p90 ribosomal S6 kinase 2 exerts a tonic brake on G protein-coupled receptor signaling

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Communicated by Erminio Costa, University of Illinois, Chicago, IL, January 23, 2006 (received for review November 1, 2005)

G protein-coupled receptors (GPCRs) are essential for normal central nervous system function and represent the proximal site(s) of action for most neurotransmitters and many therapeutic drugs, including typical and atypical antipsychotic drugs. Similarly, protein kinases mediate many of the downstream actions for both ionotropic and metabotropic receptors. We report here that genetic deletion of p90 ribosomal S6 kinases (RSKs) potentiates GPCR signaling. Initial studies of 5-hydroxytryptamine (5-HT2A) receptor signaling in fibroblasts obtained from RSK2 wild-type (+/+) and knockout (−/−) mice showed that 5-HT2A receptor-mediated phosphoinositide hydrolysis and both basal and 5-HT-stimulated extracellular signal-regulated kinase 1/2 phosphorylation are augmented in RSK2 knockout fibroblasts. Endogenous signaling by other GPCRs, including P2Y-purinergic, PAR1-thrombinergic, β1-adrenergic, and bradykinin-B receptors, was also potentiated in RSK2-deficient fibroblasts. Importantly, reintroduction of RSK2 into RSK2−/− fibroblasts normalized signaling, thus demonstrating that RSK2 apparently modulates GPCR signaling by exerting a “tonic brake” on GPCR signal transduction. Our results imply the existence of a novel pathway regulating GPCR signaling, modulated by downstream members of the extracellular signal-related kinase/mitogen-activated protein kinase cascade. The loss of RSK2 activity in humans leads to Coffin–Lowry syndrome, which is manifested by mental retardation, growth deficits, skeletal deformations, and psychosis. Because RSK2-inactivating mutations in humans lead to Coffin–Lowry syndrome, our results imply that alterations in GPCR signaling may account for some of its clinical manifestations.

Results

Identification of RSK2 as a 5-HT2A Receptor-Interacting Protein. As part of a larger effort to identify novel GPCR regulators, we performed a yeast two-hybrid screen of a human brain cDNA library (see Supporting Text, which is published as supporting information on the PNAS web site) using the third intracellular loop (i3) of the 5-HT2A receptor as bait. A large number of putative 5-HT2A receptor-interacting proteins were identified (see Table 2, which is published as supporting information on the PNAS web site, for details) and one clone, 33.5, which encoded a portion of the C-terminal kinase domain of RSK2, was selected for further study (Fig. 1 A and B; Table 2).

The site of interaction between RSK2 and the 5-HT2A receptor i3 loop bait was identified by making serial deletions of the i3 loop and monitoring the interaction with the RSK2 target by two-hybrid

Conflict of interest statement: No conflicts declared.

Abbreviations: GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; RSK, p90 ribosomal S6 kinase; ERK, extracellular signal-regulated kinase; 5-HT, 5-hydroxytryptamine; HEP, human embryonic kidney; FLAG-5-HT2A, human FLAG-tagged 5-HT2A receptor; PI, phosphoinositide; i3, third intracellular loop; CLS, Coffin–Lowry syndrome.

