G protein-coupled Receptor 30 (GPR30) Forms a Plasma Membrane Complex with Membrane-associated Guanylate Kinases (MAGUKs) and Protein Kinase A-anchoring Protein 5 (AKAP5) That Constitutively Inhibits cAMP Production*

Received for publication, March 19, 2014, and in revised form, June 19, 2014. Published, JBC Papers in Press, June 24, 2014, DOI 10.1074/jbc.M114.566893

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Background: GPR30 plays important roles in cardiometabolic regulation and cancer.

Results: GPR30 forms a complex with a MAGUK and AKAP5 that constitutively inhibits cAMP production independently of Gs and Gi/o and retains receptors in the plasma membrane.

Conclusion: The GPR30-MAGUK-AKAP5 complex mediates receptor signaling.

Significance: These results present a new mechanism by which a receptor inhibits cAMP production.

GPR30, or G protein-coupled estrogen receptor, is a G protein-coupled receptor reported to bind 17β-estradiol (E2), couple to the G proteins Gs and Gi/o, and mediate non-genomic estrogenic responses. However, controversies exist regarding the receptor pharmacological profile, effector coupling, and subcellular localization. We addressed the role of the type I PDZ motif at the receptor C terminus in receptor trafficking and coupling to cAMP production in HEK293 cells and CHO cells ectopically expressing the receptor and in Madin-Darby canine kidney cells expressing the native receptor. GPR30 was localized both intracellularly and in the plasma membrane and subject to limited basal endocytosis. E2 and G-1, reported GPR30 agonists, neither stimulated nor inhibited cAMP production through GPR30, nor did they influence receptor localization. Instead, GPR30 constitutively inhibited cAMP production stimulated by a heterologous agonist independently of Gs and Gi/o receptors. siRNA knockdown of native GPR30 increased cAMP production. Deletion of the receptor PDZ motif interfered with inhibition of cAMP production and increased basal receptor endocytosis. GPR30 interacted with membrane-associated guanylate kinases, including SAP97 and PSD-95, and protein kinase A-anchoring protein (AKAP) 5 in the plasma membrane in a PDZ-dependent manner. Knockdown of AKAP5 or St-Ht31 treatment, to disrupt AKAP interaction with the PKA RIIβ regulatory subunit, decreased inhibition of cAMP production, and St-Ht31 increased basal receptor endocytosis. Therefore, GPR30 forms a plasma membrane complex with a membrane-associated guanylate kinase and AKAP5, which constitutively attenuates cAMP production in response to heterologous agonists independently of Gi/o and retains receptors in the plasma membrane.

GPR30 is a G protein-coupled receptor (GPCR) that is currently attracting considerable attention for important roles in cardiometabolic regulation and cancer. The receptor was named G protein-coupled estrogen receptor following reports that it binds 17β-estradiol (E2) with high affinity (1, 2) and mediates non-genomic estrogenic responses via the G proteins Gs (3) and Gi/o in vitro (1–5). However, the receptor pharmacological profile, effector coupling, and subcellular localization are controversial (6–10), indicating that a number of receptor details are missing.

PDZ domains are protein-protein recognition modules present in some proteins that bind C-terminal short, linear sequences that may be divided into three types, including type I (X(-S/T)-X-Ø), type II (X-Ø-X-Ø), and type III (X(D/E)-X-Ø) (11). Some GPCRs contain PDZ motifs at their C termini that regulate receptor signaling and trafficking (12). GPR30 contains a conserved C-terminal canonical type I PDZ motif, -SSAV, and this motif has been shown recently to interact with postsynaptic density 95 (PSD-95) and to be important for receptor plasma membrane localization (13). PSD-95 is a neuronal protein and the most studied member of the membrane-associated guanylate kinase (MAGUK) family of PDZ domain proteins. These proteins serve as scaffolds to organize events in signal transduction, cell adhesion, and membrane trafficking at specialized cell-cell junctions (14). Several MAGUKs also interact via a unique domain with protein kinase A-anchoring protein (AKAP) 79/150 or AKAP5 (15, 16), which is known to interact with the RII regulatory subunit of PKA (17). Furthermore, AKAP5-RII interacts with, phosphorylates, and inhibits adenylate cyclase (AC) (17, 18). Therefore, AKAP5 exists in complexes that compartmentalize the regulation of cAMP production and signaling.

