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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 *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), but was impaired 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 LysoTraker, a lysosome marker, was observed after short-term (45 min) and prolonged (20 h) agonist exposure, respectively. Rhodamine-[Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2] (MTII), a fluorescent derivative of an MC4R agonist, was found to cointernalize with MC4R-GFP into intracellular vesicles. No significant receptor recycling or segregation from the ligand was observed 60 min after removal of the agonist. In contrast, an antagonist rhodamine-Ac-Cys-Glu-His-(D-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 (G_s) 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 (Sebhat et al., 2002). These major breakthroughs have raised

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ABBREVIATIONS: MSH, melanocyte-stimulating hormone; MCR, melanocortin receptor; GPCR, G protein-coupled receptor; GFP, green fluorescent protein; HS014, Ac-Cys-Glu-His-(D-Nal)-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp-NH_2; MTII, [Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2]; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; SHU9119, [Ac-Nle-c[Asp-His-D-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.

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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 (Claing 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 resensitization of β_2-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 β_2-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 β_2-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 intrinsically 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 from Bachem (Torrance, CA). SHU9119 and MTII were purchased from Peninsula Laboratories (San Carlos, CA). Lab-Tek II chamber coverglasses were from Nalge Nunc International (Naperville, IL). ^{125}I-NDP-MSH was purchased from PerkinElmer Life Sciences. (Boston, MA). AlphaScreen 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 (Swedesbord, 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% CO_2, 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/20 mM EDTA, 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 30,000*g* 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 (K_i) 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 $CaCl_2$, 1 mM $MgSO_4$, 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 ~0.15 nM [^{125}I]NDP-MSH and progressively higher concentrations of the competing agents in Millipore Multiscreen GF/C 96-well filter plates precoated with 0.1% polyethylenimine. Nonspecific binding was determined with 1 μM unlabeled NDP-MSH. At the end of the incubation, free ^{125}I-NDP-MSH was separated from the membrane-bound ^{125}I-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 × 10^4/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 coverglasses 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.

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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 radio 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}-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

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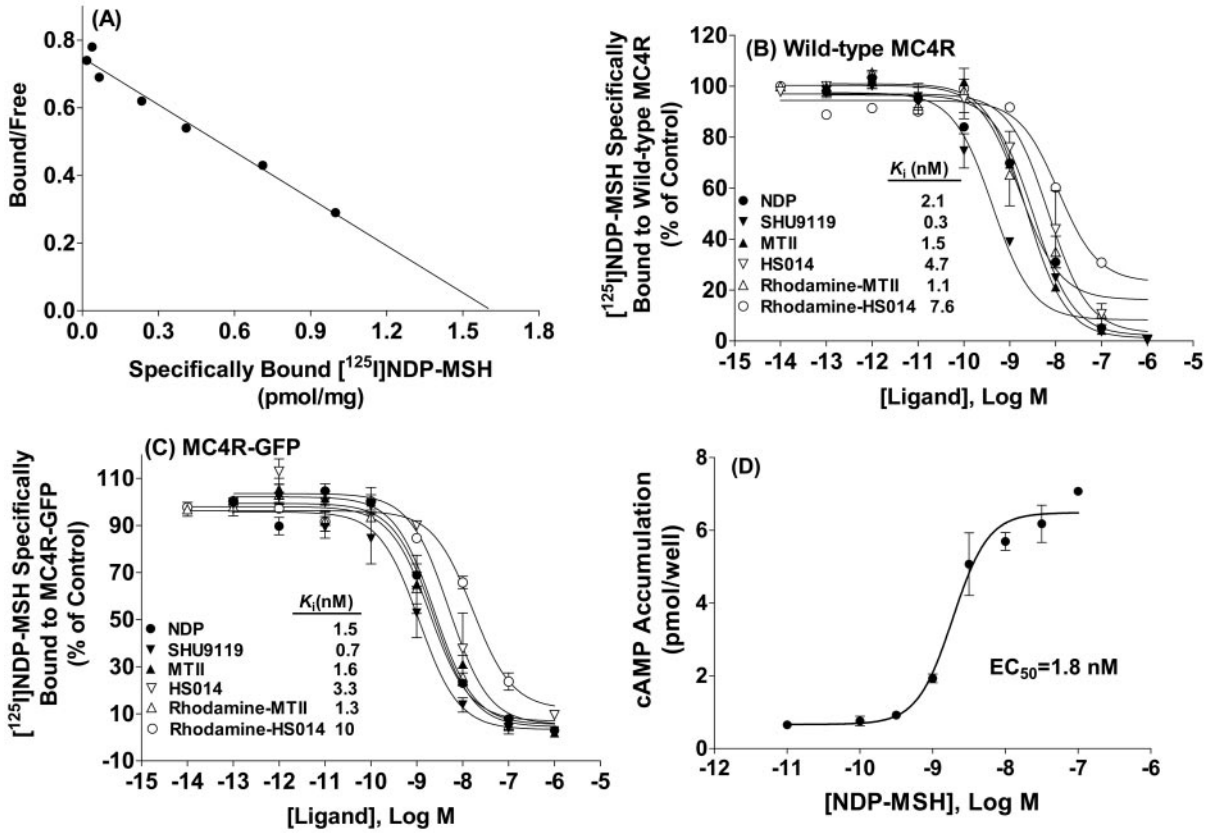

