Constitutive Cholesterol-dependent Endocytosis of Melanocortin-4 Receptor (MC4R) Is Essential to Maintain Receptor Responsiveness to α-Melanocyte-stimulating Hormone (α-MSH)\(^*\)\(^\text{1,2,3}\)

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**Background:** MC4R is essential for energy homeostasis and cycles continuously.

**Results:** MC4R internalization is blocked by clathrin and cholesterol depletion, reducing receptor response to α-MSH, which is partially recovered by mutations at Thr-312/Ser-329.

**Conclusion:** Constitutive internalization of MC4R is cholesterol-dependent and required for receptor function.

**Significance:** These findings provide a potentially novel mechanism by which hypothalamic cell cholesterol content can affect appetite control.

Melanocortin-4 receptor (MC4R) is a G-protein-coupled receptor expressed in the hypothalamus where it controls feeding behavior. MC4R cycles constitutively and is internalized at the same rate in the presence or absence of stimulation by the agonist, melanocyte-stimulating hormone (α-MSH). This is different from other G-protein-coupled receptors, such as β₂-adrenergic receptor (β₂AR), which internalizes more rapidly in response to agonist stimulation. Here, it is found that in immortalized neuronal Neuro2A cells expressing exogenous receptors, constitutive endocytosis of MC4R and agonist-dependent internalization of β₂AR were equally sensitive to clathrin depletion. Inhibition of MC4R endocytosis by clathrin depletion decreased the number of receptors at the cell surface that were responsive to the agonist, α-MSH, by 75%. Mild membrane cholesterol depletion also inhibited constitutive endocytosis of MC4R by ~5-fold, while not affecting recycling of MC4R or agonist-dependent internalization of β₂AR. Reduced cholesterol did not change the MC4R dose-response curve to α-MSH, but it decreased the amount of cAMP generated per receptor number indicating that a population of MC4R at the cell surface becomes nonfunctional. The loss of MC4R function increased over time (25–50%) and was partially reversed by mutations at putative phosphorylation sites (T312A and S329A). This was reproduced in hypothalamic GT1-7 cells expressing endogenous MC4R.

The data indicate that constitutive endocytosis of MC4R is clathrin- and cholesterol-dependent. MC4R endocytosis is required to maintain MC4R responsiveness to α-MSH by constantly eliminating from the plasma membrane a pool of receptors modified at Thr-312 and Ser-329 that have to be cycled to the endosomal compartment to regain function.

MC4R\(^4\) is a G-protein-coupled receptor (GPCR) expressed in the brain, which is central to the control of food intake. In this respect, intraventricular administration of the agonist α-MSH suppresses feeding (1–4). Moreover, MC4R knock-out in mice leads to obesity syndrome with hyperphagia, hyperglycemia, and hyperinsulinemia (5). Although MC4R is expressed in many areas of the brain (6), it controls feeding behavior at specialized sites. Correspondingly, it has been reported that restored MC4R expression in the paraventricular hypothalamus and amygdala of MC4R-deficient mice is sufficient to control food intake (7). The importance of MC4R in food intake regulation is highlighted by the discovery that most of the known genetic causes of obesity are due to mutations in MC4R (8–10). Following agonist stimulation, GPCRs are desensitized by a mechanism that classically includes phosphorylation of the receptor at the C terminus, with recruitment of the clathrin adaptors β-arrestin1 and β-arrestin2. β-Arrestins bind both directly to clathrin and to the clathrin adaptor AP-2, thereby clustering the receptors into clathrin-coated vesicles to be endocytosed (11–13). In hypothalamic GT1-7 and human...
embryonic kidney (HEK) 293 cells, MC4R is desensitized following exposure to the agonist α-MSH (14–16). Consistent with these reports, we have found that in immortalized neuronal Neuro2A cells (N2A cells) and in hypothalamic GT1-7 cells, MC4R expression at the plasma membrane is decreased by exposure to the agonist (17). However, we have also found that in these cells MC4R is internalized at the same rate in the absence and in the presence of the agonist, indicating that the process is independent of receptor activity. In addition, agonist-dependent disappearance of MC4R from the cell surface appeared to occur by blocking recycling of a fraction of internalized receptor back to the plasma membrane, rather than by inducing endocytosis (17). The intracellular traffic of MC4R along the endosomal route appears to play an important role in the function of the receptor. In this respect, prolonged exposure to α-MSH promotes its localization to lysosomes (15). Moreover, trafficking of intracellular MC4R back to the cell surface rather than to lysosomes underlies the ability of mutations of attractin and Mahogunin Ring Finger-1 (Mgrn1) to rectify the obesity in mice overexpressing agouti-signaling protein, an antagonist of MC4R (18).

In addition to MC4R, other GPCRs, including the thyrotropin receptor (19), M2 muscarinic receptor (20), β2AR (21), and the thrombin receptor (22, 23), are also internalized constitutively, albeit in most cases at a decreased rate rather than in the presence of the agonist and by binding to other adaptors rather than β-arrestin. For example, β2AR, which is internalized rapidly in response to agonist stimulation by a classical β-arrestin clathrin-dependent mechanism (11, 12, 24, 25), is also internalized constitutively although less rapidly by a clathrin-independent pathway (21). For some GPCRs, constitutive endocytosis is essential for function. In this respect, the thrombin receptor is activated by proteolysis, and the existence of a protected pool of receptors in the endosomal compartment makes possible the recovery of cell responsiveness to thrombin (26). In the case of the type 1 cannabinoid receptor, constitutive internalization of the receptor occurring in the somatodendritic compartment of the neuron appears to be required for axonal targeting of the receptor (27–30). Our observation that MC4R is constitutively internalized raises the question of whether this process is relevant for MC4R function. Here, we find that constitutive endocytosis of MC4R is clathrin-dependent, but it occurs by a different mechanism than that of β2AR by being less sensitive to depletion of β-arrestins and by being dependent on the level of membrane cholesterol. Inhibition of MC4R constitutive internalization, either by reducing clathrin expression or by depletion of membrane cholesterol, decreased signaling in response to α-MSH in neuronal and hypothalamic cells, indicating that the process is essential for MC4R function.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Lipofectamine 2000 and Amplex Red cholesterol assay kit were purchased from Invitrogen. The following antibodies were used: mouse monoclonal anti-GFP antibodies, rat monoclonal anti-HA antibody (3F10), peroxidase (POD)-conjugated anti-hemagglutinin (HA) antibody (3F10), fluorescein-conjugated rat monoclonal anti-HA antibody (3F10), secondary POD-conjugated anti-mouse IgG, protease inhibitor mixture (Complete Mini), and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) tablets were from Roche Applied Science; α-MSH, 3-isobutyl-1-methylxanthine (IBMX), mouse monoclonal anti-clathrin heavy chain clone td1, and methyl-β-cyclodextrin (MβCD) were from Sigma; BCA protein assay reagent, secondary POD-conjugated anti-rabbit IgG and secondary POD-conjugated anti-goat IgG were from Pierce; tetramethylrhodamine-Tf was from Molecular Probes (Eugene, OR); FITC-conjugated anti-mouse IgG, Cy3- and Cy5-conjugated anti-rat IgG and Cy3-conjugated antismouse IgG were from Jackson ImmunoResearch (West Grove, PA); enhanced chemiluminescence detection kits were from PerkinElmer Life Sciences. Enhanced green fluorescent protein expression vector pEGFP-N2 was from Clontech. POD-conjugated human transferrin (TI) was from Rockland Immunochemicals, Inc. (Gilbertsville, PA); formaldehyde (16%) was from Ted Pella Inc (Redding, CA). Mouse monoclonal antibodies against μ-adaptin antibodies were from BD Transduction Laboratories (San Jose, CA); DAB substrate kit for peroxidase was from Vector Laboratories (Peterborough, United Kingdom); direct cAMP EIA kit was from Enzo Life Science Inc. (Farmingdale, NY); ELISA kit was from Enzo Life Science Inc.; embryonic mouse hypothalamic cell line, N-42, was purchased from Cedarlane Laboratories (Burlington, Ontario, Canada).

