Biochemical and Biophysical Research Communications **289,** 44–50 (2001)
doi:10.1006/bbrc.2001.5926, available online at http://www.idealibrary.com on IDEAL®

Endogenous Melanin-Concentrating Hormone Receptor SLC-1 in Human Melanoma SK-MEL-37 Cells

Yumiko Saito,*[,1] Zhiwei Wang,† Kimiko Hagino-Yamagishi,‡ Olivier Civelli,† Seiichi Kawashima,§ and Kei Maruyama*

**Department of Pharmacology, Saitama Medical School, Morohongo, Saitama 350-0495, Japan; †Department of Pharmacology, University of California, Irvine, California 92612; ‡Department of Morphological Biochemistry and §Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113-8613, Japan*

Received October 16, 2001

Melanin-concentrating hormone (MCH) is a hypothalamic neuropeptide that regulates several physiological functions. The orphan G protein-coupled receptors SLC-1 and MCHR2 were recently found to bind MCH with high affinity. We show here that the human melanoma cell line SK-MEL-37 expresses SLC-1 mRNA but not MCHR2 by RT-PCR analysis and immunofluorescence studies. Using Chinese hamster ovary cells and 293 cells overexpressing SLC-1 by cDNA transfection, it was shown that SLC-1 coupled to both $G\alpha_i/G\alpha_o$ and $G\alpha_q$ proteins. In SK-MEL-37 cells, MCH inhibited forskolin-stimulated cyclic AMP accumulation and induced mitogen-activated protein kinase (MAPK) in a pertussis toxin-(PTX)-sensitive manner. The MAPK activity leads to the production of phosphorylated forms of p42/p44 MAPK. However, an increase in the intracellular free Ca^{2+} concentration was not elicited by MCH in SK-MEL-37 cells. These results show that SLC-1 is coupled only to PTX-sensitive $G\alpha_i/G\alpha_o$ in SK-MEL-37 cells. This study provides for the first time a skin-derived cellular model to analyze the molecular mechanism of the MCH signaling pathway. © 2001 Academic Press

Key Words: **G protein-coupled receptor; melanin-concentrating hormone; cyclic AMP; calcium influx; mitogen-activated protein kinase.**

Melanin-concentrating hormone (MCH) is a cyclic peptide first isolated from salmon pituitary glands (1) and subsequently found to be present in the mammalian brain (2). The rat MCH is predominantly synthesized by neurons of the lateral hypothalamic area with projections widely distributed throughout the brain (3). This extensive terminal distribution is implicated in a broad array of physiological functions, including the regulatory role of MCH in feeding (4, 5).

An orphan G protein-coupled receptor called SLC-1 was identified as a cognate receptor of MCH (6–10). This receptor is expressed in the brain at high levels (7, 9, 11, 12) and is also expressed in peripheral tissues including eye, tongue and skeletal muscle at low to moderate levels (9). Cells transfected with SLC-1 responded to MCH by inhibition of forskolin-stimulated cyclic AMP (cAMP) production (7–10) and activating mitogen-activated protein kinase (MAPK) (13, 14). Pretreatment with a pertussis toxin (PTX), which blocks $G\alpha_i/G\alpha_o$ protein, reversed the MCH-stimulated inhibition of cAMP production and MAPK activation (7–10, 13, 14). MCH activation induced also an increase in intracellular free Ca^{2+} levels (7–10), which is attenuated, but not abolished, by PTX pretreatment in cells overexpressing SLC-1 (13, 14). Very recently, several groups identified a novel MCH receptor MCHR2 (15–19). MCH stimulates an increase in intracellular Ca^{2+} levels in a PTX-insensitive manner in cells overexpressing MCHR2 (15–19). These results indicate that SLC-1 couples to both PTX-insensitive $G\alpha_q$ and PTX-sensitive $G\alpha_i/G\alpha_o$ protein, and that MCHR2 couples to PTX-insensitive $G\alpha_q$ protein in receptor-overexpressing cells. However, the coupling in cells naturally expressing MCH receptors is unknown and it may differ from that of these overexpressing systems.

