Inhibition of G Protein-coupled Receptor Kinase Subtypes by Ca\(^{2+}\)/Calmodulin*

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G protein-coupled receptor kinases (GRKs) are implicated in the homologous desensitization of G protein-coupled receptors. Six GRK subtypes have so far been identified, named GRK1 to GRK6. The functional state of the GRKs can be actively regulated in different ways. In particular, it was found that retinal rhodopsin kinase (GRK1), but not the ubiquitous βARK1 (GRK2), can be inhibited by the photoreceptor-specific Ca\(^{2+}\)-binding protein recoverin through direct binding. The present study was aimed to investigate regulation of other GRKs by alternative Ca\(^{2+}\)-binding proteins such as calmodulin (CaM). We found that Gβγ-activated GRK2 and GRK3 were inhibited by CaM to similar extents (IC\(_{50}\) = 2 μM), while a 50-fold more potent inhibitory effect was observed on GRK5 (IC\(_{50}\) = 40 nM). Inhibition by CaM was strictly dependent on Ca\(^{2+}\) and was prevented by the CaM inhibitor CaM-Bd. Since Gβγ, which is a binding target of Ca\(^{2+}\)/CaM, is critical for the activation of GRK2 and GRK3, it provides a possible site of interaction between these proteins. However, since GRK5 is Gβγ-independent, an alternative mechanism is conceivable. A direct interaction between GRK5 and Ca\(^{2+}\)/CaM was revealed using CaM-conjugated Sepharose 4B. This binding does not influence the catalytic activity as demonstrated using the soluble GRK substrate casein. Instead, Ca\(^{2+}\)/CaM significantly reduced GRK5 binding to the membrane. The mechanism of GRK5 inhibition appeared to be through direct binding to Ca\(^{2+}\)/CaM, resulting in inhibition of membrane association and hence receptor phosphorylation. The present study provides the first evidence for a regulatory effect of Ca\(^{2+}\)/CaM on some GRK subtypes, thus expanding the range of different mechanisms regulating the functional states of these kinases.

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1 The abbreviations used are: GRK, G protein-coupled receptor kinase; CaM, calmodulin; CaM-Bd, calmodulin-inhibiting peptide; Gβγ, βγ subunits of heterotrimeric G protein; NCS, neuronal calcium sensors; ROS, rod outer segment(s); TSH, thyroid-stimulating hormone; PAGE, polyacrylamide gel electrophoresis.

2 According to the order of their discovery (1). Of these six subtypes, rhodopsin kinase corresponds to GRK1, βARK1 to GRK2, and βARK2 to GRK3. Based on sequence homology, these six GRK subtypes are classified into three subgroups (1): GRK1 is alone in the first group, GRK2 and GRK3 form the second group, while GRK4, -5, and -6 constitute the third subgroup. With the exception of GRK1 and GRK4, which are specifically localized in retina and pineal gland (GRK1) and testis (GRK4), the other four subtypes are ubiquitous.

In homologous desensitization of G protein-coupled receptors, the binding of an agonist to its receptor induces a complexity of events to result in phosphorylation of the agonist-bound receptor by GRK. The phosphorylated receptor would then display increased affinity for an arrestin protein (2), the binding of which to the phosphorylated receptor prevents any further coupling between the receptor and G proteins, and thus the receptor is rendered desensitized (inactivated) (1, 2). A critical determinant factor for efficient receptor phosphorylation by GRK is the colocalization of the kinase and its receptor substrate on the plasma membrane. The mechanisms involved have been extensively studied. For GRK1, receptor activation causes farnesylation of the C-terminal tail of the kinase, facilitating its localization to the membrane (3, 4). For GRK2 and GRK3, which are highly homologous, membrane localization is shown to be mediated by binding to the βγ subunits of heterotrimeric G proteins (Gβγ) via their pleckstrin homology domains (5, 6). Palmitoylation appears to be the mechanism for GRK4 and GRK6 (7, 8), while electrostatic bonds between the basic C-terminal domain of the kinase and acidic phospholipids in the membrane are hypothesized to mediate membrane localization of GRK5 (1, 9).

