0022-3565/05/3133-1281–1288$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 313:1281–1288, 2005

Vol. 313, No. 3
83337/1199548
Printed in U.S.A.

Functional Selectivity of Melanocortin 4 Receptor Peptide and Nonpeptide Agonists: Evidence for Ligand-Specific Conformational States

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Received January 10, 2005; accepted February 17, 2005

ABSTRACT

Agonists of the melanocortin 4 (MC4) receptor have potential pharmaceutical benefit in the treatment of obesity and sexual dysfunction. In this study, we have compared the ability of a number of peptide and nonpeptide agonists to activate a FLAG-tagged human MC4 (FMC4) receptor, as measured by both cAMP accumulation and calcium mobilization using a fluorometric imaging plate reader (FLIPR). In addition, we have analyzed the ability of these agonists to cause receptor internalization, as measured by fluorescence-activated cell sorting analysis. The endogenous agonist α-melanocortin-stimulating hormone (α-MSH) increased cAMP accumulation, calcium mobilization, and receptor internalization in a dose-dependent manner in human embryonic kidney 293 cells expressing the FMC4 receptor. The activity of the other agonists varied considerably in these assays, and overall, the potency and intrinsic activity of the agonists in the cAMP accumulation assays did not correlate with their potency or intrinsic activity in either the FLIPR or receptor internalization assays. Agonists could be clearly separated into two functional classes based on their structure. Peptide agonists β-MSH, des-acetyl-α-MSH, and [Nle^4, D-Phe^7]-α-melanocortin-stimulating hormone exhibited 80 to 112% of the maximal α-MSH response in cAMP accumulation and 62 to 96% in FLIPR assays and were able to cause 75 to 118% of receptor internalization induced by α-MSH. Conversely, although the nonpeptide agonists exhibited 73 to 149% of the α-MSH response in the cAMP accumulation assays, they were significantly impaired in the FLIPR (7–40%) and receptor internalization (−5–38%) assays. These findings demonstrate an important difference in activation and internalization of the MC4 receptor by nonpeptide versus peptide agonists and provides evidence of agonist-specific conformational states.

The melanocortin 4 receptor (MC4R) is a member of the seven transmembrane receptor family that initiate signal transduction through activation of heterotrimeric G proteins. There are four additional members of the melanocortin receptor subfamily, distinguishable by their distribution and physiology. Melanocortin receptors are all activated by one or more pro-opiomelanocortin-derived peptides, which include α-, β-, and γ-melanocortin-stimulating hormone (MSH). In addition, the melanocortin receptors are regulated by the antagonists/inverse agonists, AGRP and agouti (Lu et al., 1994; Ollmann et al., 1997).

The melanocortin receptors all signal through Gα_s, which stimulates the production of cAMP by adenylate cyclase. The MC3 receptor also functions through phospholipase C to increase intracellular calcium (Konda et al., 1994). Likewise, the MC4 receptor mediates an increase in intracellular calcium, but reportedly through a cholera toxin-sensitive pathway rather than phospholipase C (Mountjoy et al., 2001).

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Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.105.083337.

ABBREVIATIONS: MC4R, melanocortin 4 receptor; MSH, melanocortin-stimulating hormone; MTII, Melanotan II [Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH$_2$]; SHU9119, [Ac-Nle-c[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH$_2$]; NDP-MSH, [Nle^4, D-Phe^7]-α-MSH; AGRP, agouti-related protein; HEK, human embryonic kidney; FMC4R, FLAG-tagged MC4R; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FBS, fetal bovine serum; FLIPR, fluorometric imaging plate reader; FACS, fluorescence-activated cell sorting; ANOVA, analysis of variance; 7TM, seven transmembrane; THIQ, *N*-[(3*R*)-1,2,3,4-tetrahydroisoquinolinium-3-ylcarbonyl]-(1*R*)-(4-chlorobenzyl)-2-[4-cyclohexyl-4-(1*H*-1,2,4-triazol-1-ylmethyl)piperidin-1-yl]-2-oxoethylamine; NBI 55886, 4-{(2*R*)-[1,2,3,4-tetrahydro-(3*R*)-isoquinolinecarboxamido]-3-(4-chlorophenyl)propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]phenyl}piperazine; NBI 56297, 4-{(2*R*)-[1,2,3,4-tetrahydro-(3*R*)-isoquinolinecarboxamido]-3-(4-chlorophenyl)propionyl}-1-{2-[*trans*-(2,5-dimethyl)-1-piperazinylmethyl]phenyl}piperazine; NBI 56453, 4-{(2*R*)-[1,2,3,4-tetrahydro-(3*R*)-isoquinolinecarboxamido]-3-(4-chlorophenyl)propionyl}-1-{2-[*N*-methyl-*N*-(pyridin-3-ylmethyl)aminomethyl]phenyl}piperazine; NBI 58702, 4-{(2*R*)-[1,2,3,4-tetrahydro-(3*R*)-isoquinolinecarboxamido]-3-(4-chlorophenyl)propionyl}-1-{2-[1-(piperidin-3-ylamino)ethyl]phenyl}piperazine; NBI58704, 4-{(2*R*)-[1,2,3,4-tetrahydro-(3*R*)-isoquinolinecarboxamido]-3-(4-chlorophenyl)propionyl}-1-{2-[1-(pyrrolidin-3-ylamino)ethyl]phenyl}piperazine.

MC4R activation of the mitogen-activated protein kinase pathway is, however, mediated by inositol triphosphates (Daniels et al., 2003; Vongs et al., 2004). In addition to activating signaling pathways, α-MSH binding to the MC4 receptor activates regulatory pathways, which include receptor internalization (Gao et al., 2003; Shinyama et al., 2003). This is of potential interest because not only is internalization involved in receptor regulation, recent findings have suggested that it may contribute to signaling because receptors can interact with downstream effectors once internalized (Daaka et al., 1998; Holstein et al., 2004).

