Caveolin-1 Interacts with 5-HT<sub>2A</sub> Serotonin Receptors and Profoundly Modulates the Signaling of Selected G<sub>α</sub><sub>q</sub>-coupled Protein Receptors*<sup>[S]</sup>

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5-Hydroxytryptamine 2A (5-HT<sub>2A</sub>) serotonin receptors are important for a variety of functions including vascular smooth muscle contraction, platelet aggregation, and the modulation of perception, cognition, and emotion. In a search for 5-HT<sub>2A</sub> receptor-interacting proteins, we discovered that caveolin-1 (Cav-1), a scaffolding protein enriched in caveolae, complexes with 5-HT<sub>2A</sub> receptors in a number of cell types including C6 glioma cells, transfected HEK-293 cells, and rat brain synaptic membrane preparations. To address the functional significance of this interaction, we performed RNA interference-mediated knockdown of Cav-1 in C6 glioma cells, a cell type that endogenously expresses both 5-HT<sub>2A</sub> receptors and Cav-1. We discovered that the in vitro knockdown of Cav-1 in C6 glioma cells nearly abolished 5-HT<sub>2A</sub> receptor-mediated signal transduction as measured by calcium flux assays. RNA interference-mediated knockdown of Cav-1 also greatly attenuated endogenous G<sub>α</sub><sub>q</sub>-coupled P2Y purinergic receptor-mediated signaling without altering the signaling of PAR-1 thrombin receptors. Cav-1 appeared to modulate 5-HT<sub>2A</sub> signaling by facilitating the interaction of 5-HT<sub>2A</sub> receptors with G<sub>α</sub><sub>q</sub>. These studies provide compelling evidence for a prominent role of Cav-1 in regulating the functional activity of not only 5-HT<sub>2A</sub> serotonin receptors but also selected G<sub>α</sub><sub>q</sub>-coupled receptors.

The serotonin (5-hydroxytryptamine) 2A receptor (5-HT<sub>2A</sub>)<sup>[1]</sup> a member of the “rhodopsin-like” (family A) G-protein-coupled receptors (GPCRs) (1), mediates the actions of many (2) but not all hallucinogens (3, 4) and is the target of a number of commonly prescribed therapeutic agents, including atypical antipsychotics, antidepressants, and anxiolytics (5). As with other GPCRs, elucidating the mechanisms of signal transduction and regulation for 5-HT<sub>2A</sub> receptors is likely to be of great relevance for the rational design of novel medications (6–8). In particular, one point where the regulation of GPCR signaling converges is the endocytic pathway (8, 9), and therefore, this pathway is likely to play a role in various functions involving the 5-HT<sub>2A</sub> receptor.

Prototypically, GPCRs are internalized via an integrated process involving arrestins and G-protein receptor kinases, a process that has been most elegantly elucidated for the β-adrenergic receptor (10), although many other proteins are also involved in the intracellular trafficking of GPCRs (6, 11). For example, we have uncovered a cell type-specific, arrestin-independent, dynamin-dependent mechanism of 5-HT<sub>2A</sub> receptor regulation (12, 13). In light of recent studies demonstrating that β-adrenergic receptors, in particular, are associated with caveolae and caveolin in their native milieu (14, 15), we hypothesized that 5-HT<sub>2A</sub> receptors might also be associated with caveola-enriched membrane specializations and that this interaction might functionally modulate 5-HT<sub>2A</sub>-mediated signal transduction.

Caveolae are small flask-shaped invaginations of the plasma membrane (16) containing high levels of cholesterol and glycosphingolipids and are initially characterized by the presence of the protein Cav-1 (17). Caveolae differ biochemically from other specialized subdomains of the plasma membrane (16). A family of caveolin proteins has been identified that includes caveolin-1, caveolin-2, and caveolin-3 (Cav-1, –2, and –3, respectively (18)), with Cav-1 and Cav-2 being expressed ubiquitously. Caveolin function is critically dependent on Cav-1 because caveola are not formed in Cav-1 knockout mice (19); conversely, caveolin-deficient cells acquire caveola when transfected with Cav-1 (20).

In prior studies, we found that caveolae were not normally required for the membrane targeting of 5-HT<sub>2A</sub> receptors heterologously expressed in either NIH 3T3 (21) or HEK-293 (12) cells, although a recent study (22) indirectly implicated caveolae in 5-HT<sub>2A</sub>-mediated signal transduction in vascular smooth muscle cells. Even though prior studies have not implicated Cav-1 as a modulator of 5-HT<sub>2A</sub> receptor signaling, Cav-1 has been indirectly implicated as a regulator of GPCR signaling via unknown mechanisms (19).

