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Regulation of GTP-binding state in RalA through Ca^{2+} and calmodulin

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Abbreviations: SV, synaptic vesicle; CaM, calmodulin; GEP, guanine nucleotide exchange protein; GAP, GTPase activating protein; GDI, guanine nucleotide dissociation inhibitor; GTPγS, guanosine 5-(3-O-thio)triphosphate; DTT, dithiothreitol

Abstract

RalA GTPase, a member of Ras superfamily proteins, shows alternative forms between the active GTP-binding and the inactive GDP-binding states. Ral-specific guanine nucleotide exchange factor such as RalGDS interacts with activated Ras and cooperates with Ras indicating that Ral can be activated through Ras signaling pathway. Another activation path for Ral are through Ca^{2+}-dependent but Ras-independent manner. In this study, studies were carried out to examine possible effects of Ca^{2+} and calmodulin, Ca^{2+}-binding protein, directly on the GTP/GDP-binding state to recombinant unprenylated GST-RalA proteins. The results showed that Ca^{2+} stimulated the binding of GTP to RalA, whereas it reduced the binding of GDP to RalA. However, it does not involve a high affinity association of Ca^{2+} with RalA. Ca^{2+}/calmodulin stimulated the GTPase activity of RalA. These results indicate that Ca^{2+} alone activates RalA by stimulating GTP-binding to RalA and Ca^{2+}/calmodulin inactivates RalA by increasing the activity of RalGTPase.

Keywords: RalA, GTP-binding, Ca^{2+}, calmodulin

Introduction

RalA belongs to Ras superfamily, small GTP-binding proteins. Ras-related small GTP-binding proteins serve as key regulators of cellular functions such as cell growth, differentiation, cytoskeletal reorganization, membrane trafficking, and membrane fusions (Bokoch and Der, 1994). Ral is composed of two highly similar proteins, RalA and RalB. They have been placed in the Ras subfamily. Like all GTPases, Ral proteins cycle between the active GTP bound and inactive GDP bound states (Faig *et al.*, 1996). This cycle is mediated by a specific guanine-nucleotide-exchange factor (Ral-GEF or Ral-GDS), which activates GTPases by promoting the replacement of GDP with GTP, and GTPase-activating proteins (GAPs), which inactivate GTPases by enhancing GTP hydrolysis (Bos, 1998).

Signal pathway through RalA is composed of several downstream effector proteins. The Activated RalA binds to RLIP-1 and this binding requires an intact effector domain of RalA (Jullien-Flores *et al.*, 1995). RLIP-1 also bears a region of homology with GTPase-activating protein (GAP) domains that are involved in the regulation of GTPases of the Rho family. RLIP-1 shows a GAP activity acting upon Rac1 and Cdc42, but not RhoA (Jullien-Flores *et al.*, 1995), indicating that Ral may have a function in regulating the cytoskeleton through its interaction with RLIP1. Subsequently, this RLIP1 binds to POB1, which is tyrosine-phosphorylated in COS cells upon stimulation with EGF and makes a complex with EGF receptor (Ikeda *et al.*, 1998). Ral also binds to phospholipase D (PLD) and thus may play a role in signaling through phospholipids including phosphatidic acid (PA) (Jiang *et al.*, 1995).

In the upstream, RalA is activated by different Ral GEFs, RalGDS, Rgl, and Rlf which bind specifically to the GTP-bound form of several Ras-like GTPases (Spaargaren and Bischoff, 1994; Kikuchi *et al.*, 1994; Wolthuis *et al.*, 1996). Ral activation is a direct downstream effect of growth factor-induced Ras activation (Wolthuis *et al.*, 1998). Introduction of the dominant-negative mutant RalN28 inhibited Ras- and Raf-induced cell transformation (Urano *et al.*, 1996). On the contrary, the inhibition of Ras activation by dominant-negative Ras or pertussis toxin has little effect on Ral-GTP levels (Hofer *et al.*, 1998). Furthermore, Ral is activated by the Ca^{2+} ionophore ionomycin, and activation by lysophosphatidic acid (LPA) or EGF can be blocked by a phospholipase C (PLC) inhibitor. These indicate that Ral can be activated by Ca^{2+}-dependent and Ras-independent pathway (Hofer *et al.*, 1998).

