Ca\(^{2+}\)/Calmodulin Causes Rab3A to Dissociate from Synaptic Membranes*

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The GTPase Rab3A has been postulated to cycle on and off synaptic membranes during the course of neurotransmission. Moreover, a Rab guanine nucleotide dissociation inhibitor has been shown to cause Rab3A to dissociate from synaptic membranes in vitro. We demonstrate here that Ca\(^{2+}\)/calmodulin also can cause Rab3A to dissociate from synaptic membranes in vitro. Like Rab guanine nucleotide dissociation inhibitor, it forms a 1:1 complex with Rab3A that requires both the lipidated C terminus of Rab3A and the presence of bound guanine nucleotide. In addition, a synthetic peptide corresponding to the Lys\(^{62}\)-Arg\(^{80}\) sequence of Rab3A can prevent the dissociating effect of each protein and disrupt complexes between each protein and Rab3A. However, Ca\(^{2+}\)/calmodulin's effect differs from that of Rab guanine nucleotide dissociation inhibitor not only in being Ca\(^{2+}\)-dependent but also in having a less stringent requirement for GDP as opposed to GTP and in involving a less complete dissociation of Rab3A. The functional significance in vivo of Ca\(^{2+}\)/calmodulin's effect remains to be determined; it may depend in part on the relative amounts of Ca\(^{2+}\)/calmodulin and Rab guanine nucleotide dissociation inhibitor that are available for binding to Rab3A in individual, activated nerve termini.

The opening of voltage-gated Ca\(^{2+}\) channels in active zones of nerve terminals causes a brief, localized influx of Ca\(^{2+}\) followed by the secretion of neurotransmitters (1–3). The molecular basis of this effect is still unclear, but increased concentrations of intracellular Ca\(^{2+}\) may act at several levels to trigger fast fusion of pre-docked synaptic vesicles with the synaptic plasma membrane, promote endocytosis of the vesicle membranes and subsequent vesicle reformation, and mobilize additional vesicles to release sites (1, 4). Proteins that bind Ca\(^{2+}\) probably mediate many of these actions, and a number of candidate proteins have been identified. They include rabphilin (5, 6); the α-, β-II-, and γ isoforms of protein kinase C (7); and dynamin (8), all of which show Ca\(^{2+}\)-dependent binding to acidic phosphoglycerides. They also include calmodulin (CaM)\(^9\), synaptotagmin (10–13), and calcineurin (14), which bind Ca\(^{2+}\) directly. CaM that contains bound Ca\(^{2+}\) (Ca\(^{2+}\)/CaM) can activate CaM kinase II and calcineurin (9), and both enzymes may play important regulatory roles (15–18). Furthermore, Ca\(^{2+}\)/CaM appears to be required for secretion in adrenal chromaffin cells (19–21). However, how Ca\(^{2+}\)- and Ca\(^{2+}\)/CaM-dependent reactions are integrated to promote and optimize synaptic responses remains to be determined.

In the present investigation we examined the effects of Ca\(^{2+}\) and CaM on the behavior of Rab3A, a low molecular mass, di-geranylgeranylated, guanine nucleotide-binding protein that is attached to neurotransmitter-containing synaptic vesicles (22, 23). Previous investigators had shown that depolarization of rat brain synaptosomes causes a reduction in the contents of both Rab3A and a related guanine nucleotide-binding protein, Rab3C, in crude synaptic vesicles (Refs. 24 and 25 but see Ref. 26 for a conflicting view). Furthermore, action of a Rab guanine nucleotide dissociation inhibitor protein (Rab GDI) had been implicated because of its known ability to form a complex with Rab3A and cause it to dissociate from synaptic membranes in cell-free experiments (27–29). While exploring the possibility that increased concentrations of Ca\(^{2+}\) might affect the Rab3A dissociation process, we discovered that Ca\(^{2+}\)/CaM also can cause Rab3A to dissociate from synaptic membranes. Studies of the mechanism of this effect and its relation to that of Rab GDI are described below.\(^\text{2}\)