Here we report that both transiently transfected 5-HT<sub>2A</sub> receptors associate with Cav-1 in HEK-293 cells and endog-
enously expressed 5-HT\textsubscript{2A} receptors associate with Cav-1 in C6 glioma cells and rat brain synaptic membrane preparations. We also report that the RNAi-mediated knockdown of Cav-1 has profound functional consequences for 5-HT\textsubscript{2A}-mediated signal transduction in C6-glioma cells, a cell type that endogenously expresses both Cav-1 and 5-HT\textsubscript{2A} receptors. Most importantly, screening of C6 glioma cells for other G\textsubscript{i}/H9251q-coupled receptors showed that knockdown of Cav-1 modulated the signaling of purinergic receptors but not of PAR-1 thrombin receptors in C6 glioma cells, suggesting that only a subset of G\textsubscript{i}/H9251q-coupled receptors is regulated by Cav-1.

EXPERIMENTAL PROCEDURES

cDNA Constructs and Reagents—The construct coding for FLAG-tagged wild type (wt) rat 5-HT\textsubscript{2A} receptor (FLAG-5-HT\textsubscript{2A}) (12) was modified by addition of an amino-terminal and cleavable signal peptide sequence (MRTIALSYIFCLVFA; see Ref. 23) from influenza hemagglutinin and has been described elsewhere (24). The caveolin-1-myc cDNA was a gift from Gary Landreth (Case Western Reserve University), and the constitutively active G\textsubscript{i}/H9251q mutant (Q229L) was from David Siderovski (University of North Carolina, Chapel Hill). Thrombin receptor-activating peptide (TRAP) was a gift from Paul Di Corleto (Lerner Research Institute, Case Western Reserve University). All constructs containing inserts in the appropriate orientation were verified.

FIG. 1. Cav-1 co-localizes with native 5-HT\textsubscript{2A} receptors independently of agonist exposure. For these experiments HEK-293 cells were transiently co-transfected with FLAG-5-HT\textsubscript{2A} + cav-1-myc (A–F). At 48 h following transfection, cells were placed in serum-free media for a minimum of 18 h and then exposed to vehicle (A–C) or 10 \mu M 5-HT (D–F) for 5 min. This was followed by dual-label immunofluorescent confocal microscopy (see “Experimental Procedures”). Representative images from one of three independent experiments are shown. FLAG-tagged native 5-HT\textsubscript{2A} receptors are shown in the red channel (A and D) and cav-1-myc is shown in the green channel (B and E). The merged images are in C and F. Scale bars are shown in all panels. G shows the quantitative analysis of percent receptor internalization in the presence and absence of Cav-1 in response to vehicle and or agonist 5-HT (10 \mu M) for 5 min. **, *p < 0.05; ns, no statistically significant difference.
Cav-1-myc - + - - + +
FLAG-5-HT$_{2A}$ - + + + + +
GFP - + - + + +
5-HT (5 min) - - - - - -

Fig. 2. Cav-1 interacts with native 5-HT$_{2A}$ receptors in HEK-293 cells. For these experiments, HEK-293 cells were either untransfected (1st and 2nd lanes) or transiently co-transfected with Cav-1 (3rd and 4th lanes), FLAG-5-HT$_{2A}$ + GFP (5th and 6th lanes; treated with vehicle), FLAG-5-HT$_{2A}$ + GFP (7th and 8th lanes; treated with 5-HT for 5 min), FLAG-5-HT$_{2A}$ + Cav-1 (9th and 10th lanes; treated with vehicle), and FLAG-5-HT$_{2A}$ + Cav-1 (11th and 12th lanes; treated with 10 µM 5-HT for 5 min). FLAG-tagged native 5-HT$_{2A}$ receptors were immunoprecipitated (IP) by a monoclonal FLAG antibody conjugated to Sepharose beads. A polyclonal Cav-1 antibody detected Cav-1myc, and a polyclonal FLAG antibody on Western blots detected FLAG-5-HT$_{2A}$. A polyclonal G$_{q}$ antibody was used to probe for G-protein as lysate control. Representative immunoblots (IB) from a single experiment that has been replicated three times with equivalent results are shown. A and B, immunoblots from immunoprecipitates. C and D, immunoblots from cell lysates.

