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Melanocortin 5 receptor activates ERK1/2 through a PI3K-regulated signaling mechanism

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Summary

Melanocortin 5 receptor (MC5R) is a G-protein coupled receptor (GPCR) with high affinity for α -MSH. To unravel some of the downstream cell-signaling pathways activated by this receptor, HEK293 cells were transiently and stably transfected with a vector encoding green fluorescent protein (GFP)-tagged MC5R. In these cells the receptor was correctly addressed to the cell surface and was functional, as shown by the MC5R-induced formation of intracellular cAMP. In fact, the MC5R agonist α -MSH induced a 10 or 16 fold increase (transient or stable cells, respectively) above the cAMP levels found in unstimulated cells. Moreover, in cells stably expressing MC5R-GFP, α -MSH promoted ERK1/2 phosphorylation in a dose-dependent manner ($EC_{50}=7.3nM$) with the maximal effect occurring after 5min of agonist incubation. The signaling pathway conveyed through ERK1/2 is not linked to cAMP, since the phosphorylation of these kinases is unchanged by the inhibition of adenylyl cyclase. Also, ERK1/2 activation is not significantly affected by PKA, PKC and Akt/PKB specific inhibitors. However, α -MSH-induced ERK1/2 activation is abolished by the PI3K inhibitors wortmannin and LY294002. Altogether, these findings demonstrate that MC5R signals through a PI3K-regulated Akt-independent pathway leading to downstream activation of ERK1/2. The involvement of these MAPK suggests that MC5R could be implicated in cellular proliferation or differentiation mechanisms.

Keywords: Melanocortin 5 receptor; PI3K; ERK1/2; α -MSH; GPCR; Akt/PKB

Introduction

The melanocortin 5 receptor belongs to a five member-family of G protein coupled receptors (MC1R-MC5R) and has a preferential affinity for α -melanocyte-stimulating hormone (α -MSH), followed by adrenocorticotrophic hormone (ACTH) and β -MSH (Gettings, 2006). MC5R has a broad tissue distribution in almost all eukaryotic organisms. In fact, northern blot analysis, RT-PCR or ribonuclease protection assays have shown MC5R expression in brain, sebaceous glands, adrenal gland, kidney, lymph nodes, uterus, esophagus, spleen, thymus, testis, ovary, muscle, adipocytes, mast cells, lymphocytes, liver, lung, mammary glands, bone marrow, skin, stomach, duodenum and exocrine glands (lacrimal, prostate, seminal, pancreatic, preputial, Harderian) (for review see Millington, 2006; Catania et al., 2004).

The physiological role of MC5R is still a matter of debate, but targeted deletion of the receptor resulted in sebaceous gland deficient secretion (Chen et al., 1997). MC5R function also seems to be related with stress response. Indeed, it was observed an up-regulation of MC5R on the rat adrenal cortex as a consequence of a chronic stress (Pignatelli et al., 2000).

Regarding the signaling mechanism of the melanocortin receptors (MCRs), it is accepted that after agonist activation, the $G\alpha$ proteins stimulate cAMP production via adenylyl cyclase leading to protein kinase A (PKA) activation. Although this is the most studied signaling pathway, it was also described that stimulation of some MCRs by α -MSH activates the mitogen-activated protein kinases family (MAPK), NF- κ B and JAK/STAT signaling cascades (Catania et al., 2004). In fact, in MC3R and MC4R transfectants, NDP- α -MSH induces extracellular signal-regulated kinase 1 and 2 (ERK1/2) phosphorylation via phosphatidylinositol 3-kinase (PI3K) (Vongs et al., 2004, Chai et al., 2007). A slightly different result was obtained with another agonist of these receptors such as melanotan II, which induces an increase of phospho-MAPK levels in COS-1 cells transfected with MC4R but not with MC3R (Daniels et al., 2003). Furthermore, it was recently demonstrated that MC2R mediates an ACTH-induced ERK1/2 activation in H295R cells by a mechanism independent of PKA (Janes et al., 2008). ACTH also induces a rapid phosphorylation of p38 kinase and ERKs in human keratinocytes (Park et al., 2007) and modulates ERK activation in adrenal gland (Ferreira et al., 2004). There is also evidence that α -MSH binding to MC5R activates the JAK/STAT pathway in lymphocytes (Buggy, 1998) and increases the production of cAMP and calcium in HEK293 cells stably transfected with MC5R (Hoogduijn et al., 2002; Mountjoy et al., 2001).

