The Natural Inverse Agonist Agouti-related Protein Induces Arrestin-mediated Endocytosis of Melanocortin-3 and -4 Receptors*

Received for publication, June 22, 2006; in revised form, October 12, 2006. Published, JBC Papers in Press, October 14, 2006, DOI 10.1074/jbc.M605982200

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Agouti-related protein (Agrp), one of the two naturally occurring inverse agonists known to inhibit G protein–coupled receptor activity, regulates energy expenditure by decreasing basal and blocking agonist–promoted melanocortin receptor (MCR) signaling. Here we report that, in addition to its inverse agonistic activities, Agrp exhibits agonistic properties on the endocytosis pathway of melanocortin receptors. Sustained exposure of human embryonic kidney 293 cells to Agrp induced endocytosis of the MC3R or the MC4R. The extent and kinetics of Agrp-promoted endocytosis were similar to the endocytosis induced by melanocortins. Using the bioluminescence resonance energy transfer (BRET) assay, we further showed that after binding of Agrp both MCRs interacted with β-arrestins. In line with this observation, in COS-7 cells co-expression of β-arrestins enhanced Agrp-induced MCR endocytosis, whereas in human embryonic kidney 293 cells co-transfection of β-arrestin–specific small interference RNAs diminished Agrp-promoted endocytosis. This new regulatory mechanism was likewise detectable in a cell line derived from murine hypothalamic neurons endogenously expressing MC4R, pointing to the physiological relevance of Agrp-promoted receptor endocytosis. In conclusion, we demonstrated that Agrp does not solely act by directly blocking MCR signaling but also by reducing the amount of MCR molecules accessible to melanocortins at the cell surface. This β-arrestin–dependent mechanism reveals a new aspect of MCR signaling in particular and refines the concept of G protein–coupled receptor antagonism in general.

Ligand–promoted translocation of a given receptor protein from the plasma membrane into the cell interior (receptor endocytosis) represents an important step in the desensitization process of most G protein–coupled receptors (GPCRs).1

1 This study was supported by the “Bundesministerium für Bildung und Forschung” as part of the NGFN-2 (Nationales Genomforschungsnetzwerk; N2NVI-S30T09) network. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: GPCR, G protein–coupled receptor; Agrp, agouti-related protein; BRET, bioluminescence resonance energy transfer; BSA, bovine serum albumin; CRE, CAMP–response element; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; Ex, Xpress epitope; Fluc, firefly luciferase; GABA_\text{A}_{\text{Bz}}, γ-aminobutyric acid receptor; HEK, human embryonic kidney; MCR, melanocortin receptor; YFP, yellow fluorescent protein.

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(1–4). Because it was proposed that agonist–promoted signaling is required to initiate the molecular processes leading to GPCR endocytosis, an overwhelming number of reports have attributed ligand–induced receptor endocytosis exclusively to ligands with positive intrinsic activities (agonists). However, for a few members of the GPCR superfamily, including the 5-hydroxytryptamine 2A receptor (5), the endothelin A receptor (6), the cholecystokinin receptor (7), and the parathyroid hormone receptor (8), receptor endocytosis promoted by ligands with no intrinsic activity (antagonists) has also been observed. Due to the synthetic nature of the ligands used in these studies, no physiological significance could be attributed to this phenomenon.

Agouti and agouti-related protein (Agrp) are the only known naturally occurring peptides that block GPCR signaling and thus act as natural antagonists (9–12). Both peptides diminish signaling of melanocortin receptors (MCRs), a family of GPCRs responsive to peptides such as the α-melanocyte-stimulating hormone (α-MSH). Agouti has been shown to regulate skin pigmentation by inhibiting MC1R signaling in melanocytes (13, 14), whereas Agrp controls body weight by blocking MC3R and/or MC4R activity in hypothalamic neurons (11). The physiological relevance of Agpt-mediated inhibition of MCR signaling has recently been emphasized by the description of an age-related lean phenotype of Agpt-deficient mice (15). Initial data indicated that agouti and Agrp mediate their physiological effects by acting as competitive antagonists. Further studies revealed that both ligands also act as inverse agonists because binding of these peptides to both MCRs decreased basal, agonist–independent receptor signaling (16, 17). However, previous reports have suggested that the antagonistic activities of agouti described to date cannot account for all inhibitory effects of this peptide on MC1R signaling (18, 19). In fact, it has been proposed that still unknown agouti–promoted signaling pathways lead to MC1R desensitization probably due to receptor endocytosis. However, the molecular mechanism initiating putative agouti–induced MC1R endocytosis remained unclear, and the physiological importance of this process is still unsettled. In the case of the MC4R, melanocortin–promoted receptor endocytosis has been reported in overexpressing cell systems such as HEK-293 cells (20–22). Effects of Agrp on MC4R endo-

tor, α-MSH, α-melanocyte-stimulating hormone; PBS, phosphate-buffered saline; Fluc, Renilla luciferase; siRNA, small interference RNA; YFP, yellow fluorescent protein.
Agrp-promoted MCR Endocytosis

cytosis have not yet been described. To date, no data considering ligand-promoted endocytosis of the MC3R are available.