The abbreviations used are: GPCR, G protein-coupled receptor; MAGUK, membrane-associated guanylate kinase; AKAP, A kinase-anchoring protein; AC, adenylate cyclase; MDCK, Madin-Darby canine kidney; β1AR, β1 adrenergic receptor; RIA, radioimmunoassay; FSK, forskolin; PGE2, prostaglandin E2; ISO, isoproterenol; Ab, antibody.
Here we investigated the role of the GPR30 PDZ motif in receptor plasma membrane localization and regulation of cAMP production and addressed the involvement of MAGUKs and AKAP5 in these events. Our results show that GPR30 forms a PDZ motif-dependent plasma membrane complex with a MAGUK and AKAP5 that constitutively inhibits cAMP production independently of Gi/o and retains the receptor in the plasma membrane.

EXPERIMENTAL PROCEDURES

Cell Culture and DNA Constructs—HEK293 cells, MDCK cells, and CHO-K1 cells (ATCC) were grown in phenol red-free DMEM supplemented with 10% FBS in 5% or 10% CO2 at 37 °C. In some experiments, FBS was replaced with charcoal-stripped FBS. N-terminally FLAG- and HA-tagged human GPR30 cDNA in pcDNA3.1 were made as described previously (19). A GPR30 cDNA construct, in which the four C-terminal residues in GPR30 (GYSA) were deleted (GPR30∆H9004SSAV), was produced by PCR. Human FLAG-tagged PSD-95, human FLAG-tagged β1-adrenergic receptor (β1AR), and PKA RIIβ-GFP cDNAs were obtained from Addgene (Cambridge, MA). Canine GPR30-specific and scrambled siRNAs were obtained from Eurofins MWG Operon (Edelsberg, Germany), and human AKAP5-specific and scrambled shRNAs in pcDNA6.1 vectors were obtained from Dr. S. Bahouth (University of Tennessee Health Science Center, Memphis, TN).

TransIT-LT1 (Mirus Bio LLC, Madison, WI) was used to transiently transfect HEK293 cells and MDCK-Cre cells, and a nucleofection protocol (Amaxa Inc., Gaithersburg, MD) was used to transiently transfect HEK293 cells and CHO-K1 cells. Cells transiently transfected with a plasmid-containing receptor construct were always compared with cells transfected with empty plasmid alone (mock). HEK293 cells stably expressing FLAG-tagged mouse GPR30 were generated and maintained as described previously (19). MDCK cells were transfected with the p6CRE/luc vector using Lipofectamine reagent (Invitrogen). Single colonies were chosen and propagated in the presence of selection-containing medium (medium plus hygromycin B) to generate a clonal stable cell line (MDCK-Cre).

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were done as described previously (19). Proteins were immunoprecipitated with mouse M2 FLAG antibody-agarose (Sigma-Aldrich, St. Louis, MO) and goat GPR30 antibody (R&D Systems, Minneapolis, MN), pan-MAGUK antibody (Merck Millipore, Billerica, MA), AKAP5 antibody (BD Biosciences), and SAP97 antibody (Santa Cruz Biotechnology) coupled to protein G-Sepharose (GE Healthcare). Proteins were immunoblotted with mouse M2 FLAG
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**FIGURE 2. Constitutive GPR30 inhibition of cAMP production requires the receptor C-terminal PDZ motif.** A, schematic of the GPR30 structure indicating the C-terminal PDZ motif and N-terminal N-glycosylation sites. B, HEK cells transfected with GPR30 or GPR30ΔSSAV were immunoprecipitated (IP) with M2 FLAG antibody (Ab) beads, treated without (−) and with (+) PNGase, and immunoblotted (IB) with M2 FLAG Ab. Molecular weight (M.) standards (arrows on the left) and major receptor species, as described by Sanden et al. (19), are indicated (arrows on the right), and the results are representative of experiments performed at least three times. C, HEK cells transfected without (Mock) and with GPR30 or GPR30ΔSSAV were treated with 1 μM FSK, and cAMP production was measured in real time with the GloSensor assay. D, CHO cells transfected without and with GPR30 or GPR30ΔSSAV were treated with 1 μM PGE2 or 1 μM FSK, and cAMP production was measured with RIA. E, MDCK-Cre cells transfected without and with GPR30 or GPR30ΔSSAV were treated with 1 μM FSK, and cAMP production was measured with the Cre promoter reporter assay. F, HEK cells transfected without and with GPR30 or β1AR treated with 1 μM FSK or 10 μM ISO, and cAMP production was measured in real time with the GloSensor assay. G, HEK cells transfected with β1AR without and with GPR30 or GPR30ΔSSAV were treated with 0.1 μM dobutamine, and cAMP production was measured in real time with the GloSensor assay. C–E, the values are mean ± S.E. of at least three independent experiments, with each data point performed in at least triplicates. F and G, the results are representative of at least three independent experiments, with each data point being the mean ± S.E. of 16 measurements. E–G, the results are shown as relative light units (RLU). ***, p < 0.01; ***, p < 0.001; ns, not significant.