** Constructs**—HA-MC4R-GFP and HA-MC4R plasmids were described earlier (17). Human β2AR cDNA in the pcMV-SPORT6 vector was obtained from Open Biosystems (Rockville, MD). The HA epitope (YPYDVPDYA)-tagged β2AR was generated by PCR amplification using Pfx-Platinum polymerase (Invitrogen) using β2AR in the pcMV-SPORT6 plasmid as template. The forward primer, 5’-CTCAAGCCTCGAGCCACCATGTTATCCTTAATGATGTGGCCTGCTATTAACCCGCGGGAACCCCGACGGC-3’, was designed with a HindIII site at the N terminus (underlined) and the HA epitope (in boldface) after the starting ATG codon. The reverse primer 5’-CGGTGATCCCAGTTGACTGGGAGTCAAGAAGGCGGAAAGCTTTCAAAGAGATCATCTGTGGC-3’ for S329A, and 5’-CTGGAGGGCCATTGTGAATGACTGGGAGTCAAGAAGGCGGAAAGCTTTCAAAGAGATCATCTGTGGC-3’ for S329A and S330A were used. The MβCD was from Sigma-Aldrich (St. Louis, MO). The HA epitope (YPYDVPDYA)-tagged β2AR was generated by PCR amplification using Pfx-Platinum polymerase (Invitrogen) using β2AR in the pcMV-SPORT6 plasmid as template. The forward primer 5’-CTCAAGCCTCGAGCCACCATGTTATCCTTAATGATGTGGCCTGCTATTAACCCGCGGGAACCCCGACGGC-3’, was designed with a HindIII site at the N terminus (underlined) and the HA epitope (in boldface) after the starting ATG codon. The reverse primer 5’- CGGTGATCCCAGTTGACTGGGAGTCAAGAAGGCGGAAAGCTTTCAAAGAGATCATCTGTGGC-3’ for S329A, and 5’-CGGTGATCCCAGTTGACTGGGAGTCAAGAAGGCGGAAAGCTTTCAAAGAGATCATCTGTGGC-3’ for S330A were used. The MβCD was from Sigma-Aldrich (St. Louis, MO).

HA-MC4R-GFP mutants T312A, S329A, and S330A were directed by PCR amplification using QuikChange site-directed mutagenesis kit (Stratagene). The primers used were as follows: 5’-CGGAGTCAAGAACTGAGAAAAGCCTTTCAAAGAGATCATCTGTGGC-3’ for T312A; 5’-CTGGAGGGCCATTGTGAATGACTGGGAGTCAAGAAGGCGGAAAGCTTTCAAAGAGATCATCTGTGGC-3’ for S329A; and 5’- CGGTGATCCCAGTTGACTGGGAGTCAAGAAGGCGGAAAGCTTTCAAAGAGATCATCTGTGGC-3’ for S330A.

The single, double, and triple mutants at putative phosphorylation sites were generated using HA-MC4R-GFP, HA-MC4R-GFP T312A, and HA-MC4R-GFP T312A/S329A as templates, respectively. All mutations were confirmed by sequencing.

**Cell Culture and Transfection**—N2A, GT1-7, N-42, and HEK 293 cells were cultured in DMEM with 10% fetal bovine serum and penicillin/streptomycin. N2A cells were transiently transfected with the indicated plasmids using Lipofectamine 2000 and following the manufacturer’s instructions. Experiments were carried out 48 h after transfection. Prior to all experi-
ments, cells were washed twice with DMEM without serum and incubated for 1 h in the same medium. For the small interfering RNA (siRNA) experiments, siRNA specific to murine clathrin heavy chain (AACCGETACGAGCATAATAT) (31), human clathrin heavy chain (GAGCATGTGACCGCTGGCC) (31, 32), and to the human AP-2 μ subunit (GTGGATGCCTTTCCGGGCTCA) (33, 34) were purchased from Qiagen. Non-targeting siRNA pool (siControl) was purchased from Dharmacon, Inc. (Chicago, IL). Cells were transfected with 100 nM siRNA using Lipofectamine 2000. Stably transfected N2A cells expressing HA-MC4R-GFP have already been described (17).

**Drug Treatment**—To deplete cell cholesterol, N2A cells were washed three times with DMEM and incubated in DMEM for 1 h at 37 °C. N2A cells were then treated with 3 mM MβCD for the indicated period.

**Quantification of Unesterified Cell Cholesterol Content**—Total unesterified cell cholesterol was measured using the Amplex Red cholesterol assay kit, following the manufacturer’s instructions (Invitrogen).

**Gel Electrophoresis and Immunoblotting**—Separation of proteins by SDS-PAGE, immunoblotting with the indicated primary antibodies and secondary POD-conjugated antibodies using enhanced chemiluminescence detection, and protein determination were performed as described previously (35). Cells were lysed by scraping cells from 60-mm diameter plates in 0.4 ml of sample buffer containing protease inhibitors. Samples were sonicated three times for 2-s periods and loaded onto SDS-PAGE.

**Fluorescence Microscopy**—Images were captured using a Photometrics CoolSnap HQ camera controlled by IP lab software (Scanalytics, Inc., Fairfax, VA).

**Co-localization of Internalized β2AR and MC4R with Endocytosed Tf**—For these experiments, N2A cells were transiently transfected either with HA-β2AR or HA-MC4R. Cells were washed three times with DMEM and incubated in the same medium at 37 °C for 1 h. Cells were incubated with 50 μg/ml tetramethylrhodamine-Tf at 4 °C for 1 h to label the endogenous Tf receptor TFR (red fluorescence) and fixed in the presence of detergent (0.2% Triton X-100) prior to staining with anti-HA fluorescence-conjugated antibodies (green fluorescence) to label HA-β2AR or HA-MC4R.