The present study was initiated to study the agonist-promoted endocytosis of the MC3R. MC3R endocytosis in HEK-293 cells induced by agonists could be detected by cell fractionation and surface ELISA experiments. Further analyzing the effects of inverse agonists on MC3R endocytosis, we surprisingly observed that Agrp has the ability to induce receptor endocytosis. This new regulatory mechanism of MCR signaling was not restricted to the MC3R because Agrp-promoted receptor endocytosis was also detectable in cells expressing the MC4R. In COS-7 cells, co-expression of β-arrestins substantially increased Agrp-induced endocytosis, whereas in HEK-293 cells co-transfection of β-arrestin-specific siRNAs inhibited Agrp-promoted endocytosis of both MCRs, indicating that β-arrestins are involved in the MCR endocytosis induced by the inverse agonist. In line with these findings, bioluminescence resonance energy transfer technique experiments revealed that binding of Agrp promoted the interaction of β-arrestins with both MCRs. Finally Agrp-induced MC4R endocytosis could also be observed in GT1-1 cells endogenously expressing MC4R, pointing to the physiological relevance of this new regulatory mechanism. In conclusion, the naturally occurring inverse agonist Agrp promoted the endocytosis of the MC3R and the MC4R in an arrestin-dependent manner, revealing a new mechanism by which Agrp fine tunes the responsiveness of MCR-expressing cells to melanocortins.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin/streptomycin, and l-glutamine were purchased from PAA, Inc. (Pasching, Austria). Metafectene was obtained from Biontex (München, Germany). Coelenterazine H was from Biaffin (Kassel, Germany), and anti-mouse horseradish peroxidase-conjugated secondary antibodies from sheep were purchased from Amersham Biosciences. [3H]Adenine was from PerkinElmer Life Sciences. The human Agrp fragment (amino acids 86–132) was obtained from the Peptide Institute, Inc. (Osaka, Japan). α-MSH was purchased from Sigma.

Eucaryotic Expression Vectors—For bioluminescence resonance energy transfer (BRET) experiments, expression vectors encoding the MC3R or MC4R fused to the yellow fluorescent protein (YFP) were generated as follows. PCR fragments containing the entire coding sequences excluding the stop codon of the human MC3R or MC4R were subcloned into the pcDNA3.1 vector harboring the sequence of YFP in a way that fused the 3′-end of the receptor cDNA to the 5′-end of the YFP cDNA. This procedure resulted in an in-frame fusion of the receptors with YFP separated by a 7-amino acid linker. To obtain fusion proteins with the Renilla luciferase (Rluc), PCR fragments containing the entire coding sequences excluding the stop codons of MCR were subcloned into the pHyc-Z YFP plasmid (PerkinElmer Life Sciences) in a way that fused the receptor sequence to the 5′-end of Rluc, resulting in an in-frame fusion of the receptor with Rluc separated by a 16-amino acid linker. For ELISA experiments, we fused the anti-Xpress peptide (Asp-Leu-Tyr-Asp-Asp-Asp-Lys) from Invitrogen to the N-terminus of MCR. To this end, PCR fragments containing the entire coding sequences of the human MC3R or MC4R were subcloned into the pcDNA4 vector (Invitrogen) in a way that fused the anti-Xpress peptide to the 5′-end of the MCR. pcDNA3.1-GABA-BR2-YFP, pcDNA3.1-β-arrestin-2-YFP, pcDNA3.1-β-arrestin-2-Rluc constructs were kindly provided by Dr. Bouvier (Montréal, Canada) and are described elsewhere (23, 24). The pAD-CRE-Fluc plasmid was kindly provided by Dr. Himmler, Bender GmbH (Vienna, Austria), and the pcDNA3.1-β-arrestin-YFP construct was kindly provided from the laboratory of Dr. Loewe (Würzburg, Germany).

Cell Culture and Transfection—HEK-293, COS-7, and GT1-1 cells (kindly provided by Dr. Weiner, University of California, San Francisco, CA) were cultured in DMEM supplemented with 10% fetal bovine serum and 2 mM l-glutamine. For transient expression of recombinant proteins, cells were seeded at a density of 2 × 10^4 cells in 10-cm dishes, cultured for 24 h, and then transfected with the appropriate amount of plasmid DNA using the Metafectene reagent according to the manufacturer’s protocol. Twenty-four hours post-transfection, cells were detached and seeded in new dishes as required for the following experiment.

cAMP Accumulation—To determine agonist-promoted cAMP accumulation, ~200,000 HEK-293 cells transfected with various MCR fusion protein constructs were seeded in 12-well dishes coated with 0.1% poly-L-lysine 24 h prior to the experiment and labeled for 2–4 h in serum-free DMEM containing 2 μCi/ml [3H]adenine. Cells were stimulated for 45 min at 37°C in DMEM containing 2.5 μM isobutylmethylxanthine along with various concentrations of α-MSH or Agrp. The reaction was terminated by removing the medium and adding ice-cold 5% trichloroacetic acid to the cells. [3H]cAMP was then purified by sequential chromatography over Dowex resin and aluminum oxide columns.

Firefly Luciferase Reporter Gene Assay—A pAD-CRE-Fluc plasmid containing the coding sequence of the firefly (Photinus pyralis) luciferase (Fluc) under the control of the CRE promoter was co-transfected with various MCR fusion proteins in HEK-293 cells using the Metafectene reagent according to the manufacturer’s protocol. Twenty-four hours after transfection, cells were seeded in 12-well dishes coated with 0.1% poly-L-lysine. After 12 h, cells were serum-starved for 12 h and stimulated in serum-free DMEM with increasing concentrations of various ligands. After 12 h of ligand stimulation, cells were lysed, and Fluc activity was determined using a luciferase reporter system (Promega, Mannheim, Germany) according to the manufacturer’s protocol in a PolarSTAR plate reader from BMG (Offenburg, Germany).