Fig. 1. Pharmacological characterization of the human MC4R-GFP in HEK-293 cells. A, Scatchard plot of a saturation isotherm of ^{125}I-NDP-MSH binding to membranes from HEK-293 stably expressing human MC4R-GFP. Membrane suspensions (5 μg/well) were incubated with increasing concentrations (0.03–5 nM) of ^{125}I-NDP-MSH for 2 h at room temperature. Nonspecific binding was determined in the presence of 1 μM NDP-MSH. Each point is the mean ± S.E.M of triplicate determinants. B and C, displacement of ^{125}I-NDP-MSH binding to membranes prepared from HEK-293 cells stably expressing human wild type (B) or MC4R-GFP (C). ^{125}I-NDP-MSH (~0.13 nM) and membrane suspensions were incubated with progressively increasing concentrations of unlabeled melanocortin receptor ligands for 2 h at room temperatures. Nonspecific binding was determined in the presence of 1 μM NDP-MSH. Each point is the mean ± S.E.M of data pooled from at least three experiments, each assayed in duplicates. D, effect of melanocortin receptor agonist NDP-MSH on cAMP content in HEK-293 cells stably expressing MC4R-GFP. HEK-293 cells were incubated for 30 min with various concentrations of NDP-MSH at 37°C. Cyclic AMP levels were determined as described under *Materials and Methods*.

Fig. 2. Colocalization of the MC4R-GFP (in green) with the cell surface marker rhodamine red concanavalin A (in red) in HEK-293 cells. HEK-293 cells stably expressing the human MC4R-GFP were washed with ice-cold PBS and stained with 100 μg/ml rhodamine red concanavalin A for 2 min at 4°C. Confocal images show the colocalization of MC4R-GFP (green) and rhodamine concanavalin A (red) on cell surface, which is shown in yellow.

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_{50} 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 adenylyl 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 rhodamine-transferrin (colocalization shown in yellow) in the absence of agonist treatment. This suggests that a small fraction of MC4R-GFP could also undergo spontaneous endocytosis. Forty-five minutes after treatment with NDP-MSH, more significant colocalization of MC4R-GFP-containing vesicles with fluorescently labeled transferrin was observed (Fig. 7).

We subsequently assessed whether a portion of internalized receptors can be delivered to lysosome for degradation. Cells were incubated for 20 h with 100 nM LysoTracker Red, which is known to accumulate specifically in lysosomes (Tarasova et al., 1997). After acute (45 min) agonist treatment, there was very little colocalization of the MC4R-GFP with the LysoTracker Red; however, after prolonged (20 h) agonist treatment, the MC4R-GFP exhibited significant accumulation in LysoTracker Red-containing lysosomes (Fig. 8).