antibody (Sigma-Aldrich, 1:1000), goat GPR30 antibody (1:200), pan-MAGUK antibody (1:2000), SAP97 antibody (1:200), and AKAP5 antibody (1:1000).

**Enzymatic Deglycosylation**—Deglycosylation was done by treating immunoprecipitates with 500 units PNGase F (New England Biolabs, Ipswich, MA) in 10 mM Tris-HCl (pH 7.4) for 2 h at 37 °C.

**Immunofluorescence Microscopy**—Immunofluorescence microscopy of HEK293 cells was done as described previously (19, 20). In live antibody staining, we took advantage of the fact that mouse M1 FLAG antibody (Sigma-Aldrich, 1:500) and goat GPR30 antibody (1:100) specifically label the receptor extracellular N-terminal FLAG epitope and N-terminal domain, respectively. Therefore, “feeding” live cells with these antibodies for 30 min at 37 °C monitored exclusively cell surface receptor-antibody complexes and complexes that had undergone endocytosis. Cells were then fixed and permeabilized. In fixed staining, to monitor total cellular receptors, cells were fixed and permeabilized and then incubated with mouse M1 FLAG antibody (1:500) or goat GPR30 antibody (1:100) for 1 h at 22 °C. In all experiments, receptors were then visualized by incubating fixed cells with secondary Alexa Fluor 488-labeled goat anti-bodies or mouse IgG2b antibodies (Invitrogen). For colocalization, fixed and permeabilized cells were also incubated with rabbit early endosomal antigen 1 (EEA1) antibody (Sigma-Aldrich, 1:200), pan-MAGUK antibody (1:2000), and AKAP5 antibody (1:1000) and then with secondary Alexa Fluor 568-labeled anti-mouse IgG1 or anti-rabbit antibodies (Invitrogen). DAPI was used for nuclear staining. Images were collected using a Nikon Eclipse confocal fluorescence microscope.

**Flow Cytometry**—Resuspended HEK293 cells were incubated with mouse anti-M1 FLAG antibody (1:200) or mouse IgG (DAKO, Glostrup, Denmark) for 20 min with or without 0.1% saponin/PBS (Sigma-Aldrich) at room temperature to detect the intracellular and cell surface expression of receptors, respectively. Cells were then washed with PBS with Ca²⁺/Mg²⁺ and resuspended in PBS with phycoerythrin-labeled goat anti-mouse antibody (DAKO, 1:2000) as a secondary antibody, with or without 0.1% saponin/PBS for 20 min at room temperature in the dark. The cells were then washed with PBS, centrifuged at 2000 × g for 5 min, and then the pellet was resuspended in PBS and analyzed directly by flow cytometry. The specificity of the secondary antibody was tested by omitting the primary antibody. The cells were analyzed using a BD FACSCanto.
cytometer and FACSDiva software (BD Biosciences). Forward and side scatter measurements were attained with gain settings in linear mode. In all experiments, binding was calculated after subtracting the background fluorescence of the control antibody.