Fig. 3. Cav-1 interacts with native 5-HT$_{2A}$ receptors in C6 glioma cells and rat brain homogenates. For these experiments cell lysate was prepared from various cell lines, and equivalent amounts of protein were loaded for all sets in duplicate. Representative immunoblot from a single experiment that has been replicated three times with equivalent results shows the expression of Cav-1 in HEK-293 cells, C6 glioma cells, A75R5, 3T3, and a 3T3 cell line stably expressing 5-HT$_{2A}$ receptors (GP62; A). B, 5-HT$_{2A}$ receptors were immunoprecipitated (IP) with Cav-1 in C6 glioma cells. The top panel shows that 5-HT$_{2A}$ receptors were immunoprecipitated by monoclonal Cav-1 antibody (2nd lane) preconjugated to protein-A/G beads. The 2nd panel from the top shows robust Cav-1 detection in the immunoprecipitate in the presence of Cav-1 antibody (2nd lane (+)). The 3rd and 4th panels from the top represents the immunoblots used to detect Cav-1 and 5-HT$_{2A}$ receptor proteins, respectively, in the total C6 cell lysate. C, 5-HT$_{2A}$ receptors were immunoprecipitated with Cav-1 from rat brain synaptic membrane preparations. Top panel shows 5-HT$_{2A}$ receptors were co-immunoprecipitated with Cav-1 antibody and protein A/G-agarose (2nd lane) but not with protein A/G-agarose beads alone (−). The 2nd panel from the top shows that Cav-1 was found in the immunoprecipitates (lane 2). The 3rd and 4th panels from the top shows immunoblots (IB) used to detect Cav-1 and 5-HT$_{2A}$ receptors, respectively, from total membrane lysates. Shown are representative immunoblots from a single experiment that has been replicated three times with equivalent results.

Caveolin-1 Associates with and Regulates 5-HT$_{2A}$ Receptors

Transfection of HEK-293 Cells and C6 Glioma Cells—Human embryonic kidney 293 (HEK-293) cells and C6 glioma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, penicillin (100 units/ml), and streptomycin (100 mg/ml) (Invitrogen) at 37 °C and 5% CO$_{2}$. Transient transfection of HEK-293 cells with FuGENE 6™ (Roche Applied Science) using a FuGENE to DNA ratio of 5:1 was performed according to the manufacturer's recommendations. A total of 6 µg of DNA was used in each co-transfection, 3 µg of which were FLAG-5-HT$_{2A}$, 2 µg of constitutively active G-protein (G$_{q,*}$) and 3 µg of which were DNA encoding Cav-1myc or GFP. In case of triple transfections with FLAG-5-HT$_{2A}$ receptor, Cav-1myc, G$_{q,*}$, and/or GFP, 2 µg of each plasmid DNA was used. C6 glioma cells were transfected using LipofectAMINE® (Invitrogen). A total of 24 µg of DNA was used with 60 µl of LipofectAMINE® as recommended by the manufacturer.

Immunocytochemistry, Agonist-mediated Internalization, and Image Quantification—Dual-labeling immunocytochemistry was performed essentially as described before (12), and images of cells treated with vehicle and/or agonist 5-HT (10 µM) were acquired digitally using a Zeiss 410 confocal microscope (Oberkochen, Germany) without saturation of pixel intensities. Images were subsequently analyzed for percent receptor internalization using MetaView software (Universal Imaging) as detailed previously (24).

Co-immunoprecipitation and Immunoblot—Co-immunoprecipitation and immunoblotting were performed essentially as detailed previously (24, 25). For visualization and immunoprecipitation of Cav-1, a polyclonal Cav-1 antibody (1:2000) (BD Transduction Laboratories) was used, and a monoclonal M2-anti-FLAG antibody preconjugated to agarose beads was used to immunoprecipitate FLAG-tagged 5-HT$_{2A}$ receptors. Wheat germ agglutinin (WGA) preconjugated to agarose beads (Sigma) was used to concentrate native 5-HT$_{2A}$ receptors from C6 glioma cells after solubilization in 1% CHAPS. A rabbit polyclonal FLAG antibody (1:1500) (Sigma) was used to detect FLAG-5-HT$_{2A}$ receptors. A monoclonal mouse Cav-1 antibody and protein A/G-agarose were used to co-immunoprecipitate native receptor and Cav-1 from C6 glioma cells and rat brain synaptic membrane preparation. Rat brain synaptic membranes were prepared from rat frontal cortex as described previously (26). The native 5-HT$_{2A}$ receptor was detected by using a polyclonal 5-HT$_{2A}$ carboxyl-terminal antibody (27), a gift from Jon Backstrom (Vanderbilt University). The G$_{q,*}$ in immunoprecipitates, the constitutively active G$_{q,*}$ mutant, and the endogenous wt G$_{q,*}$ in cell lysates were detected by a rabbit polyclonal G$_{q,*}$ antibody (1:2000) (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoblots shown are representative of at least three independent experiments.