Intracellular Trafficking and Recycling of the Ligand and the Receptor. As shown earlier, the rhodamine-conjugated agonist MTII and antagonist HS014 retain the ability to bind to MC4R or MC4R-GFP with relatively high affinity. This provides us useful tools for concurrent analysis of intracellular trafficking of both the receptor and the ligand. As shown in Fig. 9A, after 45 min of incubation, rhodamine-MTII was internalized together with the MC4R-GFP as demonstrated by the appearance of yellow intracellular vesicles. No cell surface labeling with red fluorescence was observed. A very small number of intracellular vesicles were found to contain only ligand-associated red fluorescence.

To further study the recycling of the ligand and the receptor and to determine whether agonist-induced MC4R-GFP trafficking is reversible, HEK-293 cells were treated with 20 nM rhodamine-MTII for 45 min, and the agonist was then removed from the medium. As shown in Fig. 9B, 60 min after washout of the agonist, the majority of fluorescently labeled agonists remained associated with MC4R-GFP in intracellular vesicles. There was somewhat an increase in the number of vesicles containing only red fluorescent agonist. Nevertheless, no significant recycling of MC4R-GFP from intracellular vesicles to the plasma membrane was observed.

In contrast to the agonist rhodamine-MTII, the antagonist rhodamine-HS014 did not undergo MC4R receptor-mediated endocytosis. As shown in Fig. 10, rhodamine-HS014 mainly bound to and colocalized with MC4R-GFP on the cell surface as indicated by the yellowish color but did not stimulate MC4R-GFP internalization.

Discussion

The goal of the present study is to determine the effect of agonist exposure on the subcellular distribution and trafficking of MC4R as well as its ligands. To facilitate visualization of receptor movement within intracellular compartments, we have constructed an MC4R-GFP chimeric receptor. The same approach has recently been used to study the cellular distributions of several other G protein-coupled receptors (for review, see Kallal and Benovic, 2000). Despite the fact that the native human MC4R has a very short C-terminal tail (28 amino acids), attachment of the bulky 27-kDa GFP polypeptide to the C terminus of receptor was found not to alter the pharmacological and functional characteristics of the human MC4R in extensive ligand binding and second messenger

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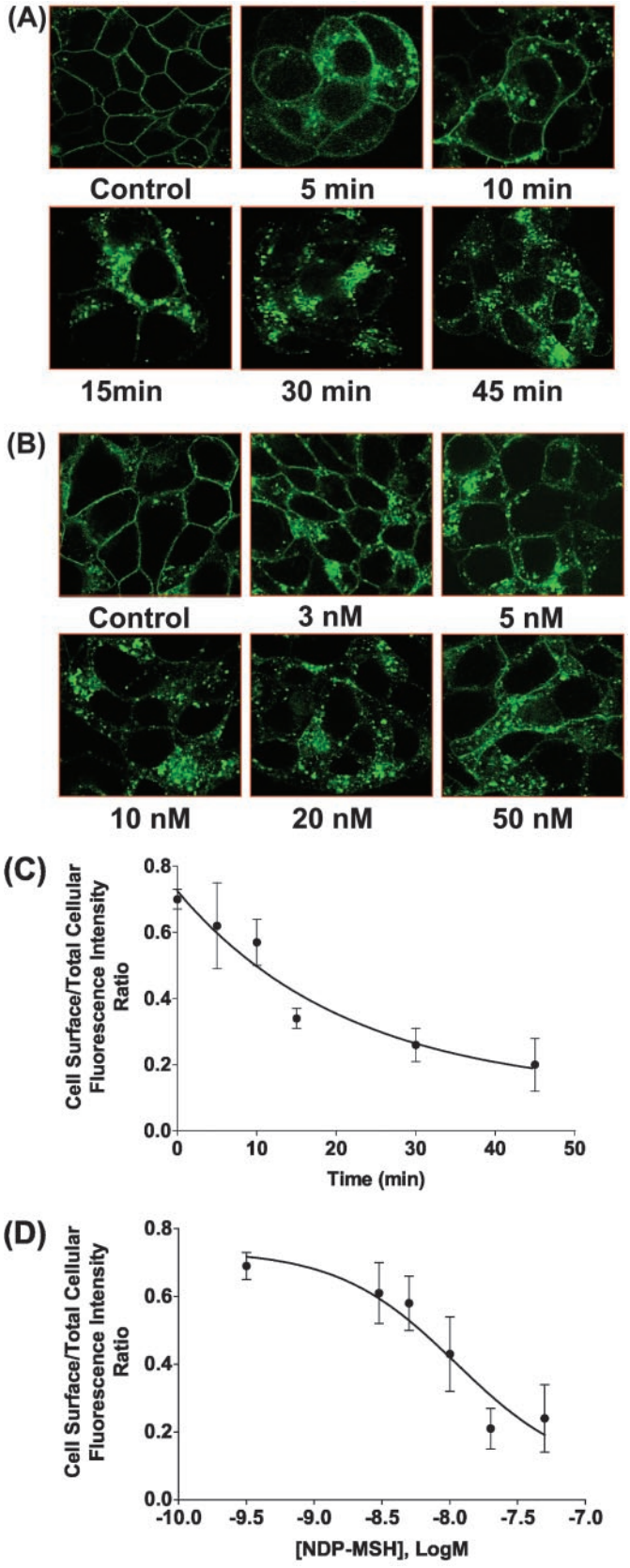