**cAMP Production**—Accumulation of cAMP in CHO-K1 cells and HEK293 cells was measured by radioimmunoassay (RIA) as described previously by us (21). Briefly, cells in 24-well plates were washed twice with Hanks’ balanced salt solution containing 20 mM HEPES (pH 7.4). Cells were then incubated with the phosphodiesterase inhibitor rolipram (25 μM) with or without stimulus for 15 min. Incubations were terminated by aspiration and addition of 500 μl of ice-cold absolute ethanol. The ethanol extracts from individual wells were then dried under a gentle air stream and reconstituted in 100 μl of 50 mM sodium acetate (pH 6.2). The cAMP content of each 100-μl sample was determined by RIA.

Production of cAMP in HEK293 cells was also measured using the GloSensor cAMP assay according to the instructions of the manufacturer (Promega, Madison, WI). Briefly, cells in 96-well plates (20,000 cells/well) were incubated with the GloSensor cAMP reagent. Following addition of the stimulus, cAMP production was measured as luminescence.

**Cre Promoter Activity**—Cre activity was measured in MDCK-Cre cells. Cells in 96-well plates (20,000 cells/well) were grown in medium overnight and then in serum-free medium for ~20 h. After washing in the same medium, cells were incubated with 25 μM rolipram for 5 min, after which various stimuli were added for 22 h. Incubations were terminated by aspiration and addition of 10 μl/well reporter lysis buffer (Promega). Follow-
riginating addition of 35 μl/well luciferin reagent (Biothema, Handen, Sweden) and ATP, Cre promoter activity was measured as luminescence.

Data Analysis—Data are presented as mean ± S.E. Where appropriate, paired analysis with Student’s t test or one-way analysis of variance with Bonferroni’s post hoc test was used for statistical comparisons. p < 0.05 was regarded as statistically significant. Data analysis was performed using the Prizm program (GraphPad, La Jolla, CA).

RESULTS

GPR30 Constitutively Inhibits cAMP Production—To investigate whether GPR30 regulates cAMP production, we transiently expressed N-terminally FLAG-tagged human GPR30 (GPR30) in HEK293 cells and CHO cells, two well described model systems. First, we assessed GPR30-dependent effects by treating HEK293 cells with the proposed GPR30 agonists E2 (0.1 μM) and G-1 (1 μM) (22). Neither agonist increased cAMP production (Fig. 1A) nor decreased cAMP production stimulated by forskolin (FSK) (1 μM) (Fig. 1B) in either GPR30- or mock-transfected cells. The same results were obtained with CHO cells transiently expressing the receptor (data not shown).

Next, we took an unbiased approach and assessed whether GPR30 regulated cAMP production constitutively, i.e. in the absence of agonist. To do so, cAMP production in cells expressing the receptor was compared with that in mock-transfected cells. GPR30 expression had no effect on basal cAMP production in either HEK293 cells or CHO cells (data not shown). On the other hand, GPR30 drastically decreased FSK-stimulated cAMP production, as determined in HEK293 cells (Fig. 1C). The inhibition was not caused by a serum-derived factor because the same effect was observed after growing cells in phenol red-free and charcoal-stripped serum or keeping them in serum-free medium for 24 h (data not shown). GPR30 also decreased both prostaglandin E2 (PGE2)- and FSK-stimulated cAMP production in CHO cells (Fig. 1D). Consistent with these observations, siRNA knockdown of native GPR30 in MDCK cells stably expressing a p6CRE/luc construct (MDCK-Cre) significantly increased FSK-stimulated Cre promoter activity (Fig. 1E). Pretreatment of CHO cells expressing GPR30 with 100 ng/ml pertussis toxin for 24 h had no effect on GPR30 inhibition of cAMP production, whereas pertussis toxin reversed the inhibitory effect of 100 nM 5-carboxyamidotryptamine through endogenous G(i)-coupled 5-HT1B receptors (Fig. 1F), as reported previously (21). Therefore, GPR30 constitutively inhibits cAMP production independently of G(i/o).

Constitutive GPR30 Inhibition of cAMP Production Requires the Receptor C-terminal PDZ Motif—To address the functional role of the type I PDZ binding motif (-SSAV) at the receptor C terminus (Fig. 2A), an N-terminally FLAG-tagged receptor construct lacking this motif (GPR30ΔSSAV) was expressed and compared with GPR30. Both constructs expressed equally well with the same peptide profile in HEK293 cells (Fig. 2B, lanes 1 and 3). Furthermore, the constructs exhibited the same unique peptide profile as that observed previously with the mouse receptor following receptor N-deglycosylation with PNGase (Fig. 2B, lanes 2 and 4) (19).