Since MCH functions as a regulator of pigmentary changes in teleost fish skin (1, 20), it is important to investigate whether mammalian skin-derived cells express the functional receptor for MCH. Specific binding sites of a MCH analogue were found on cells isolated from skin (21, 22), but the presence of MCH receptor mRNA and protein in such cells has not been reported. In the present study, we detected expression of the

[1] To whom correspondence and reprint requests should be addressed. Fax: (81)-492-76-1585. E-mail: yumisait@saitama-med.ac.jp.

0006-291X/01 $35.00

endogenously functional SLC-1 in the human melanoma cell line SK-MEL-37. This receptor is associated with a signaling pathway which inhibits forskolin-induced cAMP production and induce MAP kinase activation in a PTX-sensitive manner, but not calcium mobilization. Thus, SK-MEL-37 cells represent the first skin-derived system to investigate the signal transduction cascades coupled to SLC-1.

MATERIALS AND METHODS

Cell culture. The following cell lines were obtained from Drs. N. Tai and I. Kawashima (Department of Tumor Biology, Tokyo Metropolitan Institute of Medical Science): G361, VMRC-MELG, CRL-1579, and M12. Bowes was a gift from Dr. H. Ashino (Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science). SK-MEL-19, SK-MEL-28 and SK-MEL-37 cells were a gift from Dr. R. Ueda (Nagoya City University Medical School, Nagoya). All cell lines were cultured in RPMI 1640 with 20 mM Hepes and 10% heat-inactivated fetal calf serum.

Polymerase chain reaction cloning of human SLC-1 cDNA. Full-length (1.1-kb) cDNA encoding the human SLC-1 was cloned by nested polymerase chain reaction (PCR) from a human whole brain cDNA library (Clontech, Palo Alto, CA). The first set of primers was 5′-AGCTCAGCTCGGTTGTGGGA-3′ and 5′-TTCTCAGCATCTCT-CTCCCGGT-3′. PCR was performed using 35 cycles of 94°C (30 s), 50°C (30 s), and 72°C (90 s). The second set of primers was 5′-TGTGAATTCAGGCGACCGGCACTGGCTGGAT-3′ and 5′-CGTCT-CGAGGTGCCCTGACTTGGAGGTGTGCA-3′. The conditions for the second PCR were the same as those for the first except the annealing temperature was 60°C. The resulting full-length SLC-1 cDNA PCR fragment was subcloned into pcDNA3.1 (+) (Invitrogen, Carlsbad, CA) using the *Eco*RI and *Xho*I sites, and the fragment was verified by sequencing.

RNA extraction and expression analysis. Total RNA was extracted from cells using a kit (RNAzol, Biotex, Houston, TX) in accordance with the instructions of the manufacturer. For reverse transcription-polymerase chain reaction (RT-PCR), first-strand cDNA was produced by Super Script II reverse transcriptase (Gibco BRL, Rockville, MD) using 5 μg of total RNA and 0.5 μg of oligo $(dT)_{12-18}$. PCR was performed using cDNA template equivalent to 50 ng of total RNA, 200 μM dNTP, 1 μM of each primer, 2 mM $MgCl_2$, and 2 U *Taq*-DNA-polymerase (Promega) in a total volume of 20 μl using 30 cycles of 94°C (60 s), 65°C (60 s), and 72°C (60 s). PCR products were resolved on a 1.0% gel and visualized by ethidium bromide staining. Primer sequences were 5′-CACTGGCTGGATG-GACCTGGA-3′ and 5′-CTCATCACGGCCATGGATGCCAAT-3′ for human SLC-1 sense primers. For human SLC-1 antisense primers, 5′-CACAGAGCACGATGTACACAAAGG-3′ and 5′-GGAAGTATCA-GGTGCCTTTGCTTTCTG-3′ were used. For human MCHR2, the sense primer was 5′-AGGCCACGAACAATGAATCCATTTC-3′, and the antisense primer was 5′-CATGTCTAGACTCATGGTGATCCAT-3′.