Fig. 3. Time course and dose dependence of agonist induced internalization of MC4R-GFP. A, HEK-293 cells stably expressing MC4R-GFP were exposed to 20 nM NDP-MSH for various lengths of time. Confocal images show the distribution of receptors (green) at the selected times after agonist stimulation. B, HEK-293 cells stably expressing MC4R-GFP were treated with indicated concentrations of agonist NDP-MSH for 45 min, and cellular distribution of receptors was examined by confocal microscopy. C and D, semiquantitative analysis of time course (C) and concentration dependence (D) of agonist induced MC4R-GFP internalization. Digitized fluorescence intensity on the cell surface membrane and total cellular fluorescence intensity were quantitated as described under *Materials and Methods*. The subcellular distribution of MC4R-GFP was expressed as a ratio of cell surface fluorescence intensity/total cellular fluorescence intensity. A decrease of radio indicates receptor internalization.

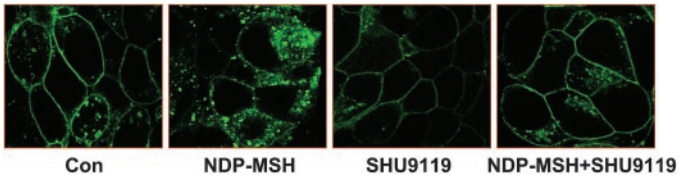

Fig. 4. Blockade of agonist induced internalization of MC4R-GFP by the antagonist SHU9119. HEK-293 cells stably expressing MC4R-GFP were stimulated with 20 nM NDP-MSH for 45 min in the absence or presence of 200 nM SHU9119 and imaged by confocal microscopy to localize the MC4R-GFP.

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, Shinyama 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 Shinyama 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_{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_{50} value (1.80 nM) in stimulation of cAMP accumulation. Although 3 nM NDP-MSH could cause ≥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_{50} value of ~20 nM (data not shown). However, in internalization studies, α-MSH, at concentrations of ≤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

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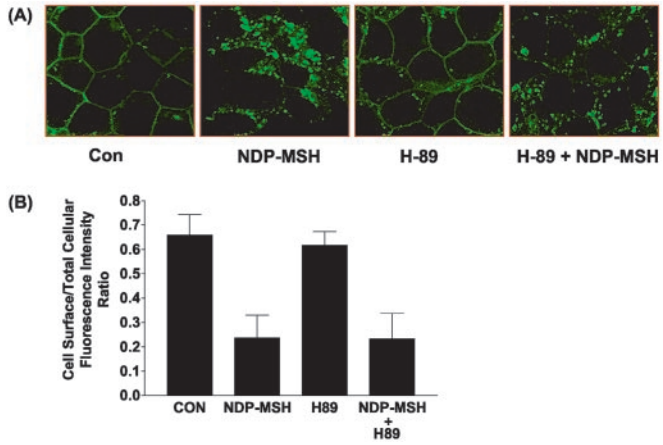