β-Arrestin Recruitment in Living Cells Monitored by BRET—For the detection of β-arrestin recruitment by MCR in living cells, BRET assays were performed as described previously (25, 26). Briefly 24 h after transfection of plasmids encoding the cDNA of MCR-YFP and/or β-arrestin-2-Rluc or of β-arrestin-1-YFP and MCR-Rluc fusion proteins, cells were detached using PBS containing 2 mM EDTA and washed with BRET puffer (PBS, pH 7.4, containing 1 mM CaCl2 and 1 mM MgCl2). The cell number was determined by measuring the extinction
of the cell suspension at 600 nm. Approximately 100,000 cells were placed in 96-well dishes and stimulated for 45 min at room temperature with 1 μM α-MSH or 100 nM Agrp. BRET measurements were started 2 min after addition of 5 μM (final concentration) RLuc substrate coelenterazine H. After degradation of the substrate by the energy donor RLuc, light is emitted with an emission peak at 480 nm. The energy acceptor YFP is excited by non-radiative energy transfer if YFP is located within a distance of less than 100 Å from the energy donor (which is achieved by the ligand-promoted translocation of β-arrestin from the cytosol to the plasma membrane). Thus, as a result of the receptor-arrestin interaction, fluorescence is re-emitted by YFP with a peak at 525 nm. Light intensity emitted at 440–500 and 510–590 nm was detected using the PolarSTAR plate reader. The ratio of the light intensities at 510–590 over 440–500 nm is defined as the BRET signal. BRET signals were then corrected by subtracting the background of the BRET measurement detected in cells expressing only RLuc.

Enzyme-linked Immunosorbent Assay to Detect Cell Surface Receptors—In the case of HEK-293 cells, 24 h after transfection Ex-MC3R- or Ex-MC4R-expressing cells were detached and seeded in 12-well dishes (~200,000 cells/well) coated with 0.1% poly-L-lysine. After 24 h, cells were stimulated at 37 °C for various time periods with 1 μM α-MSH or 100 nM Agrp in DMEM to induce receptor endocytosis. Dishes were then placed on ice, and cells were washed twice with ice-cold PBS containing 1% BSA. After blocking unpecific binding sites for 5 min on ice with the same buffer, Xpress epitope-MCR fusion proteins on the cell surface were detected by incubating the cells with 400 ng/ml anti-Xpress antibody (Invitrogen) in PBS containing 1% BSA for 1 h at 4 °C. After washing the cells twice with PBS containing 1% BSA, cells were fixed for 15 min with 3–10% paraformaldehyde at room temperature. Then cells were washed twice with PBS and incubated for 45–60 min with anti-mouse horseradish peroxidase-conjugated secondary antibodies from sheep (1:2,000) in PBS with 1% BSA at room temperature. Thereafter cells were washed twice for 20 min with PBS containing 1% BSA and once with pure PBS. The substrate o-phenylenediamine dihydrochloride (Sigma) was added according to the manufacturer’s instructions. After 5–10 min, the reaction was stopped with 3 N HCl, and extinction was measured at 492 nm. Data are presented as absolute values, or receptor endocytosis is given as the percentage of the signal measured at 492 nm. Data are presented as absolute values, or corrected by subtracting the background of the BRET measurement detected in cells expressing only RLuc.

Cell Fractionation by Ultracentrifugation through Sucrose Cushions—MCR endocytosis was also assessed after cell fractionation as described previously (26, 27). Briefly 24 h after transfection of cells with receptor-RLuc fusion proteins cells were seeded on 10-cm dishes and, after an additional 24-h incubation time, stimulated for 45 min at 37 °C with 1 μM α-MSH or 100 nM Agrp in DMEM. Cells were detached on ice with 2 ml of ice-cold PBS containing 2 mM EDTA, and the total membrane fraction was prepared as follows. Cells were homogenized in ice-cold buffer (5 mM Tris/HCl, pH 7.4, 2 mM EDTA, 5 mg/ml leupeptin, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor) using a Polytron (Ultraturrax T24, IKA) for 10–15 s at maximum speed. Lyesates were centrifuged at 500 × g for 10 min at 4 °C. The resulting supernatant containing the total membrane fraction was then placed on the top of a sucrose cushion (35%) and centrifuged at 150,000 × g for 90 min. The light endosomal fraction containing internalized receptor (RLuclight) was found at the 0–35% interface, whereas the heavy membrane fraction (RLucheavy) sedimented at the bottom of the tube. The heavy membrane fraction was resuspended in 5 mM Tris/HCl, pH 7.4, and the light endosomal fraction was collected and again centrifuged at 200,000 × g for 60 min. The resulting pellet was also resuspended in 5 mM Tris/HCl, pH 7.4, and the total amount of protein in each fraction was determined using the method of Bradford. The amount of receptor-RLuc fusion proteins in each fraction was determined by measuring RLuc activity using the PolarSTAR plate reader and normalized to the total amount of protein. Endocytosis was calculated as a percentage using the following equation:

\[
\text{Endocytosis} = \left( \frac{\text{RLuc}_{\text{heavy}} - \text{RLuc}_{\text{light}}}{\text{RLuc}_{\text{heavy}}} \right) \times 100
\]