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indicating that 5-HT2A receptors and RSK2 associate precipitated with FLAG-5-HT2A receptors (Fig. 2A, lanes 3 and 4), indicating that 5-HT2A receptors and RSK2 associate in vitro. The association of FLAG-5-HT2A and RSK2 was unaltered by agonist exposure (Fig. 2A, compare lanes 3 and 4), indicating that 5-HT2A receptors and RSK2 associate in an agonist-independent manner in HEK-293 cells. Coimmunoprecipitation studies were also done in two different cellular milieu where 5-HT2A receptors and RSK2 are constitutively expressed (C6-glioma cells and rat brain synaptic membranes), to determine whether RSK2 and the 5-HT2A receptor associate in vivo. To verify the specificity of the interaction, immunoprecipitations were performed by using protein-A/G agarose beads in the absence (-) or presence (+) of a monoclonal anti-RSK2 antibody. Fig. 2B, first image, shows that 5-HT2A-like immunoreactivity was present in C6-glioma cell immunoprecipitates only when the RSK2 antibody was used (compare lanes 1 and 2). Fig. 2B, second image, shows the immunoprecipitation of RSK2 in the presence of the monoclonal RSK2 antibody but not with protein A/G beads alone, confirming the specificity of the immunoprecipitation. Fig. 2B, second and fourth images, shows that equivalent levels of 5-HT2A-like immunoreactivity and RSK2 were present in the lysates from which the immunoprecipitations were performed. These data indicate that RSK2 and 5-HT2A receptors endogenously associate in C6-glioma cells. Similar results were obtained by using rat brain synaptic membrane.

We also examined the distribution of RSK2 in rat brain in two regions rich in 5-HT2A receptors: the prefrontal cortex and the globus pallidus. Fig. 2D–F show that 5-HT2A receptors and RSK2 were colocalized in the globus pallidus. Fig. 2G–I show that 5-HT2A receptors and RSK2 were also colocalized in layer V pyramidal neurons in the prefrontal cortex. Fig. 2J–L show higher magnifications of layer V pyramidal neurons in the prefrontal cortex and indicate an overlapping and punctate distribution of 5-HT2A receptors and RSK2. Together, these data show that 5-HT2A receptors and RSK2 have overlapping cellular and subcellular distributions in rat brain.

RSK2 Modulates GPCR Signaling. To determine the functional significance of this interaction, we obtained fibroblasts from RSK2+/+ and RSK2−/− mice (14). We performed microarray studies to identify endogenously expressed GPCRs in these cell lines (Table 3, which is published as supporting information on the PNAS web site) and assessed signaling at a subset of the identified receptors. As shown in Fig. 3A–C and Fig. 5, which is published as supporting information on the PNAS web site, deletion of RSK2 augments agonist-stimulated intracellular Ca2+ release for 5-HT2A-serotonergic, P2Y-purinergic, and PAR1-thrombinergic receptors and also potentiated agonist-stimulated β1-adrenergic receptor cAMP accumulation.

We next examined 5-HT2A receptor signaling in both cell lines in greater detail. In both RSK2−/− and RSK2+/+ fibroblasts, 5-HT induced an increase in phosphoinositide (PI) accumulation that was abolished by the selective 5-HT2A antagonist (2,3-dimethoxyphenyl)-1-[2-fluoroethyl]ethyl]-4-piperidyl]methanol (MDL110,907) (Fig. 3D), indicating that RSK2+/− and RSK2−/− fibroblasts express functional 5-HT2A receptors. Quantitative RT-PCR studies (see Supporting Text for details) disclosed that both cell lines expressed equivalent amounts of 5-HT2A mRNA (Fig. 3E). We also found that 5-HT2A-mediated PI hydrolysis was augmented in the RSK2−/− fibroblasts (Fig. 3D). To determine whether this was due to an enhanced surface expression of 5-HT2A receptors, we prepared stable lines expressing FLAG-5-HT2A and performed surface biotinylation studies. We found that stable lines of RSK2−/− and RSK2+/+ fibroblasts overexpressing FLAG-5-HT2A receptors displayed enhanced 5-HT2A-mediated maximal PI accumulation (Fig. 3F), but no difference in the cell surface expression of FLAG-5-HT2A receptors (as determined by cell surface biotinylation) was measured (Fig. 3G). These data indicate that the alterations in signaling are not due to differences in 5-HT2A receptor expression.