**EXPERIMENTAL PROCEDURES**

**Materials**—CaM was obtained from Calbiochem and freshly dissolved in 50 mM HEPES, pH 7.4, for each experiment. CaCl\(_2\), Suprapur grade, was from EM Science. BS\(_3\) was obtained from Pierce. Rab3A peptides Lys\(^{62}\)-Arg\(^{80}\), Ala\(^{2}\)-Asn\(^{18}\), and Glu\(^{177}\)-Asp\(^{195}\) (Table I) and the Rab GDI peptide, Gly\(^{21}\)-Ser\(^{45}\) (GIMSVNGKKVLHMDRNPYYGGESSS), were synthesized by the University of Washington Biopolymer Facility. The CaM kinase II peptide Leu\(^{290}\)-Ala\(^{309}\) (Table I) was from LC Laboratories. Stock solutions of peptides were prepared in Me\(_2\)SO and then added to incubation mixtures at final Me\(_2\)SO concentrations of <5%, GDP, GTP-S, and unmyristoylated Rab3A were from Calbiochem. Rab GDI was purified from bovine brain as described (29), except that all buffers used after the ammonium sulfate precipitation step contained 10% glycerol, 0.25 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml each of aprotinin and leupeptin, and 1 μg/ml pepstatin A. All other purchased chemicals were reagent grade from Sigma, and all procedures were performed at 4 °C unless otherwise indicated.

**Preparation of Synaptosomes**—Two different methods were used to prepare synaptosomes from cerebral cortex of nonhuman primates (Macaca nemestrina), obtained from the tissue distribution program of the Regional Primate Research Center at the University of Washington. In method 1, 50 g of cortex was sliced in ice-cold buffer A (320 mM sucrose; disso
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2 mM EGTA; 0.1 mM phenylmethylsulfonyl fluoride; 1 μg/ml each of leupeptin, aprotinin, and pepstatin A; and 2 μg/ml each of leupeptin and aprotinin. Then a gray matter-enriched portion, diluted with 10 volumes of buffer A, was homogenized successively with an Oster blender and a Potter-Elvehjem homogenizer. Finally, a crude synaptosomal pellet (90 % of the total homogenate volume, referred to as g × gav, where gav is g force at tube half-length) was prepared and purified on a Ficoll gradient using a modification of the methods of Fischer von Mollard et al. (24) and Barrie et al. (31). In method 2, synaptosomes were used primarily for depolarization studies were prepared from 15 g of prefrontal and temporal cortex. The tissue was minced with a razor blade in ice-cold buffer A (with 5 mM HEPES, pH 7.4, containing 0.1 mM EDTA, rinsed twice with buffer, and homogenized in a Beckman SW50.1 rotor at 16,000 × gav for 20 min at 4 °C). The volume of 100 ml was added to a low-speed-rotating Potter-Elvehjem homogenizer (0.25 mm clearance; Ref. 32). Then a synaptosome-enriched fraction was prepared as described (24).

Preparation of Synaptosomal Lysates and Lysate Subfractions—For most studies synaptosomal lysates were prepared and successively centrifuged to yield a synaptosomal plasma membrane-enriched fraction (28,000 × gav, pellet), a crude synaptic vesicle fraction (178,000 × gav, supernatant), and a corresponding high speed supernatant as described (33) except that EGTA (2 mM) was added to all buffers. The crude synaptic vesicle fraction, which contained Rab3A-enriched membranes (REM), was resuspended in 2–3 ml of buffer containing 300 mM glycine, 176,000 × g (REM (30)), was similarly analyzed. For cross-linking experiments, REM (15–25 μg of protein) were washed twice by centrifugation in 50 mM HEPES, pH 7.4, containing 100 mM NaCl, 1 mM MgCl₂, 1 mM DTT, and protease inhibitors as described for buffer B. The washed membranes were mixed with Ca²⁺/CaM in 25 μl of buffer and incubated for 30 min at 30 °C as described in the figure legends. The incubation mixtures were centrifuged for 30 min at 100,000 × gav; the supernatants were washed for 30 min at 30 °C with freshly prepared 1 mM β-sarcin (40), and the reactions were quenched with Triton x-100 and analyzed as described above. A similar treatment procedure was used in all other cross-linking experiments.

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Radiolabeling of CaM and CaM+125I-CaM-Binding Experiments—CaM (225 μg) in 50 μl of 50 mM HEPES buffer, pH 7.6, was modified by reaction with 0.5 μCi of 125I-labeled Bolton-Hunter reagent (2200 Ci/ mmol; NEN Life Science Products) according to the manufacturer’s directions. The iodinated CaM was then separated from unreacted reagent with the use of a Bio-Gel P-6 DG (Bio-Rad) spin column as described (42). After centrifugation, a mixture of 50 μl of 125I-labeled CaM (10 nmol) and 50 μl of REM (450 μg) was incubated for 45 min at 37 °C in the presence of 100 μM CaCl₂ and 0.1 mM GDP. After incubation the mixture was centrifuged for 30 min at 100,000 × gav, half of the supernatant was treated with BS⁺, and half was reserved as control. An aliquot of each half (50 μl) was then immunoprecipitated with 50 μl of anti-Rab3A IgG (1 μg) for 24 h at 0 °C. After immunoprecipitation, 40 μl of immobilized protein A on Trisacryl beads (Pierce; 50% slurry), which had been pretreated with bovine serum albumin, were added, and the sample was mixed for 20 h at 4 °C in a tube rotator. The protein A beads were then washed four times by a procedure that involved suspension in 50 mM HEPES, pH 7.4, containing 100 mM NaCl, 0.1 mM CaCl₂, 10 μl of Tween 20 ml, and 2 ml of SDS/PAGE sample buffer (4×), and subjected to SDS/PAGE analysis as described below.