**Quantification of the Effect of MβCD on HA-MC4R and HA-β2AR Localization by Fluorescence Microscopy**—N2A and GT1-7 cells transiently transfected with HA-β2AR and HA-MC4R were washed three times with DMEM and incubated in DMEM for 1 h at 37 °C. Cells were then kept in DMEM for 1 h at 37 °C with or without addition of 3 mM MβCD. Cells were then incubated for 1 h at 4 °C in the same medium in the presence or absence of the agonist and with anti-HA antibodies (25 milliunits/ml). Cells were then incubated at 4 °C (0 min) or transfected at 37 °C for the indicated time intervals (15 and 30 min and 1 h) to allow internalization of anti-HA antibodies/HA-tagged receptor complexes. Cells were then washed, fixed, and stained using a nonpermeabilizing condition by incubation in PBS containing 4% formaldehyde for 20 min. Fixed cells were incubated for 1 h with FITC-conjugated anti-rat antibodies (1:100). Cells were then washed four times with PBS and incubated 30 min with permeabilization solution (PBS, 0.2% Triton, 100 mg/ml ovalbumin, and 0.01% azide). Permeabilized cells were then further incubated for 1 h with Cy3-conjugated anti-rat antibodies (1:100). Transfected cells expressing the receptors were then incubated with the Cy3 fluorescence (HA-MC4R or HA-β2AR at the plasma membrane, green fluorescence) with the Cy3 fluorescence (HA-MC4R or HA-β2AR at the plasma membrane and in endosomes) was measured using the ImageJ software version by Wayne Rasband (National Institutes of Health, Bethesda) as described before (36). After segmentation of the green fluorescence and red fluorescence, the regions of interest were analyzed by using the intensity co-localization analysis plugin in the ImageJ software, which measures the Mander’s overlap coefficient M1 (37).

**Cholesterol and MC4R Function**
Internalization of HA-β_{2}AR, HA-MC4R, and HA-MC4R-GFP by Enzyme-linked Immunoassay—N2A cells transiently transfected with HA-β_{2}AR and HA-MC4R or stably transfected with HA-MC4R-GFP were washed three times with DMEM and incubated in DMEM for 1 h at 37 °C. Cells were further incubated for 30 min at 4 °C in the same medium in the presence or absence of the agonist and for another 30 min at 4 °C with the addition of POD-conjugated anti-HA antibodies (25 milliunits/ml). Cells were washed two times with ice-cold DMEM and one time with DMEM at room temperature. Cells were transferred at 37 °C for the indicated time intervals to allow internalization of the POD-conjugated anti-HA antibodies/HA-tagged receptor complexes. Cells were transferred on ice, washed, and fixed in nonpermeabilizing conditions by incubation in PBS containing 4% formaldehyde at 4 °C for 10 min. Cells were washed, and POD activity was determined as described above.

Recycling of HA-MC4R-GFP by Enzyme-linked Immunoassay—Recycling assay was carried out as described previously (17). Briefly, to measure recycling of wild-type HA-MC4R-GFP and mutated HA-MC4R-GFP T312A/S329A in the presence and absence of the agonist, transiently transfected N2A cells were pretreated with 10 μM cycloheximide for 1 h at 37 °C and incubated for 30 min at 4 °C with or without α-MSH. Cells were further incubated in the presence of anti-HA-POD antibodies (25 milliunits/ml) and with 10 μM cycloheximide with or without α-MSH for 1 h at 37 °C. Cells were washed and stripped of surface-bound anti-HA-POD antibody by incubating with DMEM at pH 2.5 for 10 min at 4 °C. After stripping, cells were washed three times with DMEM and transferred to 37 °C in DMEM for the indicated time points to allow internalized receptor to re-appear at the cell surface. Cells were transferred on ice, fixed, and washed. Total internalized POD-labeled receptor was measured at time 0 by incubating cells permeabilized with 0.2% Triton X-100 with the ABTS substrate. Receptors bound to anti-HA-POD re-appearing at the cell surface were measured at the indicated time points by incubating unpermeabilized cells with ABTS. MC4R recycling is expressed as the percentage of total internalized receptor that re-appears at the cell surface over time. To measure recycling of HA-MC4R-GFP in stably transfected N2A cells stably expressing HA-MC4R-GFP with and without cholesterol depletion, cells were incubated in the presence of anti-HA-POD antibodies and with 10 μM cycloheximide for 1 h at 37 °C. Cells were further incubated for 20 min at 37 °C in the same medium in the presence or absence of 3 mM MβCD. Cells were washed and stripped of surface-bound anti-HA-POD antibody, and recycled receptor was measured as described above.

Immunoelectron Microscopy—N2A cells stably expressing HA-MC4R-GFP (3 × 60-mm diameter plates per condition) were washed three times with DMEM and incubated in DMEM for 1 h at 37 °C in the absence and in the presence of 3 mM MβCD. Cells were transferred at 4 °C, and the medium was aspirated and replaced with DMEM containing POD-conjugated anti-HA antibodies (25 milliunits/ml) at 4 °C. After 1 h of incubation, cells were transferred at 37 °C for 20 min. Cells from were then harvested in PBS solution containing 1 mM EDTA, centrifuged at 600 × g for 15 min at 4 °C, and fixed with 5 ml of 100 mM sodium phosphate, pH 7.4, containing 4% paraformaldehyde. After three washes with 100 mM sodium phosphate, pH 7.4, cells were incubated overnight with DAB using the DAB substrate kit for peroxidase and following the manufacturer’s instructions. DAB staining was then intensified by the methenamine silver-gold reaction procedure (38).

Determination of TfR at the Cell Surface by Enzyme-linked Assay—To measure the amount of TfR at the cell surface in different conditions, N2A cells stably expressing HA-MC4R-GFP were preincubated for 1 h at 37 °C with either no additions or 0.45 mM sucrose or MβCD. Cells were transferred at 4 °C and incubated with DMEM containing 25 mg/ml TF-POD. Cells were washed with DMEM three times, fixed with 4% paraformaldehyde, and incubated with POD substrate as described above.

Assay to Determine cAMP—Cells were washed with DMEM and incubated with the same medium containing 0.5 mM IBMX for 10 min and then stimulated with 100 nM α-MSH or 1 mM forskolin for 15 min at 37 °C. The medium was aspirated, and intracellular cAMP was measured by using the Direct cAMP EIA kit from Enzo Life Sciences following the manufacturer’s instructions and keeping at all steps 0.5 mM IBMX. Optical density data were analyzed by using GraphPad Prism version 5.0 software (nonlinear regression curve) to obtain the concentration of cAMP in the samples. Samples from the same experiment were used to determine protein concentration by using BCA protein assay reagent. For determination of EC_{50} data were analyzed by using the sigmoid dose-response curve.

Statistical Analysis—Data from three or more independent experiments are expressed as mean ± S.D. Data were compared by using the Student’s t test and one-way analysis of variance.