The functional state of the GRK/arrestin machinery can be actively regulated via intracellular messenger pathways in different ways. These include changes in kinase activity through covalent modification (10) and/or modifications in their expression levels (11, 12). Such alterations result in modified potency of receptor desensitization and subsequently changes in receptor-mediated functions, as exemplified by the effects of GRK2 overexpression or inhibition on myocardial function in transgenic mice (13). More recently, permanent overexpression of β-arrestin 1 in FRTL5 cells has been shown to inhibit the mitogenic activity of thyroid-stimulating hormone (TSH) (14). In some settings regulation of GRK can also be mediated by Ca\(^{2+}\)-binding proteins. It has been recently demonstrated that in the presence of Ca\(^{2+}\), GRK1 is inhibited by the photoreceptor-specific recoverin through direct binding (15, 16). Recoverin is a member of the family of neuron-specific proteins named neuronal calcium sensors (NCS); several other members of this family are also able to inhibit GRK1 in a calcium-dependent manner (17). Inhibition by recoverin does not appear to be a general phenomenon for the GRK family since it has no inhibitory activity on GRK2 (15). However, regulation of other GRK
subgroups by alternative Ca\(^{2+}\)-binding proteins cannot be discounted. In particular, the ubiquitous calcium-binding protein calmodulin (CaM) binds G\(\beta\gamma\) in a Ca\(^{2+}\)-dependent manner (18, 19) and can therefore be envisaged to compete with GRK2 and GRK3 for G\(\beta\gamma\), resulting in inhibition of their activity.

As an extension to our work on intracellular regulation of GRK (10–12), we conducted the present study to test this hypothesis. It was observed that while Ca\(^{2+}\)/CaM did inhibit GRK2 and GRK3, it demonstrated a dramatically greater potency in inhibiting GRK5 through direct binding to the kinase.

**EXPERIMENTAL PROCEDURES**

**Overexpression and Purification of GRK Subtypes**—Baculovirus constructs for human GRK2 and bovine GRK5 were gifts of Drs. H. H. LeVine and R. J. Lefkowitz, respectively. Baculovirus for GRK3 was produced using the Bac-to-Bac baculovirus expression system. Sf9 cells cultured in TNM-FH insect medium were infected at a multiplicity of infection of 3. After 60 h of infection, cells were harvested, cytosolic preparation was obtained, and GRKs were purified by sequential S-Sepharose and heparin-Sepharose column chromatography (20, 21).

**Rod Outer Segment (ROS) Preparation and Phosphorylation Assays**—These were carried out essentially as described (22). Briefly, ROS containing >90% pure rhodopsin were isolated from bovine retina by stepwise sucrose gradient sedimentation, and endogenous rhodopsin kinase was denatured by treatment with 5 M urea. The ROS phosphorylation mixture contains 50 \(\mu\)M [\(\gamma\)-\(^{32}\)P]ATP, 20 mM Tris, 8 mM MgCl\(_2\), 3 mM EDTA, 5 mM NaF, 12 mM NaCl at pH 7.4. Concentration of rhodopsin varied according to the requirements. For GRK1 and GRK5, 2 \(\mu\)g was used. For GRK2 and GRK3, 0.6 \(\mu\)g was used so that no phosphorylation could be observed without adding exogenous G\(\beta\gamma\) (100 nM unless otherwise stated) into the reaction mixture. G\(\beta\gamma\) purified from bovine brain were provided by Dr. H. LeVine (23). Unless otherwise stated, GRK concentration was 5 nM. Reaction was carried out for 30 min at 30 °C in the presence of light, stopped with SDS/sample buffer, and electrophoresed on 10% SDS-PAGE followed by autoradiography. Phosphorylation was quantified by InstantImager (Packard). In phosphorylation reactions in which casein instead of ROS was used as the substrate, 20 \(\mu\)g of casein was used per reaction for 1 h at 30 °C.

**Immunoblotting**—Protein samples in SDS sample buffer were separated by SDS-PAGE on 10% polyacrylamide gels. The proteins were transferred onto nitrocellulose paper, and blots were incubated with suitable antibodies, followed by alkaline phosphatase-conjugated goat anti-rabbit antibodies, and developed by the addition of the alkaline phosphatase substrate BCIP and NBT, according to the manufacturer’s instructions. Quantitative analysis of the immunoreactive bands was conducted using an LKB Ultrascan XL Enhanced Laser Densitometer.