There has been much research in understanding the physiology of the MC4 receptor because agonists of this receptor are of potential pharmaceutical benefit in the treatment of both obesity and sexual dysfunction. The MC4 receptor is a major regulator of energy homeostasis. Activation of the MC4 receptor by MTII reduces food intake in rodents, and conversely, antagonism of the receptor by SHU9119 increases food intake (Fan et al., 1997). In addition, MC4 receptor knockout mice display hyperphagia and accelerated weight gain (Huszar et al., 1997), and overexpression of AGRP results in obesity (Butler et al., 2001). MC4 receptor agonists have also been shown to stimulate male erectile activity (Van der Ploeg et al., 2002) and female arousal (Pfaus et al., 2004).

Owing to the therapeutic potential of the MC4 receptor, considerable efforts have focused on developing peptide and nonpeptide agonists. The mechanisms of how these ligands bind to the receptor have been explored in detail (Nickolls et al., 2003). However, much less is known of how they impact receptor signaling and receptor regulation. Until recently, compounds acting at 7TM receptors were divided into one of four categories; they were either considered full or partial agonists, antagonists, or inverse agonists. However, this traditional view has been challenged by recent evidence that ligands at the same G protein-coupled receptor can cause markedly different degrees of activation for different effector pathways. This evidence includes studies on the serotonin 2C receptor (Berg et al., 1998), the β_2-adrenergic receptor (Wenzel-Seifert and Seifert, 2000), and the muscarinic acetylcholine receptor (Akam et al., 2001), also see Kenakin (2002) and Hermans (2003) for reviews of the topic. This may be of particular importance when designing nonpeptide agonists because compounds could either mimic the effects of the endogenous agonist, or have the ability to selectively activate one pathway over another. In the work presented here, we have investigated the ability of a number of peptide and nonpeptide agonists to activate adenylate cyclase, mobilize intracellular calcium, and internalize a FLAG-tagged MC4 receptor expressed in HEK-293 cells.

Materials and Methods

Materials. Radiolabeled [^{125}I]NDP-MSH was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). α-MSH, β-MSH, NDP-MSH, and SHU9119 were obtained from Peninsula Laboratories (Belmont, CA). AGRP(83-132) and des-acetyl-α-MSH were obtained from Phoenix Pharmaceuticals (Belmont, CA). The nonpeptide agonist THIQ (Van der Ploeg et al., 2002) and those from our own compound library (Pontillo et al., 2004) were synthesized by the Chemistry Department at Neurocrine.

Construction of FLAG-Tagged Melanocortin 4 Receptors. Human MC4 receptor cDNA in pcDNA3.1 was used as a template for creating FLAG-tagged MC4R (FMC4R). PCR primers designed to add an EcoRI site and a FLAG tag to the 5′ end of the receptor and a XhoI site to the 3′ end were added with the template DNA to PCR supermix (Invitrogen, Carlsbad CA), and PCR was performed as per the manufacturer's instructions (95°C 1 min, 52°C 1 min, and 72°C 2 min ×25). The PCR product was ligated into the EcoRI/XhoI sites of pcDNA3.1.

Transient Transfection. HEK-293 cells were maintained in DMEM with 10% FCS. Twenty-four hours prior to transfection, cells were seeded at 2×10^6 cells/100-mm dish. Eight micrograms of FMC4R DNA were transfected using Polyfect (QIAGEN, Valencia CA) according to the manufacturer's instructions. Cells were either assayed for cAMP accumulation or calcium flux after 48 h. The effect of pertussis toxin on signaling pathways was determined by preincubation of cells for 20 h with 100 ng/ml pertussis toxin.

Membrane Preparation. Cells were washed once with PBS. Then 5 ml of ice-cold buffer (20 mM HEPES, 1 mM $MgCl_2$, 1 mM EDTA, pH 7.4) was used to disrupt the cell monolayer in each 100-mm dish. This was transferred to a Dounce glass/glass homogenizer. The cells were homogenized at 4°C by 40 strokes of the Dounce homogenizer. The homogenate was centrifuged for 10 min at 1700*g*; the supernatant was collected and centrifuged at 48,000*g* (4°C) for 1 h. The resulting pellet was resuspended in buffer, and aliquots were stored at −80°C. Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad, Hercules CA) using BSA as standard.

Iodinated Radioligand Saturation Binding Assays. Cell membranes (5 μg of protein) were incubated in duplicate with [^{125}I]SHU9119, at concentrations ranging from 0.001 to 5 nM in a total volume of 100 μl of buffer [25 mM HEPES, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.5% BSA, 1% bacitracin (1 Complete EDTA-free protease inhibitor tablet/50 ml; Roche Diagnostics, Indianapolis, IN) pH 7.0]. Nonspecific binding was determined by the inclusion of 1 μM SHU9119. The reaction was initiated by the addition of membranes, and the plates were incubated at 25°C for 2 h. The reaction was terminated by rapid filtration using a vacuum harvester with five 100-μl washes of ice-cold wash buffer (25 mM HEPES, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.1% BSA, and 500 mM NaCl, pH 7.0). The filters were soaked in 50 μl of scintillation fluid, and the amount of radioactivity present was determined by liquid scintillation counting.

Ligand Competition versus [^{125}I]SHU9119 and [^{125}I]NDP-MSH Binding. Cell membranes (2–10 μg of protein) were incubated with 0.3 nM iodinated radioligand and various concentrations of competitor ligand, in duplicate, in a total volume of 100 μl of buffer [25 mM HEPES, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 0.5% BSA, and 1% bacitracin (1 Complete EDTA-free protease inhibitor tablet/50 ml) pH 7.0]. Nonspecific binding was determined by the inclusion of 1 μM SHU9119. The reactions were initiated, incubated, and terminated as described above.

cAMP Accumulation Assay (AlphaScreen). Compounds or cAMP standard (1 fmol to 10 nmol) were diluted in DMEM containing 1 mM 3-isobutyl-1-methylxanthine, but without phenol red or FBS. Agonist (5 μl of 2× concentration) was added to the appropriate wells of a 384-well plate. Cells were removed from tissue culture plates using enzyme-free cell dissociation buffer, washed, and resuspended in DMEM containing 1 mM 3-isobutyl-1-methylxanthine without phenol red or FBS at a density of 600 cells/μl. Anti-cAMP acceptor beads were added to the cell suspension at a concentration of 75 μg/ml, and 5 μl of this mixture was added to each well of the 384-well plate. The assay was incubated in the dark at 37°C for 30 min and then terminated by the addition of 15 μl of detection buffer containing 16.7 nM biotinylated-cAMP probe and 33.3 μg/ml streptavidin donor beads, 5 mM HEPES, 0.3% Tween 20, and 0.1% BSA. Plates were incubated in the dark for 1 h before being read on the Alpha program on a Fusion microplate reader (PerkinElmer Life and Analytical Sciences).