Preparation of Rab3A-Depleted Synaptic Membranes and Transfer of Rab3A to These Membranes—To generate Rab3A-depleted membranes, REM (30 μg) were incubated for 30 min at 30 °C with 1.6 μg Rab GDI in 50 μl of buffer B (but without CaCl₂), pelleted by centrifugation for 30 min at 100,000 × gav, and suspended in 5 μl of buffer B (but without CaCl₂). To study the transfer of Rab3A to these membranes, they were mixed with medium containing Rab3A-BS⁺/CaM complex (prepared by incubating REM with buffer containing 75 μM CaCl₂ and 100 μM CaCl₂) and incubated for 30 min at 30 °C in the presence or absence of one of the peptides listed in Table I. Then the incubation mixtures were subjected to centrifugation and analyzed as described above.

Depolarization Assays—The ability of synaptosomes to secrete glutamate was measured after KCl-induced depolarization (24) or treatment with 4-aminopyridine + β-phorbol dibutyrate (31). A modification of the method previously described (24) was used. Briefly, control and depolarized samples were incubated for 10 min at 37 °C and then placed in ice water for 2 min and centrifuged for 2 min at 15,000 × gav. The amount of NADPH that had been produced was determined by measuring the absorbance of the supernatant at 360 nm on a Beckman DU 640 spectrophotometer using 390 nm as the reference wavelength.

Other Methods—SDS-PAGE was performed as described by Laemmli (45) but with 14% gels. In some cases, EGTA was added to the samples just before they were boiled. Proteins were transferred from SDS-PAGE gels to Immobilon P membranes for 30 min at 20 V in 20 mM Tris/HCl, pH 8.3. Analysis of Rab3A or transferred for 60 min to identify cross-linked products of higher molecular mass. Immunoblots were performed as described (35). Protein concentrations were determined with the Bradford method (44) (Bio-Rad) or, for SDS-containing samples, the micro-BCA method (Pierce). Free calcium ion concentrations were varied in the presence of 1 mM EGTA, on the basis of established binding constants (45).
**RESULTS**

**Ca$_2^+$/CaM Causes Rab3A to Dissociate from Membranes**—The initial aim of this investigation was to determine whether Ca$^{2+}$ and CaM influence the dissociation of Rab3A from synaptic membranes. To examine this possibility, we isolated synaptosomes from samples of macaque cerebral cortex, lysed the synaptic membranes, and prepared Rab3A-enriched membranes (REM) from the lysates by ultracentrifugation. To examine this possibility, we isolated synaptosomes from samples of macaque cerebral cortex, lysed the synaptic membranes, and prepared Rab3A-enriched membranes (REM) from the lysates by ultracentrifugation. Then we suspended the REM in medium containing Ca$^{2+}$-riched membranes (REM) from the lysates by ultracentrifugation and prepared Rab3A-enriched membranes from samples of macaque cerebral cortex, lysed the synaptic membranes. To examine this possibility, we isolated synaptosomes from samples of macaque cerebral cortex, lysed the synaptic membranes, and prepared Rab3A-enriched membranes (REM) from the lysates by ultracentrifugation. Then we suspended the REM in medium containing Ca$^{2+}$ and/or various other additives, incubated the mixtures for 30 min at 30 °C, separated the membranes from the medium by centrifugation, and separately measured the amounts of Rab3A recovered in the membrane and supernatant fractions. The results of these experiments demonstrated that medium containing both Ca$^{2+}$ and CaM, i.e. a Ca$^{2+}$/CaM complex, caused Rab3A to dissociate from the membranes but that medium containing either 100 μM Ca$^{2+}$ or 60 μM CaM alone did not (Fig. 1). The dissociation of Rab3A occurred in the absence of added ATP, and half-maximal effects were observed when the concentrations of Ca$^{2+}$ and CaM were about 0.5 and 20 μM, respectively. Maximal dissociation of Rab3A (approximately 65%) was obtained when the concentrations of Ca$^{2+}$ and CaM were about 10 and 65 μM, respectively (data not shown).

