Ca\textsuperscript{2+}/Calmodulin Stimulates GTP Binding to the Ras-related Protein Ral-A*

(Received for publication, March 2, 1999, and in revised form, March 24, 1999)
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Ral-A is a Ras-related GTP-binding protein that has been suggested to be the downstream target of Ras proteins and is involved in the tyrosine kinase-mediated, Ras-dependent activation of phospholipase D. We reported recently that Ral-A purified from human erythrocyte membrane binds to calmodulin in a Ca\textsuperscript{2+}-dependent manner at a calmodulin binding domain identified near its C-terminal region (Wang, K. L., Khan, M. T., and Roufogalis, B. D. (1997) J. Biol. Chem. 272, 16002–16009). In this study we show the enhancement of GTP binding to Ral-A by Ca\textsuperscript{2+}/calmodulin. The stimulation up to 3-fold by calmodulin was Ca\textsuperscript{2+}-dependent, with half-maximum activation occurring at 180 nM calmodulin and 80 nM free Ca\textsuperscript{2+} concentration. The present work supports a regulatory role of Ca\textsuperscript{2+}/calmodulin for the activation of Ral-A and suggests a possible direct link between signal transduction pathways of Ca\textsuperscript{2+}/calmodulin and Ral-A proteins.

Calmodulin is a ubiquitous, highly conserved Ca\textsuperscript{2+} sensor protein that translates the Ca\textsuperscript{2+} signal into a wide variety of cellular processes. In response to an increase in the intracellular concentration of Ca\textsuperscript{2+}, calmodulin undergoes a conformational change that results in binding to its target proteins and acts as a trigger to modify their functions. Calmodulin-binding proteins therefore play an important role in intracellular Ca\textsuperscript{2+} signaling and in various physiological functions, including glycogen metabolism, secretion, muscle contraction, actin/cytoskeletal organization, and cell division (1, 2). Ras proteins are integral to signal transduction of extracellular signals to the nucleus, thus regulating a diverse spectrum of intracellular processes (3, 4). Accumulating evidence indicates the existence of a direct link between signaling pathways of Ca\textsuperscript{2+}/calmodulin and Ras proteins. RasGRF, a neuronal Ras-guanine nucleotide releasing factor, has been shown to be activated in response to Ca\textsuperscript{2+} via direct binding of calmodulin (5). In rat pheochromocytoma PC12 cells, Ca\textsuperscript{2+} influx through voltage-sensitive Ca\textsuperscript{2+} channels induces neurite growth via activation of Rac, which in turn activates the serine/threonine protein kinase, Raf (6, 7). Peppelenbosch et al. (8) have reported that epidermal growth factor-induced Ca\textsuperscript{2+} influx is mediated by Rac proteins. Rapid activation of Rap1 in human platelets has been shown to be mediated by an increased intracellular Ca\textsuperscript{2+} concentration (9). In addition, an increasing number of Ras-related proteins and Ras protein effectors have been identified as calmodulin-binding proteins, supporting the involvement of Ca\textsuperscript{2+}/calmodulin in regulation of Ras-related GTPase function. IQGAP1, a novel RasGAP-related and Ras-binding protein, has been found to bind to calmodulin and the interaction between IQGAP1 and Cdc42 is modulated by Ca\textsuperscript{2+}/calmodulin (10).

Ras proteins represent a distinct family of Ras-related GTP-binding protein. RafGDS was found to interact with ras p21 specifically and to function as an effector target in Ras signaling pathways, inducing cellular transformation in parallel with activation of the Raf/mitogen-activated protein kinase cascade (11–13). Ral-A has been shown to be involved in the tyrosine kinase-mediated activation of phospholipase D, suggesting that the signaling pathway from Ras to Ral leads to the regulation of phospholipid metabolism (14). Raf may also have a functional role in regulating the cytoskeleton through its interaction with the effector protein RalGDI (Ral interacting protein) and Cdc42 (15). In addition, the diverse subcellular localization of Ral-A, not only in plasma membrane, but also in endocytic vesicles, synaptic vesicles (16, 17), and specialized secretory organelles (18), suggests that Raf proteins may be involved in exo- or endocytosis and membrane traffic. However, the physiological activation of Raf by extracellular stimuli is largely unknown.