Calcium Mobilization (FLIPR). HEK-293 cells were transfected with FMC4R as described above. Twenty-four hours after

transfection, cells were plated at a density of 5×10^3 cells/well in black poly-D-lysine-coated clear-bottom 96-well plates in DMEM containing 10% FCS. Forty-eight hours after transfection, cells were loaded with Fluo-4 (Invitrogen) by replacement of the media with fresh DMEM containing 10% FCS, 2.5 mM probenecid, and Fluo-4. Cells were incubated for 1 h at 37°C before washing with 200 μl/well Hanks' balanced salt solution containing 2.5 mM probenecid. Intracellular calcium mobilization was measured in an ImageTrak by the addition of ligands 10 s after the start of the assessment period. Images were acquired once every 2 s for 100 s.

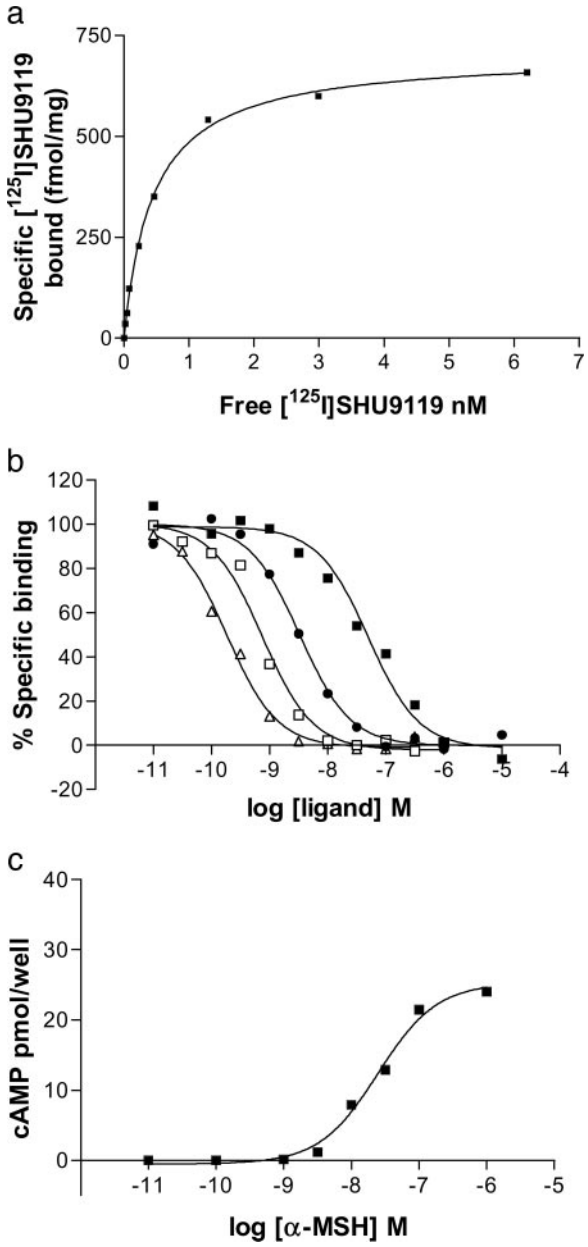

Fig. 1. a, saturation analysis of [^{125}I]SHU9119 binding to membranes prepared from HEK-293 cells expressing the FMC4 receptor. Assays were performed as described under *Materials and Methods*, and data were best fitted by a model describing a single binding site from which K_d and B_{max} values were obtained (see text). b, ligand/[^{125}I]SHU9119 competition binding to membranes prepared from HEK-293 cells expressing the FMC4 receptor. Ligands are represented by the following symbols: (●) NDP-MSH, (■) α-MSH, (□) AGRP(83-132), and (△) SHU9119. Competition binding was performed as described under *Materials and Methods*. The data were best fitted by a one binding site model from which K_i values were determined (see text); these were not appreciably different from those exhibited by the wild-type receptor. Graphs are representative of three independent experiments. c, α-MSH stimulation of cAMP accumulation in HEK-FMC4R cells. AlphaScreen assays were performed as described under *Materials and Methods*, and data were fitted best by an equation describing a sigmoidal dose-response curve from which an EC_{50} value was obtained (see text). Graphs are representative of three independent experiments.

Receptor Internalization (FACS Analysis). Twenty-four hours before experimentation, cells were plated in six-well plates at a density of 5×10^4 cells/well. On the day of experimentation, the media were removed and replaced with media containing a given concentration of agonist (0.01 nM to 1 μM). Cells were incubated at 37°C for 20 min unless otherwise stated. At the end of this incubation, the plates were placed on ice and cells were washed twice with ice-cold medium without FBS. Then anti-FLAG (M2) antibody (Sigma-Aldrich, St. Louis, MO) was added at a dilution of 1:500 in medium without FBS. Plates were placed on a slowly rotating platform shaker (<75 rpm) at 4°C for 1 h. Cells were then washed twice with ice-cold medium without FBS. Anti-mouse AlexaFluor488 (Invitrogen) at a dilution of 1:250, in medium without FBS, was added to each well. Plates were incubated for a further hour on a slowly rotating platform shaker (<75 rpm) at 4°C. Cells were then washed twice with ice-cold PBS and dissociated using 0.4 ml of ice-cold PBS containing 5 mM EDTA. Dissociated cells were added to 0.1 ml of 4% v/v formaldehyde in PBS in FACS tubes, and samples were read using a FACS Caliber (BD Biosciences PharMingen, San Diego CA).