Nevertheless, despite the several studies regarding the involvement of diverse pathways in MCRs activation, the signaling mechanisms activated by MC5R are still poorly understood. In this study, we present evidence that in HEK293 cells expressing MC5R-GFP, α -MSH induces the activation of ERK1/2 mainly by a mechanism that requires PI3K but seems to be independent of adenylyl cyclase and PKA, protein kinase B or Akt (PKB/Akt) and protein kinase C (PKC).

Materials and Methods

Materials

Forskolin, α -MSH, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinoline sulfonamide (H-89), 3-isobutyl-methyl-xanthine (IBMX), 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002), 2',5'-dideoxyadenosine (2',5'-DDA) and wortmannin were obtained from Sigma-Aldrich. DMEM/Ham's F-12 medium, glutamine and fetal bovine serum were purchased from Biochrom AG. 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl) maleimide (GF109203X) and Akt inhibitor VIII were purchased from Calbiochem.

Constructs

Total RNA from Wistar Rattus norvegicus adrenal gland was isolated using the RNeasy® Mini kit (Qiagen) following the manufacturer instructions. The residual genomic DNA which could be contaminating the RNA was degraded with a RNase-free DNase (Qiagen). The entire open reading frame (ORF) of rat MC5R was amplified by RT-PCR using the specific primers 5'-ggaagatctgccaccatgaactcctcgtctcac-3' and 5'-ccggaattctatactgccaaggagcgta-3' which were designed in accordance with the published sequence (Griffon et al., 1994). The rat MC5R ORF was cloned in the pCR2.1-TOPO vector (Invitrogen™) following the manufacturer instructions. The resultant recombinant plasmid was digested with *Eco*RI and *Bgl*II restriction enzymes and the MC5R insertion was sub-cloned in the eukaryotic expression vector pEGFP-N1 (Clontech). To produce the non-tagged MC5R, the rat MC5R ORF was amplified by RT-PCR using the primers 5'-ggaaagcttgccaccatgaactcctcgtctcac-3' and 5'-ccgggatccatactgccaaggagcgta-3' and was cloned in the pCR®-Blunt II-Topo® vector (Invitrogen™). The MC5R insert was obtained by digestion of the recombinant plasmid with *Hind*III and *Bam*HI restriction enzymes and was sub-cloned on pcDNA3.0 vector (Invitrogen™). The pMC5R-EGFP and pMC5R-pcDNA vectors were subsequently sequenced to insure that no errors were induced by PCR reactions. The isolation of pure plasmids was performed using the StrataPrep® EF Plasmid Midiprep Kit (Stratagene) and these DNAs were used on transfection experiments.

Cell culture and transfection

HEK293 cells were cultured in DMEM/Ham's F-12 medium supplemented with 10% fetal bovine serum and 2mM glutamine in 5% CO₂/humified atmosphere at 37°C until 70-80% confluence was attained for use. HEK293 cells were transiently transfected with 0,4µg of pMC5R-EGFP, pMC5R-pcDNA or pEGFP-N1 alone, as a control, in serum free medium for 16h using Effectene Reagent (Qiagen), as recommended by manufacturer instructions. HEK293 cells stably expressing MC5R-GFP or GFP alone were selected by the addition of 0,6µg/µl Geneticin (GIBCO).

RT-PCR analysis

The isolation and purification of total RNA from HEK293 cells transfected with pMC5R-EGFP or pEGFP-N1 and the MC5R RT-PCR reactions were performed as described above. As control a 496 bp sequence of human GAPDH was amplified using the primers pair 5'-ggtgaaggtcggagtcacacg-3' and 5'-caaagttgtcatggatgacc-3'.

Cell treatment

HEK293 cells expressing GFP, MC5R or MC5R-GFP were incubated in culture medium without serum for 16h. Two hours prior experiments the medium was replaced.