RESULTS

Constitutive Internalization of MC4R Occurs Faster Than Constitutive Internalization of β_{2}AR—Attachment of hemagglutinin (HA) and GFP tags to the N and C termini of MC4R, respectively, does not change the ability of the receptor to bind to α-MSH and signal (14, 15). By using both HA-MC4R and HA-MC4R-GFP, we have found that the receptor is internalized at the same rate in the absence and presence of α-MSH (17). To visualize internalization of MC4R and of another GPCR, β_{2}AR, in the absence and presence of agonist, neuroblastoma Neuro2A (N2A) cells expressing HA-MC4R or HA-β_{2}AR were incubated with fluorescein-conjugated anti-HA antibodies for 1 h at 4 °C and then transferred to 37 °C for the indicated time in the continuous presence of the antibodies, with and without α-MSH or isoproterenol, respectively (Fig. 1A). After 15 min of incubation at 37 °C, anti-HA-HA-MC4R complexes appeared in a perinuclear compartment (Fig. 1A, left panel, arrows) at similar abundance regardless of the presence of the agonist. In the absence of agonist, the anti-HA-HA-β_{2}AR complexes were clearly detectable in the perinuclear compartment at the 1-h time point, consistent with the report that β_{2}AR is endocytosed constitutively (21). However, in the presence of isoproterenol, the intracellular anti-HA-HA-β_{2}AR complexes were more abundant at the 15-, 30-, and 60-min time points (Fig. 1A, right panel, arrows). We followed quantitatively constitutive HA-MC4R and HA-β_{2}AR internal-
ization by a biochemical assay, where the receptors at the surface of live cells are labeled at 4 °C and then transferred to 37 °C for the indicated time in the absence or presence of α-MSH and isoproterenol (Iso), respectively. Cells were washed, fixed with 4% formaldehyde in PBS, and images captured as described, see “Fluorescence Microscopy” under “Experimental Procedures.” Bar in right panel = 10 μm. B, panel i, N2A cells were transiently transfected either with HA-β2AR or with HA-MC4R and incubated with POD-conjugated anti-HA antibodies at 4 °C, washed, and transferred at 37 °C. Internalization of HA-MC4R was carried out as described, see Internalization of HA-β2AR, HA-MC4R, and HA-MC4R-GFP by Enzyme-linked Assay under “Experimental Procedures,” and data are expressed as percentage of initial amount of receptor; panel ii, relative amounts of HA-MC4R and HA-β2AR at the cell surface are detected by the immunoassay of panel i and expressed as OD/mg of protein. C, panel i, N2A cells expressing HA-MC4R (upper panel) and HA-β2AR (lower panel) were incubated for 1 h at 37 °C with and without the agonist in the presence of 50 μg/ml tetramethylrhodamine-Tf to label endogenous TfR. Cells were washed, fixed, permeabilized, and stained with fluorescein-conjugated anti-HA antibodies. Images were captured as in A. Arrows indicate transfected cells. Bar, 10 μm. D, N2A cells expressing HA-MC4R and HA-β2AR were incubated for 1 h at 37 °C with and without the agonist. Cells were transferred on ice and the amount of receptor at the cell surface was measured as described, see “Determination of HA-β2AR, HA-MC4R, or HA-MC4R-GFP at the Cell Surface by Enzyme-linked Assay” under “Experimental Procedures.” Statistical significance, *** p < 0.001.
nist induced a significantly more pronounced decrease of HA-β₂AR than of HA-MC4R (~2-fold, Fig. 1D). These experiments indicate that the dynamics and cell distribution of β₂AR and MC4R in the absence of agonist exposure are different, with MC4R having faster constitutive internalization and increased intracellular localization as compared with HA-β₂AR. It also appears that the net loss of cell surface MC4R and β₂AR in response to agonist stimulation is also different, with that of MC4R being less pronounced.

Constitutive Internalization of MC4R and Agonist-dependent Internalization of β₂AR Are Equally Sensitive to Clathrin Depletion—We have found that, in unstimulated N2A cells, MC4R localizes to clathrin-coated pits, suggesting that constitutive internalization of the receptor occurs by this route (17). To test whether reduced clathrin expression inhibited MC4R internalization, murine clathrin heavy chain (CHC) expression was decreased in the N2A cells stably transfected with HA-MC4R-GFP (Fig. 2A, panel i) by an already validated siRNA (31). In these cells the rate of receptor internalization was reduced (by at least 3-fold) as compared with the control (Fig. 2A, panel ii), indicating that the process is indeed dependent on clathrin. To determine whether constitutive clathrin-dependent endocytosis of MC4R occurs also in unspecialized cells, we used transiently transfected HEK 293 cells. When HA-MC4R was expressed in the HEK 293 cells, the receptor was already rapidly endocytosed in the absence of α-MSH, and the rate of receptor internalization remained unchanged in the presence of the agonist (supplemental Fig. S1B). Conversely, when HA-β₂AR was expressed in HEK 293 cells, the receptor was internalized more rapidly in response to incubation with isoproterenol (supplemental Fig. S1B). Thus, fast constitutive endocytosis of MC4R, independent of binding to the agonist, also occurs in nonspecialized cells. When CHC expression was decreased in HEK 293 by using previously validated siRNA targeted to the human orthologue (31, 32), the rate of HA-MC4R internalization was significantly reduced (by ~2-fold, supplemental Fig. S1C). These experiments indicate that constitutive internalization of MC4R occurs by a clathrin-dependent mechanism both in neuronal and unspecialized cells. Agonist-dependent internalization of β₂AR also occurs by a clathrin-dependent mechanism (12, 39, 40). We reasoned that if constitutive internalization of MC4R occurs by a clathrin-dependent route as does agonist-dependent internalization of β₂AR, then reduced levels of the protein would lead to a similar degree of inhibition of the rates at which MC4R and β₂AR are internalized. In transiently transfected N2A cells, the extent of inhibition of HA-MC4R constitutive internalization was the same as that of agonist-induced HA-β₂AR internalization (Fig. 2B), indicating that both processes are similarly dependent on clathrin.

Inhibition of MC4R Endocytosis by Clathrin Depletion Leads to Accumulation of Nonfunctional MC4R at the Cell Surface—The physiological relevance of constitutive cycling of MC4R is unknown. Because silencing of CHC inhibits endocytosis of MC4R, we asked whether this would affect the distribution and function of the receptor. MC4R is a Gₛ-coupled receptor, which activates adenylyl cyclase, thereby increasing the intracellular concentration of cAMP. Forskolin, which directly activates

![Figure 2. Constitutive internalization of MC4R is clathrin-dependent. A, panel i, N2A cells stably expressing HA-MC4R-GFP were left untransfected or were transiently transfected with either control siRNA or siRNA targeted to CHC. Cell lysates were analyzed by Western blot with antibodies against CHC and actin. Panel ii, HA-MC4R-GFP internalization in N2A cells stably expressing HA-MC4R-GFP was measured as in Fig. 1B. *** p < 0.001. B, N2A cells were transiently co-transfected with either HA-MC4R or HA-β₂AR and with either control siRNA or siRNA targeted to CHC. Approximately 48 h after transfection, HA-MC4R and HA-β₂AR internalization was measured as in Fig. 1B. The % inhibition due to CHC silencing was calculated at the 15-min time point by the following ratio: (reduction of receptor at the plasma membrane in control siRNA sample − reduction of receptor at the plasma membrane in CHC siRNA sample)/(reduction of receptor at the plasma membrane in control siRNA sample) x 100. ns, non-significant.]
CHOLESTEROL AND MC4R FUNCTION

Reduced clathrin expression induces loss of MC4R function. A, N2A cells stably expressing HA-MC4R-GFP were transfected with control siRNA or siRNA targeted to CHC and were preincubated for 24 h at 37 °C with or without 2 mM PBA. Approximately 48 h after transfection, the amount of cell surface receptor was measured as described, see “Determination of HA-M4CR, HA-MC4R, or HA-MC4R-GFP at the Cell Surface by Enzyme-linked Assay” under “Experimental Procedures” and expressed as OD per mg of protein. B, N2A cells stably expressing HA-MC4R-GFP and treated as in A were washed and incubated in DMEM containing 0.5 mM IBMX for 10 min and then incubated in the absence or presence of 100 nM α-MSH for 15 min. The amount of cAMP induced by exposure to α-MSH was measured as described, see “Assay to Determine cAMP” under “Experimental Procedures,” and normalized to the amount of HA-MC4R-GFP at the cell surface. ns, non-significant.

