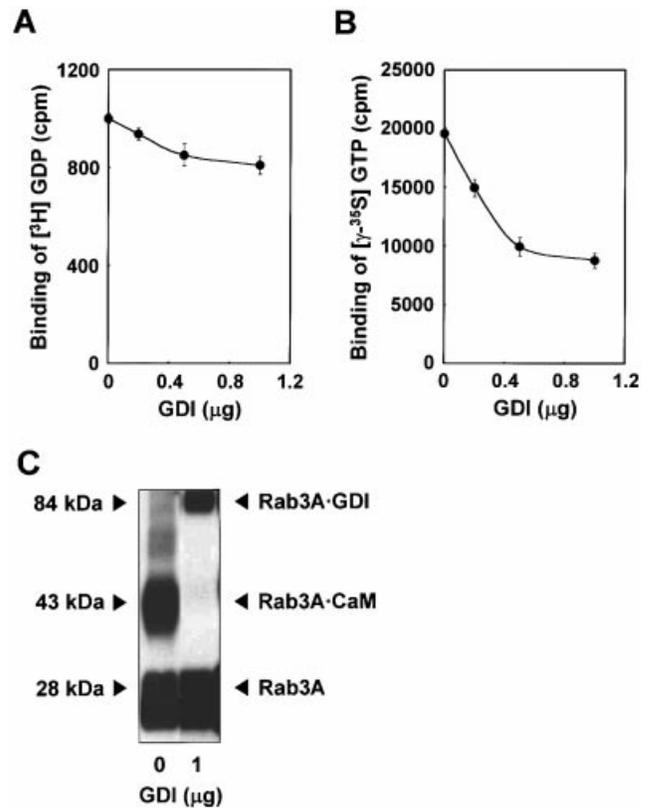


Figure 6 Inhibition of GDP and GTP-binding to Rab3A–Ca²⁺/CaM by GDI

LP2 membranes (80 μg of protein) were incubated with 20 μg of CaM in 50 μl of the reaction buffer for 30 min at 30 $^{\circ}\text{C}$ to obtain Rab3A–Ca²⁺/CaM. The supernatant (4 μg of CaM/10 μl) was incubated with GDI in 50 μl of the GTP-binding buffer at 30 $^{\circ}\text{C}$ for 30 min, and then 0.1 μM [³H]GDP (A) or [γ -³⁵S]GTP (B) was added and allowed to react for 10 min at 30 $^{\circ}\text{C}$. The reaction of guanine nucleotide binding was stopped by the addition of 1 ml of ice-cold Stop buffer. The values are expressed as means \pm S.E.M. ($n = 3$). The supernatant (4 μg of CaM/10 μl) was incubated with 1 μg of GDI, and then the sample was treated with BS³ for cross-linking and analysed by Western blotting using anti-Rab3A antibody (C).

Ca²⁺/CaM exchanges GDP in the Rab3–GDI complex for GTP in the Rab3A–Ca²⁺/CaM complex

An earlier report describing the competition of Ca²⁺/CaM with GDI for binding to Rab3A [21] suggested that a possible effect of Ca²⁺/CaM could involve consequential nucleotide binding to Rab3A in a complex with GDI. To explore such a possibility, GDI–Rab3A complex obtained from LP2 was pre-incubated with comparatively high concentrations of Ca²⁺/CaM to replace GDI on the Rab3A complex (5.5–44 μM CaM), and then [³H]GDP or [γ -³⁵S]GTP was added to measure the binding of guanine nucleotides to Rab3A. Ca²⁺/CaM was indeed found to increase the binding of [³H]GDP and [γ -³⁵S]GTP (Figures 5A and 5B respectively) to GDI–Rab3A complex in a CaM concentration-dependent manner. The competition between Ca²⁺/CaM and GDI towards Rab3A was monitored as the CaM concentration was increased. The Rab3A–GDI complex was disrupted, and Rab3A–CaM complex was concomitantly formed, depending on the concentration of CaM in the presence of Ca²⁺, whereas Rab3A–CaM complex was not formed in the absence of Ca²⁺ (Figure 5C).