RNAi-mediated Knockdown of Cav-1—A DNA vector-based siRNAi was designed to stably knock down the expression of Cav-1 in C6 glioma cells by Genscript. Briefly, a commercial algorithm for designing a specific siRNA was used (www.genscript.com/siRNA/app/raimi). The length of the siRNA target, GC % range, and the rat cDNA sequence of Cav-1 were entered into the algorithm and potential siRNA targets
using 1° was used to generate cRNA by using a Bioarray High Yield ENZOkit Qiagen columns and storage overnight at 1°. The reaction was terminated by addition of EDTA followed by cDNA clean up using additional 5 min in the presence of T4 DNA polymerase. The reaction mixture was incubated for 2 h at 16 °C and an additional 5 min in the presence of T4 DNA polymerase. The reaction was terminated by addition of EDTA followed by cDNA clean up using Qiagen columns and storage overnight at ~20 °C. In vitro transcription was used to generate cRNA by using a Bioarray High Yield ENZOKit (Affymetrix). Clean up of RNA samples was used to clean up cDNA and/or RNA as required using the manufacturer’s protocol. This was followed by cDNA synthesis using an oligo(dT) primer coupled to T7 RNA polymerase promoter. Reverse transcription of RNA was done using Superscript II reverse transcriptase in a 20-μl reaction at 42 °C for 1 h. Second strand synthesis was carried out immediately in the presence of Escherichia coli DNA polymerase I, RNase H, and DNA ligase. The reaction mixture was incubated for 2 h at 16 °C and an additional 5 min in the presence of T4 DNA polymerase. The reaction was terminated by addition of EDTA followed by cDNA clean up using Qiagen columns and storage overnight at ~20 °C. The amplification (antibody) solution mixture was as follows: 50 mM Tris, pH 8.1, 100 mM KOAc, 30 mM MgOAc) and then placed on ice. Preconditioning for hybridization was done in 1× hybridization mixture (100 mM MES, 1 mM [Na], 20 mM EDTA, 0.01% Tween 20). Herring sperm and acetylated bovine serum albumin were added to a final concentration of 0.1 and 0.5 mg/ml, respectively. A 15-μl aliquot of a 20× mixture of in vitro transcripts of bacterial genes bioB, bioC, bioD, and cre were added to the mixture to give final concentrations of 1.5, 5, 25, and 100 pM, respectively. Control oligonucleotide was added to a final concentration of 50 pM. The amount of fragmentation reaction containing 15 μg of cRNA was added to the mixture, and the remaining volume was made up with molecular biology grade water. Preconditioning of the array chip was done in stringent buffer B (10 mM MES, 0.1 N [Na], 0.01% Tween), which was also used in the protocol. The streptavidin/phycoerythrin stain mixture was as follows: 50 mM MES, 0.5 mM [Na], 0.025% Tween 20, 2 mg/ml acetylated bovine serum albumin, 10 μg/ml streptavidin phycoerythrin. The amplification (antibody) solution mixture was as follows: 50 mM MES, 0.5 mM [Na], 0.025% Tween 20, 2 mg/ml acetylated bovine serum albumin, 0.1 mg/ml normal goat IgG, 3 mg/ml biotinylated antibody. All chips were scanned twice. For data analysis, images obtained were converted into Microsoft Excel format using MAS5.0 software (Affymetrix). All chips were scaled to mean target intensity of 1500. Af- fymetrix present and absent calls were used. For comparisons between

chips, genes with 2-fold differences in expression compared with controls were considered to be significantly differentially expressed.

**Radioligand Binding and Second Messenger Studies**—Phosphoinositide hydrolysis assays with the constitutively active Gαs were performed as described previously (24). Kinetic binding parameters (Kd and Bmax) were determined from binding assays with [3H]ketanerzide, and the results were replicated in at least three separate experiments as detailed previously (24). Phosphoinositide hydrolysis data were analyzed by nonlinear regression using Prism 3.0 software (GraphPad, San Diego, CA), and saturation-binding data were analyzed by using GraphPad Prism. Measurements of p42/44 ERK phosphorylation were performed as described previously by using total p42/44 ERK for normalization (28). Measurements of intracellular calcium mobilization on a Molecular Devices Flexstation were performed essentially as described recently (29), with response normalized to the maximum with each agonist (5-HT, ATP, and/or TRAP).

**Statistical Analysis**—Statistical significance for all studies was determined using a Student t test, and statistical significance was defined as p < 0.05.

**RESULTS**

**5-HT2A Receptors Interact with Cav-1**—We initially investigated whether 5-HT2A receptors and cav-1-myc can associate in vitro by co-transfecting HEK-293 cells with FLAG-tagged 5-HT2A receptors (FLAG-5-HT2A) and MHC-tagged Cav-1 (Cav-1-myc). As shown in Fig. 1, A–C, FLAG-5-HT2A and Cav-1 were co-localized on both the cell surface of HEK-293 cells and in Cav-1-enriched intracellular vesicles. Most interestingly, following agonist administration to induce internalization, little additional 5-HT2A receptor internalization was induced in cells co-expressing Cav-1 (Fig. 1, D–F; see Fig. 1G for quantification).