Adenylate cyclase, induced the same amount of cAMP per mg of protein in cells with or without silencing of clathrin (data not shown), indicating that activity of the enzyme is not impaired in the clathrin-silenced cells. We reasoned that if reduced endocytosis of MC4R impaired the function of the receptor by inhibiting its internalization, then the amount of MC4R at the cell surface would increase, and the amount of cAMP generated per receptor would decrease. To test that, we first determined whether in the stably transfected N2A cells expressing HA-MC4R-GFP an increase of the cell surface receptor corresponds to increased generation of cAMP. We have found that incubation with 4-phenylbutyric acid (PBA), a chemical chaperone, increases folding of wild-type and mutated HA-MC4R-GFP in transiently transfected N2A cells with consequent increase of total and surface expression of the receptor (36). Consistent with these observations, incubation of N2A cells stably expressing HA-MC4R-GFP with PBA led to an ~80% increase of receptor at the cell surface (Fig. 3A) with a concomitant 80% increase of cAMP production as compared with the untreated control, so the amount of cAMP generated per receptor number remained the same (Fig. 3B). This indicates that the level of cAMP generated in N2A cells stably expressing HA-MC4R-GFP in response to the agonists is dependent upon the amount of signaling receptors at the plasma membrane. Reduced levels of CHC in the N2A cell stably expressing HA-MC4R-GFP led to an ~50% increase of receptors at the cell surface (Fig. 3A), consistent with the finding that silencing of CHC inhibits MC4R internalization. However, the amount of cAMP generated per receptor number was reduced by ~75% (Fig. 3B). These data indicate that inhibition of MC4R internalization by CHC silencing leads to accumulation of nonfunctional MC4R at the cell surface.

MC4R Constitutive Internalization Is Less Dependent on β-Arrestins and AP-2 than Agonist-dependent Internalization of β2-AR—Following binding to the agonist and signaling, internalization of most GPCRs, including β2-AR, is initiated by phosphorylation by G-protein-coupled receptor kinases with formation of high affinity binding sites to which β-arrestins bind (13). The β-arrestin bound to the GPCR interacts with both AP-2 and clathrin to internalize β2-AR (39, 41, 42). Similarly, it has been found that MC4R internalization in the presence of α-MSH and AgRP is also dependent on β-arrestin in HEK 293 cells and COS-7 cells (14, 43). Here, we tested whether MC4R internalization, in the absence of agonist, is also dependent on β-arrestin. To this end, we used mouse embryonic fibroblasts (MEFs) derived from wild-type (WT) or β-arrestin1/2 null mice, where β2-AR internalization in response to agonist is profoundly impaired (44). When these cells were transfected with HA-M4CR and HA-β2-AR, the surface expression levels of these receptors were comparable, with HA-β2-AR ~2-fold more abundant than that of HA-MC4R (data not shown). In these cells, constitutive internalization of HA-MC4R was clearly detectable, with ~50% of the receptor disappearing from the plasma membrane in 30 min in the absence of the agonist (Fig. 4A). Thus, constitutive internalization of MC4R is reproduced in the MEF, as we observed earlier in unspecialized HEK 293 cells. In the MEF with β-arrestin1/2 knock-out, constitutive internalization of HA-MC4R and agonist-induced endocytosis of HA-β2-AR were both inhibited, but the effect on HA-β2-AR internalization was more pronounced than that on HA-MC4R internalization (60 and 20% inhibition, respectively, at the 30-min time point).

Silencing of the μ subunit of AP-2 in HEK 293 cells inhibited efficiently clathrin-dependent endocytosis of TRβ, whereas endocytosis of EGF, which is internalized by a different set of clathrin adaptors, was impaired less efficiently (33). We therefore used the HEK cells to determine whether this already validated siRNA, by reducing expression of the μ subunit of AP-2, also inhibited constitutive and agonist-dependent HA-MC4R and HA-β2-AR internalization. Reduced expression of the AP-2 μ subunit inhibited agonist-dependent internalization of HA-β2-AR by ~40% and constitutive internalization of HA-MC4R by 20% (Fig. 4B). Together, the experiments of Fig. 4 indicate that, at least in unspecialized cells, β-arrestin and AP-2 function in MC4R internalization, consistent with previous reports (14, 43). However, they do so at a reduced extent as compared with agonist-dependent internalization of β2-AR, suggesting differences in the mechanism by which two processes occur.

Reduced Cell Cholesterol Inhibits Constitutive Internalization of MC4R with Accumulation of the Receptor at the Plasma Membrane and Depletion of the Endosomal Pool—Some forms of clathrin-dependent endocytosis such as internalization of amyloid precursor protein appear to be very sensitive to the level of cholesterol (45). When used at elevated concentrations...
Cholesterol and MC4R Function

FIGURE 4. MC4R constitutive internalization is less dependent on β-arrestins and AP-2 than agonist-dependent internalization of β2AR. A, panels i and ii, MEFs with double knock-out of β-arrestin1 and β-arrestin2 and wild-type control were transiently transfected with HA-MC4R and HA-β2AR. HA-MC4R and HA-β2AR internalization was measured as in Fig. 1B. Statistical significance, **, p < 0.01; ***, p < 0.001. Panel iii, % inhibition by double knock-out of β-arrestin1 and β-arrestin2 (DKO-Arr) was calculated at the 30-min time point by the following ratio: (reduction of receptor at the plasma membrane in control MEF sample—reduction of receptor at the plasma membrane in MEFs with double knock-out of β-arrestin1 and β-arrestin2)/(reduction of receptor at the plasma membrane in control MEF sample) × 100. B, panel i, HEK 293 cells were transfected with either control siRNA or siRNA targeted to AP-2 μ subunit. Cells were harvested—72 h after transfection, and lysates (20 μg per lane) were analyzed by Western blot (WB) with antibodies against AP-2 μ subunit and CHC; panel ii, HEK 293 cells were co-transfected with either HA-MC4R or HA-β2AR with either control siRNA or siRNA targeted to AP-2 μ subunit. Approximately 72 h after transfection, cells expressing HA-MC4R were preincubated for 1 h at 4 °C with anti-HA-POD, washed, and then transferred to 37 °C for 0 and 30 min. Cells expressing HA-β2AR were preincubated with and without 10 μM isoproterenol together with anti-HA-POD, washed, and then transferred to 37 °C in the continuous presence of isoproterenol. Receptor internalization was measured as in Fig. 1B after 30 min of incubation at 37 °C. Panel iii, % inhibition by AP-2 μ subunit silencing was calculated by the following (reduction of receptor at the plasma membrane in control siRNA sample—reduction of receptor at the plasma membrane in AP-2 μ siRNA sample)/(reduction of receptor at the plasma membrane in control siRNA sample) × 100. Statistical significance is as in A. DKO-Arr, double knock-out of β-arrestin1 and β-arrestin2.

(10 mM), the cholesterol-depleting drug MβCD disrupts clathrin-dependent endocytosis (46), although at lower concentrations (3 mM), MβCD appears to inhibit specifically lipid raft internalization (47, 48). Incubation of N2A cells with 3 mM MβCD for 1 h reduced the level of cell nonesterified cholesterol by ~45% as compared with that of the untreated control (Fig. 5A, panel i). In these conditions, clathrin-dependent endocytosis of transferrin receptor (TfR), which cycles constitutively between the plasma membrane and endosomes (49), still occurred (supplemental Fig. S2B). Incubation of N2A cells with 3 mM MβCD for 1 h did not change the amount of TfR at the cell surface, again indicating that clathrin-dependent endocytosis was not inhibited by exposure to the drug (supplemental Fig. S2A). Differently, acute exposure to hypertonic media (0.45 M sucrose), a treatment that disrupts formation of clathrin-coated pits (50), impaired constitutive internalization of TfR and led to a 2-fold accumulation of TfR at the plasma membrane. These experiments indicate that mild cholesterol depletion does not impair clathrin-dependent endocytosis of transferrin receptor.