Fig. 5. Effect of a protein kinase A inhibitor H89 on agonist-induced internalization MC4R-GFP. A, HEK-293 cells stably expressing MC4R-GFP were preincubated at 37°C with 10 μM H89 for 30 min before stimulation with 20 nM NDP-MSH and imaged by confocal microscopy to localize the MC4R-GFP. B, semiquantitative analysis of confocal images shown in A. Digitized fluorescence intensity on the cell surface membrane and total cellular fluorescence intensity were quantitated as described under *Materials and Methods*. The subcellular distribution of MC4R-GFP was expressed as a ratio of cell surface fluorescence intensity/total cellular fluorescence intensity.

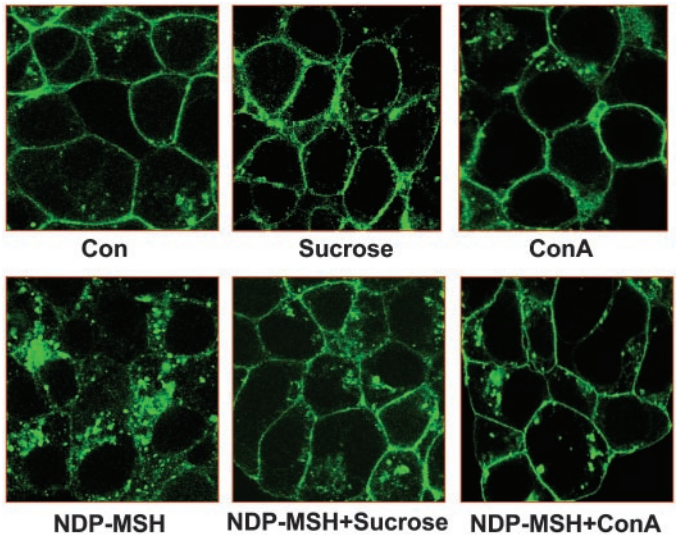

Fig. 6. Effects of hypertonic sucrose and concanavalin A on agonist-induced internalization of MC4R-GFP. HEK-293 cells stably expressing MC4R-GFP were preincubated at 37°C with either 0.5 M sucrose or 0.25 mg/ml concanavalin A for 30 min before stimulation with 20 nM NDP-MSH. The cellular distribution of MC4R-GFP was then examined by confocal microscopy.

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 μM) 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 μM H89 had no effect, both 1 and 10 μM 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

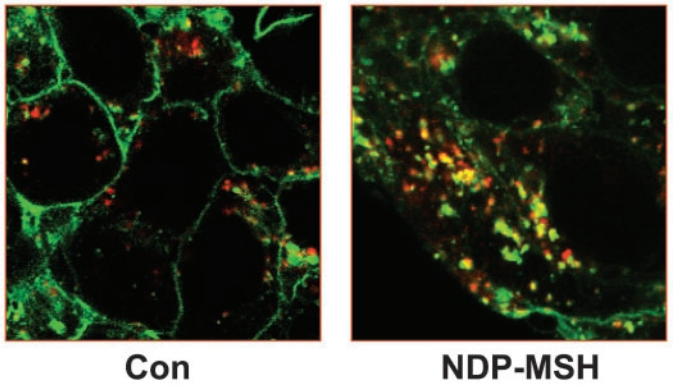

Fig. 7. Colocalization of the MC4R-GFP (in green) with the endosomal marker tetramethylrhodamine transferrin (in red) after 45 min agonist treatment. HEK-293 cells stably expressing MC4R-GFP were pretreated with 250 μg/ml tetramethylrhodamine transferrin and then stimulated with 20 nM NDP-MSH for additional 45 min. GFP-associated fluorescence (green) and tetramethylrhodamine transferrin fluorescence (red) were examined by confocal microscopy. Note the prominent colocalization (yellow) of MC4R-GFP and tetramethylrhodamine transferrin in the early endosomal compartment.