We have reported that Ral-A, purified from human erythrocyte plasma membrane, can bind to calmodulin in a Ca\textsuperscript{2+}-dependent manner, and its calmodulin binding domain has been identified and characterized near its C-terminal region (19). The Ca\textsuperscript{2+}/calmodulin regulation of Ral-A was strongly supported by the evidence that calmodulin could block Raf-A phosphorylation by PKA, PKG, and PKC and that Ral-A was phosphorylated by CaM kinase II in vitro. In this study we report the effect of Ca\textsuperscript{2+}/calmodulin on GTP binding activity of Ral-A. Up to 3-fold Ca\textsuperscript{2+}/calmodulin-dependent stimulation of GTP binding to Ral-A was observed. The results raise the possibility that calmodulin may be a potential effector for Ral-A GTPase activation and serve as a molecular switch in response to changes of intracellular Ca\textsuperscript{2+} concentration over the physiological range.

**Experimental Procedures**

Materials—Packed red blood cells were obtained from the Red Cross blood bank, Sydney (New South Wales, Australia). [\textsuperscript{32}P]GTP (3000 Ci/mmol) was purchased from NEN Life Science Products. Calmodulin was obtained from Calbiochem. Calmodulin-agarose was from Sigma. Mag-Fura-2 tetratopassium salt was obtained from Molecular Probes (Eugene, OR). Sephadex G-25 was purchased from Pharmacia Biotech (Uppsala, Sweden). Spin columns were obtained from Promega (Madison, WI). Other materials and chemicals were the highest grade available from commercial sources.

Protein Purification—Ral-A protein was purified from human red blood cell plasma membrane as described previously (19). Briefly, calmodulin-depleted human plasma membranes were prepared according to the protocol of Roufogalis et al. (20).

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* This work was supported by the National Health and Medical Research Council of Australia. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Printed in U.S.A.
to the method of Wang et al. (20). The membrane solute in 200 mM KCl, 1 mM MgCl₂, 200 μM CaCl₂, 20 mM Tris-HCl, pH 7.4, 0.55% (w/v) Triton X-100, and 20% (v/v) glycerol was applied to a calmodulin agarose column and the column was washed extensively with washing buffer (200 mM KCl, 1 mM MgCl₂, 200 μM CaCl₂, 20 mM Tris-HCl, pH 7.4, 0.1% (w/v) Triton X-100, and 20% (v/v) glycerol) until no protein was detected in the wash. The protein was eluted from the column by a gradient of increasing concentrations of EDTA from 2 to 5 mM in the elution buffer (200 mM KCl, 20 mM Tris-HCl, pH 7.4, 0.1% (w/v) Triton X-100, and 20% (v/v) glycerol). The fractions were analyzed by 6–14% SDS-polyacrylamide gel electrophoresis and stored at -80 °C.