Data Analysis. Data were analyzed using PRISM (GraphPad Software Inc., San Diego, CA) and statistical significance determined using one-way ANOVA followed by Tukey post hoc analysis. $p < 0.05$ determined statistical significance. K_i values were determined from IC_{50} values using the method of Cheng and Prusoff (1973).

Results

Development of FLAG-Tagged MC4 Receptor. The goal of this study was to determine the functional properties of a wide variety of agonists acting through the MC4 receptor. To facilitate our ability to measure one of these properties, receptor internalization, the MC4 receptor was tagged with the FLAG epitope at the N terminus (FMC4R). Saturation binding studies indicated that [^{125}I]SHU9119 bound specifically to FMC4R expressed in HEK-293 cells with a B_{max} of 700 fmol/mg and a K_d of 0.5 nM (Fig. 1a). To ensure appropriate pharmacology of the FMC4 receptor compared with the wild-type receptor, binding of agonist and antagonist peptides was assessed in competition versus [^{125}I]SHU9119 (Fig. 1b). The FMC4 receptor showed no appreciable differ-

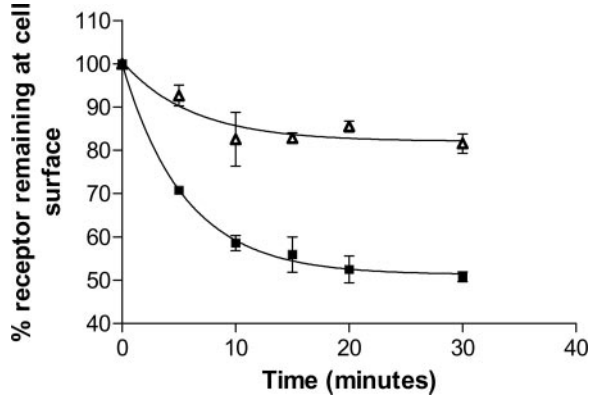

Fig. 2. Time-dependent reduction in the level of FMC4 receptor detectable at the cell surface by FACS analysis upon exposure to MC4 receptor agonists. Agonists are represented by the following symbols: (■) α-MSH and (△) NBI 58702. Data are mean ± S.E.M. of three independent experiments.

ences in ligand binding affinities compared with previously published values for the wild-type receptor (Nickolls et al., 2003). pK_i± S.E.M. (K_i nanomolar concentration) (n = 3) values for ligands were as follows: α-MSH 7.42 ± 0.16 (38.0), NDP-MSH 8.62 ± 0.17 (2.20), AGRP(83-132) 9.41 ± 0.16 (0.389), and SHU9119 9.72 ± 0.11 (0.191). Function as assessed by the stimulation of cAMP accumulation was also not appreciably affected by the addition of the FLAG-tag with the EC_{50} value of 21.4 nM for α-MSH, which was similar to the value of 14.8 nM obtained for the wild-type receptor (Nickolls et al., 2003) (Fig. 1c).

α-MSH-Mediated MC4 Receptor Internalization and Calcium Mobilization. The ability of the MC4 receptor to undergo receptor internalization in response to agonist has been documented (Gao et al., 2003; Shinyama et al., 2003) and is considered to be an important regulatory mechanism in receptor desensitization. We assessed the ability of the FMC4 receptor to undergo agonist-dependent internalization using FACS analysis. α-MSH (1 μM) was able to induce a time-dependent decrease in the amount of receptor present at the cell surface to a maximum of 56% receptor remaining (Fig. 2). The peptide antagonists SHU9119 and AGRP(83-132) were not able to induce receptor internalization; indeed, there was a slight increase in cell surface fluorescence to 107 ± 5 and 113 ± 4%, respectively, after cells were incubated with these compounds for 20 min at a concentration of 0.1 μM. The ability of the MC4 receptor to mobilize calcium has also been reported (Mountjoy et al., 2001). We therefore assessed the ability of the FMC4 receptor to mobilize calcium. A dose-dependent increase in calcium mobilization was observed upon addition of α-MSH with an EC_{50} value of 130 nM (Fig. 3), which was not affected by pretreatment with pertussis toxin.

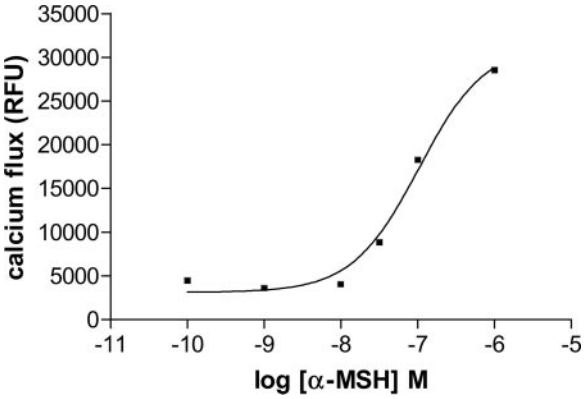

Fig. 3. Stimulation of intracellular calcium mobilization by α-MSH in HEK-293 cells expressing the FMC4 receptor. FLIPR experiments were performed as described under *Materials and Methods*, and data were fitted best by an equation describing a sigmoidal dose-response curve. The graph is representative of six independent experiments.

Peptide and Nonpeptide Agonists Have High Binding Affinity and Potency and Intrinsic Activity in cAMP Accumulation Assays. A number of MC4 receptor peptide and nonpeptide (Fig. 4) agonists were selected to allow the functional properties of individual agonists to be assessed. These were the endogenous peptides α-, β-, γ-, and des-acetyl-α-MSH, the synthetic peptide NDP-MSH, the nonpeptide agonist THIQ (Van der Ploeg et al., 2002), and five compounds from our in-house library (Pontillo et al., 2004). All compounds were able to bind to the MC4 receptor as determined in ligand/[^{125}I]NDP-MSH competition binding assays (Fig. 5) and exhibited a range of affinity values (Table 1). All agonists were able to stimulate FMC4 receptor-mediated accumulation of cAMP with an intrinsic activity between 73 and 149% of the maximal α-MSH response (Fig. 6A; Tables 2 and 3). The peptide agonists showed much stronger correlation (r^2 = 0.98) between binding affinity and functional potency than the nonpeptide agonists (r^2 = 0.62).

