Agonist-Dependent Internalization of the Human Melanocortin-4 Receptors in Human Embryonic Kidney 293 Cells

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ABSTRACT
A chimeric protein comprised of melanocortin-4 receptor (MC4R) and the green fluorescent protein (GFP) was created for studying receptor/ligand localization and trafficking. The ligand binding affinities and second messenger stimulation induced by MC4R-GFP closely resembled those of the wild-type receptor, suggesting functional integrity of the chimeric protein. As observed with a confocal microscope, in human embryonic kidney (HEK)-293 cells MC4R/GFP was distributed evenly along the cell membrane. Addition of [Nle^4-D-Phe^7]α-melanocyte-stimulating hormone (NDP-MSH), a peptide MC4R agonist, induced receptor translocation into intracellular compartments in a time- and concentration-dependent manner. [Ac-Nle-c[Asp-His-D-Nal(2)-Arg-Trp-Lys]-NH$_2$] (SHU9119), a potent MC4R antagonist, completely inhibited NDP-MSH-mediated internalization. MC4R-GFP internalization was unaffected by a protein kinase A inhibitor (SHU9119), a potent MC4R antagonist, completely inhibited NDP-MSH-mediated internalization. MC4R-GFP internalization was unaffected by pretreatment with inhibitors of endocytosis through clathrin-coated pits, hypertonic sucrose, or concanavalin A. Time-dependent colocalization of MC4R-GFP with rhodamine-transferrin, an early endosome marker, and with LysoTracker, a lysosome marker, was observed after short-term (45 min) and prolonged (20 h) agonist exposure, respectively. Rhodamine-[Ac-Nle-c[Asp-His- D-Nal(2)-Arg-Trp-Lys]-NH$_2$] (MTII), a fluorescent derivative of an MC4R agonist, was found to colocalize with MC4R-GFP into intracellular vesicles. No significant receptor recycling or segregation from the ligand was observed even after removal of the agonist. In contrast, an antagonist rhodamine- Ac-Cys-Glu-His-(o-Nal)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp-NH$_2$ (HS014) bound to and colocalized with MC4R-GFP on the cell surface and did not stimulate receptor internalization. In sum, these results suggest that MC4R is subject to agonist-dependent endocytosis via clathrin-coated pits. Prolonged agonist exposure directs MC4R into lysosomes, possibly for degradation. Receptor and ligand recycling is not efficient for MC4R in HEK-293 cells.

Melanocortins are a group of pro-opiomelanocortin-derived peptides that are comprised of adrenocorticotropin and the α, β, and γ-melanocyte-stimulating hormones (MSHs). These bioactive peptides act as endogenous agonists for cell surface melanocortin receptors (MCRs) and play a variety of physiological roles in steroidogenesis, pigmentation, anti-inflammation, control of food intake, energy expenditure, and sexual behavior (Abdel-Malek, 2001; MacNeil et al., 2002). Molecular cloning studies have revealed the existence of five subtypes of melanocortin receptors termed MC1R, MC2R, MC3R, MC4R, and MC5R, which form a distinct family of G protein coupled receptors (GPCRs). All five melanocortin receptors activate adenylyl cyclases via stimulatory G proteins (Gs) and thereby elevate intracellular cAMP (Abdel-Malek, 2001). Nonetheless, the five subtypes of receptors are distinguishable by their tissue distribution, physiological function, and their ability to recognize various melanocortin peptides.

Interest in melanocortin receptors has intensified in recent years, mainly due to the therapeutic potential of these receptors as small molecule drug targets for a variety of human diseases (Butler and Cone, 2002; MacNeil et al., 2002). Of particular interest is the MC4R as a target for antiobesity therapeutics. Numerous studies have been aimed at understanding the complex pharmacology and physiology of this receptor. This research has led to the identification of MC4R as a major regulator of eating behavior and body weight in mice and humans, and also suggests a potential role of MC4R in stimulation of male erectile activity both in rodent and humans (Wessells et al., 2000; Van der Ploeg et al., 2002). More importantly, small molecule agonists with potent and selective action on MC4R have been identified by high throughput screening from our group as well as others (Sebhah et al., 2002). These major breakthroughs have raised ABBREVIATIONS: MSH, melanocyte-stimulating hormone; MCR, melanocortin receptor; GPCR, G protein-coupled receptor; GFP, green fluorescent protein; HS014, Ac-Cys-Glu-His-(o-Nal)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp-NH$_2$; MTII, [Ac-Nle-c[Asp-His-o-Phe-Arg-Trp-Lys]-NH$_2$]; H89, N-[2-(p-bromocinnamylamino)ethyl]-5-isooquinolinesulfonyamide; SHU9119, [Ac-Nle-c[Asp-His-o-Nal(2')]-Arg-Trp-Lys]-NH$_2$; NDP-MSH, [Nle^4-D-Phe^7]-α-melanocyte-stimulating hormone; PBS, phosphate-buffered saline; PKA, protein kinase A; HA-hMC4R, hemagglutinin-tagged human MC4R.
exciting hopes for the development of better treatments for obesity and sexual dysfunctions in the near future.