**Fluorescence Concentration Measurement**—The fractions containing purified Ral-A were pooled. To determine the precise concentration of EDTA in the protein sample, excess CaCl₂ was added to the sample and Mag-Fura-2, a Ca²⁺-sensitive fluorescence probe, was used to measure the free Ca²⁺ available. This method is based on the fact that Mag-Fura-2 displays shifts in its excitation spectra upon calcium binding and the ratio of the fluorescence intensities at the characteristic maxima for completely bound and unbound forms is indicative of the concentration of Ca²⁺ present (21). The fluorescence ratio measurements of Mag-Fura-2 ([I₅₀₀]/[I₃₆₅]) in the sample buffer were conducted using a Luminescence Spectrometer (Perkin-Elmer model LS 50B). Excitation was measured at 331 nm when Mag-Fura-2 was completely bound to calcium, and 385 nm when Mag-Fura-2 was unbound to calcium, with emission at 510 nm. Ca²⁺ concentration was calculated from the ratio ([I₅₀₀]/[I₃₆₅]) calibration data using the program in the “Intracellular Biochemistry Application” (Perkin-Elmer model LS 50B). EDTA concentration in the protein sample was then obtained by subtracting the measured free Ca²⁺ concentration from the total added CaCl₂ concentration. When the Ca²⁺ dose-response curve for calmodulin stimulation of Ral-A-GTP binding activity was performed, various external CaCl₂ concentrations were added to yield the free Ca²⁺ concentrations required, as calculated by a computer program from Fabiato et al. (22).

This program calculates the free ionic concentrations resulting from specified total concentrations of cations (Ca²⁺ and Mg²⁺) and ligands (EDTA) that have been used in the assay.

**Guanine Nucleotide Binding**—GTP binding activity of Ral-A was determined using a spin column filtration assay. Approximately 1.2 ml of Sephadex G-25 filtration matrix pre-equilibrated in 200 mM KCl and 20 mM Tris-HCl, pH 7.4, was added to individual spin columns. The assembly was then centrifuged at 800 × g in a Jouan CR 4–11 centrifuge (St-Nazaire Cedex, France) with a E4 swinging bucket rotor for 5 min. A second centrifugation is necessary to remove remaining buffer. 200 μl of Ral-A preparation (about 20 pmol) was incubated for the required time at 37 °C with 1 μCi of [α-³²P]GTP (3000 Ci/mmol) in the assay buffer containing 200 mM KCl, 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and required CaCl₂ in the absence and presence of calmodulin. Controls were obtained by omission of Ral-A in the assay mixture. Aliquots of 60 μl were withdrawn in triplicate and directly applied to the center of the spin column matrix. The assembly was subjected to centrifugation at 800 × g for 5 min at 4 °C. Unincorporated free [α-³²P]GTP remained in the matrix and is effectively removed from [α-³²P]GTP-bound Ral-A. 5 ml of scintillation fluid was added to the flow-through collected from the spin columns, and the radioactivity of [α-³²P]GTP-bound protein was measured by scintillation counting in a liquid scintillation analyzer (1900A, TRI-CARB, Packard Pty. Ltd.). The [α-³²P]GTP binding activity of Ral-A was determined as the radioactivity of the sample minus the radioactivity of the relevant control and expressed as nanomoles of [α-³²P]GTP/milligrams of protein.

**RESULTS AND DISCUSSION**

Since Ral-A, purified from human erythrocyte membrane, has been described as a Ca²⁺/calmodulin-binding protein (19), we investigated a possible role of Ca²⁺/calmodulin to modulate the GTP binding of Ral-A. To test this hypothesis [α-³²P]GTP binding activity of Ral-A was determined using a spin column filtration assay in the absence and presence of calmodulin. Although a nitrocellulose filtration assay is commonly used to measure [α-³²P]GTP binding activity of Ras-related GTPases, this method is displaced by addition of increasing amounts of [α-³²P]GTP. Fig. 1 shows that Ca²⁺/calmodulin enhances the [α-³²P]GTP binding over the concentration range examined. Based on Fig. 1B, the [α-³²P]GTP binding stoichiometry of Ral-A is calculated to be 0.11 mol of GTP-bound per mol of Ral-A and 0.2 mol of GTP-bound per mol of calmodulin in the absence and presence of Ca²⁺/CaM, respectively. However, the stoichiometry could be underestimated in the current experimental conditions, since Fig. 2 shows the [α-³²P]GTP binding of the omission of Ca²⁺ completely abolished the stimulatory effect of calmodulin. Ca²⁺ alone does not affect GTP binding to Ral-A, indicating that GTP binding of Ral-A is not directly activated by Ca²⁺, but rather requires calmodulin in its Ca²⁺-bound form. Fig. 1B shows that the binding of GTP to Ral-A in the presence and absence of Ca²⁺/calmodulin is time-dependent and that Ca²⁺/calmodulin stimulates the initial rate of GTP binding.
Ral-A has not reached saturation over the \([\alpha-\text{32P}]\text{GTP}\) concentration range examined. Nevertheless, accumulating evidence shows that only a small amount of Ral, in the order of 10\%, is in the active GTP-bound form in intact cells (23, 24). Based on these observations we suggest that most of the Ral-A purified from human erythrocyte may be in its inactive GDP-bound form and Ca\(^{2+}\)-dependent calmodulin binding to Ral-A may accelerate the displacement of the bound GDP, thereby promoting GTP binding. The displacement of GDP may be incomplete under the conditions used.