**Mechanism of the Effects of Ca$^{2+}$/CaM**—To examine the mechanism of the Rab3A-dissociating effect of Ca$^{2+}$/CaM, we first incubated REM in the presence of medium that contained Ca$^{2+}$/CaM and then recovered the medium and subfractionated it by sucrose gradient ultracentrifugation. Upon measuring the content of Rab3A in the subfractions, we detected a peak of material that had an apparent molecular mass of about 40 kDa (Fig. 2A). This peak could be distinguished easily from the peak of Rab3A-containing material detected in sucrose gradient ultracentrifugation experiments with a high speed supernatant fraction from a synaptosomal lysate (Fig. 2A). The peak from the lysate supernatant had a considerably larger apparent molecular mass and probably corresponded to a complex of Rab3A and Rab GDI (28).

In subsequent studies we incubated REM with Ca$^{2+}$/CaM, recovered the medium, and added the cross-linking agent, BS$^3$, to it. Then we analyzed the cross-linked material by SDS-PAGE and Western blotting with an antibody to Rab3A. In agreement with the sucrose gradient experiments, the results revealed the presence of Rab3A-containing material that had an apparent molecular mass of 43 kDa (Fig. 2B, lane 2). The combined results of these experiments suggested that Ca$^{2+}$/CaM could form a 1:1 molar complex with Rab3A.

To obtain additional evidence concerning this possibility, we incubated REM with Ca$^{2+}$/CaM that contained $^{125}$I-labeled CaM, recovered the medium, and added BS$^3$ to it. Then we immunoprecipitated Rab3A-containing material, analyzed it by SDS-PAGE, and identified $^{125}$I-CaM-containing bands by
with 10 mM EGTA greatly reduced the amount of the complex required for binding to Ca$^{2+}$, providing evidence that the lipidated C terminus of Rab3A is a binding site for Ca$^{2+}$ and CaM. 

The fact that no complex of recombinant Rab3A with Ca$^{2+}$/CaM was observed in the presence of 1 mM EGTA decreased the electrophoretic mobility of the Rab3A/CaM complex (Fig. 3, compare lanes 2 and 3), whereas incubation with 10 mM EGTA greatly reduced the amount of the complex that could be detected (Fig. 3, compare lanes 2 and 4). The binding of Ca$^{2+}$ to CaM is known to increase its electrophoretic mobility (49; see also Fig. 4, lanes 8 and 9 and Fig. 7B, lanes 1 and 2). Therefore, both results provided evidence for the formation of a Rab3A Ca$^{2+}$/CaM complex.

To investigate the role of hydrophobic interactions in forming the Rab3A Ca$^{2+}$/CaM complex, we used two different approaches. First, we incubated REM with Ca$^{2+}$/CaM, recovered the medium, and incubated aliquots of it in the presence of different concentrations of Ca$^{2+}/$CaM. The results revealed that incubation of the medium in the presence of 1 mM EGTA decreased the electrophoretic mobility of the Rab3A-CaM complex (apparent molecular mass 43 → 50 kDa) (Fig. 3, compare lanes 2 and 3), whereas incubation with 10 mM EGTA greatly reduced the amount of the complex that could be detected (Fig. 3, compare lanes 2 and 4). The binding of Ca$^{2+}$ to CaM is known to increase its electrophoretic mobility (49; see also Fig. 4, lanes 8 and 9 and Fig. 7B, lanes 1 and 2). Therefore, both results provided evidence for the formation of a Rab3A Ca$^{2+}$/CaM complex.

Among the many proteins that form complexes with Ca$^{2+}$/CaM is CaM kinase II (50). Furthermore, a basic- and hydrophobic amino acid-containing binding site for Ca$^{2+}$/CaM on this kinase, Leu290-Ala309 (Table I), has been identified (50, 51). Rab3A also contains a sequence that is enriched in basic and hydrophobic amino acids, Lys$^{62}$-Arg$^{85}$. To investigate the possibility that the Rab3A Lys$^{62}$-Arg$^{85}$ sequence might include a binding site for Ca$^{2+}$/CaM, we synthesized a peptide that corresponded to it (Table I) and then compared the effects of this peptide with those of other synthetic peptides in the following experiments.