Reverse Transcription-Polymerase Chain Reaction—Total RNA from GT1-1 or as a control from HEK-293 cells (data not shown) was isolated using the TriFast reagent (PeqLab, Erlenagen, Germany). First strand synthesis was carried out with random hexamers as primers (pdN6, Amersham Biosciences) using REVERTAID reverse transcriptase (MBI-Fermentas, Saint Leon-Roth, Germany). Products were amplified using mouse MC3R- (forward, 5′-TGGGCAACCTATATATCCACA-3′; reverse, 5′-CCCTTC-ATGCAAGGATGTC-3′), mouse MC4R- (forward, 5′-TTCCCT-CCACCTCTTGAA-3′; reverse, 5′-GGGGAAACAAAAAG- TTG-3′), mouse β-arrestin-1- (forward, 5′-CTCAAGCATGAG- GACACGAA-3′; inverse, 5′-ATTTCAGCGTGTGCACCCCTT- 3′), mouse β-arrestin-2- (forward, 5′-GACCAATTTCTCTGAG- ACGAG-3′; reverse, 5′-TTAACCTTGGAACACTTGCG- CATG-3′), or, as a control, mouse β-actin (forward, 5′-CCAACTGGTGGA- AAAAGATGACC-3′; reverse, 5′-TGTTGAGCTGACAGAGGCA- TAC-3′)-specific primer pairs. PCRs were carried out using the following conditions: initial denaturation for 3 min at 94 °C, 45 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min followed by a final extension at 72 °C for 7 min.

Specific Gene Knock-out by siRNAs—The small RNA interference technique was used as described before (28, 29). siRNAs used for specifically knocking down β-arrestin-1 (5′-AAAGC- CUUCUGCAGGGAAGA-3′) or β-arrestin-2 (5′-AAUUCUC- CGGAACGUGUCAGCU-3′) expression were identical to
**TABLE 1**

Ligand-promoted cAMP production in HEK-293 cells expressing various MC3R or MC4R fusion proteins

In HEK-293 cells cAMP accumulation was detected after transient expression of MCR fused C-terminally to either YFP or RLuc or N-terminally to an 8-amino acid sequence serving as an epitope tag (Ex). For cAMP accumulation assays cells were labeled with [3H]adenine, and [3H]AMP was purified by sequential chromatography over Dowex resin and aluminum oxide columns. For reporter gene assays cells were co-transfected with a reporter gene construct harboring the firefly luciferase gene under the control of the CRE promoter. Dose-response curves of both ligands were drawn from data obtained in three (CRE-Fluc) or two (cAMP) independent experiments carried out in triplicate. EC50 values are expressed as the mean ± S.E. of these experiments. To determine Fmax values cAMP accumulation measured under basal conditions was defined as 100%. α-MSH-induced increase and Agrp-promoted decrease of cAMP production are given in percentage of the basal level.

<table>
<thead>
<tr>
<th></th>
<th>Ex-MC3R</th>
<th>MC3R-YFP</th>
<th>MC3R-RLuc</th>
<th></th>
<th>Ex-MC4R</th>
<th>MC4R-YFP</th>
<th>MC4R-RLuc</th>
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<tr>
<td></td>
<td>α-MSH</td>
<td>Agrp</td>
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<td>EC50</td>
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<tr>
<td>cAMP</td>
<td>ns</td>
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<td>%</td>
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<td>ns</td>
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<tr>
<td>CRE-Fluc</td>
<td>0.04</td>
<td>1.1</td>
<td>0.26</td>
<td>2.0</td>
<td>0.26</td>
<td>2.0</td>
<td>0.26</td>
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<tr>
<td></td>
<td>2.3 ± 0.3</td>
<td>67 ± 13</td>
<td>2.0 ± 1.7</td>
<td>72 ± 2</td>
<td>0.04 ± 0.06</td>
<td>72 ± 2</td>
<td>0.26 ± 0.04</td>
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<td></td>
<td>0.29 ± 0.02</td>
<td>13 ± 4</td>
<td>0.21 ± 0.01</td>
<td>240 ± 31</td>
<td>0.26 ± 0.02</td>
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<td></td>
<td>0.03 ± 0.02</td>
<td>11 ± 3</td>
<td>0.21 ± 0.01</td>
<td>240 ± 31</td>
<td>0.26 ± 0.02</td>
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</table>

**RESULTS**

**Agrp Inhibits Basal cAMP Production in HEK-293 Cells Expressing Various MCR Fusion Proteins**—Originally Agrp was described to be an antagonist of various MCR subtypes, including the MC3R and the MC4R. Further studies revealed that Agrp not only antagonizes melanocortin-promoted MCR signaling but also inhibits basal, agonist-independent receptor activity, defining Agrp as an inverse agonist (17). To validate receptor fusion proteins used in the present study, we monitored cAMP accumulation by anion exchange chromatography and cAMP production after co-transfection of a reporter gene construct encoding the Fluc protein under the control of the CRE promoter. Both the cAMP accumulation and the CRE-Fluc reporter gene assay revealed that neither fusion of RLuc or YFP to the C terminus nor fusion of the Xpress epitope to the N terminus of MCR inhibited receptor signaling and that no dramatic alterations in the potency or efficacy of the agonist (α-MSH) or the inverse agonist (Agrp) was observed (Table 1).

It is noteworthy that in this and all following experiments a fragment of human Agrp (amino acids 86–132) was used. This Agrp fragment has been reported to occur naturally after cleavage of the full-length peptide by protein convertase 1 or 3 and to exhibit the most potent inverse agonistic activities of all known Agrp fragments (32).