Interestingly, deletion of the PDZ motif in GPR30 blunted the ability of this receptor to inhibit cAMP production, regardless of whether cAMP production had been increased with FSK in HEK293 cells (Fig. 2C), FSK or PGE2 in CHO cells (Fig. 2D),
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or FSK in MDCK-Cre cells (Fig. 2E). Furthermore, deletion of the PDZ motif did not introduce either E2- or G-1 stimulation or inhibition of cAMP production (data not shown). In contrast to GPR30, FLAG-tagged β1AR, a G1-coupled GPCR that also contains a C-terminal type 1 PDZ motif (-ESKV), did not constitutively inhibit FSK-stimulated cAMP production in HEK293 cells (Fig. 2F). Furthermore, although GPR30 inhibited cAMP production in response to isoproterenol (ISO) stimulation of endogenous β2AR in these cells, β1AR enhanced the ISO response, as expected (Fig. 2F). Also, when GPR30 and β1AR were coexpressed, GPR30 inhibited cAMP production in response to the β1-selective agonist dobutamine, whereas GPR30ΔSSAV was less efficacious (Fig. 2G). Therefore, GPR30 constitutively inhibits cAMP production in a PDZ-dependent manner.

**GPR30 Requires the C-terminal PDZ Motif for Plasma Membrane Retention**—To determine the role of the GPR30 PDZ motif in receptor subcellular localization, HEK293 cells expressing GPR30 or GPR30ΔSSAV were stained with M1 FLAG antibodies or GPR30 antibodies, both directed toward extracellular N-terminal receptor epitopes, and then imaged by confocal immunofluorescence microscopy. Fixed staining, in which receptors were stained following cell fixation to monitor all cellular receptors, showed that GPR30 and GPR30ΔSSAV distributed similarly both intracellularly and in the plasma membrane (Fig. 3A, Fixed), and neither distribution was influenced by E2 or G-1 (data not shown). Live staining, in which live cells were fed primary antibody for 30 min at 37 °C prior to cell fixation to selectively monitor cell surface receptor-antibody complexes and complexes that had internalized during this time period, showed that GPR30 was present primarily in the plasma membrane with limited basal endocytosis (Fig. 3A, Live). On the other hand, live staining of GPR30ΔSSAV was primarily intracellular, indicating that, although this construct also reached the cell surface, it was subject to considerably higher basal endocytosis compared with GPR30 (Fig. 3A, Live). Flow cytometry analysis confirmed that the steady-state cell surface level of GPR30ΔSSAV was significantly lower than that of GPR30 (Fig. 3B). Therefore, the GPR30 PDZ motif is also important for retaining receptors in the plasma membrane.

Fig. 3C shows that the limited amount of internalized GPR30 observed following live staining partially colocalized with the early endosomal marker EEA1, indicating that it proceeds via typical receptor-mediated endocytosis. Interestingly, deletion of the PDZ motif increased colocalization with this marker, suggesting that this motif may also participate in postendocytic receptor sorting.

**The GPR30 C-terminal PDZ Motif Forms a Complex with MAGUKs and AKAP5**—Immunoblotting of HEK293 cell lysates shows that these cells express MAGUKs with which the GPR30 PDZ motif could potentially interact (Fig. 4A, lanes 1 and 2). Immunoprecipitation of mouse GPR30 from HEK293 cells stably expressing this receptor specifically coprecipitated a native MAGUK of about 110 kDa (Fig. 4A, lane 4). Transiently expressed human GPR30 coprecipitated a MAGUK of a slightly larger size (Fig. 4A, lane 6) that was not observed when expressing GPR30ΔSSAV (Fig. 4A, lane 7). The size of this MAGUK was very similar to that coprecipitated by human β1AR in these cells, which was reported to interact through its PDZ motif with SAP97 (Fig. 4A, lane 9) (23). Indeed, both human GPR30 and β1AR coprecipitated SAP97 from these cells (Fig. 4A, lanes 11 and 12). Although the prototypic neuronal MAGUK PSD-95 is not expressed in HEK293 cells (23), this protein also coprecipitated with GPR30 in a PDZ motif-dependent manner when coexpressed with the receptor (Fig. 4A, lanes 14 and 15). Consistent with the above results, immunoprecipitation of native MAGUKs and, specifically, SAP97 coprecipitated GPR30 (Fig. 4B, lanes 2 and 5) but not GPR30ΔSSAV (Fig. 4B, lanes 3 and 6). HEK293 cells also express AKAP5 (Fig. 4C, lanes 1 and 2), and GPR30 also co-precipitated this protein (Fig. 4C, lane 4),