First, we incubated REM with Ca$^{2+}$/CaM in the presence of each peptide and then measured the amount of Rab3A that dissociated to the medium. The results revealed that a 100 μM concentration of the Rab3A Lys$^{62}$-Arg$^{85}$ peptide or of a peptide corresponding to the CaM kinase II Leu290-Ala309 sequence blocked the Rab3A-dissociating effect of Ca$^{2+}$/CaM (Fig. 5A); half-maximal values were observed at concentrations of 42 and 18 μM, respectively. In contrast, 100 μM concentrations of peptides that respectively corresponded to regions near the Rab3A N terminus or unmodified C terminus (Rab3A Ala$^{2}$-Asn$^{18}$ or Glu$^{177}$-Asp$^{198}$; Table I) had no effect.

Second, after incubating REM with Ca$^{2+}$/CaM, we recovered the medium and incubated aliquots of it with the Rab3A Lys$^{62}$-Arg$^{85}$ peptide, the CaM kinase II Leu290-Ala$^{309}$ peptide, or the Rab3A Ala$^{2}$-Asn$^{18}$ peptide, then added BS$^{3}$ to each incubation mixture, and analyzed the content of cross-linked Rab3A-Ca$^{2+}$/CaM complex. Both the Rab3A Lys$^{62}$-Arg$^{85}$ peptide and the CaM kinase II Leu290-Ala$^{309}$ peptide reduced the amount of complex that could be detected, but the Rab3A Ala$^{2}$-Asn$^{18}$ peptide had no effect (Fig. 6A).

Third, we incubated the Rab3A Lys$^{62}$-Arg$^{85}$, Ala$^{2}$-Asn$^{18}$, or Glu$^{177}$-Asp$^{198}$ peptides with Ca$^{2+}$/CaM in the presence of Ca$^{2+}/$CaM, then added BS$^{3}$, and analyzed the products by SDS-PAGE. The results showed that the Lys$^{62}$-Arg$^{85}$ peptide could form a Ca$^{2+}$-dependent complex with CaM but the other Rab3A peptides could not (Fig. 7). Taken together, the results of these experiments provided strong evidence that Ca$^{2+}$/CaM promotes the dissociation of Rab3A from synaptic membranes by binding to amino acids within the Rab3A Lys$^{62}$-Arg$^{85}$ sequence.

Transfer of Rab3A from Rab3A Ca$^{2+}$/CaM to Membranes—Having shown that the Rab3A Lys$^{62}$-Arg$^{85}$ and CaM kinase II Leu290-Ala$^{309}$ peptides could separately disrupt Rab3A-Ca$^{2+}$/CaM complexes, we examined the possibility that disruption of the complexes might promote the transfer of Rab3A to membranes. We did this by incubating REM with Ca$^{2+}$/CaM to...
TABLE I
Amino acid sequences of peptides used in CaM binding studies

| Protein source | Peptide notation | Peptide sequence |
|----------------|-----------------|-----------------|
| CaM kinase II  | Leu<sup>209</sup>-Ala<sup>209</sup> | LKKFNARRKIKGAILTTMLA |
| Rab3A          | Lys<sup>62</sup>-Arg<sup>85</sup> | KTIYRNDERIKLQWDAGQEBYR |
| Rab3A          | Ala<sup>2</sup>-Asn<sup>18</sup> | ASATDSRYGKEDSSQDN-C |
| Rab3A          | Glu<sup>177</sup>-Asp<sup>195</sup> | C-ERLVVDVICERSMSESLTAD |

FIG. 5. Effects of Rab3A and CaM kinase II peptides on the Ca<sup>2+</sup>/CaM- or Rab GDI-induced dissociation of Rab3A from REM. A, aliquots of REM (15 μg of protein) were incubated for 30 min at 30 °C in 50 μl of buffer B containing 100 μM CaCl<sub>2</sub>, 50 μM CaM, and 100 μM concentrations of a Rab3A peptide (Lys<sup>62</sup>-Arg<sup>85</sup>, Ala<sup>2</sup>-Asn<sup>18</sup>, or Glu<sup>177</sup>-Asp<sup>195</sup>) or the CaM kinase II peptide, Leu<sup>209</sup>-Ala<sup>209</sup>. The medium was then recovered and analyzed as described in Fig. 1. Following Western analysis of supernatant and pellet fractions and densitometry of the Rab3A containing band, the absorbance value obtained for each supernatant fraction was expressed as a percentage of the total Rab3A recovered in each sample. Error bars represent standard errors of mean values from at least two experiments; * denotes standard errors of <1.5%. B, aliquots of REM (15 μg of protein) were incubated for 30 min at 30 °C in 50 μl of buffer B that contained purified Rab GDI (1 μM) and a 100 μM concentration of the indicated peptide but no CaCl<sub>2</sub>. The incubation mixtures were then centrifuged and analyzed as described as described above. Error bars as in A. Single-letter amino acid codes are used.