Recent study indicated that RalA is a calmodulin (CaM)-binding protein and RalA may be associated with Ca^{2+}-dependent intracellular signaling pathways (Wang *et al.*, 1997). Ca^{2+}/CaM also affected the dissociation of the RalA from synaptic vesicles (Park *et al.*, 1999). In this study, examination of Ca^{2+} or Ca^{2+}/CaM affects on

both the GDP- or GTP-binding to RalA and the GTPase activity of RalA showed that Ca^{2+} indeed stimulated the binding of GTP and reduced the binding of GDP to RalA. In addition, we observed that Ca^{2+}/CaM stimulated the GTPase activity of RalA.

Materials and Methods

Materials

CaM was purified from bovine brain. Supra-grade $CaCl_2$ was purchased from EM Science. Anti-RalA antibody was purchased from Transduction Laboratories. Anti-Rab3A antibody and peroxidase-conjugated anti-IgG antibodies were purchased from Santa-Cruz. PVDF membrane was purchased from Millipore. GDPβS and $GTP_{\gamma}S$ were from Calbiochem. Bovine serum albumin (BSA) and other reagents were purchased from Sigma.

Preparation of glutathione s-transferase (GST)-fusion proteins

pGEX4T1-Rab3A was constructed using *Eco*RI and *Sal*I restriction enzyme sites, and pGEX4T3-RalA and pGEX2T-RalB were obtained from Dr. S. H. Ryu at Pohang University of Science and Technology in Korea. To purify the proteins, *E. coli* DH5α containing the plasmids was cultured with 0.1 mM isopropylthio-β-D-galactoside (IPTG). Bacteria were disrupted by sonication and GST-Rab3A, GST-RalA, and GST-RalB were purified with glutathione (GSH)-sepharose beads (Pharmacia).

Purification of CaM

CaM was purified from bovine brain with a slight modification of a phenyl-superose column (Gopalakrishna *et al.*, 1982). Bovine brain was homogenized by Warring blender in one volume of 30 mM Hepes pH 7.4, 2 mM EDTA, 2 mM EGTA, 1 mM $CaCl_2$, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 2 μg/ml leupeptin, and followed by Potter Elvehjem homogenization. The homogenized sample was centrifuged at 66,000 *g*, for 30 min at 4°C. Supernatant was adjusted to a $CaCl_2$ concentration of 5 mM, heated at 85°C for 3 min, and cooled on ice. Heat-denatured materials were removed by centrifugation at 12,000 *g* for 30 min at 4°C, and the clear supernatant was loaded on a 50 ml bed volume of phenyl-Sepharose column pre-equilibrated in buffer I (30 mM Hepes pH 7.4, 1 mM $CaCl_2$ and 0.1 mM DTT). After washing with 10 column volumes of the buffer I, a 5 column volumes of buffer II (30 mM Hepes pH 7.5, 200 mM NaCl, 1 mM $CaCl_2$ and 0.1 mM DTT) was applied to the column for subsequent washing. The bound CaM was eluted with buffer III (30 mM Hepes pH 7.4, 2 mM EGTA and 0.1 mM DTT). CaM fractions were pooled together and concentrated to 1.5 ml using ultrafiltration method with PM10 membrane (Amicon). Glycerol was added to a final concentration of 5% and aliquots of CaM were stored at -70°C. Coomassie staining of the SDS-PAGE gel run with purified CaM showed a single band.