Despite these significant advances, little is known regarding the potential for molecular regulation of MC4R function through ligand-mediated internalization, trafficking, responsiveness, and turnover. Agonist-induced internalization is a prominent feature of many G protein-coupled receptors and serves a variety of important physiological functions in the regulation of receptor activity and signal transduction (Claring et al., 2002). Endocytosis influences receptor function in ways that seem to be variable among G protein-coupled receptors. Internalization is apparently the predominant mechanism of desensitization of the secretin receptor and somatostatin receptor (Holtmann et al., 1996; Beaumont et al., 1998; Mundell and Kelly, 1998). Conversely, internalization has clearly been shown to be obligatory for desensitization of β2-adrenergic receptor and M3 muscarinic receptor (Yu et al., 1993; Edwardson and Szekeres, 1999). In addition, recent findings suggest that internalized receptors may act as scaffolds for regulatory proteins, which continue to engage with the receptor in intracellular signaling. For instance, activation of mitogen-activated protein kinases by β2-adrenergic receptor requires prior translocation of the receptor into clathrin-coated vesicles, in which specific interactions between the receptor and the appropriate downstream effectors occur (Daaka et al., 1998). The differences in agonist-mediated receptor trafficking and compartmentation in specialized microdomains may explain the signaling specificity of multiple GPCRs apparently coupled to the same type of G protein in the same cell type. Hence, internalization is an important process involved in both temporal and spatial regulation of G protein-coupled receptor-mediated signal transduction.

Agonist-promoted GPCR internalization, however, is a very complex process that remains to be completely understood. Significant heterogeneity exists in the endocytic pathways used by various G protein-coupled receptors. For example, whereas β2-adrenergic receptor is internalized via dynamin-dependent clathrin-coated pits (Zhang et al., 1996), agonist-induced endocytosis of bradykinin B2 receptor proceeds through caveolae (de Weerd and Leeb-Lundberg, 1997). Furthermore, an alternative and ill-defined atypical pathway has recently been described for M2 muscarinic receptor (Roseberry and Hosey, 2001). It is now widely believed that the mechanism of agonist-induced GPCR internalization varies among cell types and among individual receptors.

In the present study, we generated a chimeric receptor MC4R-green fluorescent protein (GFP) in which the C terminus of MC4R was fused to the N terminus of GFP. The extrinsically fluorescent MC4R-GFP allows us to directly visualize subcellular localization and trafficking of the MC4R in the presence or absence of agonist exposure. Moreover, in conjunction with the use of a red fluorescence-labeled MC4R agonist and antagonist, we have also examined the intracellular trafficking and recycling of the ligand and the receptor.

**Materials and Methods**

**Materials.** Tetramethylrhodamine-concanavalin A, tetramethylrhodamine-transferrin, and LysoTracker Red were purchased from Molecular Probes (Eugene, OR). Rhodamine-HS014, HS014, and rhodamine-MTII were purchased from Phoenix Pharmaceuticals (Belmont, CA). α-MSH, H89, sucrose and concanavalin A were purchased from Sigma; G418 and LipofectAMINE Plus reagent were purchased from Invitrogen (Carlsbad, CA). NDP-MSH-α-MSH was purchased (Torrance, CA). SHU9119 and MTII were purchased from Peninsula Laboratories (San Carlos, CA). Lab-Tek II chamber coverslips were from Nalge Nune International (Naperville, IL). 125I-NDP-MSH was purchased from PerkinElmer Life Sciences (Boston, MA). Alphagenes cAMP kit was purchased from PerkinElmer Life Sciences (Montreal, Canada). The SPA CAMP kit was purchased from Amersham Biosciences Inc. (Piscataway, NJ). Formalin was purchased from Biochemical Sciences (Swedesboro, NJ).