Then cells were treated with various concentrations of α -MSH at 37°C for the length of time indicated in Results. For studies of inhibition of the different signaling pathways, several drugs were tested at concentrations that were based on cellular half-maximal inhibitory concentration (IC₅₀) values derived from the available literature (Davies et al., 2000). Thus, cells stably expressing GFP or MC5R-GFP were incubated with vehicle or with different concentrations of the following inhibitors (2',5'-DDA, H-89, GF109203X, wortmannin, LY294002 and Akt inhibitor VIII), for 30min, before addition of 1 μ M α -MSH during 5min. Then, cells were washed with ice-cold PBS and lysed with a cell lysis buffer (50mM Tris-HCl pH 7.6, 10mM NaCl, 5mM EDTA and 0.5% Triton X-100) supplemented with phosphatase and protease inhibitor cocktails (Sigma-Aldrich). Lysates were sonicated and the protein concentrations were determined using the Bradford protein assay (BioRad).

Fluorescence microscopy

To detect the GFP-tagged MC5R expression and cellular distribution, transient or stable transfected HEK293 cells were washed with PBS and fixed with 4% formaldehyde for 20min at room temperature. Images were captured with an ApoTome fluorescence microscope (Zeiss).

cAMP assay

After serum starvation, cells transiently or stably expressing MC5R-GFP or GFP were treated for 10min with 50 μ M forskolin or 1 μ M α -MSH in the presence of 1mM IBMX (phosphodiesterase inhibitor) at 37°C. The medium was removed and cells were immediately lysed in 0.1M HCl. Intracellular cAMP was quantified following the manufacturer instructions using the enzyme immunoassay kit from Sigma-Aldrich.

Western-blotting analysis

Twenty micrograms of cell lysate were separated in a 10% polyacrylamide gel using the Laemmli discontinuous buffer system (Laemmli, 1970) and transferred onto a nitrocellulose membrane (BioRad). The blots were blocked with 5% non-fat dried milk in TBSt (Tris buffer saline with 0.1% Tween-20) and incubated with anti phospho-ERK1/2, phospho-Akt (Ser473) or Akt antibodies (Cell Signaling) 1:1000 in 10% normal goat serum in TBSt, overnight at 4°C. After thorough washing, the anti-ERK1/2 and Akt (phospho and total) primary antibodies were detected with anti-rabbit or anti-mouse-HRP conjugates (1:5000), respectively, for 1 h at room temperature. The bound antibodies were detected using enhanced chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce) and exposed to Pierce CL-XPosure film. Antibodies against pERK1/2 were then stripped incubating the membranes with 10% SDS for 30min at room temperature. Following blocking, the membranes were incubated overnight with anti-ERK1/2 antibodies (Cell Signaling), 1:1000 at 4 °C. The signal detection was performed as previously described.

Data analysis

Digital images of Western blots were analyzed by densitometry using Scion Image and the dose-response analysis was performed using GraphPad Prism 5 software. Each result represents the mean $\pm$ S.D. of at least three experiments. All data were analyzed using the Student's *t* test or one-way ANOVA followed by Bonferroni's post hoc test.

Results

Expression of MC5R-GFP in HEK293 cells

The study of MC5R-mediated signal transduction mechanisms was undertaken by use of an heterologous expression system. HEK293 cells were transiently and stably transfected with a cDNA construct encoding the rat MC5R fused to GFP. This system was chosen because primary cell cultures expressing MC5R have a multitude of other melanocortin receptors, which also respond to the MC5R agonist α -MSH. Otherwise, HEK293 cells have been used by several authors to over-express MC5R (although in a non-tagged form) and in this system MC5R revealed to have properties similar to other melanocortin receptors (Hoogduijn et al., 2002; Mountjoy et al., 2001).

MC5R expression was first characterized by RT-PCR analysis in cell transfectants. HEK293 cells transiently transfected with the pMC5R-EGFP, as well as the stably transfected clone, expressed MC5R mRNA (Fig 1A).