Preparation of LP2 membrane fraction from rat brain

Synaptosomes were prepared from rat brain by using the Ficoll gradient method (Huttner *et al.*, 1983; Fischer von Mollard *et al.*, 1991). Crude synaptic vesicle fraction, which contained Rab3A-enriched membranes (LP2 membrane fraction), was prepared through lysis of synaptosomes in hypotonic solution (Park *et al.*, 1997)

Dissociation of small-GTPases from synaptic vesicle membrane by Ca^{2+}/CaM

LP2 membranes (60 μg protein) were preincubated for 10 min at 30°C in 50 μl of buffer (50 mM Hepes pH 7.4, 0.5 mM $MgCl_2$, 1 mM DTT, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1 mM $CaCl_2$) in the presence of 10 mM EDTA, or 0.1 mM GDP, or GTP, and incubated with 40 μg CaM for 30 min at 30°C. The reaction mixtures were centrifuged for 30 min at 100,000 *g*, at 4°C in a Beckman TLA45 rotor. The supernatants and the pellets in 50 μl of the same buffer were run on a SDS-PAGE and the proteins were transferred to PVDF membranes (Park *et al.*, 1997). RalA was measured by Western blot analysis using anti-RalA antibody.

Binding of GDP or GTP and GTPase activity

For the binding of GDP or GTP, RalA was incubated with GDP or GTP in 50 ml of the buffer A (10 mM Hepes pH 7.4, 0.5 μM $MgCl_2$, 1 mM DTT, 1 mM DMPC, and 0.5 mg/ml BSA) at 30°C for 10 min. For the GTPase assay, 50 μl reaction buffer B (10 mM Hepes pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, and 1 mM GTP) was added and incubated for various periods at 30°C (Kikuchi *et al.*, 1988). Termination of GDP or GTP-binding was performed by adding 1 ml of ice-cold buffer C (20 mM Hepes pH 7.4, 100 mM NaCl, 25 mM $MgCl_2$). The stopped reaction mixtures were filtered immediately on a NC45 membrane (Schleicher & Schuell) and the membranes were washed 5 times with 1 ml of cold stop buffer C, and the membranes were dried in air. Radioactivity of dried membranes was measured in 5 ml cocktail (Beckman, Ready Safe) using a liquid scintillation counter (Beckman LS5000TD).

Results

RalA was dissociated from synaptic vesicle membranes by Ca^{2+}/CaM. Excess EGTA inhibited the dissociation of RalA by Ca^{2+}/CaM (Figure 1), suggesting Ca^{2+} is essential for the activation of CaM and only activated CaM can

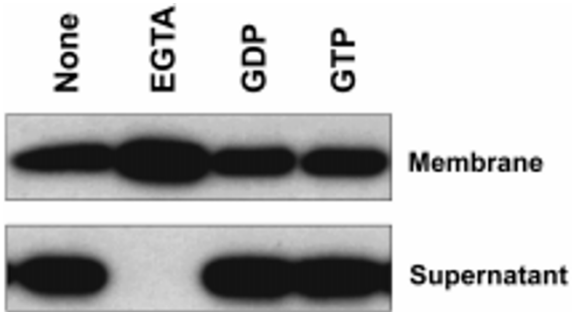

Figure 1. Dissociation of RalA from synaptic vesicle membrane by Ca^{2+}/CaM. LP2 membranes (60 μg protein) were preincubated for 10 min at 30 °C in 50 μl of buffer (50 mM Hepes pH 7.4, 0.5 μM $MgCl_2$, 1 mM DTT, 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 0.1 mM $CaCl_2$) in the presence of 10 mM EDTA, or 0.1 mM GDP, or GTP, and incubated with 40 μg CaM for 30 min at 30°C. The reaction mixtures were centrifuged for 30 min at 100,000 *g* in a Beckman TLA45 rotor. The supernatants were saved and the pellets were resuspended in 50 μl of the same buffer with brief sonication and vortexing. The aliquots of the supernatants and pellets were run on a SDS-PAGE and the proteins were transferred to PVDF membranes. RalA was visualized by Western blot analysis using anti-RalA antibody.