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### Figure 5. The GPR30-MAGUK-AKAP5 complex is localized in the plasma membrane.

- **A.** HEK cells transfected with GPR30 were subjected to live staining (Live) with GPR30 antibodies. Following fixation and permeabilization, cells were stained with pan-MAGUK antibodies, AKAP5 antibodies, or DAPI. B. HEK cells transfected with GPR30 and PSD-95 were subjected to fixed staining (Fixed) and live staining (Live) with GPR30 antibodies. C. HEK cells transfected with GPR30ΔSSAV and PSD-95 were subjected to fixed staining and live staining with GPR30 antibodies. Following fixation and permeabilization, cells were stained with pan-MAGUK antibodies or DAPI. The results are representative of experiments performed at least three times, and the individual and merged images (Merge) were collected using a Nikon Eclipse confocal microscope, ×60 objective, and 50-μm zoom. The arrows indicate protein colocalization (yellow).
whereas GPR30ΔSSAV did not (Fig. 4C, lane 5). Therefore, GPR30 forms a PDZ-dependent complex with both a MAGUK and AKAP5.

We also assessed whether native GPR30 in MDCK cells (Fig. 4D, lanes 1 and 3) forms a complex with a native MAGUK and AKAP5. Indeed, immunoprecipitation of native MAGUKs and AKAP5 from these cells coprecipitated a small but significant amount of native GPR30 (Fig. 4D, lanes 4 and 5). Consistent with these results, immunoprecipitation of native GPR30 coprecipitated native MAGUKs (Fig. 4D, lanes 6 and 7). Therefore, both recombinant and native GPR30 form complexes with a MAGUK and AKAP5.

The GPR30-MAGUK-AKAP5 Complex Is Localized in the Plasma Membrane—To address the subcellular localization of the GPR30-MAGUK-AKAP5 complex, the proteins were imaged by confocal microscopy in HEK293 cells. Ectopically expressed GPR30 colocalized with native MAGUKs and AKAP5 specifically in the plasma membrane (Fig. 5A, arrows). To improve imaging of MAGUKs, GPR30 was coexpressed with PSD-95. Both fixed and live staining showed that GPR30 colocalized with MAGUK/PSD-95 exclusively in plasma membrane (Fig. 5B, arrows). In contrast, only limited, if any, colocalization was observed between GPR30ΔSSAV and MAGUK/PSD-95 following either fixed or live staining (Fig. 5C). Therefore, the GPR30-MAGUK-AKAP5 complex is localized in the plasma membrane. Consistent with these results, a PKA regulatory subunit IIβ (RIIβ)-GFP fusion protein (24) colocalized with GPR30 in the plasma membrane, whereas it did not colocalize with GPR30ΔSSAV (Fig. 6, arrows).

The GPR30-MAGUK-AKAP5 Complex Inhibits cAMP Production—We then investigated whether AKAP5 mediates GPR30 inhibition of cAMP production. To this end, HEK293 cells were nucleofected with PKA RIIβ-GFP (RIIβ-GFP) together with GPR30 or GPR30ΔSSAV were subjected to live staining with M1 FLAG antibodies. The results are representative of experiments performed at least three times. The individual and merged images (Merge) were collected using a Nikon Eclipse confocal microscope, ×60 objective, and 50-μm zoom. The arrows indicate cells coexpressing PKA RIIβ-GFP either with GPR30 or GPR30ΔSSAV.