Immunofluorescence studies. SLC-1 antibody was purchased from Bachem. This antibody was raised in chicken against the carboxy-terminal portion of the molecule, which is identical in rat and human SLC-1. The cells were fixed for 20 min in 4% paraformaldehyde in 0.1 M phosphate-buffered saline, and blocked in 5% BSA. Then the cells were incubated overnight with chicken anti-SLC-1 antibody (1:200), washed, incubated for 1 h with a FITC-conjugated rabbit anti-chicken IgY (1:100, Sanbio, Uden, The Netherlands), washed again, and mounted.

Measurement of cAMP production. SK-MEL-37 cells were dislodged from flasks using trypsin, plated in 24-well plates, and incubated for 48 h. Cells were then washed once with cAMP buffer (Hanks-buffered salt solution containing 20 mM Hepes) and preincubated for 10 min in cAMP buffer containing 0.5 mM isobutylmethylxanthine at 37°C (9). The cells were then incubated with test agents for 10 min at 37°C. Reactions were terminated with HCl after the immediate removal of the medium by aspiration, and the level of cellular cAMP was measured using a sensitive radioimmunoassay kit (Yamasa, Chiba, Japan) in accordance with the manufacturer's protocol.

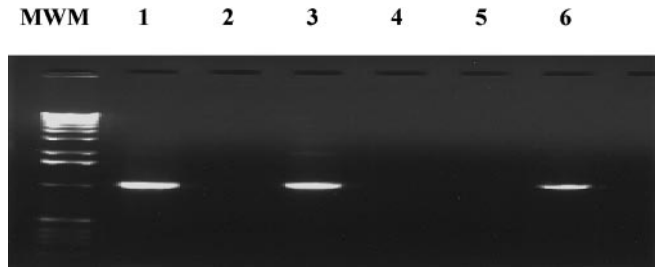

FIG. 1. Expression of SLC-1 MCH receptor in human melanoma SK-MEL-37 cells. Total RNA of 1 μg was used for cDNA synthesis (lanes 1, 2, 4, and 5). PCR with human SLC-1 and MCHR2 specific primers was performed with cDNA corresponding to 50 ng total RNA. Total RNA was reverse transcribed in the presence of superscript II enzyme (lanes 1 and 4). As control, no enzyme was added (lanes 2 and 5). Lanes 3 and 6 show positive control, which uses 0.1 pg SLC-1/pcDNA3.1 (+) or 0.1 pg MCHR2/pcDNA3.1 (−) as template for PCR analysis, respectively.

Assay of MAPK activity. Cells were incubated in 30-mm plates and serum-starved for 24 h in medium containing 0.5% FBS. Subsequently, cells were stimulated with MCH as indicated for 5 min at 37°C, washed with PBS, and lysed at 4°C with 0.2 ml RIPA buffer [10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin]. Cell lysates were centrifuged and the supernatant was assayed for MAPK activity by measuring the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into the synthetic substrate peptide (Amersham, Buckinghamshire, UK) (13).

Western blotting. Cell lysates were prepared as in the assay for MAPK activity. Samples were transferred to Hybond-ECL membranes (Amersham–Pharmacia-Biotech Europe GmbH, Freiburg, Germany) and blocked with 5% BSA in TBS [20 mM Tris–HCl (pH 7.5), 150 mM NaCl] for 1 h at 37°C. Monoclonal anti-p42/p44 antibody (Amersham–Pharmacia-Biotech Europe GmbH) at a 1:1000 dilution was used for incubation for 3 h at room temperature in 2% BSA in TTBS [TBS + 0.2% Tween]. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG was used at 1:1000 in TTBS for 1 h incubation at room temperature. Immunoblots were developed using an ECL reaction (Amersham–Pharmacia-Biotech Europe GmbH).