In this cell system, MC5R was expressed as a GFP fusion protein, with the fluorescent tag located at its C-terminal, which enables the study of the receptor localization by fluorescence microscopy. A MC5R construct containing the GFP at the N-terminus was also produced but fluorescence microscopy analysis revealed that the protein was poorly expressed and strongly retained inside the cell (data not shown). Cells transfected with the empty vector pEGFPN1 revealed a diffuse green fluorescence distributed through-out the cell (Fig 1B). On the contrary, MC5R-GFP was detected at cell surface suggesting that the receptor was correctly targeted to the plasma membrane (Fig 1C, D and E). A high and distinct cytoplasmic fluorescence was also visualized in pMC5R-EGFP transfected cells resembling a Golgi-apparatus and/or endoplasmic reticulum localization. In stably transfected cells, the receptor did not accumulate so strongly on intracellular compartments and MC5R-containing secretory vesicles were now clearly distinguishable (Fig 1D, E). This data confirms that at least part of the MC5R-GFP was correctly folded and well translocated to the plasma membrane both in transiently and stably transfected cells.

α -MSH-mediated cAMP production in MC5R-GFP expressing HEK293 cells

To confirm the functionality of the MC5R-GFP located at the plasma membrane, we tested for α -MSH-induced formation of intracellular cAMP. As illustrated in Fig 2, pEGFPN1-transfected HEK293 cells do not respond to α -MSH stimulation, which confirms the absence of endogenous melanocortin receptors expression in this cell system. However, when these cells are treated with the adenylyl cyclase activator forskolin there is a strong stimulation of the cAMP production (about 400 fold above the control). The incubation of transiently and stably transfected cells for 10min with MC5R agonist results in a 10 or 16 fold increase, respectively, above the cAMP levels found in un-stimulated cells. In conclusion, stable cell cultures respond slightly better than transient transfected cells and, for this reason, were used on the subsequent experiments.

Activation of ERK1/2 in stably MC5R-GFP transfected HEK293 cells

Although GPCRs classically signal through Gas-cAMP-PKA pathway, some alternative signal transduction mechanisms have been described (for review see New and Wong, 2007). Therefore, the involvement of the MAP-kinase cascades on MC5R-

mediated signal was investigated. MC5R-GFP and GFP expressing HEK293 cells were incubated with α -MSH (Fig 3A, lanes +) or with vehicle (Fig 3A, lanes -) and the stimulation of ERK1/2 was assessed by western-blotting analysis. In pEGFPN1 transfected cells, α -MSH did not activate ERK1/2 (Fig 3A, lanes GFP). However both ERK1 and ERK2 were strongly and specifically phosphorylated by α -MSH in the presence of MC5R-GFP (Fig 3A, lanes MC5R-GFP). In order to exclude some non-specific effect of the GFP tag on the MC5R functional mechanism, the non-tagged MC5R was transiently expressed on HEK293 cells. As for MC5R-GFP, ERK1/2 was activated after α -MSH stimulation of the MC5R expressing cells (Fig 3A, lanes MC5R). Though the absolute amount of phosphorylated ERK2 was greater than that of ERK1, the relative increases in phosphorylated proteins between control and α -MSH stimulated cells were found to be similar. For this reason, the quantification of ERK1/2 phosphorylation was jointly performed in the following experiments.

To determine if ERK1/2 activation occurs in a dose-responsive manner, MC5R-GFP expressing cells were incubated with increasing concentrations of α -MSH (1nM to 10 μ M) (Fig 3B). The analysis of ERK1/2 phosphorylation by western-blotting revealed that it was dose-dependent with an EC₅₀ of 7.3nM, in agreement with published data for hMC5R-stimulated cAMP production (Bednarek et al., 2007). The maximum ERK1/2 activation was obtained with 1 μ M α -MSH, with an increase of around 8-fold above the basal levels.

For the time-course study, MC5R-GFP transfected cells were incubated with 1 μ M α -MSH for several time-points (5-180min). Western-blotting analysis revealed that ERK1/2 is promptly activated, with maximal intensity 5min after stimulus (Fig 3C). A cyclic pattern or a sustained effect may operate on ERK1/2 signaling by α -MSH since 180min after stimulus, pMC5R-EGFP transfected cells maintained ERK1/2 phosphorylated levels higher than control. In subsequent experiments, cells were treated with 1 μ M α -MSH for 5min.