Peptide Agonists, but Not Nonpeptide Agonists, Exhibit High Intrinsic Activity in Calcium Mobilization Assays. The ability of the MC4 receptor agonists to mobilize calcium was determined (Fig. 6B). The peptide agonists were able to cause the mobilization of calcium with similar intrinsic activity to that of α-MSH, with the exception of γ-MSH, which was a partial agonist in both assays. The potency of α-,

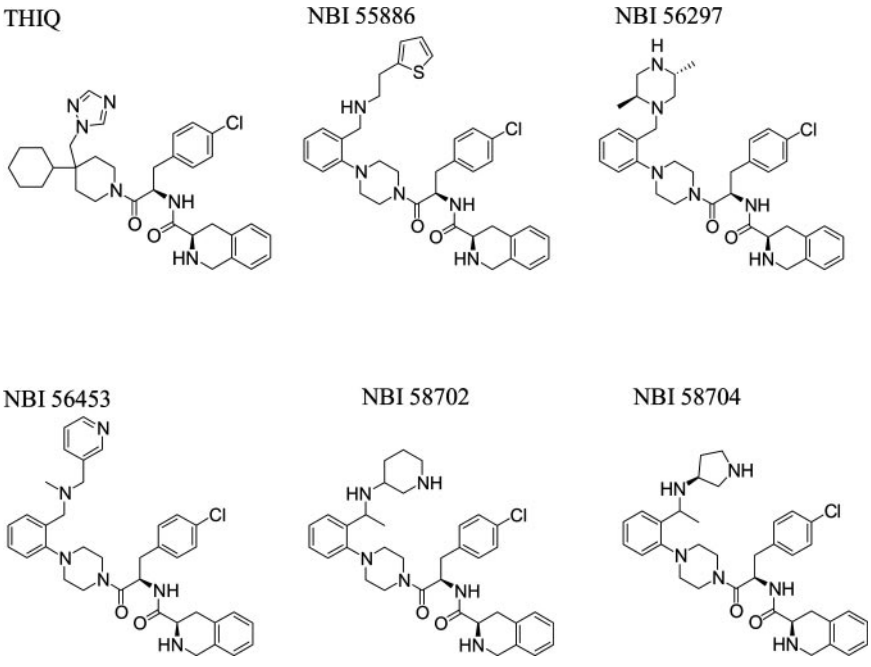

Fig. 4. Structures of the small molecule agonists used in this study.

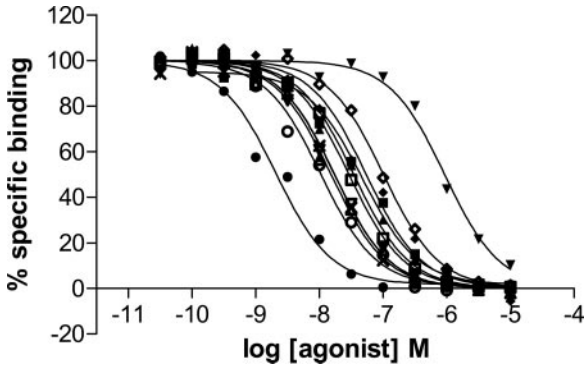

Fig. 5. Binding of agonists to membranes prepared from HEK-293 cells expressing the MC4 receptor. Ligand/[^{125}I]NDP-MSH] competition experiments were performed as described under *Materials and Methods*, and data were best fitted by an equation describing a single binding site. Data are mean ± S.E.M. of three independent experiments. Agonists are represented by the following symbols: (●) NDP-MSH, (■) α-MSH, (▲) β-MSH, (▼) γ-MSH, (◆) des-acetyl-α-MSH, (○) THIQ, (✕) NBI 55886, (◇) NBI 56453, (□) NBI 56297, (△) NBI 58702, and (▽) 58704.

TABLE 1

Binding affinities of MC4 receptor agonists to the MC4 receptor expressed in HEK-293 cells

Agonist/[^{125}I]NDP-MSH competition binding was performed as described under *Materials and Methods*. Data were fitted best by a one binding site model from which K_i values were determined. Data are mean ± S.E.M. of three individual experiments.

	pK_I ± S.E.M.	K_i
		nM
α-MSH	7.30 ± 0.08	50.1
β-MSH	7.73 ± 0.05	18.6
γ-MSH	6.23 ± 0.02	589
Des-acetyl-α-MSH	7.52 ± 0.06	30.2
NDP-MSH	8.40 ± 0.14	3.98
THIQ	7.97 ± 0.06	10.7
NBI 55886	8.02 ± 0.10	9.55
NBI 56297	7.70 ± 0.06	20.0
NBI 56453	7.13 ± 0.09	74.1
NBI 58702	8.20 ± 0.20	6.31
NBI 58704	7.87 ± 0.03	13.5

β-, γ-, and des-acetyl-α-MSH was approximately 10-fold lower, and the potency of NDP-MSH was approximately 100-fold lower in the calcium mobilization assays compared with the cAMP accumulation assays; these differences were significant in all cases ($p < 0.05$, Tukey post hoc analysis). NDP-MSH was the only peptide agonist to exhibit significantly lower intrinsic activity in the calcium mobilization assay compared with the cAMP accumulation assay ($p < 0.05$, Tukey post hoc analysis). Conversely, very little mobilization of calcium was observed in response to the six nonpeptide agonists. The intrinsic activities of all the nonpeptide agonists, with the exception of NBI 55886, were significantly lower in the FLIPR assays compared with the cAMP accumulation assays ($p < 0.05$, Tukey post hoc analysis). Conversely, there were no significant differences between the potencies of nonpeptide agonists in FLIPR and cAMP accumulation assays.