RESULTS

Human Melanoma SK-MEL-37 Cells Express SLC-1 mRNA

RT-PCR analysis showed that SLC-1 mRNA was expressed in human SK-MEL-37 cells (Fig. 1, lane 1). DNA sequencing of the transcript from SK-MEL-37 cells showed that it corresponded to the human SLC-1 sequence. Recently the second MCH receptor MCHR2 was identified (15–19), but its expression was not de-

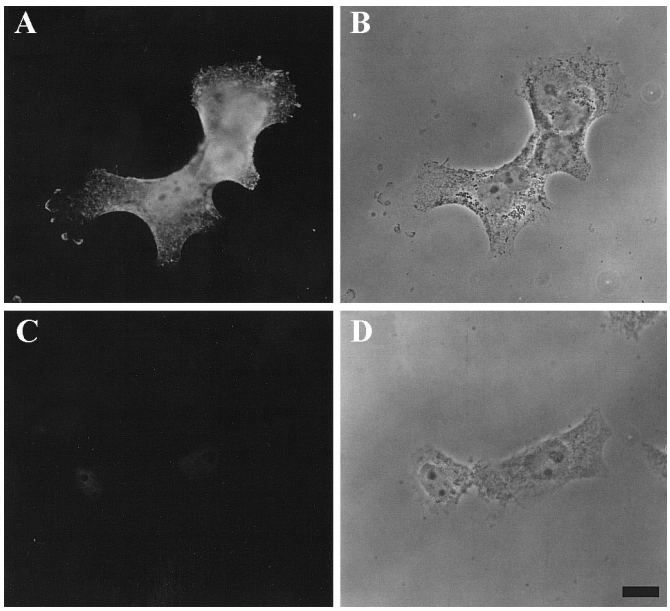

FIG. 2. Fluorescence immunohistochemistry on SK-MEL-37 cells. SLC-1 immunoreactive on cell surface was detected as described under Materials and Methods (A). The corresponding phase images are presented (B). No staining was seen if antibody was preincubated with an excess of the SLC-1 peptide NH_2-VSNAQT-ADEERTESKGT-COOH (C and D). Calibration bar represents 10 μm.

tected in SK-MEL-37 cells by RT-PCR analysis (Fig. 1, lane 4). Various human melanoma cell lines (G361, VMRC-MELG, CRL-1579, M12, Bowes, SK-MEL-19, and SK-MEL-28) were subjected to RT-PCR or Northern blot analysis but a positive signal for SLC-1 and MCHR2 was not detected (data not shown).

Cell Surface Expression of SLC-1 Protein in SK-MEL-37 Cells

Positive immunofluorescence staining for SLC-1 was observed in SK-MEL-37 cells (Figs. 2A and 2B). Staining was mainly observed on the cell surface, with some cytoplasmic staining also observed. Negative controls using preabsorbed antibody with C-terminal peptide were negative (Figs. 2C and 2D), suggesting specific immunoreactivity to SLC-1 protein.

MCH Inhibited Forskolin-Stimulated cAMP Accumulation in SK-MEL-37 Cells

SLC-1 couples to $G\alpha_i/G\alpha_o$ and its activation attenuates cellular cAMP accumulation in SLC-1 transfected CHO or 293 cells (7–10). As shown in Fig. 3A, MCH evoked a response in the form of inhibition of forskolin-stimulated cAMP accumulation in a dose-dependent manner with an estimated EC_{50} value of 28.1 $\pm$ 3.2 nM. Pretreatment of cells with 200 ng/ml pertussis toxin (PTX), a blocker of G_i/G_o, completely abolished MCH-induced inhibition of cAMP accumulation (Fig. 3A). MCH and α-melanocyte-stimulating hormone (α-MSH) have opposite effects on skin color in teleost fishes (20) and exert antagonistic influences on various physiological functions including feeding behavior (14, 23). We found that α-MSH stimulated cAMP production in SK-MEL-37 cells which could not be inhibited by MCH, at concentrations up to 10 μM (Fig. 3B). Although 10 μM ATP induced a robust Ca^{2+} influx in SK-MEL-37 cells, MCH did not affect intracellular free Ca^{2+} concentration using the FLIPR (fluorometric imaging plate reader) detection system or calcium imaging processor with CCD camera (data not shown).