We next performed co-immunoprecipitation studies in HEK-293 cells co-transfected with FLAG-5-HT2A and Cav-1 to determine whether 5-HT2A receptors and Cav-1 interact. As can be

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**Caveolin-1 Associates with and Regulates 5-HT2A Receptors**

C6 glioma cells were transfected with pRNA-U6.1/Neo-RNAi-Cav-1 vector, and clones were stably selected (see “Experimental Procedures” for details). A, representative immunoblot (IB) of five clonally isolated cell lines screened for the presence of Cav-1 (equal amount of protein was loaded in duplicates). B, representative immunoblot for RNAi clone showing knockdown of protein Cav-1 with respect to wt C6 glioma cell lysates. Samples were loaded in duplicate and also probed for total Gαs and RSK1 to assess nonspecific knockdown of unrelated proteins. C, representative immunoblot for RNAi clone showing knockdown of protein Cav-2 with respect to wt C6 glioma cell lysates. Equal amounts of protein were loaded in duplicate and, as a control, probed for total Gαs in the samples.

**FIG. 4.** RNAi-mediated knockdown of Cav-1. For these experiments C6 glioma cells were transfected with pRNA-U6.1/Neo-RNAi-Cav-1 vector, and clones were stably selected (see “Experimental Procedures” for details). A, representative immunoblot (IB) of five clonally isolated cell lines screened for the presence of Cav-1 (equal amount of protein was loaded in duplicates). B, representative immunoblot for RNAi clone showing knockdown of protein Cav-1 with respect to wt C6 glioma cell lysates. Samples were loaded in duplicate and also probed for total Gαs and RSK1 to assess nonspecific knockdown of unrelated proteins. C, representative immunoblot for RNAi clone showing knockdown of protein Cav-2 with respect to wt C6 glioma cell lysates. Equal amounts of protein were loaded in duplicate and, as a control, probed for total Gαs in the samples.
seen, FLAG-tagged 5-HT\textsubscript{2A} receptors were co-immunoprecipitated robustly with Cav-1 (Fig. 2, 9th to 12th lanes), indicating that 5-HT\textsubscript{2A} receptors and Cav-1 associate with each other and are present in a complex in vitro. To establish the specificity of the interaction, we showed that cells co-transfected with Cav-1 and empty vector yielded minimal pull-down of Cav-1 (Fig. 2, 3rd and 4th lanes). Furthermore, no Cav-1 was pulled down from untransfected cells (Fig. 2, 1st and 2nd lanes) or from cells co-expressing 5-HT\textsubscript{2A} receptors and GFP (Fig. 2, 5th and 6th lanes). The interaction of FLAG-5-HT\textsubscript{2A} and Cav-1 was unaf-
fects by agonist exposure (Fig. 2, compare 9th to 12th lanes). These findings demonstrated that Cav-1 forms a complex with 5-HT2A receptors in an agonist-independent fashion.

We also examined whether 5-HT2A receptors and Cav-1 are associated in a milieu in which both 5-HT2A receptors and Cav-1 are endogenously expressed. Several cell lines reported to express 5-HT2A receptors were tested including an aortic smooth muscle cell line (A7R5 (30)), NIH 3T3 cells (31), and C6 glioma cells (13). As controls, we also examined HEK-293 cells, which express little Cav-1, and GF-62 cells, which we have demonstrated previously (21) express little Cav-1. We found that C6 glioma cells expressed the highest levels of Cav-1, and these were therefore chosen for further study.

Co-immunoprecipitation studies were then performed in C6 glioma cells and rat brain synaptic membrane preparations wherein endogenous Cav-1 was immunoprecipitated, and the immunoprecipitates were probed for endogenous 5-HT2A-like immunoreactivity. Fig. 3B shows that 5-HT2A receptors were co-immunoprecipitated with Cav-1 in C6 glioma cells, and Fig. 3C shows co-immunoprecipitation of native 5-HT2A receptors by Cav-1 from solubilized rat brain synaptic membrane preparations. As controls to verify the specificity of the interaction, 5-HT2A receptors were immunoprecipitated with protein-A/G beads conjugated to agarose in the absence (−) and/or presence of a monoclonal Cav-1 antibody (+). Fig. 3B, top panel, 1st lane, shows no immunoreactivity to 5-HT2A receptor antibody, and the 2nd lane shows that 5-HT2A receptors were co-immunoprecipitated with Cav-1. The 2nd panel from the top of Fig. 3B shows the 1st lane with minimal Cav-1 immunoreactivity (e.g. negative control lane), and the 2nd lane shows a large amount of Cav-1 was detected when Cav-1 was immunoprecipitated (e.g. positive control lane). The 3rd panel from the top of Fig. 3B shows representative immunoblots from cell lysates to detect total Cav-1. The bottom panel of Fig. 3B shows 5-HT2A-like immunoreactivity in C6 glioma lysates.