Mild cholesterol depletion by MβCD increased significantly the amount of HA-MC4R-GFP localized at the cell surface by the immunoblot (Fig. 5A, panel ii), differently than what was observed with TfR. Direct observation of HA-MC4R-GFP, by fluorescence microscopy, showed that after incubation with MβCD and hypertonic medium, there was an increased pool of receptors localized at the plasma membrane (Fig. 5A, panel iii, arrowheads). Additionally, depletion of the intracellular pool of receptors was observed (Fig. 5A, panel iii, arrows). To visualize at the ultrastructural level whether cholesterol depletion inhibited MC4R traffic to endosomes, transiently transfected N2A cells expressing HA-MC4R-GFP were treated with anti-HA-POD at 37 °C to label the population of cycling receptors and processed for electron microscopy. In cells incubated without MβCD or agonist, anti-HA-HA-MC4R-GFP complexes corresponding to silver-gold depositions were visible as sites of electron dense dots at the plasma membrane and, most abundantly, in perinuclear vesicles (Fig. 5B, panels i–iii, arrowheads and arrows, respectively). These data are consistent with the immunofluorescence of Fig. 1, indicating that the receptor internalizes rapidly to endosomes in the absence of agonist stimulation. Conversely, in the MβCD-treated cells, most of the anti-HA-HA-MC4R-GFP complexes were at the cell surface (Fig. 5B, panels iv–vi, black arrowheads), although vesicles in the intracellular localization (Fig. 5B, black arrows), including those adjacent to the plasma membrane (panel vi, white arrowhead), had very little, if any, HA-MC4R-GFP immunoreactivity. Thus, cholesterol depletion, under conditions that do not affect TfR distribution, induces accumulation of MC4R at the plasma membrane and depletion of the endosomal pool of the receptor.
Reduced Cell Cholesterol Impairs MC4R Traffic Specifically at the Internalization Step—The fraction of a receptor that cycles between the plasma membrane and the endosomes is determined by the rate at which it is endocytosed and recycled back to the cell surface (49). MC4R undergoes constitutive endocytosis and recycling from endosomes back to the plasma membrane (17). Thus, accumulation of the receptor at the plasma membrane by cholesterol depletion may also result, in addition from impaired endocytosis, from increased recycling of internalized MC4R back to the plasma membrane. Cholesterol depletion inhibited internalization of POD-conjugated anti-HA-HA-MC4R-GFP complexes by at least 5-fold (Fig. 6A), consistently with the immuno-EM showing reduced labeling of endocytosed vesicles (Fig. 5B). In contrast, recycling of POD-conjugated anti-HA-HA-MC4R-GFP complexes back to the plasma membrane remained unchanged after cholesterol depletion (Fig. 6B). These data indicate that cholesterol depletion impairs traffic of MC4R specifically at the internalization step.
pared with cells exposed to MβCD, consistent with the conclusion that cholesterol depletion inhibits internalization of MC4R (Fig. 6C).

Cell Cholesterol Depletion Affects Constitutive Internalization of MC4R and Not Agonist-dependent Internalization of β2AR—To investigate whether the inhibition of internalization by cholesterol depletion is specific to MC4R rather than shared with other GPCRs, we compared the effect of MβCD on plasma membrane expression of HA-MC4R and HA-β2AR in transiently transfected N2A cells. When N2A cells transiently transfected with HA-MC4R were preincubated with MβCD for 1 h, the amount of HA-MC4R at the cell surface was increased (Fig. 7A, 0 min time point) and internalization of HA-MC4R from the plasma membrane was inhibited (Fig. 7A, 30-min time point), in agreement with the data of Figs. 5 and 6. Conversely, pretreatment of cells transiently expressing HA-β2AR with MβCD did not change the cell surface expression of HA-β2AR or the extent of isoproterenol-induced internalization of the receptor (Fig. 7B).

GT1-7 cells are immortalized hypothalamic neurons that have endogenous MC4R (14, 17, 51–53). To compare the effect of cholesterol depletion on MC4R and β2AR endocytosis, we carried out antibody feeding experiments. GT1-7 cells expressing HA-MC4R and HA-β2AR were incubated with anti-HA antibody alone and with anti-HA antibodies in the presence and absence of isoproterenol, respectively. Cells were then transferred at 37 °C for 1 h to allow labeling of HA-tagged receptors at the plasma membrane and to be internalized to endosomes. Cells were fixed in nonpermeabilizing conditions and stained with a nonsaturating concentration of secondary FITC-labeled secondary antibody to visualize the pool of the anti-HA:HA-tagged receptor complexes at the plasma membrane (Fig. 7C, green fluorescence) and then permeabilized and stained with Cy3-conjugated secondary antibody to label the pools of anti-HA:HA-tagged receptor complexes at the plasma membranes and at the intracellular localization. In cells without cholesterol depletion, intracellular Cy3 staining (Fig. 7C, red fluorescence, merged image, red arrows) of HA-MC4R that did not co-localize with plasma membrane staining of the receptor (green fluorescence) corresponds to the internalized receptor and was significantly increased already at the 15-min time point. In cholesterol-depleted cells, HA-MC4R internalized less efficiently at all time points as compared with untreated cells. Conversely, internalization of HA-β2AR was not significantly different in the absence or in the presence of cholesterol depletion at all time points (Fig. 7D, red fluorescence, merged image). Similar results were obtained in the N2A cells (supplemental Fig. S3). These experiments indicate that the effect of cholesterol on MC4R internalization is specific to this GPCR and occurs in hypothalamic cells in addition to neuronal cells.