To further assess the influence of Ca\(^{2+}\) on calmodulin stimulation of GTP binding, a Ca\(^{2+}\) dose-response curve was carried out. Since Ral-A was purified by elution from a calmodulin agarose column by a gradient EDTA elution buffer, the EDTA concentration in Ral-A was required for the accurate determination of free Ca\(^{2+}\) and was determined as described under “Experimental Procedures.” A computer program (22) was then used to calculate the free Ca\(^{2+}\) concentration in the final assay buffer when EDTA, Ca\(^{2+}\), and Mg\(^{2+}\) were present. Fig. 3A shows the -fold calmodulin stimulation of Ral-A GTP binding activity as a function of increasing concentration of free Ca\(^{2+}\) in the presence of 240 nM calmodulin. The data in Fig. 3 shows -fold stimulation at 1.1 \mu M CaCl\(_2\) as a function of increasing concentrations of free Ca\(^{2+}\). B shows -fold stimulation at 240 nM calmodulin as a function of increasing concentrations of calmodulin. The data in A and B was fit to the Hill equation \(B = B_{\text{max}}[C]/(K_\gamma + [C])\), where \(K_\gamma\) for free Ca\(^{2+}\) is 80.5 nM, with a correlation coefficient of 0.992. Inset, -fold activation of \([\alpha-\text{32P}]\text{GTP}\) binding as a function of concentrations of CaCl\(_2\) or calmodulin on a logarithmic scale.

Fig. 2. Dependence of \([\alpha-\text{32P}]\text{GTP}\) binding to Ral-A on GTP concentrations. GTP binding activity of Ral-A was determined using a spin column filtration assay as described under “Experimental Procedures.” 200 \mu l of Ral-A preparation (about 20 pmol) was incubated with 0.5 \mu M GTP and various concentrations of \([\alpha-\text{32P}]\text{GTP}\) (3000 Ci/mmol) in the absence and presence of CaCl\(_2\) and calmodulin at 37 °C for 90 min. Aliquots of 60 \mu l were withdrawn in triplicate and directly applied to the spin column matrix. The flow-through was collected from the spin columns, and \([\alpha-\text{32P}]\text{GTP}\) binding to Ral-A was measured by scintillation counting. Results are expressed as \([\alpha-\text{32P}]\text{GTP}\) bound protein scintillation counts (counts/min) ± S.D. ○ absence of Ca\(^{2+}\)/calmodulin; ● presence of 360 nM calmodulin and final free Ca\(^{2+}\) of 1.1 \mu M.