**Agrp Promotes Endocytosis of MCR Expressed in HEK-293 Cells**—After sustained exposure to agonists, most GPCRs undergo receptor endocytosis that results in a rapid loss of binding sites from the cell surface (3, 4). For the MC4R recombinantly expressed in HEK-293 cells, it has been shown that this receptor subtype is endocytosed in an agonist-dependent manner (20, 21). However, to our knowledge, no such data are available for the MC3R. To investigate agonist-promoted receptor endocytosis, we fused the Xpress epitope to the N terminus of the MC3R (Ex-MC3R) and performed cell surface ELISA experiments with HEK-293 cells recombinantly overexpressing this fusion protein. As shown in Fig. 1, A and B, stimulation of these cells with the agonist α-MSH (1 μM) for 45 min significantly reduced the amount of Ex-MC3R molecules accessible to the specific antibody against the Xpress epitope, revealing that the MC3R undergoes agonist-promoted endocytosis. Although ligand-promoted GPCR endocytosis has almost completely been attributed to agonists, we next wondered whether the inverse agonist Agrp is also able to regulate MC3R endocytosis. To our surprise, incubation of Ex-MC3R-expressing cells with Agrp (100 nM) for 45 min reduced the number of receptor molecules expressed at the cell surface, indicative of MC3R endocytosis induced by this inverse agonist (Fig. 1, A and B). To test whether Agrp-promoted endocytosis is restricted to the MC3R, we also analyzed ligand-promoted endocytosis of an Ex-MC4R fusion protein. As expected, stimulation of Ex-MC4R-expressing cells with the agonist α-MSH reduced the MC4R density at the plasma membrane as a result of agonist-promoted MC4R endocytosis (Fig. 1, A and B) as mentioned before. Moreover as for the MC3R, stimulation of HEK-293 cells with Agrp also promoted MC4R endocytosis (Fig. 1, A and B), indicating that the inverse agonist induced endocytosis of both MCRs. Inverse agonist-promoted receptor endocytosis was not promoted by the overexpression of MCR in HEK-293 cells because, as shown in Fig. 1, C and D, the extent of MCR endocytosis decreased with increasing receptor expression levels under basal conditions. To further compare the time course of agonist- and inverse agonist-induced MCR endocytosis, we next stimulated Ex-MC3R- or Ex-MC4R-expressing cells for various times with α-MSH or Agrp and determined the extent of receptor endocytosis. In Ex-MC3R-expressing cells, both ligands induced maximal receptor endocytosis after ~20 min of ligand stimulation (Fig. 1F). The kinetics of MC4R endocytosis induced by both ligands revealed similar results (Fig. 1F). Thus, the time...
course of inverse agonist-promoted MCR endocytosis in HEK-293 cells showed no significant differences compared with the endocytosis induced by the agonist.

Cell surface ELISA experiments are suitable tools to determine the amount of a given GPCR at the plasma membrane. However, this technique does not provide direct information about the translocation of a given transmembrane protein from the plasma membrane into the cell interior. Thus, techniques based on cell fractionation and differential centrifugation have been introduced to follow translocated GPCR within the cell (26, 27, 33, 34). These studies have shown that high density membrane fractions mostly contain membranes derived from the plasma membrane, the Golgi complex and the endoplasmic reticulum, whereas low density membrane fractions contain endosomal membranes harboring endocytosed receptors. We took advantage of this technique and performed cell fractionation experiments on total membrane fractions derived from HEK-293 cells expressing MCR-Rluc fusion proteins. Fusion of MCR with Rluc enabled us to detect receptor-Rluc proteins by measuring Rluc activity in a given membrane fraction. After stimulation with α-MSH, HEK-293 cells expressing MC3R-Rluc showed a significant decrease of Rluc activity in the high density membrane fraction (Fig. 2, A and B) and inversely an increase in the low density membrane fraction, indicating

![Graph A](image1.png)  
**A**  
pcDNA4  Ex-MC4R  Ex-MC3R  
basal  α-MSH  Agrp  

![Graph B](image2.png)  
**B**  
Ex-MC4R  Ex-MC3R  
α-MSH  Agrp  

![Graph C](image3.png)  
**C**  
Ex-MC3R  α-MSH  Agrp  

![Graph D](image4.png)  
**D**  
Ex-MC4R  α-MSH  Agrp  

![Graph E](image5.png)  
**E**  
Ex-MC3R  α-MSH  Agrp  

![Graph F](image6.png)  
**F**  
Ex-MC4R  α-MSH  Agrp  

**FIGURE 1. Ligand-promoted MCR endocytosis monitored by cell surface ELISA.** Cell surface receptors of HEK-293 cells transiently expressing Ex-MC3R or Ex-MC4R were quantified by assessing the amount of Ex-MCR fusion proteins accessible to a specific anti-Xpress epitope antibody. In A one representative experiment is shown in which Ex-MCR-expressing cells were incubated with 1 μM α-MSH or 100 nM Agrp for 45 min at 37 °C. HEK-293 cells transfected with the pcDNA4 plasmid indicate the background of mock-transfected cells. Results are expressed as the mean ± S.E. of quadruplicate measurements. The asterisk indicates a significant (*, p < 0.05) difference of cells stimulated with a ligand compared with non-stimulated cells. In B α-MSH- or Agrp-induced MCR endocytosis of seven (MC3R) or eight (MC4R) independent experiments is given and expressed as percentage of ligand-promoted receptor endocytosis, whereas 100% corresponds to the A_{492} value under basal conditions minus the A_{492} value of mock-transfected cells. In C (Ex-MC3R) and D (Ex-MC4R) the same data as in B are shown but are now illustrated to show the dependence of receptor expression levels (x axes) on the extent of ligand-promoted endocytosis (y axes). The time course of ligand-induced Ex-MC3R (E) or Ex-MC4R (F) is given as percentage of receptor endocytosis. Results are expressed as the mean ± S.E. of three independent experiments carried out in quadruplicate.
**Agrp-promoted MCR Endocytosis**