Peptide Agonists, but Not Nonpeptide Agonists, Exhibit High Intrinsic Activity in Receptor Internalization Assays. We then determined the ability of agonists to induce FMC4 receptor internalization using FACS analysis (Fig. 6C). Again β-, des-acetyl-α-, and NDP-MSH were able to cause a similar degree of receptor internalization to α-MSH, with potencies between 2- and 5-fold lower than

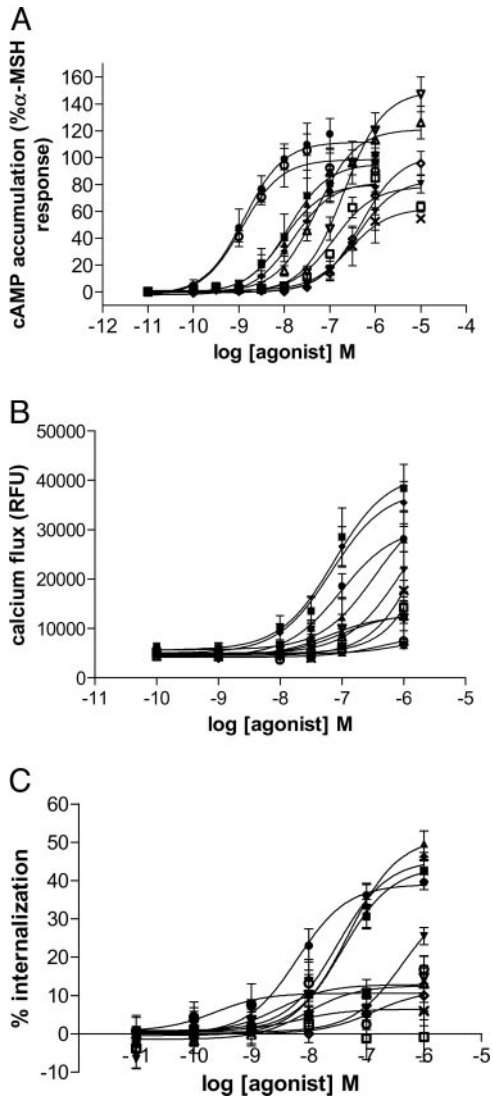

Fig. 6. Functional potency and intrinsic activity of MC4 receptor agonists in cAMP accumulation (A), calcium mobilization (B), and receptor internalization (C). Assays were performed as described under *Materials and Methods*. Data were best fitted by an equation describing a sigmoidal dose-response curve from which potency (Table 2) and intrinsic activity (Table 3) values were derived. Data plotted are mean ± S.E.M. from three (cAMP accumulation), six (calcium mobilization), or four (receptor internalization)-independent experiments. Agonists are represented by the following symbols: (●) NDP-MSH, (■) α-MSH, (▲) β-MSH, (▼) γ-MSH, (◆) des-acetyl-α-MSH, (○) THIQ, (✕) NBI 55886, (◇) NBI 56453, (□) NBI 56297, (△) NBI 58702, and (▽) NBI 58704.

observed in cAMP accumulation assays. This decrease in potency was, however, only significant in the case of NDP-MSH ($p < 0.05$, Tukey post hoc analysis). The partial agonist γ-MSH caused 75% of the α-MSH response at 1 μM, which was similar to its response in the calcium mobilization and cAMP accumulation assays. The nonpeptide agonists, however, were impaired in their ability to induce receptor internalization, with NBI 58702 exhibiting the highest intrinsic activity (38% of the α-MSH response at 1 μM). All nonpeptide agonists exhibited significantly lower intrinsic activity in the receptor internalization assay compared with the cAMP accumulation assay ($p < 0.05$, Tukey post hoc analysis). There were also clear reversals in the intrinsic activities of compounds between

TABLE 2

Potency of MC4 receptor agonists in cAMP accumulation, calcium mobilization, and receptor internalization assays

Functional properties of agonists at FMC4 receptors expressed in HEK-293 cells were measured, respectively, by AlphaScreen, FLIPR, and FACS analysis as described under *Materials and Methods*. Potency values are expressed as pEC_{50} ± S.E.M. (EC_{50} nM). Data are $n = 3$ (cAMP accumulation), $n = 6$ (calcium mobilization), and $n = 4$ (receptor internalization).

	cAMP Accumulation	Calcium Mobilization	Internalization
α-MSH[a]	7.67 ± 0.19 (21.4)	6.89 ± 0.13 (129)	7.54 ± 0.21 (28.8)
β-MSH[a]	7.95 ± 0.20 (11.2)	6.76 ± 0.12 (174)[b]	7.36 ± 0.26 (43.7)
γ-MSH	6.20 ± 0.48 (631)	>1000	>1000
Des-acetyl-α-MSH[a]	7.76 ± 0.13 (17.4)	6.96 ± 0.13 (110)[b]	7.52 ± 0.28 (30.2)
NDP-MSH[a]	8.86 ± 0.20 (1.38)	6.92 ± 0.15 (120)[b]	8.14 ± 0.13 (7.24)[c,d]
THIQ	8.88 ± 0.12 (1.32)	6.16 ± 0.92 (692)	9.09 ± 0.06 (0.813)
NBI 55886	6.48 ± 0.04 (331)	>1000	8.46 ± 0.64 (3.47)[c]
NBI 56297	6.89 ± 0.07 (129)	>1000	8.10 ± 0.44 (7.94)
NBI 56453	6.29 ± 0.13 (513)	7.34 ± 0.53 (45.7)	8.28 ± 0.66 (5.25)
NBI 58702	7.37 ± 0.06 (43)	8.62 ± 0.58 (2.40)	8.12 ± 0.54 (7.59)
NBI 58704	6.69 ± 0.11 (204)	7.75 ± 0.55 (17.8)	6.84 ± 0.23 (145)

[a] ANOVA showed a significant influence of the different assays on agonist potency.
[b] Agonist potency was significantly different between cAMP accumulation and calcium mobilization assay.
[c] Agonist potency was significantly different between cAMP accumulation and receptor internalization assays.
[d] Agonist potency was significantly different between calcium mobilization and receptor internalization assays.