Likewise, by reversing the binding order of Ca²⁺/CaM and GDI to Rab3A, the influence of GDI on guanine nucleotide

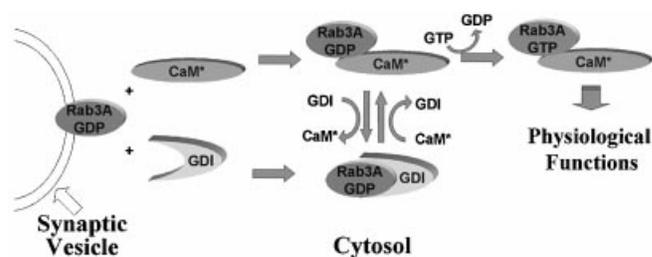
binding to Rab3A–Ca²⁺/CaM was examined. Ca²⁺/CaM–Rab3A complex obtained from LP2 was pre-incubated with GDI to replace CaM on the complex. GDI modestly decreased [³H]GDP binding to Rab3A–Ca²⁺/CaM, whereas it decreased more profoundly [γ -³⁵S]GTP-binding to Rab3A–Ca²⁺/CaM complex in a GDI-concentration-dependent manner (Figures 6A and 6B respectively). These results suggest that Ca²⁺/CaM is able to replace GDP of the Rab3A–GDI complex with GTP by the formation of a new Ca²⁺/CaM–Rab3A complex. To confirm the exchange of Rab3A–CaM complex with Rab3A–GDI complex as the GDI concentration was increased, the Rab3A–CaM complex was pre-incubated with GDI, and then cross-linked with BS³. GDI disrupted the Rab3A–CaM complex, and Rab3A–GDI complex was subsequently formed in the relatively high concentration of GDI (Figure 6C), which was in agreement with the previous study of Park and co-workers [21].

DISCUSSION

In an attempt to elucidate a possible role for Ca²⁺/CaM together with Rab3A following equimolar complex formation resulting in the dissociation of Rab3A from the membrane (Figure 1), experiments were performed to define the guanine nucleotide-binding properties and the intrinsic GTPase activity of Rab3A in a complexed state. Rab3A, when associated with Ca²⁺/CaM, was found to bind guanine nucleotides, whereas Ca²⁺/CaM had no effect on the GTPase activity of Rab3A (Figures 3 and 4). On the basis of the fact that intrinsic GTPase activity was not affected by Ca²⁺/CaM binding (Figure 3), it may be surmised that the catalytic domain of Rab3A GTPase is distinct from the binding domain of Ca²⁺/CaM in Rab3A. Although both GDP and GTP bound appreciably well to the Ca²⁺/CaM–Rab3A complex, GTP was found to be the ‘preferred’ nucleotide, relative to GDP (Figure 4).

In a comparative control study using the known Rab3A-binding protein, GDI, it was shown that the Rab3A–GDI complex did not bind with guanine nucleotides (Figure 4), in agreement with an earlier study [7] that revealed that neither GDP nor GTP could replace the nucleotides bound to the Rab3A–GDI complex. The substantially larger molecular size of GDI relative to CaM [27,31] might result in structural hindrance of the binding of guanine nucleotides in Rab3A. Ca²⁺/CaM was found to compete with GDI in terms of interaction with Rab3A in a concentration-dependent manner, resulting in the formation of a Ca²⁺/CaM–Rab3A complex that is favourable for GTP binding in the soluble state (Figure 5). Recently, it has been reported that CaM is able to bind to GTP-bound Rab3A in preference to the GDP-bound form [23]. In addition, the intracellular concentration of GTP has been shown to be higher than that of GDP [32].

Members of the Rho subfamily, another class of Ras-related GTPase, also has interconvertible inactive GDP-bound and active GTP-bound forms, and the interconversion of these GDP- and GTP-bound forms is also stimulated by GEPs and GAPs [33]. Many GEPs, GAPs and GDIs pertaining to the Rho family members have been identified [34]. Similarly to the Rho proteins, Rab3A also has many regulatory proteins, i.e. GAP, GEP and GDIs. The discovery of two isoforms of GDI that are able to bind to Rab3A [35] suggests that tissue-specific Rab protein(s) may be regulated by multiple forms of GDI in a tissue- and cell-specific manner. Subsequently, the purification and characterization of Rab3 GAP [36] and Rab3 GEP [37] proteins has been described. Overexpression of the deletion mutant of Rab3 GEP decreased the release of growth hormone [38], implicating the involvement of Rab3A GEP in the regulation of Rab3A activity