**CaM-Sepharose 4B Binding Assay**—CaM-Sepharose 4B or unconjugated Sepharose 4B gel was washed twice in 500 \(\mu\)l of water and equilibrated in binding buffer (20 mM Tris/HCl, pH 7.5, 1 mM dithiothreitol, 100 mM NaCl, 0.1% Lubrol). The gel was pelleted each time by centrifugation for a few seconds with bench top Microfuge. Each equilibrated gel pellet (60 \(\mu\)l) was resuspended in binding buffer containing various components in a total volume of 700 \(\mu\)l. In appropriate samples, GRK2, GRK5, or G\(\beta\gamma\) was present at 200, 100, or 30 nM and Ca\(^{2+}\) at 1 mM as suggested by the manufacturers of CaM-Sepharose 4B. Samples were incubated for 1 h on a rotator at 4 °C, and then the unbound materials were removed by repeating twice centrifugation and washes with 1 ml of ice-cold binding buffer. To the final gel pellet was added SDS sample buffer, heated to 95 °C to detach gel-bound materials, followed by electrophoresis on 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose paper and immunoblotted with appropriate antibodies as described above.

**Translocation Assay**—GRK5 (440 nM) was mixed in reaction buffer on ice with CaM (5 \(\mu\)M) in the absence or presence of Ca\(^{2+}\) (1 mM), followed by the addition of 6 \(\mu\)M ROS and 50 \(\mu\)M ATP to initiate the processes of translocation and phosphorylation. Incubation was for 30 min under room light at 30 °C. The samples were then centrifuged to separate the ROS membrane from the soluble fraction (48,000 \(\times\) g, 30 min, 4 °C). SDS sample buffer was added to the ROS pellets and analyzed by 10% SDS-polyacrylamide gel electrophoresis, followed by Western and immunoblotting as described above. Approximately 20% of the total GRK5 bound to the membrane in the control samples, as revealed by densitometric analysis.

**Materials**—TNM-FH insect cell medium was from Sigma; Bac-to-Bac baculovirus expression system from Life Technologies, Inc.; CaM was from Pierce; antibodies for GRK5 and pan-\(\beta\) antibodies that recognize \(\beta1-\beta4\) subtypes of the heterotrimeric G protein were from Santa Cruz Biotechnology; 5-bromo-4-chloro-3-indolylphosphate, p-toluidine salt, and nitroblue tetrazolium chloride were from Life Technologies, Inc.; cyanogen bromide-activated Sepharose 4B was from Fluka; CaM-Bd was obtained from Calbiochem; casein and all other materials were from Sigma.

**RESULTS**

To test the possibility that CaM may inhibit GRK2 activity by virtue of its ability to bind G\(\beta\gamma\) in a Ca\(^{2+}\)-dependent manner, the effect of CaM on GRK2 activity was examined in a G\(\beta\gamma\)-dependent ROS phosphorylation assay (see “Experimental Procedures”). In the absence of Ca\(^{2+}\), CaM at 1 and 20 \(\mu\)M had little or no effect on GRK2, but when Ca\(^{2+}\) (1 mM) was present, the same concentrations of CaM resulted in pronounced inhibition of the G\(\beta\gamma\)-mediated GRK2 activity (Fig. 1A). The IC\(_{50}\) value for this inhibitory action was approximately 2 \(\mu\)M. This Ca\(^{2+}\)-dependent inhibition was proven to be CaM-dependent since it was completely reversed by the CaM-inhibiting peptide (CaM-Bd) (24) derived from the CaM-dependent protein kinase II (Fig. 1B).