Generate a Rab3A-Ca<sup>2+</sup>/CaM complex, recovering the medium, and incubating aliquots of it with one or the other of the peptides in the presence of Rab3A-depleted synaptic membranes. After the incubations we separated the membranes from the medium by centrifugation and measured the contents of Rab3A in the pellet and supernatant fractions. The results demonstrated that each peptide could cause Rab3A to translocate from the medium to the membranes, whereas a control peptide had no effect (Fig. 6).

Relation between the Effects of Ca<sup>2+</sup>/CaM and Those of Rab GDI—Rab GDI can form a 1:1 complex with digeranylgeranylated Rab3A and cause it to dissociate from synaptic membranes (22, 24). To examine the relation between this effect and that of Ca<sup>2+</sup>/CaM, we first sought to determine whether the two proteins bind to similar sites on Rab3A. In one set of experiments we incubated REM with Ca<sup>2+</sup>/CaM to generate a soluble Rab3A-Ca<sup>2+</sup>/CaM complex or used the high speed supernatant from a synaptosomal lysate as a source of Rab3A-Rab GDI. Then we incubated the Rab3A-Ca<sup>2+</sup>/CaM complex for 30 min at 30 °C with Rab GDI or incubated the synaptosomal lysate supernatant under similar conditions with Ca<sup>2+</sup>/CaM. Following the incubations, we treated the incubation mixtures with BS<sup>3</sup> and analyzed the Rab3A-containing material (Fig. 7). The results of these experiments showed that Ca<sup>2+</sup>/CaM and Rab GDI could compete with each other for binding to Rab3A. In a second set of experiments we incubated REM with Rab GDI in the presence of the Rab3A Lys<sup>62</sup>-Arg<sup>85</sup> peptide, the CaM kinase II Leu<sup>209</sup>-Ala<sup>209</sup> peptide, the Rab3A Ala<sup>2</sup>-Asn<sup>18</sup> peptide, or the Rab3A Glu<sup>177</sup>-Asp<sup>195</sup> peptide. Then we recovered the medium and measured the content of Rab3A in the supernatant by immunoblotting. The Rab3A Lys<sup>62</sup>-Arg<sup>85</sup> and CaM kinase II Leu<sup>209</sup>-Ala<sup>209</sup> peptides separately blocked the Rab GDI-dependent dissociation of Rab3A from REM (Fig. 5B); half-maximal values were observed at concentrations of 46 and 41 μM, respectively (not shown). In contrast, the Rab3A Ala<sup>2</sup>-...
CaM and analyzed as described in materials with Ca²⁺/CaM (27). To determine whether guanine nucleotides also control the movement translocation of Rab3A to membranes. A supernatant that was treated the REM with Rab3A-GDI, then was treated with Ca²⁺/CaM or Rab GDI, then measured the amount of Rab3A that dissociated from the REM to the medium (Fig. 10). Only a small response to Ca²⁺/CaM was prepared as described in Fig. 3 but with 90 μM Ca²⁺/CaM. The supernatant was incubated with or without purified Rab GDI (2 μM), treated with BS 3, and analyzed by SDS-PAGE and Western blotting. The open arrowhead indicates the cross-linked, 83-kDa Rab3A-Rab GDI complex. The solid arrowhead indicates the cross-linked, 43-kDa Rab3A-Ca²⁺/CaM complex. This figure is representative of three separate experiments. B, in a parallel experiment, a high speed supernatant fraction was prepared from synaptosomal lysates and then incubated with or without CaCl₂ (final concentration, 200 μM) and CaM (140 μM). The samples were then treated with BS 3 and analyzed as above. This figure is representative of three separate experiments.

REM for 1 h at 37 °C in the absence of added guanine nucleotides, treated the REM with Ca²⁺/CaM or Rab GDI, then measured the amount of Rab3A that dissociated from the REM to the medium (Fig. 10). Only a small response to Ca²⁺/CaM or Rab GDI could be detected (compare preincubated samples with nonpreincubated controls). In contrast, Rab3A dissociated from REM that had been preincubated in the presence of GDP or GTPyS before being treated with Ca²⁺/CaM or Rab GDI, and GDP was more effective than GTPyS. Notice that Rab GDI had a more stringent requirement for GDP than Ca²⁺/CaM did.