Microarray studies were used to determine whether the changes in GPCR signaling might be due to altered expression of genes involved in second-messenger production. Similar numbers of genes were expressed [“present” calls by the Affymetrix software (see Dataset1, which is published as supporting information on the PNAS web site); see Supporting Text for details] in the RSK2+/+ and RSK2−/− fibroblasts, and roughly equivalent numbers of
Fig. 2. RSK2 interacts with 5-HT2A receptors in vitro and in vivo. (A) Coimmunoprecipitation of RSK2 with FLAG-5-HT2A receptors in transfected HEK-293 cells. HEK-293 cells were transiently cotransfected with either RSK2 and cyan fluorescent protein (CFP) (negative control) or FLAG-5-HT2A receptors. Protein lysates were prepared, and equivalent amounts of protein were loaded for all sets. Lanes 1 and 3 were treated with vehicle, and lanes 2 and 4 were treated with 10 μM 5-HT for 5 min. FLAG-tagged human 5-HT2A receptors were immunoprecipitated (IP) by monoclonal FLAG antibody conjugated to Sepharose beads. (B) Coimmunoprecipitation of native 5-HT2A receptors with RSK2 in C6-glioma cells. (C) Coimmunoprecipitation of native 5-HT2A receptors with RSK2 from rat brain synaptic membrane preparations. For experiments in native cell lines, cell lysates were prepared from either C6-glioma cells (B) or rat brain synaptic membranes (C), and equivalent amounts of protein were loaded for all sets. In both B and C, the first image shows that native 5-HT2A receptors are immunoprecipitated with monoclonal RSK2 antibody (lane 1 [+]) but not protein A/G beads alone (lane 2 [−]). The second image shows native 5-HT2A receptors present in the lysates (lanes 1 and 2). The third image shows robust RSK2 detection in the immunoprecipitate in the presence of RSK2 antibody (lane 1 [+] but not with protein A/G beads alone (lane 2 [−]). The fourth image shows RSK2 in the lysates (lanes 1 and 2). Shown are representative immunoblots (IB) from a single experiment since replicated three times with similar results. (D–L) Rat brain sections were prepared and stained as described in Materials and Methods, followed by dual-label immunofluorescent confocal microscopy. Representative images from one of three independent experiments are shown. (D–F) Globus pallidus. (G–I) Layer V prefrontal cortex. (J–L) Higher-power layer V prefrontal cortex. Native rat 5-HT2A receptors are shown in the red channel (D, G, and J), and native rat RSK2 is shown in the green channel (E, H, and K). The merged images are in F, I, and L.
which is published as supporting information on the PNAS web site). Significantly, the elevated basal levels of ERK1/2 phosphorylation were attenuated by treatment with 100 nM MDL100,907 (Fig. 4A, compare lanes M and UT; Fig. 4C), a highly selective 5-HT2A antagonist (17). These results imply that the increase in basal phosphorylation seen in RSK2−/− fibroblasts is due, at least in part, to augmented constitutive 5-HT2A receptor activity.

Discussion

In this paper, we show that RSK2 exerts a tonic brake on GPCR signaling. We also show that RSK2 can directly interact with at least one GPCR (the 5-HT2A serotonin receptor), and that this association has two significant cellular consequences on GPCR signaling. The absence of RSK2 results in an increase in agonist efficacy as measured through PI hydrolysis, without a change in agonist potency and second, in an increase in both basal and stimulated ERK phosphorylation. Intriguingly, this increase in basal ERK phosphorylation is attenuated by treatment with MDL100,907, a selective 5-HT2A antagonist. Additionally, reintroduction of RSK2 into RSK2 knockout fibroblasts normalizes 5-HT2A serotonin, PAR-1 thrombin, P2Y-purinergic, and bradykinin-B receptor-mediated signaling. Together, these findings describe a previously undescribed mode of GPCR regulation.

We have previously shown an interaction of 5-HT2A receptors with postsynaptic density protein 95 (PSD-95), a PSD-95/discs large/ZO-1 (PDZ) domain-containing scaffolding protein that interacts with the extreme C-terminal tail of the 5-HT2A receptor via a canonical type 1 PDZ ligand (18). In addition, a number of other GPCRs have previously been shown to interact with PDZ domain-containing scaffolding proteins (see ref. 19 for review).