Inhibition of MC4R Internalization by Depletion of Cell Cholesterol Leads to Accumulation of Nonfunctional Receptors at the Plasma Membrane—we have shown in Fig. 3 that inhibition of MC4R internalization by silencing of clathrin leads to...
FIGURE 7. Reduced cell cholesterol inhibits MC4R but not β2AR internalization. A, N2A cells transiently transfected with HA-MC4R were preincubated for 30 min at 37 °C with either no additions or with 3 mM MβCD. Cells were transferred at 4 °C and incubated with POD-conjugated anti-HA antibody. Cells were washed and either kept on ice (0 min) or transferred at 37 °C for additional 30 min. The amount of surface receptor was measured as in Fig. 3A and is expressed as percentage of that found at time 0 in the sample treated without MβCD. B, N2A cells transiently transfected with HA-β2AR were treated with or without 3 mM MβCD for 30 min at 37 °C. Cells were transferred at 4 °C and incubated in DMEM with POD-conjugated anti-HA antibody and with isoproterenol. Cells were washed and either kept on ice (0 min) or transferred at 37 °C in the continuous presence of 10 μM isoproterenol for an additional 30 min. The amount of receptor at the cell surface was measured as in A and is expressed as percentage of that found at time 0 in the sample treated without MβCD. C, GT1-7 cells transiently transfected with HA-MC4R were preincubated for 1 h at 37 °C with DMEM with or without 3 mM MβCD. Cells were transferred at 4 °C and incubated with anti-HA antibody for 1 h. Cells were kept on ice (0 min) or transferred at 37 °C for the indicated time points (15 and 30 min and 1 h). Immunofluorescence and quantifications of MC4R localization, shown in the graphs where each dot or square corresponds to one cell, was done as described, see “Quantification of the effect of MβCD on HA-MC4R and HA-β2AR Localization by Fluorescence Microscopy” under “Experimental Procedures.” np, nonpermeabilizing conditions; p, permeabilizing conditions. D, GT1-7 cells were transiently transfected with HA-β2AR and preincubated for 1 h min at 37 °C with DMEM or with 3 mM MβCD. Cells were transferred at 4 °C and incubated with POD-conjugated anti-HA antibody and isoproterenol for 1 h. Cells were kept on ice (0 min) or transferred at 37 °C for the indicated time points (15 and 30 min and 1 h). Immunofluorescence and quantification of MC4R internalization was done as above. Statistical significance, *, p < 0.05; **, p < 0.01; ***, p < 0.001. Bar, 10 μm. Red arrows in C and D indicate endocytosed HA-MC4R and HA-β2AR.
accumulation of nonfunctional receptors at the plasma membrane. Because cholesterol depletion by incubation with MβCD induces an acute and specific inhibition of MC4R endocytosis, we used treatment with the drug to explore whether there was also a loss of MC4R function at the cell surface and whether this increased over time. When tested immediately after cholesterol depletion, the dose-response curve and the production of cAMP per mg of protein in response to α-MSH were similar to the control (Fig. 8A). This indicates that the time interval is not sufficient to replenish cholesterol. Panel ii, in the same cells, MC4R abundance at the cell surface was increased immediately after exposure to MβCD with an additional increase after the 2-h incubation at 37 °C. Panel iii, this is in agreement with the data in Figs. 5 and 6 and indicates that inhibited endocytosis leads to progressive accumulation of MC4R at the cell surface. The amount of cAMP generated per amount of receptor expressed at the plasma membrane decreased immediately after exposure to MβCD (by ∼25%) and more so in the following 2 h of incubation at 37 °C (∼50%; *, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, non-significant).
membrane was exhausted. In this respect, when MβCD-treated N2A cells expressing HA-MC4R-GFP were further incubated at 37 °C for 2 h to deplete the endosomal pool of receptors, there was a significant decrease of cAMP generated per mg of protein in response to α-MSH (Fig. 9). Such a decrease in cAMP production per mg of protein (~25%) was also observed in the untransfected N2A cells and in immortalized hypothalamic N-42 and GT1-7 neurons, which express endogenous MC4R (54). These experiments show that reduced cell cholesterol leads over time to a net loss of response to α-MSH in cells expressing endogenous MC4R.

Accumulation of Nonfunctional Receptors at the Plasma Membrane by Inhibition of MC4R Endocytosis Is Partially Reversed by Mutations at Predicted Phosphorylation Sites—It is possible that the time-dependent loss of functional MC4R at the plasma membrane upon inhibition of constitutive internalization of the receptor is due to covalent modifications of the receptor, such as phosphorylation at the C-terminal domain. Phosphorylation of MC4R has been implicated in the mechanism by which cell surface expression of MC4R decreases upon protracted exposure to the agonist. In this respect, it has been found that MC4R mutations at predicted phosphorylation sites (Thr-312, Ser-329, and Ser-330 to Ala residues) blunts the loss of cell surface MC4R in response to protracted incubation with α-MSH in HEK 293 cells (14). Consistent with this report, we find here that in N2A cells treated with prolonged exposure to α-MSH, there is a greater loss of MC4R surface expression in the WT HA-MC4R-GFP (~40%) than of the single mutants T312A and S329A (~20%) or the double mutant T312A/S329A (~10%) (Fig. 10A). We have found that loss of MC4R upon agonist exposure is due to reduced recycling of MC4R from the endosomal compartment back to the plasma membrane, rather than to increased MC4R internalization (17). Thus, we expected that mutations at predicted phosphorylation sites would not change the rate of MC4R internalization. Consistent with this, the rate of internalization of WT HA-MC4R-GFP and of the single and double phosphorylation mutants of HA-MC4R-GFP was similar regardless of cell exposure to α-MSH (Fig. 10B). Next, we determined the effect of mutations at predicted phosphorylation sites on MC4R recycling in cells that were incubated with and without α-MSH. In the absence of the hormone, ~43% of the internalized WT HA-MC4R-GFP and mutated HA-MC4R-GFP T312A/S329A receptors recycled to the cell surface in 1 h. However, in the presence of the hormone, a significantly reduced fraction of the internalized WT HA-MC4R-GFP (~30%) recycled back to the cell surface at 1 h, whereas HA-MC4R-GFP T312A/S329A was essentially unchanged (~40%) (Fig. 10C). Thus, mutations at predicted phosphorylation sites blunt agonist-dependent retention of MC4R in the endosomal compartment.

We asked whether Thr-12 and Ser-329 were also implicated in loss of MC4R response to α-MSH when constitutive endocytosis of the receptor was inhibited. When N2A cells expressing WT HA-MC4R-GFP and HA-MC4R-GFP T312A/S329A were cholesterol-depleted by MβCD, the rate of internalization of the wild-type and mutated receptor was decreased to the same extent, indicating that cholesterol depletion affects similarly the rate of WT and mutated HA-MC4R-GFP constitutive endocytosis (Fig. 10D). Incubation with MβCD decreased significantly (p < 0.001) the amount of cAMP generated per number of WT HA-MC4R-GFP and HA-MC4R-GFP T312A/S329A at the cell surface, indicating that both WT and mutated receptors lose function upon prolonged residency at the cell surface (Fig. 10E). However, the loss of function was significantly less pronounced for the MC4R mutated at the predicted phosphorylation sites than for the wild-type receptor (~25 and ~50%, respectively). Parallel experiments with the triple HA-MC4R-GFP T312A/S329A/S330A mutant, to determine whether incomplete inhibition of MC4R phosphorylation underlined the incomplete recovery of MC4R function, gave instead the same result as the double mutant. These experiments indicate that, upon prolonged residency at the plasma membrane in the absence of agonist, modifications at T312A and S329A MC4R contribute to make MC4R unresponsive to the agonist.