The GPR30-MAGUK-AKAP5 Complex Inhibits cAMP Production—We then investigated whether AKAP5 mediates GPR30 inhibition of cAMP production. To this end, HEK293 cells were nucleofected with an AKAP5-specific shRNA vector to knock down the protein, as described previously (25), or with scrambled shRNA. AKAP5 shRNA completely prevented GPR30 inhibition of FSK- and PGE2-stimulated cAMP production 48 and 96 h post-nucleofection, respectively, whereas GPR30 inhibition still occurred with scrambled shRNA (Fig. 7A). AKAP5 shRNA had no effect on the response to either FSK or PGE2 in the absence of GPR30 (Fig. 7A). Therefore, AKAP5 mediates GPR30 inhibition of cAMP production. We then determined the effect of pretreating cells with St-Ht31 (Pro-mega), a stearated peptide that permeates the membrane and disrupts the interaction between AKAPs and PKA RII (26, 27). Consistent with the above results, 50 μM St-Ht31 attenuated GPR30 inhibition of FSK-stimulated cAMP production over that observed with 50 μM control peptide St-Ht31P (Fig. 7B and C). By contrast, St-Ht31 had no effect over that of St-Ht31P on basal or FSK-stimulated cAMP production in the absence of GPR30 (Fig. 7C, Mock).

The GPR30-MAGUK-AKAP5 Complex Retains GPR30 in the Plasma Membrane—To address whether AKAP also regulates GPR30 cell surface localization, live staining of GPR30 was done on HEK293 cells pretreated with St-Ht31 or the control peptide St-Ht31P. Treatment with 50 μM St-Ht31 increased basal receptor endocytosis, whereas treatment with St-Ht31P (50 μM) had no effect (Fig. 8A). These results show that AKAP-RII mediates the retention of GPR30 in the plasma membrane.
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DISCUSSION

Cyclic AMP is a central second messenger in cell signaling and physiology that is regulated by GPCRs through their coupling to $G_s$ and $G_{i/o}$, mediating stimulation and inhibition of AC, respectively. This study outlines a new receptor mechanism for inhibiting cAMP production (Fig. 9) where the GPR30 C-terminal type I PDZ motif enables the receptor to form a plasma membrane complex with a MAGUK and AKAP5. Through AKAP5, this complex constitutively inhibits cAMP production in response to a heterologous agonist (e.g. PGE2, ISO, and FSK) independently of $G_s$ and $G_o$, and retains the receptor in the plasma membrane. This is the first observation of a GPCR constitutively inhibiting cAMP production through such a protein complex, and, therefore, this presents a new mechanism of receptor coupling. Considering that this protein complex is capable of recruiting numerous signaling proteins, including protein kinases and phosphatases, this discovery opens up new opportunities to study receptor-regulated cAMP signaling in general as well as to resolve controversies currently surrounding GPR30 specifically.

St-Ht31 had no effect on basal $\beta_1$AR endocytosis (Fig. 8A), consistent with this receptor requiring AKAP5 for recycling following agonist stimulation (28).
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FIGURE 9. Model of GPR30-MAGUK-AKAP5-mediated inhibition of cAMP production. GPR30 (green) constitutively interacts via its C-terminal PDZ motif (SSAV) with one of the PDZ domains of a MAGUK (brown), and the MAGUK interacts with AKAP5 (blue). AKAP5, in turn, binds the PKA RII regulatory subunit (red). Agonist stimulation of a heterologous Gs-coupled GPCR (orange) stimulates (+) cAMP production, which leads to activation of AKAP5-RII in the GPR30 complex. This, in turn, leads to PKA phosphorylation and inhibition (−) of AC (yellow) and attenuation of cAMP production. AKAP5-RII also causes PKA phosphorylation of GPR30, which leads to inhibition (−) of basal receptor endocytosis and retention of the receptor in the plasma membrane.

We identified SAP97 as one endogenous MAGUK with which GPR30 interacts in a PDZ-dependent manner in HEK293 cells. Although PSD-95 is not expressed endogenously in HEK293 cells (23), we found that GPR30 also interacts with this MAGUK when coexpressed ectopically with GPR30 in these cells, as reported previously (13). Therefore, GPR30 is able to interact with more than one type of MAGUK. GPR30 adds to a growing list of GPCRs that form PDZ-dependent plasma membrane complexes with MAGUKs, including β1AR (23, 29, 30), β2AR (31), the 5HT2A receptor (32), the 5HT2C receptor (33), corticotropin-releasing factor receptor 1 (34), and the somatostatin subtype 1 receptor (35).