Signaling requirements for MC5R-mediated ERK1/2 activation

As described before, when cells stably expressing MC5R are stimulated with α -MSH there is an increase of cAMP production. cAMP in turn activates the cAMP-dependent protein kinase PKA. To determine if activation of adenylyl cyclase is fundamental to MC5R-mediated ERK1/2 phosphorylation, HEK293 cells stably expressing the receptor were incubated with 2',5'-DDA (10 μ M-1mM) before α -MSH stimulation (see Materials and Methods). As shown in Fig 4A, the inhibition of adenylyl cyclase has no effect on α -MSH-induced ERK1/2 activation. The involvement of PKA was also addressed by pre-treating MC5R-GFP expressing cells with a PKA specific inhibitor, H-89 (10 and 100 μ M). Although western-blotting analysis shows a slight decrease on ERK1/2 activation after PKA inhibition with 10 μ M H-89, this difference is not statistically significant (Fig 4B). This H-89 concentration was reported as being sufficient to block PKA activity in HEK293 cells, as indicated by the abolishment of ERK1/2 phosphorylation (Gao et al., 1999). The higher H-89 concentration used (100 μ M) induces an intracellular unspecific inhibition, since controls are also affected by this inhibitor. These results demonstrate that cAMP and PKA are not fundamental for the MC5R-induced phosphorylation of ERK1/2.

The role of other second-messenger-dependent kinases, such as PKC, in MC5R-mediated ERK1/2 phosphorylation was also tested. The PKC inhibitor GF109203X (5 and 10 μ M) was added to cell cultures 30min prior stimulation with α -MSH. As

illustrated in Fig 5, the inhibition of PKC also does not interfere with ERK1/2 phosphorylation after treatment of stably transfected cells with α -MSH.

The next step in this work involved the analysis of the PI3K/Akt signaling pathway on MC5R-induced ERK1/2 activation. With this purpose, pre-treatment of cells with wortmannin (10nM-1 μ M) or LY294002 (10-100 μ M), two PI3K inhibitors, markedly inhibited ERK1/2 phosphorylation. In fact, very low concentrations of these inhibitors (such as 10nM for wortmannin and 10 μ M for LY294002) were sufficient to significantly decrease ERK1/2 phosphorylation indicating that MC5R mediated ERK1/2 activation is conveyed through PI3K (Fig 6A and B). The use of two structurally unrelated inhibitors of the same kinase eliminates the possibility of a non-specific effect of the drugs in our cell-based system.

Although Akt acts as the main effector for PI3K, other Akt-independent effectors also play important roles in carrying out PI3K signaling. In fact, when MC5R-GFP expressing cells are stimulated with α -MSH, the Akt phosphorylation levels remain similar to control (Fig 6A and B). This result suggests that MC5R-mediated activation of ERK1/2 is independent of Akt/PKB phosphorylation. As expected, phospho-Akt levels are abolished with wortmannin and LY294002 treatment (Fig 6A and B).

To confirm this last result, the effect of the Akt inhibitor VIII on ERK1/2 activation was tested. This inhibitor interacts with the pleckstrin homology domain of Akt preventing the conformational change required for phosphorylation of this kinase (Green et al., 2008). As shown in Fig 7, even high concentrations of this inhibitor (50 μ M) do not have any effect on MC5R-mediated ERK1/2 phosphorylation after α -MSH stimulation. This result corroborates the evidence that MC5R signals through a PI3K-regulated but Akt independent pathway.

Discussion

The expression of rat MC5R with a tag (GFP) at its C-terminus was successfully attained in HEK293 cells and, as far as we know, is reported here for the first time. Since we have used a cell line from human origin, the success of our data is explained by the high similarity of the rat and human MC5R primary sequence (82% of identity). Moreover, even though rat MC5R has a very short intracellular C-terminal tail (only 12 aminoacids), the attachment of a 27 kDa GFP polypeptide to the receptor C-terminus does not impair its trafficking to the cell surface neither its ability to respond to α -MSH.