In rat brain membrane preparations 5-HT2A receptors were immunoprecipitated with protein-A/G beads conjugated to agarose without (−) or with a monoclonal Cav-1 antibody (+). As shown in Fig. 3C, top panel, 1st lane (−) shows no immunoreactivity to 5-HT2A receptor antibody, and the 2nd lane (+) shows that 5-HT2A receptors were co-immunoprecipitated with Cav-1. The 2nd panel from the top of Fig. 3C shows the 1st lane wherein minimal Cav-1 immunoreactivity to polyclonal Cav-1 antibody was detected in the negative control, and the 2nd lane shows that Cav-1 in the immunoprecipitate was detected (e.g. positive control). The 3rd panel from the top of Fig. 3C represents the immunoblots from the cell lysates to detect endogenous Cav-1. The bottom panel of Fig. 3C represents the immunoblot from rat brain membrane preparation to verify the expression of 5-HT2A receptors. Taken together, these results indicate that 5-HT2A receptors are complexed with Cav-1 in both transfected cells and in their native milieu.

RNAi-mediated Knockdown of Cav-1 in C6 Glioma Cells—We next examined whether Cav-1 functions as a physiological modulator of 5-HT2A signaling by stably suppressing the expression of Cav-1 in C6 glioma cells using RNAi-mediated gene silencing. We clonally isolated 48 separate C6 lines that survived selection, and we quantified the level of relative Cav-1 expression by Western blot analysis. Shown in Fig. 4A are representative results from five lines screened for Cav-1 expression. Fig. 4B shows representative results from one of the surviving lines, clone 2 of the several screened for Cav-1 expression. All the signaling studies were subsequently carried out with clone 2. The Cav-1 knockdown C6 glioma cells appeared to be morphologically similar to wild-type C6 cells, although they had a slower doubling time than wt C6 cells (data not shown). In our initial studies we examined the relative expression of two unrelated proteins, Goα and ribosomal S6 kinase-1 (RSK1), to determine the specificity of the RNAi-mediated knockdown of Cav-1. We also examined the expression of Cav-2, another member of caveolin family of proteins. As can be seen in Fig. 4B, both the parental and the RNAi-Cav-1 lines expressed similar amounts of Goα and RSK1. However, as shown in Fig. 4C there is a significant down-regulation in the expression of protein Cav-2 with no change in Goα protein levels suggesting that the expression of Cav-2 protein is regulated by Cav-1, as reported previously (33).

RNAi-mediated Knockdown of Cav-1 Profoundly Suppresses 5-HT2A-mediated Signaling—We next examined 5-HT2A-mediated signal transduction in wt and Cav-1 knockdown C6 cells. As shown in Fig. 5A, 5-HT2A-mediated intracellular Ca2+ mobilization was nearly abolished in Cav-1 knockdown C6 cells. To determine whether this represented a generalized effect on Goα-mediated signaling, we performed a cDNA microarray experiment to identify other Goα-coupled GPCRs that are endogenously expressed in C6 cells. As shown in Table I and in the Supplemental Material, several other GPCRs were expressed, including the P2Y purinergic receptor and the coagulation factor II receptor (PAR-1), both of which are coupled to Goα. Fig. 5B shows that the ATP-induced Ca2+ transients induced by P2Y purinergic receptor activation were also greatly attenuated in Cav-1 knockdown C6 cells. However, the TRAP-induced Ca2+ flux mediated by PAR-1 was unaffected (Fig. 5C). Estimates of agonist potency (EC50) and efficacy (Emax) values revealed that Cav-1 knockdown altered the efficacy without significantly altering potency (Table II). As a control, we also examined the response induced by the calcium ionophore A23187 response (Fig. 5D). These results indicate that Cav-1 modulates signaling of some but not all Goα-coupled receptors. The results also imply that Cav-1 knockdown does not induce a generalized dysfunction of Goα-mediated signaling.

RNAi-mediated Knockdown of Cav-1 Does Not Alter 5-HT2A Receptor Binding or Expression—To address the possibility that the impairment in 5-HT2A receptor signaling was a consequence of decreased 5-HT2A receptor expression in Cav-1 knockdown cells, we concentrated the receptor protein by using WGA-agarose and subjected the eluate to immunoblot analysis in order to estimate receptor protein expression in Cav-1 knockdown cells versus wt C6 glioma cells. Total cell lysates were also probed for Goα as a loading control. Fig. 6A shows a representative immunoblot of 5-HT2A expression in wt C6 cells.
and Cav-1 knockdown cells; as can be seen the Cav-1 knockdown cells expressed slightly more 5-HT$_{2A}$-like immunoreactivity as assessed by Western blot analysis. We also performed radioligand binding assays using [3H]ketanserin, a selective 5-HT$_{2A}$ radioligand in both sets of cells. As shown in Table II there was no significant change in maximal binding potential of 5-HT$_{2A}$ receptor in wt C6 cells and Cav-1 knockdown C6 cells (p value > 0.05) or affinity of 5-HT$_{2A}$ receptors for ketanserin. These data demonstrate that the impairment in signaling of Cav-1 there is a significant increase of G$_q$.q-coupled receptors (32). In the absence of 5-HT$_{2A}$ receptors, overexpression of Cav-1 modestly but significantly attenuated the G$_q$.q*-stimulated inositol phosphate accumulation when compared with GFP alone (p < 0.05; Fig. 8A). It has been reported previously that the association of Cav-1 with G-proteins invariably attenuates their activity. Most importantly, Cav-1 did not cause any change in the relative expression of G$_q$.q* (Fig. 8B). These findings support the hypothesis that Cav-1 modulates intracellular signaling at the level of receptor-effector coupling and not via a receptor-independent attenuation of G$_q$.q signaling.