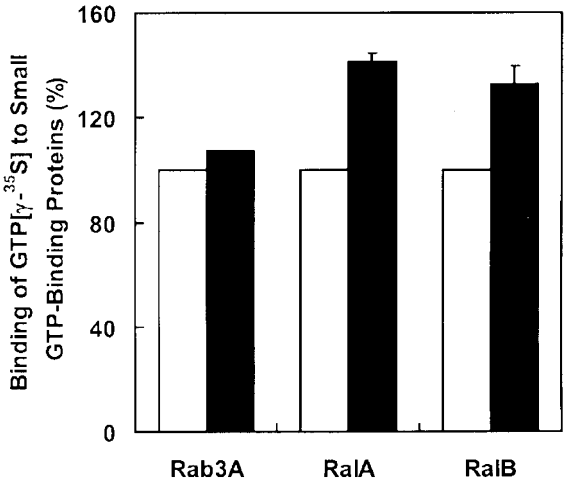

Figure 3. Stimulation of the binding of GTP to RalA and RalB by Ca^{2+}. Rab3A, RalA, and RalB (0.1 μg) was incubated with 0.1 μM [γ-^{35}S]GTP in 50 μl of the buffer A containing 1 mM $CaCl_2$ (□) or none (■) at 30°C for 10 min, respectively. GTP-binding to proteins was terminated by adding 1 ml of ice-cold buffer C. The values are expressed as a mean ± S.E. (n=3).

dissociate RalA from membranes by forming 1 : 1 complex. Although GDP-bound RalA was more readily dissociated than GTP-bound form, the difference was not significant. Ca^{2+}/CaM appear to interact directly with RalA GTPase regardless of GDP or GTP. However, it was not clear whether Ca^{2+} or CaM has an effect on the GDP- or GTP-binding of RalA. In order to examine Ca^{2+} effect on Ra1A *in vitro*, the recombinant GST-RalA was expressed in *E. coli* from pGEX2T-RalA in the presence of IPTG and purified with GSH-sepharose beads. GST-Rab3A, -RalB, -RalBN28 (dominant-negative mutant), and RalB86E (constitutive mutant) were also prepared by the same method. Purified proteins showed a single major band on SDS-PAGE (Figure 2).

Examination of the GDP- or GTP-association with recombinant RalA and RalB in the presence of Ca^{2+} showed stimulation of the [γ-^{35}S]GTP association with RalA and RalB (Figure 3) including to RalB86E (data not shown). However, Ca^{2+} did not stimulate the binding of [γ-^{35}S]GTP to Rab3A. [γ-^{35}S]GTP was rarely bound to dominant-negative mutant RalA, Ral28N both in the presence or absence of Ca^{2+}.

Ca^{2+} stimulated the binding of [γ-^{35}S]GTP to RalA in dose-dependent manner, and 0.1 mM Ca^{2+} increased the GTP-binding to RalA to an extent of about 120% (Figure 4).

In contrast, Ca^{2+} inhibited the binding of [^{3}H]GTP to RalA (Figure 5). High concentration of Mg^{2+} has been known to inhibit the guanine nucleotides to GTP-binding proteins (Hall and Self, 1986), and our results are in

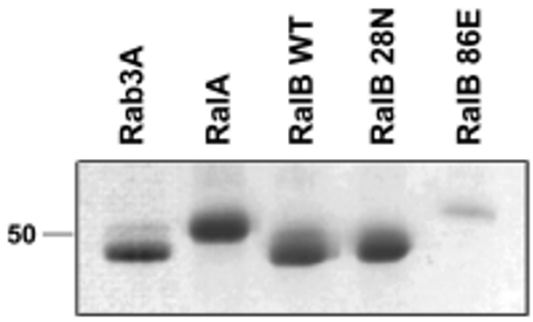

Figure 2. Preparation of glutathione s-transferase (GST)-fusion proteins. pGEX4T1-Rab3A, pGEX4T3-RalA, and pGEX2T-RalB in *E. coli* DH5α were cultured with 0.1 mM IPTG. Bacteria were disrupted by sonication and GST-Rab3A, GST-RalA, and GST-RalB proteins were purified with GSH-Sepharose beads. GST-Rab3A, -RalB, -RalBN28 (dominant-negative mutant), and -RalB86E (constitutive mutant) showed single bands.