**Construction of Human MC4R-GFP Chimera and Transfection.** GFP cDNA (BD Biosciences Clontech, Palo Alto, CA) was fused in frame to the C terminus of human MC4R with the C-terminal termination codon removed. The MC4R-GFP fragment was subcloned into pcDNA 3,1, and the sequence was verified by automatic DNA sequencing. Human MC4R-GFP was transfected into HEK-293 cells by means of LipofectAMINE Plus. The stable colonies were selected by growth of cells in 1 mg/ml G418 and were maintained in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal bovine serum and 0.5 mg/ml G418. Cells were cultured in an atmosphere of 5% CO2, 95% air and maintained at 37°C.

**Membrane Preparation.** Cell monolayers were washed with PBS (3 × 10 ml) and harvested in buffer A (10 mM HEPES/NaOH, 1.5 mM CaCl2, 1 mM MgSO4, 0.1 M NaCl, 1 mM 1,10 phenanthroline, 0.2% bovine serum albumin, and one Complete, EDTA-free protease inhibitor tablet/100 ml (Roche Diagnostics), pH 7.4), supplemented with protease inhibitors (Roche Diagnostics, Indianapolis, IN). The cells were homogenized with Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) on setting 4 for ~ 20 s, centrifuged at 30,000g and washed twice with buffer HE (10 mM HEPES/1 mM EDTA, pH 7.4) containing protease inhibitors. The pellet was resuspended in HE supplemented with 10% sucrose and frozen in aliquots at ~ 80°C. The protein concentration of membrane suspensions was determined by using the Bradford method (Bio-Rad, Richmond, CA) with bovine serum albumin as standard.

**Radioligand Binding Assays.** Competition radioligand binding assays were performed to determine the affinities (Kd) of various agonists and antagonists for human MC4R-GFP. Membrane suspensions derived from transfected HEK-293 cells stably expressing human MC4R-GFP were incubated for 2 h at room temperature in 100 μl of binding buffer [25 mM HEPES-NaOH, 1.5 mM CaCl2, 1 mM MgSO4, 0.1 M NaCl, 1 mM 1,10 phenanthroline, 0.2% bovine serum albumin, and one Complete, EDTA-free protease inhibitor tablet/100 ml (Roche Diagnostics), pH 7.0] containing ~ 15 nM [125I]NDP-MSH and progressively higher concentrations of the competing agents. In Millipore Multiscreen GF/C 96-well filter plates precoated with 0.1% polyethyleneimine. Nonspecific binding was determined with 1 μM unlabelled NDP-MSH. The assay was sampled, free [125I]NDP-MSH was separated from the membrane-bound [125I]NDP-MSH using Multiscreen vacuum manifold. Scintillation cocktail (100 μl) was added to each well. After 2-h incubation, plates were counted using Microbeta Trilux (PerkinElmer Wallac, Gaithersburg, MD).

**cAMP Measurements.** Cells (~1.5–5 × 104/well) were seeded in 96-well culture plates 1 day before the experiment. On the day of experiment, the culture medium was removed and cells were treated with appropriate compounds for 30 min. The cAMP production was determined using either alpha screen cAMP (PerkinElmer Wallac) or SPA kit (Amersham Biosciences Inc., Piscataway, NJ) according to manufacturer’s instructions.

**Immunofluorescence Microscopy and Quantification of Fluorescence Intensity.** Cells were grown in Lab-Tek II chamber coverslips 24 to 48 h before the experiments. After appropriate treatments, cells were washed with cold PBS and fixed in formalin for 20 min, and observed on an LSM 510 META laser scanning microscope (Carl Zeiss, Thornwood, NY). Fluorescence of GFP was excited using a 488-nm argon/krypton laser, detected with a band pass filter of 500 to 550 nm. Red signal was excited with a HeNe laser at 543 nm and fluorescence was detected with a 565 to 615 band pass filter.
The digitally acquired images were quantitated using Scion Image Beta 4.02 (downloaded from www.scioncorp.com). The original green fluorescence confocal images were converted to grayscale and median filtering was performed. Each pixel is assigned an intensity value ranging from 0 (black) to 255 (white). The cell surface and total cellular fluorescence intensity was measured after manually selecting the corresponding area. The subcellular distribution of MC4R-GFP was expressed as a ratio of cell surface fluorescence intensity/total cellular fluorescence intensity. A decrease of ratio indicates receptor internalization.