The GPR30-MAGUK complex also contains AKAP5. AKAPs constitute a family of proteins that share the ability to bind the PKA RII regulatory subunit (36). AKAP5 is also known to interact with SAP97 via an alternatively spliced polybasic sequence, termed i3 (37). Therefore, SAP97 potentially bridges the interaction of receptors containing type I PDZ motifs with AKAP5. Through such coupling, AKAP5 could bring PKA into close proximity with GPCRs and their effectors to regulate local cAMP signaling (17, 18). β1AR and β2AR are the only GPCR that have, so far, been shown to form such a complex, which interacts with AKAP5 via SAP97 and PSD-95 (23, 31). The ionotropic AMPA-type glutamate receptor subunit GluR1 also forms a PDZ motif-dependent complex with MAGUKs and AKAP5 (15, 16). Here we show that GPR30 is an additional GPCR with such coupling.

Deletion of the PDZ motif in GPR30 and treatment with St-Ht31 also increased basal receptor endocytosis. Again, the timeframe in which St-Ht31 caused the loss of membrane receptors suggests that this effect also involves protein-protein interaction and/or covalent modification. In β1AR, AKAP5-RII mediates agonist-promoted phosphorylation of the receptor at Ser-312 in the third intracellular loop, which is necessary for the receptor to enter a recycling pathway and resensitize the receptor following agonist-promoted internalization (28). A similar mechanism occurs with AMPA receptors where AKAP5-RII mediates phosphorylation of Ser-845 in GluR1, and this phosphorylation also favors recycling of the receptor following endocytosis (42, 43). GPR30 contains a PKA phosphorylation consensus motif at Ser-166 in the second intracellular loop. However, if GPR30 is a substrate for AKAP-RII, the consequence of such phosphorylation may be different from β1AR because St-Ht31 increased basal internalization of GPR30, whereas it had no effect on the basal localization of β1AR.

The GPR30 PDZ motif may also participate in postendocytic receptor sorting because deleting the motif apparently increased the amount of receptors colocalized with the endosomal marker EEA1. Cheng et al. (44) showed that constitutively internalized GPR30 reached recycling compartments. However, these receptors did not recycle but, instead, targeted the trans-Golgi network and proteasomal degradation.

Our results have direct implications on several of the controversies surrounding GPR30. First, the interaction of GPR30 with AKAP5 opens up new ways to try to understand receptor coupling to cAMP signaling. Second, our results address the specificity and efficacy of receptor agonists reported currently and present new avenues by which this receptor system may be targeted therapeutically. Third, we show that deleting the PDZ motif influences GPR30 subcellular localization, a clear issue of contention with some investigators claiming that GPR30 is localized exclusively in the endoplasmic reticulum (2), whereas others have shown that receptors can clearly be identified in the plasma membrane (13, 19, 45–47). Interestingly, endoplasmic reticulum localization was concluded using a GPR30 construct fused at the C terminus with GFP (2), a modification that has been shown previously to alter trafficking of both GPR30 (46) and GluR1 from this compartment (38) and that we show here.
would likely interfere specifically with PDZ-dependent GPR30 trafficking. Whether this has any implications for G-1, a substance selected using a GPR30-GFP construct (22) and used frequently to define GPR30 specificity, remains to be determined.

In summary, we show that GPR30 exists in a complex with a MAGUK and AKAP5 and that this complex allows AKAP5-RII to constitutively inhibit cAMP production in response to heterologous agonists and independently of $G_{i/o}$ and retain receptors in the plasma membrane (Fig. 9). These results present a new mechanism by which a receptor can inhibit cAMP production and, therefore, could possibly impact several cAMP-elevating agonists. Considering that GPR30 has been implicated in cancer, cardiometabolic disease, and the central nervous system, this discovery is likely to present new therapeutic opportunities in these systems.

Acknowledgments—We thank Dr. Suleiman Bahouth for sharing AKAPs and scrambled shRNA vectors and J. Daszkiewicz-Nilsson for expert technical assistance.

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