TABLE 3

Intrinsic activity of MC4 receptor agonists in cAMP accumulation, calcium mobilization, and receptor internalization assays

Functional properties of agonists at FMC4 receptors expressed in HEK-293 cells were measured, respectively, by AlphaScreen, FLIPR, and FACS analysis as described under *Materials and Methods*. Intrinsic activity values are expressed as percentage of the maximal α-MSH response (mean ± S.E.M.). Data for γ-MSH in calcium and receptor internalization assays and NBI 55886 and NBI 56297 in the calcium assays are the observed activity at 1 μM. Data are $n = 3$ (cAMP accumulation), $n = 6$ (calcium mobilization), and $n = 4$ (receptor internalization).

	cAMP Accumulation	Calcium Mobilization	Internalization
α-MSH	100	100	100
β-MSH	83 ± 13	94 ± 12	118 ± 9
γ-MSH	80 ± 7	62 ± 7	75 ± 5
Des-acetyl-α-MSH	84 ± 18	96 ± 9	107 ± 2
NDP-MSH[a]	112 ± 11	80 ± 7[b]	89 ± 5
THIQ[a]	98 ± 5	7 ± 3[b]	29 ± 7[c,d]
NBI 55886[a]	73 ± 17	40 ± 8	16 ± 2[c]
NBI 56297[a]	81 ± 6	23 ± 7[b]	−5 ± 18[c]
NBI 56453[a]	103 ± 8	7 ± 2[b]	27 ± 20[c]
NBI 58702[a]	110 ± 14	20 ± 5[b]	38 ± 16[c]
NBI 58704[a]	149 ± 15	23 ± 7[b]	34 ± 11[c]

[a] ANOVA showed a significant influence of the different assays on intrinsic activity.
[b] Intrinsic activity was significantly different between cAMP accumulation and calcium mobilization assay.
[c] Intrinsic activity was significantly different between cAMP accumulation and receptor internalization assays.
[d] Intrinsic activity was significantly different between calcium mobilization and receptor internalization assays.

the two assays with NBI 58704 > NBI 58702 > β-MSH = des-acetyl-α-MSH in the cAMP accumulation assay and β-MSH = des-acetyl-α-MSH > NBI 58704 = NBI 58702 in the receptor internalization assay. Furthermore, a time course of the decrease in cell surface fluorescence induced by 1 μM NBI 58702 showed no difference in kinetics to that induced by α-MSH (Fig. 2). Much less variation was observed when comparing potency values between cAMP accumulation and receptor internalization; only NBI 55886 exhibited a significant difference ($p > 0.05$, Tukey post hoc analysis) with higher potency in the receptor internalization assay compared with the cAMP accumulation assay.

Correlation of Intrinsic Activity Values. Correlation of intrinsic activity values revealed distinctly different profiles for peptide and nonpeptide agonists (Fig. 7). Overall, there was no correlation between the cAMP accumulation E_{max} values and either the FLIPR or internalization E_{max} values. However, the intrinsic activity of the peptide agonists, but not the nonpeptide agonists, lay close to the line of incidence in both cases. Conversely, correlation was observed ($r^2 = 0.82$) between the E_{max} values in the FLIPR and internalization assays, suggesting a common signaling mediator.

Discussion

Due to the therapeutic potential of MC4 receptor agonists a number of peptide and nonpeptide agonists have been developed for this receptor. In this study, we have investigated the ability of peptide and nonpeptide agonists to activate the MC4 receptor as measured by cAMP accumulation and calcium mobilization and also investigated their ability to regulate receptor internalization. We have discovered that the ability of individual agonists to activate MC4 receptor signaling/regulatory pathways differs. The peptide agonists exhibited high intrinsic activity in all three assays, whereas nonpeptide agonists only exhibited high intrinsic activity in the cAMP accumulation assays and were severely impaired in their ability to mobilize calcium or internalize the receptor. These data are the first to show significant differences between the ability of peptide and nonpeptide agonists to signal through and regulate the MC4 receptor and are consistent with agonist-specific conformational states.

Our results show that although there is strong correlation between binding affinity and functional potency in the cAMP assay for peptide agonists, this relationship is dissociated for nonpeptide agonists despite a similar range of affinity values. Likewise, cAMP E_{max} values for the peptide agonists, but not nonpeptide agonists, correlated with calcium mobilization and receptor internalization E_{max} values. Conversely, there was correlation between FLIPR and receptor internalization E_{max} values for both peptide and nonpeptide agonists.

Our results suggest that agonist activation of $G\alpha_s$ signaling and calcium mobilization/receptor internalization is dissociated. Receptor theory asserts that if a 7TM receptor couples more efficiently to one pathway over another, then agonists may be expected to exhibit higher intrinsic activity and/or potency at that effector due to strength of signal (Kenakin, 1995). Therefore, the ability of a drug to activate one effector more readily than another is only indicative of functional selectivity if there are reversals in intrinsic activity and/or potency between the different effectors. Clear re-