**Caveolin-1 Potentiates the Interaction between G$_q$ and 5-HT$_{2A}$ Receptors**—To investigate further the role of Cav-1 in modulation of 5-HT$_{2A}$ receptor signaling, we performed immunoprecipitation studies in HEK-293 cells co-transfected with 5-HT$_{2A}$ receptors and G$_q$.q in the presence and absence of Cav-1. Forty-eight hours post-transfection cells were serum-starved for 18 h and exposed to agonist for various times. Cells were then lysed, and 5-HT$_{2A}$ receptors were immunoprecipitated as described earlier (see “Experimental Procedures”), and G$_q$.q was detected in the immunoprecipitates using anti-G$_q$.q antibody (Fig. 9A). Immunoblot analysis showed that in presence of Cav-1 there is a significant increase of G$_q$.q detected in the immunoprecipitates under conditions of no agonist and at 2 min after agonist exposure (Fig. 9A compare the 4th and 5th with the 7th and 8th lanes). Fig. 9B shows the quantitative analysis of the net pixel intensities of bands from three independent experiments normalized to the total G$_q$.q in the lysate. The data show a significant increase in G$_q$.q in the immunoprecipitates in presence of Cav-1. These findings imply that Cav-1 plays a major role in modulating the signaling of 5-HT$_{2A}$ receptors by promoting a functional interaction between 5-HT$_{2A}$ receptors and G$_q$.q.
DISCUSSION

The major findings of the present work are that Cav-1 associates with 5-HT2A receptors in vivo and in vitro, promotes the association of 5-HT2A receptors with Gq/H1251q, and that the RNAi-mediated knockdown of Cav-1 profoundly impairs signaling of 5-HT2A receptors and selected Gq/H1251q-coupled GPCRs. Caveolin-1 has thus emerged as a novel modulator of 5-HT2A receptor signaling. We also demonstrate that the RNAi-mediated knockdown of Cav-1 expression in C6 glioma cells profoundly impairs signaling for extracellular ATP acting on P2Y purinergic receptors without altering PAR-1 thrombin receptor-mediated signaling. Taken together, these results imply that Cav-1 plays a major role in modulating the signal transduction of selected Gq/H1251q-coupled GPCRs. We also show that the impairment of signaling in Cav-1 knockdown cells was not a consequence of altered receptor or Gq/H1251q protein content. Additionally, our studies with a constitutively active Gq/H1251q imply that Cav-1 does not directly potentiate Gq/H1251q signaling. Instead, our results suggest that Cav-1 facilitates functional interactions between Gq/H1251q and selected GPCRs.

Prior studies by Razani et al. (33) in Cav-1 null mouse fibroblasts have shown that in the absence of Cav-1 there is a drastic reduction in Cav-2 levels because Cav-2 is not targeted to the membrane and is subsequently degraded intracellularly. In another study, an antisense strategy used to deplete Cav-1 in NIH 3T3 cells had no effect on expression of Cav-2 (34). In our present study we report that the stable knockdown of Cav-1 had no significant effect on the mRNA levels of Cav-2; Cav-3 was not measured because it is not expressed in C6 glioma cells (microarray data not shown). However, we report a significant reduction in Cav-2 protein expression suggesting that Cav-2 protein is degraded in Cav-1 knockdown C6 glioma cells, as...
Caveolin-1 Associates with and Regulates 5-HT2A Receptors

A

Cav-1-myc
FLAG-5-HT2A
GFP
Gqα

IP
IB

0
2
5
2

5-HT (min)

B

Fig. 9. Cav-1 potentiates 5-HT2A receptor interactions with Gqα in absence of agonist. For these experiments, Gqα was co-transfected with 5-HT2A receptors and either GFP or Cav-1 in HEK-293 cells. Cells were put in serum-free media for a minimum of 18 h, and then exposed to vehicle (0) or 10 μM 5-HT for 2 and 5 min. Lysates were prepared and subjected to immunoprecipitation (IP) using M2 FLAG preconjugated agarose ("Experimental Procedures"). A, shown is a representative immunoblot (IB) from a single experiment that has been replicated three times with equivalent results. B, quantification of the net pixel intensities of Gqα in the immunoprecipitates normalized to total Gqα. Each set was then normalized to the maximum in each set. Result shown here are the means ± S.E. from all three experiments.

shown previously (33) for mouse embryonic fibroblasts from Cav-1 knockout mice.