Table 1. RSK2 knockout augments GPCR signaling

<table>
<thead>
<tr>
<th>Agonist potency, EC50 (pEC50 ± SEM)</th>
<th>Relative agonist efficacy, Emax ± SEM</th>
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<tbody>
<tr>
<td></td>
<td>RSK2−/− fibroblast</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>678 nM (6.17 ± 0.16)</td>
</tr>
<tr>
<td>PAR-1</td>
<td>8.6 µM (5.07 ± 0.10)</td>
</tr>
<tr>
<td>P2Y</td>
<td>8.6 µM (5.06 ± 0.24)</td>
</tr>
<tr>
<td>Bradykinin-B</td>
<td>0.19 nM (9.72 ± 0.40)</td>
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Agonist potencies (EC50) and efficacies (Emax) were determined for agonist-mediated activation of PI hydrolysis, as described in Materials and Methods. pEC50 values are represented as −log of EC50 as molar concentrations. The results represent the average of four independent experiments.

*Statistically different from RSK2+/+ fibroblasts, P < 0.05.
Interestingly, RSK2 has recently been demonstrated to contain C-terminal sequences that bind PDZ domains, and the interaction of RSK2 with PDZ domain-containing proteins has been demonstrated to be important for the role of RSK2 in synaptic function (9). The possibility that PDZ domain-containing proteins, GPCRs, and RSK2 form large synaptic signaling complexes is an intriguing possibility that warrants further study. To this end, we will attempt to identify other RSK2-interacting proteins in future studies.

Prior studies with RSK2 knockout mice revealed an increase in basal or insulin- and exercise-stimulated ERK1/2 phosphorylation (13). Dufresne et al. (13) hypothesized that the augmented basal ERK1/2 phosphorylation could be due to either diminished feedback inhibition of RSK2 on the ERK/MAPK cascade or altered expression of one of the ERK phosphatases. Our studies have demonstrated a similar finding, showing an apparent dissociation between agonist treatment and basal ERK1/2 phosphorylation and an increase in 5-HT-stimulated ERK1/2 phosphorylation in the absence of RSK2. However, we suggest that the increase in basal ERK1/2 phosphorylation in RSK2 knockout mice and cells might be due to the removal of a tonic brake on GPCR signaling. That pretreatment of RSK2−/− fibroblasts with the 5-HT2A receptor-selective antagonist MDL100,907 lowered basal ERK1/2 phosphorylation to levels similar to those seen in the RSK2+/+ fibroblasts supports this notion. Additionally, microarray data indicate that global dysregulation of phosphatases or MAPK cascade members does not occur at the mRNA level (see Table 4 and Fig. 10, which are published as supporting information on the PNAS web site). These data argue that RSK2 may have a novel role in maintaining the appropriate level of basal ERK-MAP kinase cascade activity by suppressing basal and receptor-stimulated activity of 5-HT2A receptors, and perhaps other GPCRs.

The kinases participating in the phosphorylation of 5-HT2A receptors have proven elusive, despite the presence of >30 potential phosphorylation sites and the identification of serine and threonine residues involved in desensitization (16). To date, no agonist-directed phosphorylation of 5-HT2A receptors has been reported, despite continued study (16). Indeed, the association of RSK2 with 5-HT2A receptors appears to be agonist-independent, because agonist stimulation had no effect on the coimmunoprecipitation of RSK2 with 5-HT2A receptors. It is therefore possible that RSK2 constitutively phosphorlates the 5-HT2A receptor, a possibility we are currently investigating. Because 5-HT efficacy is increased in RSK2 knockout cells, it is also possible that constitutive phosphorylation of 5-HT2A receptors in wild-type cells could interfere with G protein-coupling efficiency, leading to a “presensitized” state of the receptor.