Of the six GRKs, G\(\beta\gamma\) has been shown to bind and activate only GRK2 and GRK3 through binding to their respective pleckstrin homology domains (5, 6). In an attempt to verify that this Ca\(^{2+}\)/CaM inhibitory effect was dependent on its G\(\beta\gamma\) binding activity, experiments were conducted to compare the effects of Ca\(^{2+}\)/CaM on GRK2 and GRK3, which are dependent on G\(\beta\gamma\), with those on GRK1 and GRK5, which are G\(\beta\gamma\)-insensitive. In the presence of 400 nM CaM plus Ca\(^{2+}\), both GRK2 and GRK3 were inhibited to similar extents, GRK1 was unaffected, but surprisingly GRK5 was dramatically inhibited (Fig. 2). Ca\(^{2+}\) alone had no effect on any of these four GRKs. Fig. 3A
shows the dose-response effects of Ca^{2+}/CaM on these four GRKs. Since a near maximal inhibition of GRK5 was achieved with 400 nM CaM, a detailed dose response of lower doses of CaM was performed (Fig. 3B). The inhibitory activity of CaM on GRK5 was ~50-fold more potent than on GRK2, with an IC_{50} value of ~40 nM. The effective concentration of CaM on GRK5 lies in the low range of CaM concentrations that have been shown to regulate target proteins (25), and it is more potent than the inhibitory effect of Ca^{2+}/recoverin on GRK1 (15, 16). This is suggestive of a highly relevant interaction and had therefore channeled our interest into further experiments on this phenomenon.

As for GRK2, the inhibition of GRK5 by Ca^{2+}/CaM was Ca^{2+}-dependent (Fig. 4A) and was almost completely reverted by the CaM-inhibiting peptide CaMBd (Fig. 4B). These results confirmed that the inhibitory effect was mediated by CaM. The inhibition was effectively reverted by increasing the concentration of GRK5 in the phosphorylation reaction by 40-fold (Fig. 5). One mechanism by which Ca^{2+}/CaM could inhibit GRK5 was by direct binding to GRK5, as in the case of GRK1 inhibition by Ca^{2+}/recoverin (15). To address this possibility, in vitro binding assays were performed using CaM-conjugated Sepharose 4B gel and unconjugated Sepharose 4B as negative control (Fig. 6). G\betaγ was used as a positive control for the binding assay since it has previously been shown to bind CaM-Sepharose 4B in a Ca^{2+}-dependent manner (15, 16). Both GRK5 and G\betaγ bound to CaM-Sepharose 4B in a Ca^{2+}-dependent manner, though trace amounts of both also bound in the absence of Ca^{2+}. Densitometric analysis showed that the -fold excess of CaM-Sepharose-bound protein/control-Sepharose-bound protein was 5.8 and 4.3 for GRK5 and G\betaγ, respectively (n = 2). Neither of them bound to the unconjugated Sepharose 4B gel. Similar results were obtained when the molarity ratio between CaM and the ligand was varied by 3-fold (CaM, ligand = 60 or 180:1). These binding assays document a direct, Ca^{2+}-dependent interaction between GRK5 and CaM. Unlike GRK5, GRK2 (30, 100, and 200 nM) did not bind to CaM-conjugated Sepharose 4B gel (data not shown), supporting the specificity of this interaction.

Similar to other kinases, GRK5 contains catalytic and regulatory domains. Binding of Ca^{2+}/CaM to GRK5 may result in
inhibition of either of these domains. To distinguish between these two possibilities, the phosphorylation assays were repeated in a membrane-free system using the soluble protein casein instead of ROS as substrate (26). The results shown in Fig. 7 demonstrate clearly the lack of any inhibitory effect on casein phosphorylation by GRK5. This suggested that the effect of Ca<sup>2+</sup>/CaM would most likely be exerted on non-catalytic domains, which have regulatory roles.

Since membrane localization is a prerequisite for efficient receptor phosphorylation by all GRKs, the effect of Ca<sup>2+</sup>/CaM on the binding of GRK5 to ROS membranes was examined using a centrifugation assay. GRK5 and CaM ± Ca<sup>2+</sup> were incubated together prior to the initiation of phosphorylation reaction by the addition of ROS and ATP. At the end of the reaction, ROS membrane fraction was separated from the soluble fraction by centrifugation, the quantities of membrane-bound GRK5 were analyzed by Western blotting, and rhodopsin phosphorylation was revealed by autoradiography. In the absence of Ca<sup>2+</sup>, the quantity of GRK5 bound to the membrane was unaffected by CaM. When GRK5 was exposed to CaM in the presence of Ca<sup>2+</sup>, a dramatic reduction in GRK5 translocation onto the membrane was observed (Fig. 8). Densitometric analysis showed a 10-fold lower amount of GRK5 bound to the membrane when in the presence of Ca<sup>2+</sup>/CaM. This Ca<sup>2+</sup>-dependent inhibition of GRK5 translocation was paralleled by the effects of CaM on rhodopsin phosphorylation activity by the same samples (Fig. 8).