MCH Stimulated an Increase in MAPK and Induced Production of Phosphorylated Forms of p42 and p44 MAPK

MAPK activity in cell lysates prepared from SK-MEL-37 cells was increased to 116.2 $\pm$ 7.6% of the non-stimulated control level at 1 min, 180 $\pm$ 5.7% at 5 min, 209.0 $\pm$ 11.3% at 10 min, 200.0 $\pm$ 14.5% at 15 min, and 135.7 $\pm$ 10.3% at 30 min after the addition of 300 nM MCH (three separate experiments). MAP kinase activity evoked by stimulation with MCH for 10 min was dose-dependent with an EC_{50} value of 58 $\pm$ 3.8 nM (Fig. 4A). Treatment of the cells with 200 ng/ml PTX for 18 h completely abolished the ability of MCH to activate MAPK (Fig. 4B), indicating that the response is mediated by PTX-sensitive G proteins, $G\alpha_i$ and/or $G\alpha_o$.

MAPKs, which consist of two isoforms p44 and p42, exist as unphosphorylated forms in nonstimulated cells and become activated when both tyrosine and threonine residues are phosphorylated by MAPKK. As shown in Fig. 4C, stimulation of SK-MEL-37 cells with MCH increased the appearance of phosphorylated forms of p42 and p44 MAPK in a time-dependent manner. p44 and p42 MAPKs were phosphorylated maximally after stimulation for 5 to 15 min. Figure 4D shows that the appearance of phosphorylated MAPKs is dependent on MCH concentration. Maximal phosphorylation was observed in the range from 100 nM to 1 μM MCH, and it was compatible with that stimulated by 10% FBS. Thus, the time course (Fig. 4C) and dose dependence (Fig. 4D) of MCH-induced MAPK phosphorylation paralleled with those of MCH-induced increase in MAPK activity. Depletion of cellular protein kinase C (PKC) by treatment with 1 μM phorbol 12-myristate 13-acetate for 18 h partially attenuated MCH-stimulated MAPK activation (Fig. 4B). This result suggests that MAPK activation mediated by SLC-1 is at least partially dependent on PKC activation.