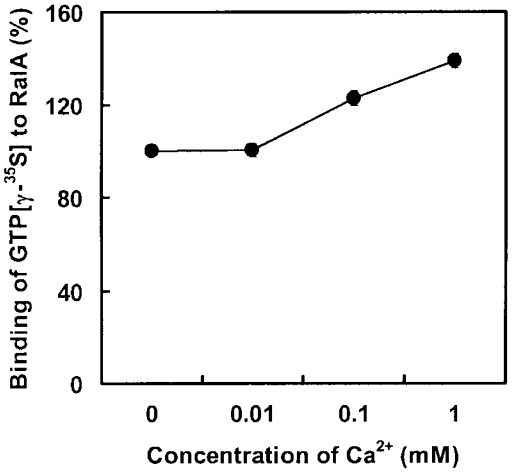

Figure 4. Effect of various concentrations of Ca^{2+} on the binding of GTP to RalA. RalA (0.1 μg) was incubated with 0.1 μM [γ–^{35}S]GTP in 50 μl of the buffer A containing various concentrations of $CaCl_2$ at 30°C for 10 min. GTP-binding to Ral A was terminated by adding 1 ml of ice-cold buffer C. The values are expressed as a mean ± S.E. (n=3). Symbols contain the S.E. values.

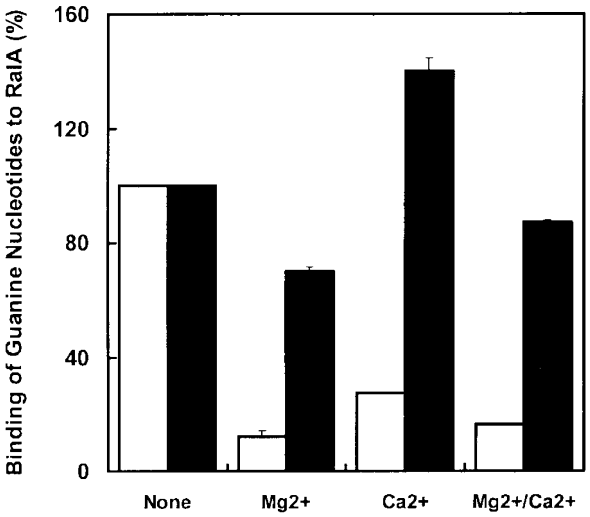

Figure 5. Effects of Ca^{2+} and Mg^{2+} on the binding of GDP or GTP to RalA . RalA (0.1 μg) was incubated with 0.1 μM [γ-^{35}S]GTP (○) or [^{3}H]GDP (●) in 50 μl of the buffer A containing 1 mM $CaCl_2$ or $MgCl_2$ at 30°C for 10 min. GTP-binding to RalA was terminated by adding 1 ml of ice-cold buffer C. The values are expressed as a mean ± S.E. (n=3).

accord to the report (Figure 5). Furthermore, Ca^{2+} could relieve the suppression of Mg^{2+} in the binding of [γ-^{35}S]GTP to RalA. This result indicates that Ca^{2+} may share entire or partial Mg^{2+}-binding site and two ions may compete each other for the binding to RalA protein. The results also raises possiblity that Ca^{2+} binding to RalA could induce enhancement of the RalA affinity for GTP. Such hypothesis was explored by measuring $^{45}Ca^{2+}$-binding to RalA using membrane-filtering method. Although this method did not show an accurate equilibrium association rate of Ca^{2+} with RalA but does indicate the absence of any high affinity binding state (data not shown). Ca^{2+} does not have a high affinity to GTP-binding proteins.