Individuals with CLS have an increased risk of cardiac abnormalities, including mitral, tricuspid, and aortic valve abnormalities, pulmonary and aortic root dilation, and cardiomyopathy, suggesting an indispensable role for RSK2 in cardiovascular function (20). PAR-1 thrombin receptors are highly expressed in the heart and have many cardiovascular functions (21). Additionally, the 5-HT2A receptor is highly expressed in vascular smooth muscle, pulmonary artery, and cardiac tissues. Because RSK2 deletion augments 5-HT2A and PAR-1 signaling, our findings imply that cardiovascular abnormalities in patients with CLS may be due to a hyperactivation of GPCR signaling.

In summary, RSK2 has emerged as a regulator of GPCRs, exerting a tonic brake on GPCR signaling. Inactivating mutations of RSK2 in humans lead to mental retardation, skeletal muscle deformities, and psychosis. Intriguingly, GPCR signaling has been implicated in signaling pathways that regulate brain, bone, and skeletal muscle development (22–26). Alterations of GPCR signaling induced by genetic deletion or inactivation of RSK2 may play a role in the pathogenesis of CLS and may be involved in other disorders manifested by dysmorphogenesis, psychosis, and/or mental retardation.
Materials and Methods

Western Blotting, Immunoprecipitation, and Cell Surface Biotinylation. Coimmunoprecipitation and immunoblotting were performed as detailed (18, 30). A monoclonal mouse RSK2 antibody (Santa Cruz Biotechnology) and protein A/G agarose were used to immunoprecipitate RSK2 from C6 glioma cells and from rat brain synaptic membrane preparations. Rat brain synaptic membranes were prepared from rat frontal cortex as described (30). Cell surface biotinylation was performed as described (32).

Immunohistochemistry and Confocal Microscopy. For immunohistochemical studies, rats were perfused with 4% paraformaldehyde, prefrontal cortex sections were prepared, and immunohistochemical localization was done as described (33). Sections were incubated in a mixture of a mouse monoclonal 5-HT2A antibody (PharMingen; 1:1,000) and a rabbit polyclonal RSK2 antibody (Upstate Biotechnology; 1:100), with subsequent incubation in the secondary antibodies goat anti-rabbit Alexa Fluor 488 (1:400; Molecular Probes) and goat anti-mouse CY3 (1:1,250; Jackson Immunoresearch). Dual-label immunofluorescence confocal microscopy was done as described (18, 30).

Second-Messenger Studies. RSK2−/− and RSK2+/− fibroblasts were split at 40,000 cells per well into 24-well plates for PI hydrolysis assays and adenylate cyclase assays or at 120,000 cells per well into six-well plates for ERK1/2 phosphorylation assays, in DMEM supplemented with 5% dialyzed FCS, and then serum-starved for 15 h before experimentation. The cells were then prepared for PI hydrolysis studies, for adenylate cyclase assays, or for measurements of ERK1/2 phosphorylation, as described (18, 30, 36). All studies were replicated at least three times and analyzed by nonlinear regression by using PRISM 4.03 software (GraphPad, San Diego). Statistical significance of the data was determined by two-tailed paired t test and was defined as P < 0.05.

We thank Dr. Jon Backstrom (Vanderbilt University Medical Center) for generously providing the ct-2A antibody, Dr. Allison Limpert for insightful comments. We thank Konstantina Angelis and the laboratory of Dr. Edward Stavnezer for technical expertise in quantitative RT-PCR. This research was supported by the Gene Expression Array Core Facility of the Comprehensive Cancer Center of Case Western Reserve University; University Hospitals of Cleveland Grant P30 CA43703; National Research Service Award predoctoral fellowship F31 MH67435 (to D.J.S.); National Institutes of Health Grants R01 MH51635, R01 MH61887, and K02 MH01366; the National Institute of Mental Health Psychoactive Drug Screening Program (to B.L.R.); and a National Alliance for Research on Schizophrenia and Depression Young Investigator Award (to W.K.K.).