Cav-1 is an integral membrane protein, which associates with numerous lipid-modified signaling molecules including Ha-Ras, c-Src, and endothelial nitric-oxide synthase, and almost invariably attenuates the activity of the signaling molecule when overexpressed (35, 36). Additionally, Cav-1 directly interacts with the EGF receptor and inhibits its activity (37), and such an interaction leads to internalization of EGF receptor via caveolar domains (38). More recent studies have shown that transcriptional up-regulation of Cav-1 in senescent cells attenuates EGF-mediated signaling thus implicating the role of Cav-1 in EGF receptor-mediated signaling and the general unresponsiveness of senescent cells to growth stimuli (39). The ability of Cav-1 to regulate oncogenes and the downstream signaling cascades has been observed in many overexpression studies wherein Cav-1 is a potent inhibitor of the Ras-p42/44 MAP kinase cascade (40). On the other hand antisense-mediated down-regulation of Cav-1 leads to hyperactivation of the p42/44 MAP kinase cascade (34). These studies suggest that Cav-1 is a modulator of multiple signaling cascades. In this regard, we found that Cav-1 knockdown in C6 glioma cells significantly elevates basal p42/44 ERK phosphorylation and impairs 5-HT2A agonist-mediated p42/44 ERK phosphorylation. Thus, Cav-1 knockdown appears to induce a dysregulation of GPCR-mediated p42/44 ERK phosphorylation.

Our results are especially intriguing in light of a recent study that has indirectly implicated caveolae in 5-HT2A-mediated signal transduction (22) in vascular smooth muscle cells. In those studies, the authors reported that 5-HT2A receptors were enriched in "lipid rafts" isolated by sucrose density gradients. We have also found that a small fraction of 5-HT2A receptors is located in the caveolar fraction when Cav-1 is overexpressed in HEK-293 cells and in C6 glioma cells where both proteins are endogenously expressed. The localization of other GPCRs such as β2-adrenergic receptors (41) and β2- and β3-adrenergic receptors in the caveolar fraction has been reported previously (42), where the role of caveolae has been implicated as trafficking centers where GPCRs translocate into or out of the caveolae. Our findings that the effect of Cav-1 knockdown is seen with at least one other Gqα-coupled GPCR family (P2Y purinergic receptors) and not with PAR-1 implies that Cav-1 selectively modulates Gqα-coupled receptor signaling, perhaps by promoting association with Gqα.

Recent in vivo studies with Cav-1 knockout mice (19) suggest an indispensable role of Cav-1 in normal life span and cardiac, pulmonary, and vascular functioning (43, 44). Our studies, wherein Cav-1 is knocked down, revealed that in the absence of Cav-1 the signalings of 5-HT2A and P2Y receptors were nearly totally abolished. Because 5-HT2A receptors represent the principal vascular smooth muscle 5-HT receptor and a main pulmonary and cardiac 5-HT receptor, and because purinergic receptors are ubiquitously expressed, our results suggest that the profound cardiovascular phenotype found in Cav-1 knockout mice may result, in part, from impaired serotonergic and purinergic signaling.

A preliminary microarray analysis of Cav-1 knockdown C6 glioma cells shows that the Cav-1 knockdown cells exhibited no global changes in gene expression compared with the parental cells, although Cav-1 but not Cav-2 mRNA was significantly diminished (not shown). Most important, mRNA levels for 5-HT2A serotonin receptors and PAR-1 receptors remained unchanged along with the relative mRNA levels for all of the various proteins involved in Gqα-mediated signaling (not shown). However, the mRNA encoding the P2Y2 purinergic receptors was absent in Cav-1 knockdown cells and present in wt C6 cells. Most intriguingly, for another Gqα-coupled P2Y receptor subtype (P2Y5), the steady-state mRNA levels remain unchanged in parental and Cav-1 knockdown cells (not shown). The selective knockdown of P2Y2 likely accounts for at least some of the decreased response to ATP seen in the Cav-1 knockdown cells. The precise role of Cav-1 in selectively regulating the expression of a single subtype of P2Y family receptors (e.g. P2Y2 and not P2Y5) will require further study.

In summary we have discovered a novel role for Cav-1 in regulating the activity of serotonergic and purinergic receptors but not thrombinergic Gqα-coupled receptors. Our results indicate that Cav-1 associates with the 5-HT2A receptors in vitro (HEK-293 and C6 cells) and in vivo (rat brain synaptic membranes) and that this interaction has profound functional significance. Given the widespread distribution of serotonergic receptors (e.g. platelets, gastrointestinal smooth muscle, uterine smooth muscle, kidneys, the cardiovascular system, and the brain) and the correspondingly ubiquitous distribution of Cav-1, it is likely that the 5-HT2A-Cav1 interactions represent a functionally significant protein-protein interaction of potentially profound biological significance.

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