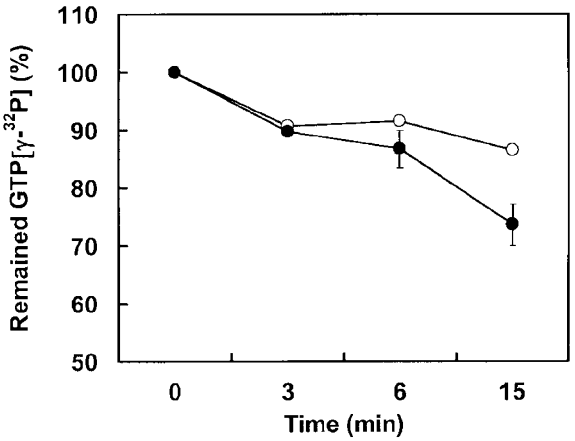

Figure 6. Ca^{2+}/CaM stimulated GTPase activity of RalA. RalA (0.1 μg) was incubated with 0.1 μM GTP[γ-^{32}P] in 50 μl of the buffer A containing 1 mM $CaCl_2$ at 30°C for 10 min in the absence (□) or in the presence (■) of 24 μg of CaM. For the GTPase assay, 50 μl of reaction buffer B was added and incubated for various periods at 30°C. The reaction of RalA GTPase was terminated by adding 1 ml of ice-cold buffer C. The values are expressed as a mean ± S.E. (n=3).

The possibility that Ca^{2+} has an effect on the GTPase of RalA was examined. Ca^{2+} or CaM alone did not change GTPase activity of RalA. However, Ca^{2+}/CaM stimulated the GTPase activity of RalA (Figure 6), although the potency of stimulation was not strong. This result suggests that Ca^{2+}/CaM forms a complex with RalA, and Ca^{2+}/CaM may change the GTPase activity of RalA. However, Ca^{2+}/CaM did not change the GDP- or GTP-binding to RalA.

Discussion

In this study, we showed that Ca^{2+} stimulated GTP-binding to RalA and Ral B and inhibited the GDP-binding to these proteins. These results indicate that Ca^{2+} directly regulates the GTP-binding states of RalA and B. Ca^{2+} concentration is known to be regulated by several factors through several signal pathways. Therefore, another Ca^{2+}-mediated effect can be included where an increased Ca^{2+} can activate RalA and B via enhancing binding of GTP and inhibition of GDP binding to RalA.

The local concentration of Ca^{2+} in nerve terminal ending may be 0.1-0.2 mM. At this concentration, Ca^{2+} significantly increased the GTP-binding to RalA (Figure 4). Except for these biochemical results, it was reported that Ca^{2+} ionophore activates Ral and PLC inhibitor, which decreases the cellular concentration of Ca^{2+}, blocks the activation of Ral induced by LPA or EGF in COS-7 cells (Hofer *et al.*, 1998).

CaM showed the opposite effect on the GTP-binding to RalA. CaM activated by Ca^{2+} slightly decreased the GTP level through activating GTPase of RalA. Consequently, it can be postulated that Ca^{2+} may stimulate the circulating of GTP/GDP turnover in RalA.

Ca^{2+}/CaM stimulation of GTP binding to RalA, purified from human red blood cell plasma membrane (Wang and Roufogalis, 1999) was also reported. Native prenylated RalA binds to CaM in a Ca^{2+}-dependent manner (Wang *et al.*, 1997). A peptide of 18 amino acid residues, with the sequence SKEKNGKKKRKSLAKRIR, was identified as a putative CaM-binding domain in RalA (Wang *et al.*, 1997), but prenyl group on the molecule is likely required for the interaction (Park *et al.*, 1997). RalA has C-terminus that end in CCIL (Kinsella *et al.*, 1991) where prenylation occurs. Native RalA forms a complex with CaM and CaM bound to RalA appears to stimulate the GTP-binding to RalA whereas recombinant non-prenylated RalA may not tightly associate with CaM. Therefore, it can be speculated that Ca^{2+} might activate recombinant unmodified naked RalA by increasing GTP-binding. However, what physiological roles does unprenylated RalA which can be activated by Ca^{2+}, play in the

cells presents very interesting question in regards to functional aspects on the post-translational modification of Ra1A or other related Ras super family proteins.

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