![Graph](image)

**FIGURE 2.** Ligand-promoted MCR endocytosis monitored by cell fractionation and differential centrifugation through sucrose cushions (35%). The presence of receptor-Rluc fusion proteins in high density (HDM) or low density (LDM) membrane fractions was monitored by measuring Rluc activity. In A one representative experiment is shown in which HEK-293 cells transiently expressing MC3R-Rluc or MC4R-Rluc, or GABAAR2-Rluc were stimulated for 45 min at 37 °C with 1 μM α-MSH or with 100 nM Agrp. Results are expressed as the mean ± S.E. of triplicate measurements. Asterisks indicate a significant (**, p < 0.01) difference of ligand-stimulated cells compared with non-stimulated cells. In B MCR endocytosis determined in five independent experiments is given as the extent of ligand-promoted receptor endocytosis in percent.

**translocation of the MC3R-Rluc from the plasma membrane to endosomal vesicles after agonist stimulation. Similarly the MC4R-Rluc fusion protein also translocated into endosomes after stimulation with α-MSH (Fig. 3, A and B), showing that both MCRs are internalized after agonist exposure. In contrast, a fusion protein of Rluc and the γ-aminobutyric acid receptor (GABAAR2), a GPCR that is also located in the plasma membrane but does not bind melanocortins, underwent no translocation into the low density membrane fraction after α-MSH stimulation (Fig. 2A), demonstrating the integrity of the sucrose cushion used in this study. Notably after Agrp treatment of cells expressing either MC3R-Rluc or MC4R-Rluc, a decrease of Rluc activity in the high density membrane fraction and a consequent increase in the low density membrane fraction could also be detected, indicating the translocation of both MCRs from the cell surface into endosomal vesicles induced by the inverse agonist (Fig. 2A and B).

In conclusion, two independent approaches, cell surface ELISA and cell fractionation experiments, clearly showed that MC3R and MC4R undergo receptor endocytosis after stimulation with Agrp. In addition, receptor endocytosis promoted by the inverse agonist exhibited amplitudes and kinetics similar to the agonist-induced endocytosis.

**Agrp-induced Recruitment of β-Arrestins by MCR**—Because agonist-induced β-arrestin recruitment is a crucial step in the internalization process of most GPCRs (35) and in particular of the MC4R (21), the observation of Agrp-promoted MCR endocytosis raised the question whether the inverse agonist is able to induce β-arrestin recruitment to MCR. To address this question, we monitored recruitment of β-arrestin-1-YFP to MCR-Rluc fusion proteins in HEK-293 cells using the BRET technique as described in previous reports (25, 26, 36, 37). As shown in Fig. 3A, stimulation with 1 μM α-MSH significantly increased the BRET signal between MC4R-Rluc and β-arrestin-1-YFP, reflecting agonist-induced translocation of β-arrestin-1 to this receptor subtype. Similarly α-MSH stimulation of HEK-293 cells co-expressing MC3R-Rluc and β-arrestin-1-YFP likewise increased the BRET signal, showing that MC3R also interacts with β-arrestin-1 in an agonist-dependent manner (Fig. 3A). In contrast, HEK-293 cells co-expressing similar levels of β-arrestin-1-YFP and GABAAR2-Rluc fusion proteins when compared with MCR-Rluc-expressing cells (indicated by the similar amount of total luminescence and fluorescence measured in these cells, Fig. 3A) showed no BRET signal, confirming the specificity of the BRET assay used in this study. Cells expressing β-arrestin-1-YFP and either MC3R-Rluc or MC4R-Rluc also exhibited a significantly increased BRET signal after stimulation with 100 nM Agrp (Fig. 3A). Increased BRET signals between β-arrestin-1-YFP and both MCR-Rluc fusion proteins promoted by Agrp are indicative of Agrp-dependent interactions between MCR and β-arrestin-1. Interestingly stimulation of HEK-293 cells co-expressing β-arrestin-2-Rluc and MCR-YFP fusion proteins with Agrp equally induced a significant BRET signal between the two chromatophores (Fig. 3B), suggesting that the inverse agonist exhibits the propensity to recruit both β-arrestin subtypes to MCR and that β-arrestins are probably involved in the internalization process initiated by Agrp.

**Agrp-induced MCR Endocytosis Requires the Expression of β-Arrestins**—It has been reported that GPCRs, e.g. the β2-adrenergic receptor, internalize poorly in COS-7 cells, which express low levels of endogenous arrestins (26, 38, 39). Therefore, COS-7 cells represent an excellent tool to analyze arrestin-independent GPCR endocytosis. Accordingly as shown in Fig. 4, A and B, α-MSH- and Agrp-promoted MC3R or MC4R endocytosis in COS-7 cells was drastically decreased compared with the endocytosis observed in HEK-293 cells under the same conditions (Fig. 2B). However, co-expression of either β-arrestin-1- or β-arrestin-2-YFP significantly increased α-MSH- and Agrp-induced endocytosis of both MCRs in COS-7 cells. This increase in α-MSH-promoted receptor endocytosis confirms a previous report showing the importance of arrestins for the agonist-induced MC4R endocytosis (21) and reveals the involvement of arrestins in agonist-induced endocytosis of the MC3R.
Furthermore increased Agrp-promoted MCR endocytosis in COS-7 cells after co-expression of β-arrestins indicates that β-arrestins play a crucial role also in the endocytosis of MCR promoted by the inverse agonist. To confirm that β-arrestins play an important role in Agrp-induced MCR endocytosis we further took advantage of siRNAs reported previously to specifically inhibit β-arrestin-1 or β-arrestin-2 expression in HEK-293 cells (30, 31). As shown in Fig. 5, A and B, co-transfection of a random siRNA or a specific siRNA against either β-arrestin-1 or β-arrestin-2 in HEK-293 cells did not affect ligand-promoted MC3R or MC4R endocytosis. However, co-transfection of both siRNAs against β-arrestin-1 and -2 together significantly inhibited α-MSH- and Agrp-promoted endocytosis of MCR. These data provide further evidence that β-arrestins initiate MCR endocytosis induced by the inverse agonist.