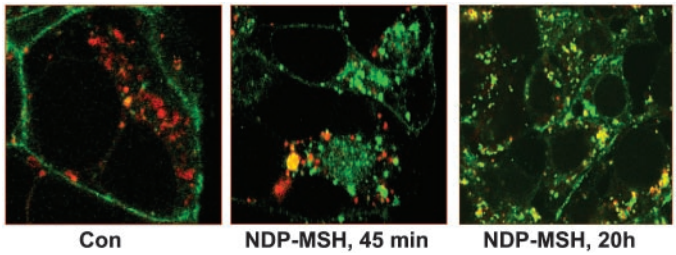

Fig. 8. Localization of MC4R-GFP in the lysosome after short-term (45 min) and prolonged agonist (20-h) treatment. HEK-293 cells stably expressing MC4R-GFP were pretreated with 100 nM LysoTracker Red and then stimulated with 20 nM NDP-MSH for either 45 min or 20 h. GFP-associated fluorescence (green) and LysoTracker fluorescence (red) were examined by confocal microscopy. Colocalization of the two shows as yellow.

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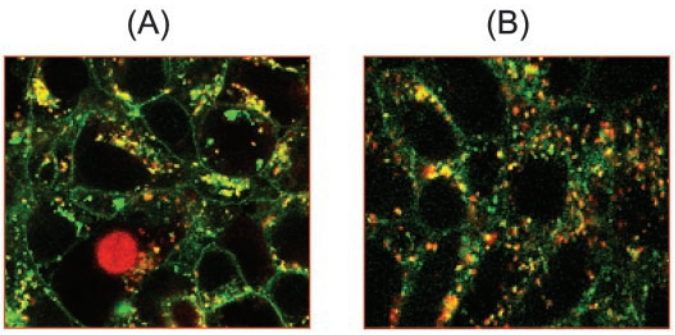

Fig. 9. Internalization of MC4R-GFP (in green) and Rhodamine-MTII (in red) complexes. A, HEK-293 cells stably expressing MC4R-GFP were treated for 45 min with 20 nM agonist rhodamine-MTII. GFP-associated fluorescence (green) and rhodamine-MTII fluorescence (red) were examined by confocal microscopy. B, cells were treated with 20 nM rhodamine-MTII for 45 min, washed extensively to remove the agonist, and further incubated in agonist-free medium for additional 60 min. Localization of receptor (green) and agonist ligand (red) was examined by confocal microscopy. Colocalization of the two shows as yellow.

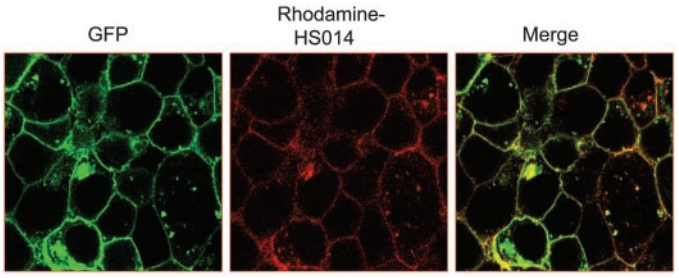

Fig. 10. Colocalization of the MC4R-GFP (in green) with the antagonist rhodamine-HS014 (in red) on the cell surface. HEK-293 cells stably expressing MC4R-GFP were incubated with 20 nM rhodamince-HS014 for 45 min. GFP-associated fluorescence (green) and rhodamine-HS014 fluorescence (red) were examined by confocal microscopy. Colocalization of the two shows as yellow.

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 β_2-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 ^{125}I-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 β_2-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 Shinyama 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-

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tion is a prerequisite for the clinical application of MC4R agonists.

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