Results

Pharmacological and Functional Properties of MC4R-GFP. To study the intracellular trafficking pathways of the human MC4R, we have constructed a chimeric MC4R with a C-terminal fusion to the GFP (MC4R-GFP). Saturation binding studies indicate that MC4R-GFP stably expressed in HEK-293 cells, bound to agonist $^{125}$I-NDP-MSH with a $B_{max}$ of ~1.6 pmol/mg membrane protein and a $K_D$ of ~1.8 nM (Fig. 1A). The pharmacological properties of the chimeric receptor MC4R-GFP seem to be indistinguishable from the wild-type receptor. The binding affinities of various agonists (NDP-MSH and MTII) and antagonists (SHU9119 and HS014) for MC4R-GFP were determined by competition binding assays and found to be quite comparable with those for the wild-type receptor (Fig. 1, B and C). In addition, we also characterized for the first time, a fluorescence-labeled peptide agonist rhodamine-MTII and peptide antagonist rhodamine-HS014 (Fig. 1, B and C). We found that tagging the agonist MTII with the rhodamine has minimal effect on their binding affinities for MC4R, whereas the binding affinity of rhodamine-conjugated HS014 is somewhat lower than that of the parent peptide (~1.8-fold and 3-fold lower for wild-type and MC4R-GFP, respectively). The capacity of MC4R-GFP to activate adenylyl cyclases was also assessed. As shown in Fig. 1D, MC4R-GFP, in response to agonist NDP-MSH stimulation, potently increased intracellular cAMP content in HEK-293 with an EC$_{50}$ value (1.8 nM) very similar to that documented for the wild-type receptor (Yang et al., 2002).

Agonist-Induced Translocation of MC4R-GFP. When MC4R-GFP expressing cells were analyzed by confocal laser scanning microscopy, the green receptors seemed to be evenly distributed on the cell surface membrane in unstimulated HEK-293 cells (Fig. 2). The predominant localization of receptor on the plasma membrane was confirmed by the colocalization of the green fluorescence of MC4R-GFP with the red fluorescence of rhodamine-concanavalin A (Fig. 2), a cell surface membrane marker. Intracellular localization of the receptor molecules was also detectable in some cells, but...
nuclear localization of the receptor was never observed. As shown in Fig. 3, A and B, addition of NDP-MSH caused a time- and concentration-dependent internalization of MC4R-GFP, characterized by the gradual appearance of MC4R-GFP in punctate intracellular structures and a concomitant reduction of the cell surface green fluorescence signal. Semiquantitative analysis of receptor internalization (indicated by a decrease of cell surface/total cellular fluorescence intensity ratio) suggested that NDP-MSH induce MC4R internalization with a $t_{1/2}$ of ~15 min and an $EC_{90}$ value of 11 nM (Fig. 3, C and D).

In contrast to the agonist NDP-MSH, SHU9119, a potent MC4R antagonist was ineffective in driving receptor internalization even when used at a very high concentration (200 nM) (Fig. 4). However, internalization of MC4R-GFP induced by 20 nM NDP-MSH was significantly inhibited by 200 nM SHU9119, suggesting that internalization was a melanocortin agonist-specific effect.

Characterization of the MC4R-GFP Internalization. Agonist-occupied MC4R is positively coupled to adenyllyl cyclases, leading to the cAMP accumulation and activation of protein kinase A (PKA). We set out to determine whether the PKA activity contributes to agonist-induced internalization of MC4R. As shown in Fig. 5, blockade of the PKA pathway by pretreatment with H89 had no effect on cellular distributions of MC4R-GFP either in the absence or presence of agonist exposure. On the other hand, the same batch of H89 effectively inhibited PKA-mediated mitogen-activated protein kinase activation in HEK-293/MC4R-GFP (data not shown), confirming that PKA activity is indeed blocked by H89 under these conditions.

Agonist-induced endocytosis of many GPCRs occurs via clathrin-coated pits, a process that can be inhibited by hypertonic sucrose (Hansen et al., 1993) and concanavalin A (Pippig et al., 1995). As shown in Fig. 6, preincubation of cells with 0.5 M sucrose or 0.25 mg/ml concanavalin A caused total ablation of agonist-induced internalization of MC4R-GFP.