Scheme 1 Scheme for recycling of GDP- and GTP-binding states of Rab3A by CaM

Rab3A·GDP in a complex with GDI can be converted into a Ca²⁺/CaM–Rab3A·GDP complex when CaM is activated by Ca²⁺ (CaM*), and then GTP can replace GDP of Rab3A, resulting in the formation of the Ca²⁺/CaM–Rab3A·GTP complex. The Ca²⁺/CaM–Rab3A·GTP complex may have physiological roles *in vivo*.

and, ultimately, in neurotransmitter release [39]. Rab3 GEP, however, has no activity with the Rab3A–GDI complex: it cannot effect the exchange of GDP with GTP for the Rab3A–GDI complex *in vitro* [37], whereas Ca²⁺/CaM can exchange GDP of Rab3A–GDI complex with GTP *in vitro* (Figure 5).

It is not clear whether Ca²⁺/CaM is able to function as a stimulator of GTP binding to Rab3A complexed with GDI *in vivo*, since CaM does not appear to retain the high affinity for binding to Rab3A as is found for GEP. Recently, the affinity of Rab3A for CaM has been reported to be relatively low (K_d of 18–22 μ M at 10⁻⁵ M Ca²⁺) [23]. Indeed, a relatively high concentration of CaM was required in the present study for stimulating GTP binding to Rab3A complexed with GDI *in vitro* (Figure 5). The levels of Ca²⁺/CaM available *in vivo* might be sufficient to satisfy this criterion, considering that the total concentration of CaM in the brain is estimated to be approx. 30 μ M [31]. In addition, it has been reported that Ca²⁺ concentration measured in the vicinity of the Ca²⁺ channels might be as high as 100 μ M [40–42]. Indeed, we found that the dissociation of Rab3A by CaM was dependent upon the concentration of Ca²⁺, and the maximal level of dissociation was found to occur at a concentration of Ca²⁺ of 10–100 μ M (Figure 1C). It is therefore possible that the Ca²⁺ concentration used in our experiments could be physiologically relevant for the mechanism of GTP binding to Rab3A complexes with GDI in the presence of CaM. Recently, it was reported that the Ca²⁺-dependent Rab3A–CaM association occurs in a similar range of Ca²⁺ ion concentrations (1–100 μ M), which is required to evoke insulin secretion from pancreatic β -cells [23]. A schematic representation of the formation of the Ca²⁺/CaM–Rab3A·GTP complex from membrane-bound Rab3A and from GDI–Rab3A complex is shown in Scheme 1. Even though the exact physiological function of GTP-bound Rab3A–Ca²⁺/CaM complex has yet to be determined unequivocally in nerve cells, there is strong evidence that Ca²⁺/CaM–Rab3A·GTP has a physiological role in PC12 cells [22]. The inhibition of secretion caused by the GTP-bound form of Rab3A requires an interaction with Ca²⁺/CaM, whereas interactions of Rab3A with Raphyllin and RIM are neither required nor sufficient to sustain the effect of Rab3A in exocytosis [22]. Although it remains to be elucidated how the Ca²⁺/CaM–Rab3A·GTP complex regulates neurosecretion, the complex might stimulate the downstream effector protein in the cytosol. If the effector protein for the Ca²⁺/CaM–Rab3A·GTP complex exists, further isolation and char-

acterization of this protein will provide a better understanding of the mechanism of the action of the Ca²⁺/CaM–Rab3A·GTP complex. Alternatively, CaM may participate in delivering Rab3A·GTP to the membranes, since vesicle-bound Rab3A is in the GTP form before exocytosis [43]. In the case of translocation of Rab3A to the membrane from the Rab3A–GDI complex, Rab3A receptor protein in the membrane is required [44]. Like the Rab3A–GDI complex, the membrane receptor or another unidentified factor might be required for the translocation of Rab3A from the Ca²⁺/CaM–Rab3A·GTP complex to the membranes.

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