**DISCUSSION**

Internalization of MC4R Is Required to Maintain the Pool of Receptors Residing at the Plasma Membrane in a Functional State—The data presented here indicate that MC4R constitutive endocytosis is both clathrin- and cholesterol-dependent and is essential for MC4R function. Constitutive internalization/recycling of MC4R appears to be required to eliminate receptors that become nonfunctional by being modified during prolonged residency at the plasma membrane (Fig. 11A). In the absence of constitutive endocytosis, nonfunctional MC4R accumulates at the plasma membrane, depleting the endosomal pool of receptors and eventually impairing the overall cell response to α-MSH (Fig. 11B). In support of these conclusions, we have presented here the following data. 1) Membrane cholesterol depletion inhibits constitutive internalization of MC4R, while leaving recycling of the receptor unchanged. This causes accumulation of MC4R at the plasma membrane and loss of MC4R localized in the endosomal compartment, as vis-

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**FIGURE 9.** Cholesterol depletion impairs α-MSH-stimulated cAMP production in untransfected hypothalamic cells. N2A cells stably expressing HA-MC4R-GFP, untransfected N2A, N-42, and GT1-7 cells were preincubated for 1 h at 37 °C with or without 3 mM MβCD and transferred at 37 °C for another 2 h. Cells were washed and incubated in DMEM containing 0.5 mM IBMX for 10 min and then incubated in presence of 100 nM α-MSH for 15 min. The amount of cAMP produced after stimulation with α-MSH was measured as in Fig. 3. The α-MSH-stimulated cAMP production by MβCD-treated cells is expressed as percentage of that generated by the same cells incubated in the absence of MβCD. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
FIGURE 10. Mutations at predicted phosphorylation sites blunt agonist-dependent MC4R sequestration and loss of MC4R function by cholesterol depletion. A, N2A cells transiently expressing wild-type HA-MC4R-GFP, HA-MC4R-GFP T312A, HA-MC4R-GFP S329A, and HA-MC4R-GFP T312A/S329A were stimulated with α-MSH for 2 h. HA-MC4R-GFP at the cell surface was measured as in Fig. 3A, and data were expressed as the percentage of plasma membrane receptors disappeared from the plasma membrane in response to α-MSH treatment. B, N2A cells transiently transfected as in A were incubated without (panel i) or with (panel ii) α-MSH and in the presence of anti-HA-POD for 30 min at 4 °C. Cells were washed and transferred at 37 °C in the continuous absence and presence of α-MSH. Receptor internalization was monitored as in Fig. 1B. C, N2A cells transiently expressing WT HA-MC4R-GFP and HA-MC4R-GFP T312A/S329A were pretreated for 60 min with cycloheximide and incubated for 30 min at 4 °C with or without α-MSH. MC4R recycling is measured and expressed as in Fig. 6B. However, in the presence of hormone, a significantly reduced fraction of the internalized WT HA-MC4R-GFP (∼30%) recycled back to the cell surface at 1 h (panel i), whereas HA-MC4R-GFP T312A/S329A was essentially unchanged (∼40%) (panel ii). D, N2A cells transiently expressing WT HA-MC4R-GFP and HA-MC4R-GFP T312A/S329A were preincubated with either no additions or MJCD at 37 °C. Internalization of α-MSH-stimulated cAMP production is expressed as in Fig. 9. E, incubation with MJCD decreased significantly (p < 0.001) the amount of cAMP generated per number of WT HA-MC4R-GFP and HA-MC4R-GFP T312A/S329A at the cell surface, indicating that both WT and mutated receptors lose function upon prolonged residency at the cell surface. Statistical significance, ***. p < 0.001.
Inhibition of MC4R Recycling

Thr-312 and Ser-329 Are Implicated in Loss of Responsiveness to α-MSH When Constitutive Endocytosis Is Inhibited—A question that remains to be answered is why MC4R at the plasma membrane is modified to a nonfunctional state in the absence of the agonist. One possibility is that this is consequent to the constitutive activity of MC4R, which appears to be important for energy homeostasis in humans (55–57). Such constitutive activity of MC4R, in conditions where constitutive endocytosis of the receptor is inhibited, may ultimately lead to loss of receptor function by a process that has steps in common with the pathway desensitization of MC4R following prolonged agonist exposure (14). Our findings that mutations at Thr-312 and Ser-329 of MC4R blunt both reduction of MC4R at the plasma membrane in response to prolonged exposure to the agonist and the loss of MC4R function consequent to inhibition of constitutive internalization of the receptor are in agreement with this possibility. The model in Fig. 11 predicts that phosphorylation at Thr-312 and Ser-329 (consequent either to an interaction of MC4R to α-MSH or to constitutive activity of the receptor or to stochastic interactions) contributes to make MC4R nonfunctional. The model further predicts that endocytosis of MC4R occurs irrespective of the functional state of the receptor. This is suggested by our earlier finding that MC4R internalization occurs by the same rate in the absence and in the presence of α-MSH (17) and by data presented here that mutations at these putative MC4R phosphorylation sites, which affect function of the receptor at the plasma membrane, do not affect its internalization. The data suggest that once modifications at Thr-312 and Ser-329 are lifted, MC4R recycles as a functional α-MSH-responsive receptor back to the plasma membrane.

Constitutive Internalization of MC4R Occurs by a Clathrin-dependent Mechanism and Is Sensitive to Cholesterol Depletion—The data presented here indicate that constitutive internalization of MC4R occurs by a process that is clathrin-dependent but less sensitive to β-arrestin and AP-2 depletion.
than agonist-dependent β2AR internalization and is specifically dependent on membrane cholesterol. Others have found that internalization of MC4R in the presence of agonist and antagonist is dependent on β-arrestin (14, 43). In this respect, overexpression of dominant-negative mutants of β-arrestin1 and -arrestin2 in the HEK 293 cells inhibited MC4R internalization in the presence of an agonist (43). Here, by using for the first time MEF with double knock-out of β-arrestin1 and -arrestin2 to study MC4R trafficking, we find that constitutive internalization of receptor is β-arrestin-dependent but to a lower extent (3-fold) than that of β2AR. However, endocytosis of MC4R appears to be specifically sensitive to the membrane cholesterol content. The effect of cholesterol depletion on MC4R internalization is likely to be due to inhibition of clathrin-dependent endocytosis of MC4R. In this respect, the extent of inhibition induced by clathrin depletion on constitutive internalization of MC4R and on agonist-dependent internalization of β2AR, a well established form of a clathrin-dependent endocytosis, is similar, indicating that both processes are equally dependent on clathrin. Most pathways of endocytosis are either clathrin-dependent or clathrin-independent, with the latter functioning in internalization of lipid rafts (58, 59). Yet there are some forms of clathrin-dependent endocytosis that are cholesterol-regulated. For example, clathrin-dependent internalization of Niemann-Pick C1-like protein (NPC1L1) is enhanced by increased cholesterol (60, 61). In addition, clathrin-dependent endocytosis of amyloid precursor protein is inhibited by a decreased level of cholesterol (45), similarly to what is observed here for MC4R. Another possibility is that MC4R internalization also occurs by pathways other than clathrin-dependent endocytosis that have yet to be investigated.

Importantly, it appears that membrane cholesterol is reduced in the hypothalamus of insulin-deficient diabetic mice and that this leads to increased food intake and weight gain (62). Here, we have found that reduced membrane cholesterol inhibits specifically MC4R constitutive endocytosis and impairs responsiveness to α-MSH, thus suggesting a new possible molecular mechanism by which changes in membrane lipid composition can affect appetite control. It will be important in the future to determine whether changes in cholesterol levels obtained by modulation of physiologically relevant pathways leads to changes in membrane traffic/signaling of MC4R.

Acknowledgments—We are grateful to Dr. Timothy E. McGraw (Weill Medical College, Cornell University) for the gift of the plasmid pCDTR with human TR; Dr. Robert J. Lefkowitz (Howard Hughes Medical Institute, Duke University Medical Center) for the gift of murine embryonic fibroblasts from transgenic mice lacking β-arrestin1 and -arrestin2; Dr. Richard I. Weiner (University of California San Francisco) for the gift of GT1-7 cells, and to Dr. Brian Storrie (University of Arkansas for Medical Sciences) for comments on the manuscript.

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