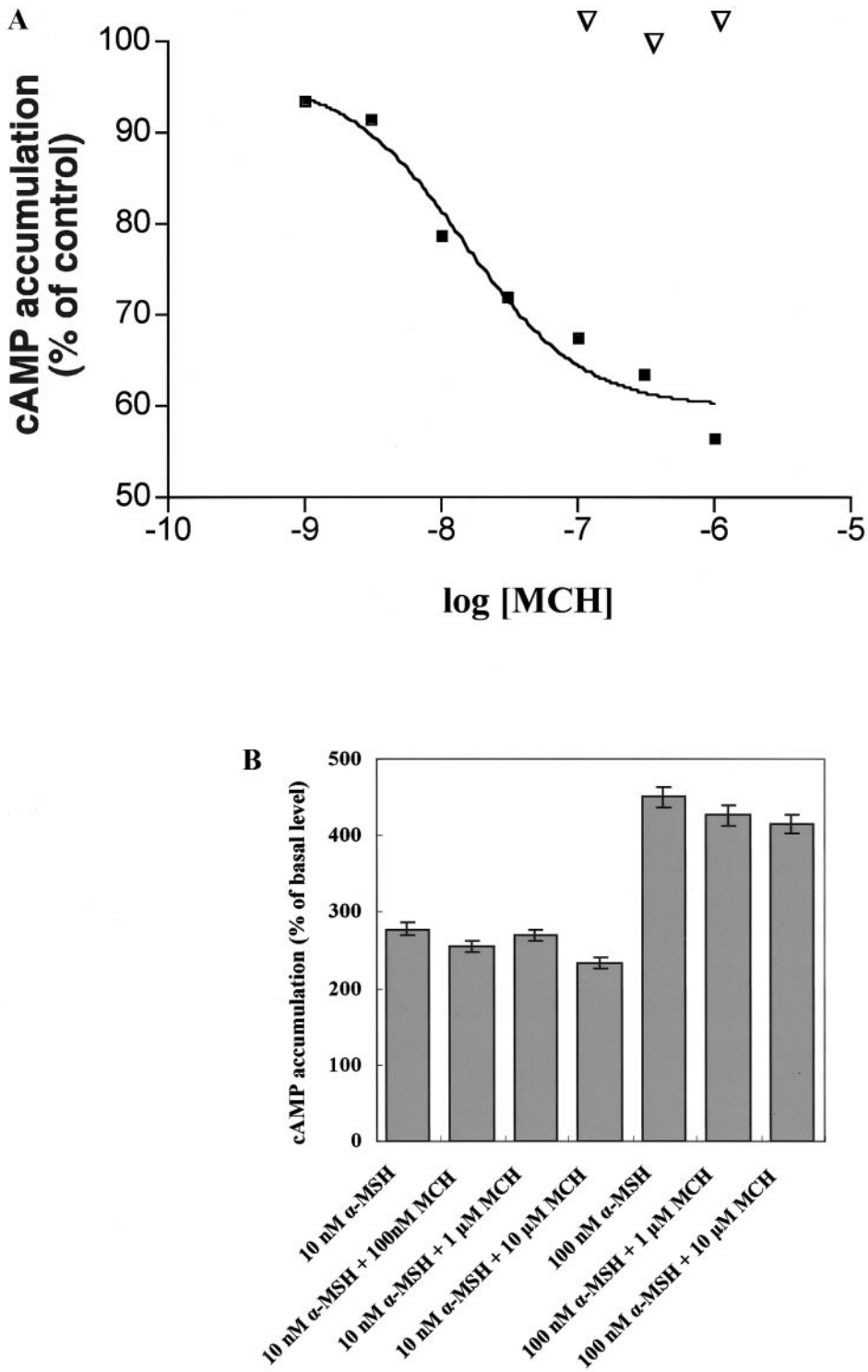

FIG. 3. Inhibition of forskolin-stimulated cAMP accumulation in SK-MEL-37 cells. (A) The dose–response curve for inhibition of forskolin-stimulated accumulation of cellular cAMP by MCH. Cells were also treated with 200 ng/ml PTX for 18 h and then stimulated with 30, 100, 300, and 1000 nM MCH, respectively (triangles). All incubations were done in triplicate and representative data are shown. (B) Effects and interaction between MCH and α-MSH on cAMP level. SK-MEL-37 cells were preincubated with α-MSH for 10 min for the indicated concentration, and treated MCH for 10 min. All incubations were done in triplicate and data were means $\pm$ SEM values from two separate experiments.