Interaction between GPCRs is multiple and complex. A significant number of these receptors are shown to form homo- or heterocomplexes, suggesting that oligomerization of GPCRs may be a quite general phenomenon. For instance, MC1R (Zanna et al., 2008; Mandrika et al., 2005; Sánchez-Laorden et al., 2006) and MC4R (Nickolls and Maki, 2006) have the ability to homodimerize and MC1R and MC3R form heterodimers when expressed in Cos-7 cells (Mandrika et al., 2005). Our results demonstrate that, in HEK293 cells, MC5R-GFP is correctly addressed to the cell-surface and is functionally active in the absence of other melanocortin receptors. Even though these results were obtained in an over-expression system, they suggest that MC5R function does not require heterodimerization with any other melanocortin receptor. If MC5R oligomerizes it should interact with itself or with other receptor from a different family of GPCRs.

The data in this paper show that, in addition to cAMP activation, the α -MSH-stimulated MC5R induces the phosphorylation of ERK1/2 in a dose-dependent manner. The maximum level of ERK1/2 activation is obtained 5min after stimulation and then 50% of activation remains even after 180min. Thus, it seems that MC5R induces a sustained ERK1/2 activation instead of a transient one. A sustained ERK1/2 response is generally related with the stabilization of immediate early genes-encoded transcription factors, such as c-fos, c-jun and c-myc, and results in an increase in target gene expression. At cellular level, ERK1/2 sustained responses are frequently associated with cell cycle progression, cellular transformation or differentiation (Murphy and Blenis, 2006). In fact, earlier reports have postulated a MC5R role in sebocyte (Zhang et al., 2006) and adipocyte (Cho et al., 2005) differentiation. Experiments are in progress to test these hypotheses in an endogenously expressing MC5R cell system, essential to attain the physiological role of the receptor.

Having established an ERK1/2 response to MC5R stimulation by α -MSH, we tried to identify the mechanism by which this signal occurs. The use of specific inhibitors for the adenylyl cyclase, PKA and PKC revealed that ERK1/2 activation is independent of the pathways in which these proteins are involved. However, the PI3K inhibitors wortmannin and LY294002 specifically abolished the ERK1/2 activation mediated by α -MSH-MC5R-GFP. Classically, PI3K activation initiates a phosphorylation cascade leading to the activation of Akt. However the data obtained in this work indicates that ERK1/2 activation is dependent of PI3K but does not require Akt phosphorylation.

One of the mechanisms of lipid kinase PI3K activation by GPCRs involves a direct binding of G $\beta\gamma$ subunits and the small GTPase Ras to PI3K (Schwindinger and Robishaw, 2001). Additionally, GPCRs can also activate PI3K pathways via receptor tyrosine kinases or integrin transactivation. PI3K then initiates a phosphorylation cascade leading to B-Raf and finally to ERKs activation.

Surprisingly, it has been recently proposed that some GPCRs do not couple to G proteins and then are able to activate ERK1/2 by a G protein-independent signaling. In

fact, Jak/STATs, Src-family tyrosine kinases, GRKs/ β -arrestins, and PDZ domain-containing proteins have been suggested to relay signals from GPCRs, independent of G proteins, which could result in ERK1/2 activation (Sun et al., 2007). The single report on MC5R signaling states that this receptor activates the Jak/STAT pathway on B-lymphocytes (Buggy, 1998) but the involvement of the ERK1/2 signaling cascade is not referred. However, there is a strong possibility that the same receptor could activate different transduction pathways when bound to different agonists or when activated in different cell systems.

In conclusion, the present study demonstrates that, after α -MSH activation, MC5R stimulates the PI3K and Raf/MEK/ERK signaling pathways in HEK293 cells. This pathway is cAMP-independent and does not involve PKA, PKC and Akt. We believe that the expression of the recombinant MC5R protein described here represents a useful tool for the biochemical characterization of the receptor, since no functional antibodies are available to this protein. Furthermore it will be of utmost importance in getting new insights on MC5R function and for the elucidation of the key signaling mechanisms transduced by this receptor.

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Legend to Figures

Fig 1. MC5R-GFP expression pattern in transiently and stably transfected HEK293 cells.

A. RT-PCR analysis of MC5R expression was performed in pEGFPN1 (GFP) and in transiently and stably pMC5R-EGFP transfected cells (MC5R-T and MC5R-S, respectively). Lanes (-) are controls without reversed transcription. HEK293 cells transiently expressing GFP (B) or MC5R-GFP (C) and stably transfected with pMC5R-EGFP (D and E) were observed with ApoTome fluorescence microscopy. Scale bar: 10 μ m.