**DISCUSSION**

The present study demonstrates for the first time Ca<sup>2+</sup>-dependent inhibitory effects of CaM on some members of the GRK family. The Gβγ-activated GRK2 and GRK3 were inhibited to similar extents, while a 50-fold more potent inhibitory effect was observed on GRK5. The mechanism of GRK5 inhibition appeared to be through direct binding to Ca<sup>2+</sup>/CaM, resulting in inhibition of membrane association and hence receptor phosphorylation.

CaM is an acidic protein that is considered the primary “decoder” of Ca<sup>2+</sup> information in the cell (25, 27), exerting many of its functions when bound to Ca<sup>2+</sup> (4 Ca<sup>2+</sup> ions per CaM molecule). Numerous proteins have been identified to be regulated by Ca<sup>2+</sup>/CaM, e.g. kinases, phosphodiesterases, calcium pumps, and adenylate cyclase. Gβγ is also a binding target of Ca<sup>2+</sup>/CaM, as demonstrated previously (18, 19) and confirmed in the present study. More recently, it has been established that the binding of Gβγ2 and Gβγ3 to Gβγ is critical in mediating the activation of these two GRK subtypes (5, 6). Therefore, Gβγ provides a possible site of indirect interaction.
between GRK2 and GRK3 with Ca\(^{2+}\)/CaM. Indeed, in the present study, using a receptor phosphorylation assay in which the G\(\beta\gamma\)-activated GRK2 and GRK3 activity was measured, we observed inhibition of GRK activity by CaM in a Ca\(^{2+}\)-dependent manner. This may be due to blockade of G\(\beta\gamma\) by Ca\(^{2+}\)/CaM, thus inhibiting GRK activity. However, this does not preclude the possibility of Ca\(^{2+}\)/CaM inhibiting the activity of GRK2 and GRK3 through alternative targets important in the functioning of these kinases.

The IC\(_{50}\) value of CaM for GRK2 is 2 \(\mu\)M. This is within the reported range of cellular CaM levels of 1–10 \(\mu\)M (28) and is similar to the IC\(_{50}\) value of Ca\(^{2+}\)/recoverin inhibition of GRK1 (15), thus suggesting a possible physiological implication for the functional interaction between CaM and GRK2, possibly through their common binding target of G\(\beta\gamma\).

The more significant finding of the present study was the unexpected observation of a potent inhibitory effect of Ca\(^{2+}\)/CaM on GRK5. Similar to GRK2 and GRK3, GRK5 inhibition by CaM was strictly dependent on Ca\(^{2+}\) and was prevented by the CaM inhibitor CaMBl. However, since GRK5 activity is not influenced by G\(\beta\gamma\) (21, 29), there must be an alternative site of interaction between GRK5 and CaM. This site does not appear to be rhodopsin since Ca\(^{2+}\)/CaM was at least 50-fold more potent in inhibiting ROS phosphorylation by GRK5 than by GRK2 and GRK3 and had no effects on GRK1. The affinity of potent in inhibiting ROS phosphorylation by GRK5 than by CaM indicates that different kinases can be specifically regulated by CaM on the adenylyl cyclase-cAMP axis, such as activation of the cyclase (28). Another study stressed the importance of temporal overlapping of the Ca\(^{2+}\) pulse with the addition of the facilitatory neurotransmitter serotonin in classical conditioning in Aplysia (34). For the adrenocorticotrophic hormone receptor, the CaM-mediated potentiation effect was attributed to an action on receptor-Gs coupling (35), which is the classical site of intervention by GRKs. Such synergistic effects of CaM on the functions of receptors may be at least partially mediated by the effect of Ca\(^{2+}\)/CaM on GRKs.

In conclusion, the present study provides the first evidence for a regulatory effect of Ca\(^{2+}\)/CaM on some GRK subtypes, thus expanding the range of different mechanisms by which the functional states of GRK/arrestin proteins can be regulated. The effect of Ca\(^{2+}\)-sensor proteins appears to be rather general, and the GRK subtype selectivity documented for recoverin and CaM indicates that different kinases can be specifically regulated in different target tissues.

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