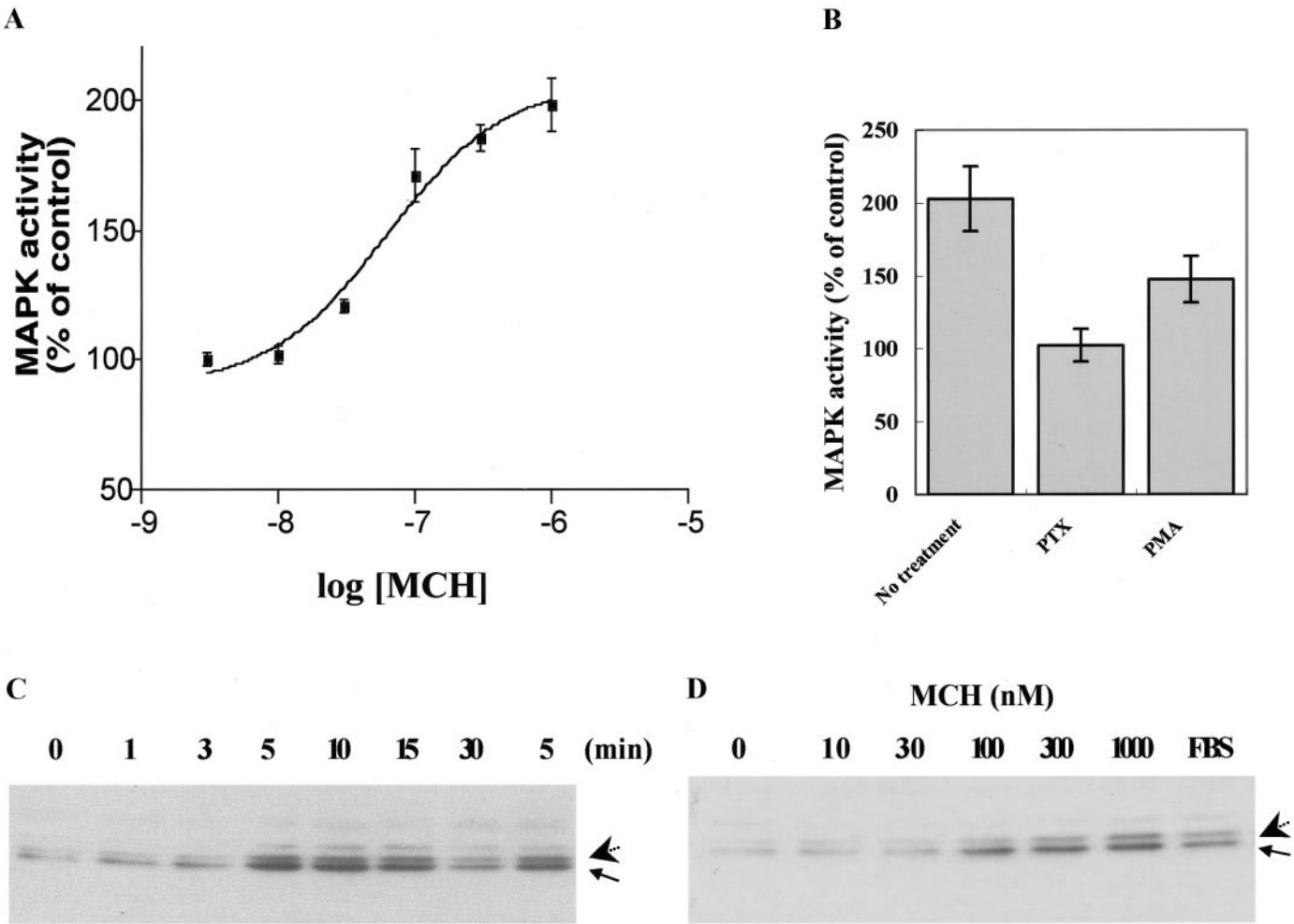

FIG. 4. MCH stimulates MAPK signaling pathway in SK-MEL-37 cells. (A) Dose–response relationship for MCH-mediated MAPK activation. Cells were stimulated by MCH at the indicated concentration for 10 min. (B) Effects of PTX and phorbol 12-myristate 13-acetate (PMA) on MCH-activated MAPK activity. SK-MEL-37 cells were treated with 200 ng/ml PTX for 18 h or 1 μM PMA for 18 h, and then stimulated with 300 nM MCH for 10 min. Data are means ± SEM values from three separate experiments. (C) Immunoblot analysis using antibody specific for the phosphorylated MAPK, p42/p44. SK-MEL-37 cells were stimulated for the indicated times with 300 nM MCH, and for 10 min with the indicated concentrations of MCH or 10% fetal bovine serum (FBS) (D). The positions of phosphorylated forms of MAPKs (p42 and p44) are indicated as an arrow and arrowhead, respectively.

DISCUSSION

The MCH/MCH receptors are predominantly expressed in the central nervous system (3, 9, 11, 12) and MCH is known to be a potentially important effector of nutritional homeostasis (4, 5). Recently the presence of MCH and SLC-1 in non-neuronal tissues was also shown (9, 24–26). It seems likely that MCH of local origin may act on nonneuronal cellular targets as described by the effect of MCH on leptin secretion in adipocytes (24). MCH got it name for its ability to induce concentration of the intracellular pigment granules in teleost fish skin (20). The possible effect of MCH on mammalian skin remains however to be studied. Thus far, specific binding of the MCH analogue [^{125}I][Phe^{13}, Tyr^{19}]-MCH on mouse B16F1 melanoma cells and human SVK14 kelatinocyte (21, 22) is the only link between MCH and mammalian skin. However, this was not corroborated by the demonstration of the presence of MCH receptor mRNA or protein in these cells. We, therefore, began to study the possible expression of MCH receptors in mammalian skin-derived cells, and show the presence of functionally active SLC-1 in the human melanoma cell line, SK-MEL-37. SK-MEL-37 cell expresses the broadest pattern of cancer-testis (CT) antigens among human melanoma cell lines (27). CT antigens, which are expressed in testis and a proportion of diverse human cancer types, are useful markers of disease progression for malignant tumors and are promising targets for cancer vaccines. There-