It is known that guanine nucleotide exchange factors, such as RalGAP, RalGDS, and RalGDI, are responsible for direct interaction with and regulation of the “on” and “off” status of Ral protein through the conformational transitions induced by the cycle of GDP/GTP exchange and GTP hydrolysis. Although Hinoi et al. (28) have demonstrated that post-translational modification of Ral enhances the action of RalGDS to stimulate GDP/GTP exchange on Ral, the modes of activation and action of RalGDS or RalGAP have not yet been clarified. The Ca\(^{2+}\)/calmodulin effect on GTP binding activity of Ral-A differs from that of Ral-A regulatory factors. The maximum stimulation of GTP binding by Ca\(^{2+}\)/calmodulin of about 3-fold is lower than the enhanced proportion of Ral-A GDP-bound form induced by RalGDS in COS-7 cells (11). RalGAP partially purified from human platelet cytosol also stimulates the intrinsic GTPase activity of Ral-A by at least 6-fold (29). These results indicate that activation by Ca\(^{2+}\)/calmodulin may be less effective than those of Ral-A regulatory factors and thus it might act in conjunction with regulatory factors in vivo.
The sensitivity of GTP binding of Ca$^{2+}$ (half-maximal activation at 80 nM) is within the range of the increase in Ca$^{2+}$ from a resting level of about 20 nM to levels of 1 μM or more in cells responding to incoming signals. Calmodulin, acting as a Ca$^{2+}$-sensor protein, is present in large excess in cells, although the free concentration of Ca$^{2+}$-bound calmodulin is limiting and essentially all the Ca$^{2+}$-bound calmodulin present in the cell must be bound to targets (30). The very low physiological levels of free Ca$^{2+}$-bound calmodulin indicates that small changes in the affinity of a typical target could significantly affect the level of its activity at a submaximal intracellular Ca$^{2+}$, as demonstrated recently for smooth muscle myosin light chain kinase activity (31). Recent evidence supports the notion that the sensitivity of Ral-A to Ca$^{2+}$ has potential physiological significance. Wolthuis et al. (24) reported that an elevation of intracellular Ca$^{2+}$ through Ca$^{2+}$/influx stimulated by thapsigargin, induced a rapid activation of Ral in platelets. In addition, Hofer et al. (32) found that endogenous levels of activated GTP-bound Ral (Ral-GTP) was increased by treatment with the Ca$^{2+}$-ionophore ionomycin in Rat fibroblasts. However, the Ca$^{2+}$ sensitivity of Ral activation has not yet been reported in these papers. Although the precise mechanism of Ral activation by Ca$^{2+}$ is unknown, it is speculated that Ca$^{2+}$-dependent calmodulin binding to Ral may have a direct effect on Ral activation (24).

Recently, a growing number of calmodulin binding proteins have been identified among Ras-related GTPases, including members of the Raf family of GTPases (including Rad, Gem, and Kir) and Rin family of GTPases (including Rin, Rit, and Ric) (33–36). However, [3H]GTP or [3H]GDP binding activity of GST/Rad was not affected by calmodulin, neither was its GTP hydrolysis activity (34), and calmodulin inhibits GTP binding to GST/Kir and GST/Gem (33). Furthermore, the effects of calmodulin on Rad, Kir, and Gem were seen in the absence of Ca$^{2+}$ (33, 34). Park et al. (37) have reported that the interaction of Ca$^{2+}$/calmodulin with Rab3A induces its dissociation from synaptic membranes. The same effect can be achieved by Rab-GDI (37), but compared with Ca$^{2+}$/calmodulin with less effect and a less stringent requirement for GDP than that of RabGDI (37). Similarly to this finding, our work has shown that the stimulatory effect of calmodulin on Ral-A GTP binding is Ca$^{2+}$-dependent and appears to be less effective than that of Ras protein regulatory effectors. While Ca$^{2+}$/Calmodulin and GDP/GTP exchange factors of this Ras protein may occur by a different mechanism and possibly in conjunction with other Ras effectors. While Ca$^{2+}$/CaM enhances the initial rate and apparent sensitivity of GTP binding to Ral-A, it needs to be determined whether this occurs via enhancement of GDP/GTP exchange, by increases in the number and/or affinity of GDP binding sites, or by displacement by calmodulin of an autoinhibitory domain from the active site (38). This work shows a direct functional regulation of Ral-A by Ca$^{2+}$/Calmod-