Fig 2. Intracellular cAMP production.

HEK293 cells expressing GFP and transiently or stably expressing MC5R-GFP (MC5R-T and MC5R-S, respectively) were mock-treated (control) or incubated for 10min with 1 μ M α -MSH or 50 μ M forskolin in the presence of 1mM IBMX. Cells were immediately lysed and the cAMP content was quantified using a competitive enzyme immunoassay kit. The data are expressed as fold increase over control cAMP levels. Statistical analysis was performed by two-tailed Student's *t* test (**P*<0.05; ****P*<0.001, versus control).

Fig 3. α -MSH induces ERK1/2 phosphorylation in HEK293 cells expressing MC5R-GFP.

After serum depletion, HEK293 cells expressing MC5R-GFP, MC5R or GFP alone were mock-treated, lanes (-), or incubated with 1 μ M α -MSH, lanes (+), for 5min (A). For dose-response analysis, MC5R-GFP expressing cells were serum-depleted and incubated for 5min with several concentrations of α -MSH (ranging from 1nM to 10 μ M) (B). In the kinetic analysis, cells expressing MC5R-GFP were treated with 1 μ M α -MSH for 5, 10, 30, 60, 120 and 180min (C). In all blots, 20 μ g of total protein were analyzed. ERK1/2 phosphorylation was determined using anti-phospho-ERK1/2 antibody. Then, blots were stripped and reblotted with an anti-total ERK1/2 antibody to determine the protein loading. Densitometry analysis of phospho and total ERK1/2 was performed. The data are expressed as fold increase over control phospho/total ERK1/2 levels. Asterisks indicate groups that are statistically significant from controls using two-tailed Student's *t* test (***P*<0.01; ****P*<0.001).

Fig 4. cAMP and PKA are not involved in MC5R-induced ERK1/2 activation

Cells expressing MC5R-GFP were serum depleted for 16h and mock-treated or incubated with several concentrations of the adenylyl cyclase inhibitor 2',5'-DDA (10 μ M-1mM) (A) or the PKA inhibitor H-89 (10 and 100 μ M) (B), 30min prior incubation with α -MSH. Then cells were harvested and 20 μ g of total protein was analyzed by western-blotting using the anti-phospho and total ERK1/2 antibodies. Densitometry analysis of the results was performed. The data are expressed as fold increase over mock-treated phospho/total ERK1/2. Statistical significance was calculated using a one-way ANOVA followed by Bonferroni's post hoc test (ns - not significant for *P*=1).

Fig 5. PKC is not linked to MC5R-mediated ERK1/2 phosphorylation

MC5R-GFP expressing cells were incubated with 5 μ M or 10 μ M of the PKC inhibitor GF109203X. Thirty minutes later, cells were stimulated with 1 μ M α -MSH. The levels

of phospho and total-ERK1/2 were determined by western-blotting and data analysis was performed as described in the legend to figure 4 (ns - not significant for $P=1$).

Fig 6. MC5R-mediated ERK1/2 activation is conveyed through PI3K

The role of PI3K on MC5R signaling was analyzed by incubating HEK293 cells expressing this receptor with two specific inhibitors of PI3K: wortmannin (10nM-1 μ m) (A) and LY294002 (10-100 μ m) (B). The ERK1/2 and Akt phosphorylation levels were determined by western-blotting. The ERK1/2 and Akt total levels were used to normalize the protein loading. Asterisks indicate groups that are statistically significant from stimulated cells in the absence of inhibitor using a one-way ANOVA followed by Bonferroni's post hoc test (** $P<0.01$; *** $P<0.001$). ns - not significant for $P=1$.

Fig 7. MC5R-mediated ERK1/2 activation is Akt independent

HEK293 cells expressing MC5R-GFP were incubated with the Akt inhibitor VIII (1-50 μ m) 30 min before α -MSH stimulation. The ERK1/2 and Akt phosphorylation levels were determined by western-blotting. The ERK1/2 and Akt total levels were used to normalize the protein loading. Statistical significance was calculated using a one-way ANOVA followed by Bonferroni's post hoc test (ns - not significant for $P=1$).

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Figure 1

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