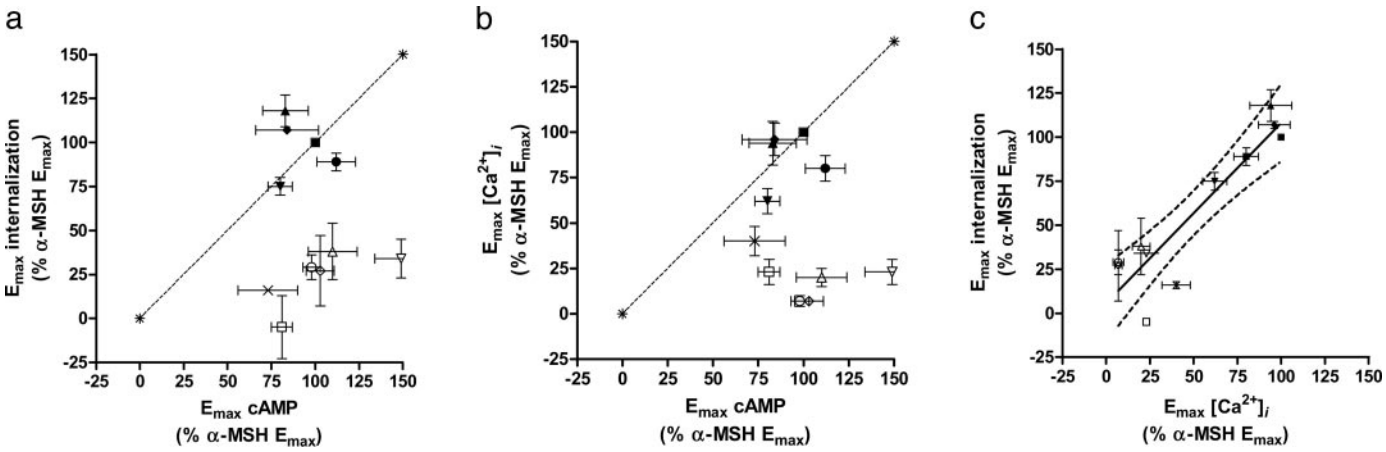

Fig. 7. Correlation of intrinsic activity of MC4 receptor agonists in cAMP accumulation, calcium mobilization, and receptor internalization assays. a, correlation between E_{max} values obtained in cAMP accumulation and receptor internalization assays. The dashed line represents the line of incidence. b, correlation between E_{max} values obtained in cAMP accumulation and calcium mobilization assays. The dashed line represents the line of incidence. c, correlation between E_{max} values obtained in calcium mobilization and receptor internalization assays. Correlation was obtained ($r^2 = 0.82$) (solid line). The dashed lines represent the 95% confidence intervals. Agonists are represented by the following symbols: (●) NDP-MSH, (■) α-MSH, (▲) β-MSH, (▼) γ-MSH, (◆) des-acetyl-α-MSH, (○) THIQ, (✕) NBI 55886, (◇) NBI 56453, (□) NBI 56297, (△) NBI 58702, and (▽) NBI 58704.

versals in intrinsic activity are observed in our investigation, for example, NBI 58704 was a superagonist (149% of the max α-MSH response) in the cAMP accumulation assay, but a very weak partial agonist in both the calcium mobilization and receptor internalization assays. Conversely, γ-MSH was a strong partial agonist, and des-acetyl-α-MSH and β-MSH were full agonists in all three assay systems.

These data therefore add to the growing consensus that receptors are able to activate different pathways dependent on the agonist bound. Over the last decade, a considerable number of studies have provided evidence that agonists differ in their ability to couple the same receptor to different G proteins, including the Drosophilia tyramine receptor (Robb et al., 1994), the calcitonin receptor (Watson et al., 2000), the D_2 dopamine receptor (Nickolls and Strange, 2003), and the α_2-adrenergic receptor (Brink et al., 2000). More recently, this phenomena has also been used to describe signaling/regulatory properties of ligands that bind to 7TM receptors that may not be wholly G protein-dependent, such as phosphorylation of the angiotensin II receptor (Thomas et al., 2000) and desensitization of the μ-opioid receptor (Blake et al., 1997).

Although peptide agonists caused marked receptor internalization, the nonpeptide agonists were severely impaired in this assay. The ability of agonists to cause receptor internalization may be important in any long-term pharmaceutical therapy, as agonist-induced internalization is ultimately linked to receptor down-regulation. Furthermore, in addition to its important role in receptor regulation, receptor internalization may also be important for signaling. There is some evidence that at the β_2-adrenergic receptor, both G protein-coupled receptor kinase and β-arrestin act as scaffold proteins for the intracellular activation of the mitogen-activated protein kinase signaling cascade (Daaka et al., 1998). Indeed, although receptor internalization is commonly allied to agonist activation, several studies have revealed that this is not always the case. In particular, antagonist occupation of both cholecystokinin receptors (Roettger et al., 1997) and CCR5 chemokine receptors (Vila-Coro et al., 1999) can result in receptor internalization. Likewise, consistent with the data in our study, endogenous peptide agonists of the μ-opioid receptor, but not the nonpeptide agonist morphine, are able to induce receptor internalization (Keith et al., 1996).

Even though the compounds from our in-house library are from the same series, they are structurally dissimilar from the nonpeptide agonist THIQ, decreasing the likelihood that the dissociation between cAMP accumulation and calcium mobilization/receptor internalization is only a property of some MC4R nonpeptide agonists. In addition, we have previously demonstrated that THIQ occupies the α-MSH binding pocket (Nickolls et al., 2003), implying that these data are not due to a vastly different mechanism of receptor activation. Similarly the catecholamines norepinephrine, epinephrine, and dopamine occupy similar binding pockets in the β_2-adrenergic receptor. Biophysical studies of this receptor have demonstrated that upon catecholamine binding the receptor undergoes transitions to two kinetically distinguishable conformational states, which correlate with biological responses (Swaminath et al., 2004). Norepinephrine and epinephrine were able to induce both rapid and slow conformational changes and were also efficient at both G_s activation and receptor internalization, whereas dopamine, which was only able to activate signaling and was inefficient at receptor internalization, only induced a rapid conformational change.

Whether these properties of nonpeptide agonists have a direct effect on their therapeutic potential has yet to be determined. Interestingly, the amount of peptide versus nonpeptide agonists required to elicit feeding in rodents does not correlate with their in vitro profile with nonpeptides exhibiting decreased potency in vivo (Cepoi et al., 2004). However, further analysis of both in vitro and in vivo data are required before deductions can be drawn.

In conclusion, we have demonstrated that the melanocortin 4 receptor can exist in agonist-specific receptor conformations. Specifically we have demonstrated that nonpeptide agonists of the MC4 receptor are unable to promote calcium mobilization or receptor internalization to the same degree as peptide agonists.

Acknowledgments

We thank Rajesh Huntley and Molly Gross for technical assistance and Chen Chen and Joseph Pontillo for compound synthesis.

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