We next sought to determine the nature of intracellular vesicles into which MC4R-GFP is translocated after agonist exposure. Cells were preloaded with tetramethylrhodamine transferrin, a classic marker for early endosomes (Richardson and Ponka, 1997). In resting cells, the MC4R-GFP and the rhodamine-transferrin were mostly separated in different compartments (Fig. 7, control) with the receptor located on the cell surface and transferrin accumulated in intracellular vesicles. In some of these cells, we saw that a small portion of vesicles contained both MC4R-GFP and rhodam-
activation studies. In addition, MC4R-GFP was predominantly expressed and distributed evenly on the cell surface in transfected HEK-293 cells, suggesting that the receptor may not be compartmentalized in any specialized microdomains under basal conditions. Agonist treatment caused a time- and concentration-dependent internalization of MC4R-GFP.

During the preparation of this manuscript, Shinya et al. (2003) published a report demonstrating that the hemagglutinin-tagged human MC4R (HA-hMC4R) underwent agonist-dependent internalization in transiently transfected HEK-293 cells using enzyme-linked immunosorbent assays. The time course of α-MSH-induced HA-hMC4R internalization is quite similar to what we observed with hMC4R-GFP, further validating the use of MC4R-GFP as a convenient model system to study ligand-mediated receptor internalization and trafficking. Nonetheless, the two reports have focused on entirely different aspects of receptor internalization. Whereas Shinya et al. (2003) investigated the involvement of receptor phosphorylation in MC4R internalization, we centered our efforts on studying the fate of both MC4R-GFP and fluorescent ligands after internalization with the use of fluorescence colocalization technique. In addition, the two reports differ significantly in the data interpretation on the involvement of PKA activation in MC4R internalization (see below).

It is worth noting that the EC\textsubscript{50} value (11 nM) of NDP-MSH to promote MC4R-GFP endocytosis is significantly higher than the apparent binding affinity \( K_D \) (1.5 nM) and the EC\textsubscript{50} value (1.80 nM) in stimulation of cAMP accumulation. Although 3 nM NDP-MSH could cause \( \geq 50\% \) of maximal cAMP response, it has only a marginal effect on receptor internalization. Similarly, α-MSH, an endogenous agonist, increased intracellular cAMP content in HEK-293/MC4R-GFP cells with an average EC\textsubscript{50} value of \( \sim 20 \) nM (data not shown). However, in internalization studies, α-MSH, at concentrations of \( \leq 30 \) nM, were not very effective in causing receptor internalization. Significant receptor internalization was observed, however, when cells were treated with 100 nM α-MSH. Based on these data, we concluded that agonist occupancy is very important for efficiently promoting MC4R internalization and hypothesized that receptor-mediated...
cAMP accumulation and subsequent PKA activation may not be sufficient or essential for this process in HEK-293 cells.

To test this hypothesis, we assessed the ability of a specific PKA inhibitor H89 to inhibit NDP-MSH-mediated MC4R-GFP internalization. We have found that H89, at a concentration \(10^{-6} M\) that is sufficient to block the PKA activity [as indicated by complete inhibition of PKA-mediated mitogen-activated protein kinase activation as reported by Gao et al. (1999)], had no detectable effect on NDP-MSH-stimulated MC4R-GFP internalization. This result is different from that reported by Shinyama et al. (2003), who detected a moderate (25%) decrease of agonist-induced HA-hMC4R internalization in the presence of H89 in HEK-293 cells. However, the effect of H89 in that study did not seem to be clearly concentration-dependent. Whereas 0.1 \(\mu M\) H89 had no effect, both 1 and 10 \(\mu M\) H89 caused the same 25% reduction of MC4R internalization. On the other hand, Shinyama et al. (2003) showed that activation of PKA by forskolin has little influence on the internalization of MC4R, and removal of potential PKA phosphorylations sites only had very moderate effect on MC4R internalization and mutant receptors still underwent significant internalization. The reason for the different results with inhibitor H89 remains unclear at the present, but it is possible that the disagreement may reflect the differences in the cellular context between stably and transiently transfected HEK-293 cells, and the differences in the sensitivity of detection systems (enzyme-linked immunosorbent assay versus GFP) used in the two reports. At the present, we could not absolutely exclude the involvement of PKA in MC4R internalization. Additional studies using PKA-deficient cell line or PKA phosphorylation-deficient MC4R-GFP mutant may help to resolve the differences and further clarify this issue. Nonetheless, in our opinion, data from both reports argue strongly that agonist-mediated MC4R internalization is largely independent of PKA activation.