**Agrp-induced MC4R Endocytosis in GT1-1 Cells**—Data presented so far support a model in which binding of the natural inverse agonist, Agrp, induces endocytosis of MCR in heterologous expression systems. To verify whether Agrp-induced receptor endocytosis also regulates MCR signaling under more physiological conditions, we took advantage of a murine hypothalamic cell line, GT1-1, that has been shown to endogenously express MCR (40). As shown in Fig. 6A, reverse transcription-PCR experiments revealed the presence of MC3R and MC4R transcripts in GT1-1 cells. α-MSH-activated kinase or the cAMP pathway further confirmed the expression of functional MCR expressed in GT1-1 cells (data not shown). Interestingly reverse transcription-PCR experiments additionally demonstrated that GT1-1 cells exhibit high levels of β-arrestin-1 but low levels of β-arrestin-2 transcripts (Fig. 6A) confirming a previous study (21). To test whether Agrp induces MCR endocytosis in GT1-1 cells, we modified the cell surface ELISA assay in a way that allowed us to analyze MC4R endocytosis in these cells (see “Experimental Procedures”). Incubation of GT1-1 cells with 1 μM α-MSH for 45 min significantly decreased the number of MC4R molecules accessible to a specific anti-MC4R peptide antibody, indicating that MC4R undergoes agonist-promoted endocytosis in these cells (Fig. 6B). Furthermore treatment of GT1-1 cells with Agrp equally reduced the number of MC4R molecules at the cell surface (Fig. 6B), demonstrating that the inverse agonist promotes endocytosis of the MC4R endogenously expressed in cells derived from hypothalamic neurons. Endocytosed receptor proteins can be either proteolytically cleaved and degraded or relocated to the plasma membrane leading to the recovery of receptor signaling (41). To analyze whether ligand-induced MC4R endocytosis is a reversible process that allows recycling of the receptor protein, we determined MC4R cell surface expression in GT1-1 cells after removal of the corresponding peptide and an additional incubation time of 60 min. As shown in Fig. 6C, removal of α-MSH or Agrp led to an almost complete relocation of MC4R proteins to the cell surface after sustained ligand exposure. Thus, Agrp-promoted MC4R endocytosis is a reversible process that temporarily reduces the number of MC4R molecules located at the cell surface and therefore transiently weakens the responsiveness of GT1-1 cells to melanocortins.

**DISCUSSION**

In the present study, we provide compelling evidence that the MC3R undergoes agonist-promoted receptor endocytosis and, in addition, that the MC3R and the MC4R are internalized after binding to the natural inverse agonist, Agrp. BRET assays, expression experiments in COS-7 cells, and siRNA approaches in HEK-293 cells further revealed that β-arrestins are involved in the endocytosis pathway induced by the inverse agonist. The analysis of GT1-1 cells additionally indicated that Agrp promotes MC4R endocytosis in cells derived from murine hypothalamic neurons.

Agonist-promoted receptor endocytosis is a widespread phenomenon among GPCRs (3, 4). Only a few members of the GPCR family, e.g. the β2-adrenergic receptor (42) or the κ opioid receptor (43), have been reported to be resistant to this regulatory process. Studies with the β2-adrenergic receptor, in particular, revealed a common pathway for agonist-promoted endocytosis by which agonist-induced receptor activation is enhanced by the binding of β-arrestins to the receptor (44).
Arrestin binding then facilitates the translocation of the receptor protein into the cell (35). For MCRs, ligand-promoted receptor endocytosis has been described for the MC2R (45, 46) and the MC4R (20–22), but no such data are available for the MC3R. Here based on data obtained by two independent experimental approaches we report that the MC3R expressed in HEK-293 cells undergoes ligand-promoted receptor endocytosis. Cell fractionation followed by differential centrifugation through sucrose cushions was first described by Hertel et al. (33) and has since then been extensively used to monitor receptor endocytosis (26). Cell surface ELISA experiments have also been successfully used to study cell surface expression and endocytosis of GPCRs (47–49). Thus, data presented herein provide compelling evidence that, like the MC2R and MC4R, the MC3R undergoes agonist-promoted receptor endocytosis. Based on the analysis of the molecular mechanisms leading to agonist-promoted MC3R endocytosis, we suggest that arrestins are involved in this regulatory process. First, using the BRET technique, we provide evidence that binding of α-MSH to the MC3R induced the recruitment of β-arrestins in living cells. BRET assays have been successfully used to monitor arrestin-receptor interactions of multiple GPCRs (25, 26, 36, 37) and thus have become an established tool to analyze the interaction of a given receptor with arrestins in living cells. Second, co-transfection of siRNAs specifically decreasing expression of β-arrestins reduced MC3R endocytosis in HEK-293 cells. Third, co-expression of β-arrestins in COS-7 cells dramatically enhanced agonist-promoted MC3R endocytosis. COS-7 cells have been shown to express much lower levels of both arrestins when compared with HEK-293 cells (38), and as a consequence, co-expression of arrestins in COS-7 cells has been shown to enhance arrestin-dependent endocytosis of various GPCRs (26, 31, 50). Thus, we clearly favor a model in which the MC3R undergoes agonist-induced endocytosis in a β-arrestin-dependent manner. However, one cannot exclude that arrestin-independent pathways are also involved in MC3R endocytosis and/or that the extent of receptor endocytosis might be different depending on the cell type used.