The mechanism of these guanine nucleotide-dependent effects remains to be determined. However, the conformation of Ras-GDP is known to differ from that of Ras-GTP (52), and guanine nucleotides are presumed to have similar effects on Rab proteins and other Ras-related proteins. Furthermore, a series of experiments with BS₃-treated REM provided direct evidence that guanine nucleotides alter the conformation of Rab3A (Fig. 11). First, analyses of untreated control REM by SDS-PAGE and Western blotting revealed a single major band of Rab3A that had an apparent molecular mass of about 28 kDa, but analyses of BS₃-treated control REM revealed two major, Rab3A-containing bands that had respective apparent molecular masses of about 28 and 23 kDa (Fig. 11, compare lanes 1 and 3). Second, REM that had been preincubated for 1 h at 37 °C in the absence of guanine nucleotides before being treated with BS₃ contained an increased amount of the 28-kDa band but a reduced amount of the 23-kDa band (Fig. 11, compare lanes 3 and 4). Third, REM that had been preincubated in the presence of GDP or GTPyS before being treated with BS₃ showed a distribution of 23- and 28-kDa bands which resembled that in BS₃-treated control membranes (Fig. 11, compare lanes 3, 5, and 6). These results suggest that treatment with BS₃ can stabilize a guanine nucleotide-dependent conformation of Rab3A that has an increased electrophoretic mobility. Thus, it seems reasonable to postulate that the Rab3A of control REM or REM that have been incubated in the presence of added GDP or GTPyS contains bound guanine nucleotides but that these nucleotides dissociate from Rab3A when REM are incubated in the absence of added GDP or GTPyS or when Rab3A is analyzed by SDS-PAGE (see also Ref. 53). Furthermore, it seems likely that Rab3A that contains bound GDP or GTP has a more compact conformation than guanine nucleotide-free Rab3A.
Fig. 10. The Ca\(^{2+}\)/CaM-induced dissociation of Rab3A from membranes is under the control of guanine nucleotides. REM (15 µg of protein/50 µl of buffer B) were preincubated for 60 min at 37 °C in the absence of added guanine nucleotides or in the presence of 1 mM GDP or 1 mM GTPyS. Then CaM (50 µM) was added (A), and the samples were incubated for 30 min at 30 °C. After incubation, supernatants were prepared and analyzed as described in Fig. 5. Control REM were incubated directly with CaM and CaCl\(_2\), and then the supernatants were prepared and analyzed for comparison. B, in a parallel experiment, Rab GDI (1 µM) was substituted for CaM. Error bars represent the standard errors of mean values from three separate experiments. Note that the GTPyS used contained 4% GDP, which may explain the Rab GDI activity observed in the sample that contained GTPyS.

does and that this compact conformation can be stabilized by BS\(^{3}\)-dependent, intramolecular cross-linking reactions.

**DISCUSSION**

The interaction of Ca\(^{2+}\)/CaM with Rab3A resembles that of Rab GDI with Rab3A in several respects. Both proteins form soluble 1:1 complexes with Rab3A and cause it to dissociate from synaptic membranes. Formation of each of the complexes requires both the lipidated C terminus of Rab3A and the presence of guanine nucleotides. Both Ca\(^{2+}\)/CaM and Rab GDI evidently bind to sites within the Rab3A Lys\(^{62}\)-Arg\(^{85}\) sequence.

The interactions of Ca\(^{2+}\)/CaM and Rab GDI with Rab3A also differ in several respects. Importantly, the interaction of Ca\(^{2+}\)/CaM with Rab3A depends on Ca\(^{2+}\). In addition, half-maximal effects of Ca\(^{2+}\)/CaM on the dissociation of Rab3A from REM or the dissociation of Rab3A from Rab3A-Rab GDI occur at concentrations of Ca\(^{2+}\)/CaM (-20 µM) that are much higher than the concentrations of Rab GDI required to dissociate Rab3A from REM or Rab3A-Ca\(^{2+}\)/CaM (<0.5 µM; data not shown). The Ca\(^{2+}\)/CaM-dependent dissociation of Rab3A from REM is less extensive than the Rab GDI-dependent dissociation of Rab3A. And the Rab3A-dissociating effect of Ca\(^{2+}\)/CaM has a less stringent requirement for GDP than does that of Rab GDI.