Agonist-induced MC4R-GFP internalization seems to proceed via clathrin-coated vesicles. The internalization was...
completely blunted by the hypertonic sucrose solution, a treatment that has been known to specifically inhibit the formation of clathrin-coated pits, and also by concanavalin A, a more general internalization inhibitor (Hansen et al., 1993; Pippig et al., 1995). Consistent with this notion, we have shown that after acute agonist exposure, internalized MC4R-GFP was colocalized with rhodamine-transferrin. The transferrin binds to cell surface transferrin receptor, constitutively internalizes into early endosome also via the clathrin-mediated endocytic process and then recycles back to the cell surface membrane (Dautry-Varsat, 1986). Extensive colocalization of GFP-MC4R with LysoTracker Red, a specific lysosome marker, was observed with prolonged, but not with acute agonist treatment. These results suggest that after endocytosis, MC4R-GFP is initially delivered into early endosomes and then sorted and transported to lysosomes in the continued presence of agonist, possibly for degradation.

The availability of a rhodamine-conjugated agonist, MTII, permitted us to simultaneously monitor the fate of both the agonist ligand and the receptor after endocytosis. We demonstrated that the agonist ligand is internalized together with the receptor as a receptor-ligand complex. It seems that MTII is very efficient (compared with antagonist rhodamine-HS014) at promoting receptor internalization, because no cell surface labeling of the receptor with the fluorescent agonist was observed after 45 min of incubation. A very small portion of intracellular vesicles was found to contain only either red fluorescent ligands or green fluorescent receptors. This indicates that some dissociation of the ligands from the receptors occurs during the endocytosis.

One of the major findings in this report is the slow recycling of MC4R and ligands after agonist-promoted receptor internalization. A significantly large amount of ligands remain associated with receptors in intracellular compartments even 60 min after removal of the agonist from the medium. This result is significantly different from that observed for most G protein-coupled receptors. For example, the recycling of cholecystokinin receptor type A (Tarasova et al., 1997), thyrotropin-releasing hormone receptor (Ashworth et al., 1995), C5a anaphylatoxin receptor (Naik et al., 1997), and β2-adrenergic receptor (Kallal et al., 1998) has been shown to be remarkably efficient with the average recycling time ranging from 20 to 60 min.

The slow or lack of receptor recycling, however, is not without precedent. It has been reported that both hCG/LH receptor and thrombin receptor are targeted to a lysosome-mediated degradative pathway instead of sorting to the recycling compartment after internalization (Ghinea et al., 1992; Hoxie et al., 1993). Another interesting example is the MC1R, which shares about 45% amino acid identity with MC4R. In murine B16 melanoma cells, prolonged treatment with 125I-NDP-MSH caused significant accumulation of the ligands in the lysosome and down-regulation of the cell surface MC1R (Wong and Minchin, 1996). Moreover, indirect biochemical evidence suggests that MC1R is also delivered to the lysosome compartment along with its ligand after endocytosis and does not seem to recycle between the endosomes and plasma membrane in B16 melanoma cells (Wong and Minchin, 1996).

The relative slow dissociation of ligand from MC4R shown in HEK-293 cells may have important implication in MC4R-mediated receptor signal transduction. Internalized ligand-receptor complex may continue to engage in the same signaling pathway or switch to a totally different pathway as demonstrated for β2-adrenergic receptor. This may help explain, at least partially, the very modest desensitization (<20%) of MC4R-mediated cAMP accumulation in HEK-293 cells after a 60 min agonist pretreatment as reported by Shinya et al. (2003) despite the fact that near-maximal receptor internalization has been observed at 60 min.

In conclusion, the current data provide strong evidence that like many other G protein-coupled receptors, the human MC4R-GFP is subject to significant agonist-promoted endocytosis. Upon acute agonist challenge, the MC4R-GFP is internalized and delivered to early endosome via a mechanism possibly involving clathrin-coated pits. Continued agonist exposure seems to cause receptors to be sorted and accumulated into lysosomes, likely for protein degradation. Unlike most GPCRs, MC4R-GFP is not efficient at recycling of the receptor and the ligand. The GFP-tagged MC4R may prove to be a powerful and perfect tool for detailed studies of the kinetics, pharmacology, molecular determinants, as well as the functional significance of both peptide and small molecule agonist-induced MC4R internalization. Because agonist-induced internalization is intimately linked to cellular events that may lead to receptor desensitization and limit the usefulness of GPCR agonists in long-term therapy, a better understanding of agonist-mediated regulation of MC4R func-
tion is a prerequisite for the clinical application of MC4R agonists.

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References


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