Although ligand-promoted receptor endocytosis has almost exclusively been attributed to ligands with positive intrinsic activities (agonists), in a few cases receptor endocytosis has also been observed for ligands with no intrinsic activity, termed antagonists (5–8). However, due to the synthetic nature of the ligands used in these studies, so far no physiological significance could be attributed to the phenomenon of antagonist-promoted receptor endocytosis. In addition, none of these antagonists has been shown to initiate the recruitment of arrestins to their cognate receptor, suggesting that these synthetic ligands are not able to activate the arrestin-mediated endocytosis pathway common for most GPCRs. Recently it has been reported that binding of some synthetic inverse agonists of the β2-adrenergic receptor induced arrestin recruitment to this receptor (51), but no receptor endocytosis mediated by these ligands could be observed. Here we report, based on BRET data, that like inverse β-adrenergic agonists Agrp promotes the interactions of β-arrestins to MC3R or MC4R but in contrast additionally promotes receptor endocytosis. Thus, the endogenous inverse agonist Agrp combines the properties of inverse β-adrenergic agonists (enhancement of arrestin recruitment) with those of the neutral antagonists mentioned before (promotion of receptor endocytosis). Considering the involvement of arrestins in Agrp-promoted MCR endocytosis, we clearly showed that in COS-7 cells co-expression of β-arrestins enhanced Agrp-induced MCR endocytosis, whereas co-transfection of β-arrestin-specific siRNAs in HEK-293 cells blocked Agrp-induced endocytosis. In conclusion, our data support an
Agrp-promoted MC4R endocytosis

FIGURE 5. Ligand-promoted MCR endocytosis in HEK-293 cells monitored by cell surface ELISA experiments. Ligand-induced MC3R (A) or MC4R (B) endocytosis was quantified by cell surface ELISA experiments in HEK-293 cells co-transfected with a randomly generated control siRNA, with a specific siRNA raised against β-arrestin-1 or β-arrestin-2, or with both siRNAs at the same time. Receptor endocytosis of cells incubated with 1 μM α-MSH or 100 nM Agrp for 45 min at 37 °C is given as the mean ± S.E. of receptor endocytosis in percent of three independent experiments carried out in quadruplicate. Asterisks indicate a significant (*, p < 0.05; **, p < 0.01) difference of cells expressing both specific siRNAs, versus cells expressing the random siRNA.

整体模型中，Agrp 通过反向调节 G 蛋白信号传导，主要由于 cAMP 途径，但同时促进 β-arrestin 招募，导致 MCR 内化。一种可能的假设是，Agrp 促进的 MCR 内化可以导致 G 蛋白的直接后果，导致 β-arrestin 招募，从而启动 MCR 内化。这可能意味着，Agrp 促进的 MCR 内化是非必需的，因为有反向调节活性的 Agrp 在 MC4R 驱动的 cAMP 生成可以检测到在总膜准备中（17）。激活时 β-arrestin 招募要求整合的能力，我们假设 Agrp 促进的 β-arrestin 招募不是对反向调节活性的先决条件。

目前，内化促进的信号传导是 Agrp 在 MCR 上的内在属性，但取决于环境，其中感受体蛋白被表达。MC3R 和 MC4R 是在下丘脑神经元中最为显著表达的 MC 细胞外信号传导细胞模型中表示的假设性神经元。GT1-1 细胞是衍生自小鼠下丘脑神经元，最近已建立为一种细胞系统，其 MC4R 上内化 MC3R 转录。GT1-1 细胞与 α-MSH 显著减少了膜上 MC4R 分子的数量，指示 MC4R 信号传导在这些细胞上被抑制。这一观察促使我们提出一种新模型，在这种模型中，内源性 MCR 表达的反向调节者减弱了 MCR-表达细胞对 melanocortins 的敏感性，不仅通过直接阻断感受体活性，而且通过部分减少 MCR 分子在达到的

整体模型中，Agrp 通过反向调节 G 蛋白信号传导，主要由于 cAMP 途径，但同时促进 β-arrestin 招募，导致 MCR 内化。一种可能的假设是，Agrp 促进的 MCR 内化可以导致 G 蛋白的直接后果，导致 β-arrestin 招募，从而启动 MCR 内化。这可能意味着，Agrp 促进的 β-arrestin 招募不是对反向调节活性的先决条件。
plasma membrane. In such a refined concept, inverse agonists not only possess short term (within seconds) antagonistic effects that require direct ligand-receptor interactions but also long term (within minutes) effects due to the translocation of the receptor protein into the cell. In contrast to short term effects that permit fast signaling recovery after dissociation of the peptide, long term effects should continue to affect receptor signaling even after the peptide has dissociated due to a much slower kinetics of recovery. Further studies will be required to investigate to which extent long term antagonistic effects modulate MCR signaling in vivo and thus contribute to the physiological regulation of the melanocortin system.

Acknowledgments—We are grateful to Drs. Ingrid Boekhoff and Tim Plant from the Institute of Pharmacology and Toxicology of the Philippus-University Marburg (Marburg, Germany) for critically reading the manuscript.

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