The precise mechanism of the Rab3A-dissociating effect of Ca\(^{2+}\)/CaM remains to be determined, but it is of interest that the Rab3A Lys\(^{62}\)-Arg\(^{85}\) peptide contains a cluster of basic amino acids toward its N terminus, while its hydrophobic amino acids are more evenly distributed (Table I). Furthermore, a helical wheel projection of the peptide’s sequence suggested that the clustered, basic amino acids may be located on one side of an amphipathic helix (not shown). The CaM kinase II Leu\(^{290}\)Ala\(^{309}\) peptide has similar characteristics (Table I and Ref. 47); and a recent crystallographic study of its interaction with Ca\(^{2+}\)/CaM has shown that the latter can “wrap around” the peptide to make close contact with its basic and hydrophobic amino acids (48). Ca\(^{2+}\)/CaM may conceivably interact with the Rab3A peptide in the same way. However, Ca\(^{2+}\)/CaM may interact differently with native Rab3A because its binding to the protein appears to require the presence of the modified C terminus. Molecular modeling studies of Rab3A might provide some insight into this issue.

Modeling studies of the GDP-bound form of human Rab5A have suggested that the Rab5A Gln\(^{60}\)-His\(^{83}\) sequence, QTVCLDDTTVKTFEIEDTAFGQHY, which is homologous to the Rab3A Lys\(^{62}\)-Arg\(^{85}\) sequence, may be partially exposed on the protein’s surface (54). The cluster of hydrophilic amino acids toward the N terminus of the Rab5A Gln\(^{60}\)-His\(^{83}\) sequence occupies an exposed position adjacent to loop 3 of the Rab5A molecule, but the hydrophobic amino acids of the sequence are generally more exposed and interact with other amino acids in the protein. If modeling studies of Rab3A suggest that the amino acids of the Lys\(^{62}\)-Arg\(^{85}\) sequence (Table I) occupy similar positions related to the protein’s surface, the possibility that the clustered basic amino acids in the sequence may be available for binding to Ca\(^{2+}\)/CaM would be worth examining.

Experimental tests of the role of individual basic amino acids in the sequence might be done by site-directed mutagenesis. A similar approach has been used to examine the regulatory role of amino acids in the Rab3A Asp\(^{77}\)-Glu\(^{82}\) sequence, which corresponds to the G2 guanine nucleotide-binding region. A Gln\(^{81}\) → Leu mutation altered the k\(_{off}\)(GDP) and k\(_{off}\)(GTPyS) of Rab3A and greatly reduced the ability of Rab3A to respond to Rab3A guanine nucleotide releasing factor (55). In addition, a recent study of a Rab6-\(\alpha\)-Ha-Ras chimera showed that the Rab6 Arg\(^{60}\) Trp\(^{72}\) sequence (RTVRLQLW), which is homologous to the Rab3A Lys\(^{62}\)-Trp\(^{72}\) sequence (KRIKLQW), includes binding sites for Rab GDI and Rab geranylgeranyl transferase (56).

It might also be of interest to examine the effects of site-directed mutagenesis within the corresponding regions of several Rab proteins. Thus, the first portion of the Rab3A Lys\(^{62}\)-Arg\(^{85}\) sequence contains five clustered basic amino acids, but the first portions of the corresponding sequences of Rab1A and Rab5A contain one and three basic amino acids, respectively. Furthermore, these differences may correlate with differences in the dissociating effects of Ca\(^{2+}\)/CaM on the three Rab proteins. In a representative experiment, we incubated REM (from synaptosomes prepared using method 2; see “Experimental Procedures”) for 30 min at 30 °C in the presence of 1 mM GDP and 80 µM Ca\(^{2+}\)/CaM. Measurements by quantitative densitometry revealed that this caused the dissociation of 55% of the membrane-bound Rab3A but only 10% of the Rab1A and 20% of the Rab5A (data not shown). Therefore, mutation experiments designed to alter the number and/or distribution of basic amino acid residues should be informative.
acids within the Lys

Interestingly, the GTPase Rad, which was recently shown to bind Ca

Ca

because its affinity for MARCKS is greatly reduced by the same

content of total CaM in brain may be

significance of the binding of Ca

m

mg/kg (56), and the concentration of Rab GDI in brain may be

nelss in nerve terminals may be as high as 100

m

phorbol ester, comparable to those observed by others for rat

and guinea pig synaptosomes (24, 75). Therefore, the macaque

synaptosomes might provide a useful model for examining the functional significance of the Rab3A-dissociating effect of Ca

2

1

in vivo, what is the significance of the differences between this effect and that of Rab GDI? Does the effect of Ca

2

/CaM complement that of Rab GDI in some unknown way? Do complementary Rab3A-dissociating effects of Ca

2

/CaM and Rab GDI increase the efficiency of synaptic vesicle recycling? These questions may suggest directions for future research.

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