fore, our present finding may set the basis to study the effect of MCH on malignant melanoma.

MCH and α-MSH produced antagonistic effects on pigmentary aggregation in teleost fish (20). This antagonism extends to the mammalian central nervous system with respect to feeding (23), the hypothalamic-pituitary-adrenal-axis (23), grooming, and auditory gating (14). Since α-MSH did not block the activation of SLC-1 by MCH in cells overexpressing SLC-1 (7, 9), α-MSH did not exert antagonistic activity by competing with MCH for SLC-1. In the present study, α-MSH-stimulated cAMP accumulation was observed in SK-MEL-37 cells, suggesting the functional presence of endogenous α-MSH receptors. MCH, at concentrations up to 10 μM, however, did not inhibit the stimulation of cAMP accumulation induced by α-MSH (Fig. 3B). Thus, our study suggested that no "cross-talk" between the two receptors occurred even in the case of endogenous coexpression of both the SLC-1 and the α-MSH receptor in the same cells. Physiological antagonism between MCH and α-MSH may result from the complex convergence of signaling pathways mediated by distinct receptors expressed in different neurons or other tissues.

Although it has been demonstrated that SLC-1 couples to $G\alpha_i/G\alpha_o$ and $G\alpha_q$ proteins in CHO or 293 cells exogenously transfected with SLC-1 (7–10, 13, 14), the signal transduction processes of SLC-1 have not been studied in the native cellular environment. The expression of SLC-1 receptors has been detected in adipocytes, insulin-producing cell lines and ciliary epithelial cells (23–25), but the signal transduction pathway via SLC-1 was not described in such cells and a native cellular model is needed to study MCH-mediated signal transduction. In this study, we provide biochemical and pharmacological evidence that MCH inhibited forskolin-stimulated cAMP accumulation and induced MAPK activity in a PTX-sensitive manner in SK-MEL-37 cells. Thus, the present study demonstrated for the first time that SLC-1 is indeed coupled to PTX-sensitive $G\alpha_i$ protein in the native cellular environment. MCH inhibited forskolin-stimulated cAMP accumulation and increased intracellular free Ca^{2+} levels in SLC-1 overexpressing cells. However, no increase in intracellular free Ca^{2+} concentration was elicited by MCH in SK-MEL-37 cells. This is not due to a lack of $G\alpha_q$ protein in this melanoma cell because ATP elicited a robust Ca^{2+} influx. The specificity of the receptor/G protein interaction may be influenced by receptor densities, stoichiometric considerations or accessory proteins found in the membrane environment (28). The response of ligands in cells expressing endogenous receptors at a low to moderate density could be different from that of a high-density expression system in receptor overexpressing cells. Thus, the receptor coupling to G protein observed in the overexpressed cells might not reflect the physiological situation in intact cells. For example, transfected angiotensin AT_1 receptor has been proposed to couple with three distinct G proteins ($G\alpha_q$, $G\alpha_i$, and $G\alpha_o$) in CHO cells (29). In the hepatoma cell line PLC-PRF-5 that expresses AT_1 receptor, however, no evidence for receptor coupling via $G\alpha_i$ to inhibit adenyl cyclase was found, while stimulation of the receptor increased Ca^{2+} influx (30). In vascular myocytes, in contrast, the angiotensin AT_1 receptor couples to $G\alpha_{13}$ but not $G\alpha_i/G\alpha_o$ nor $G\alpha_q$ protein (31). Since this may also apply to MCH receptors, it is important to find other natural cells that express endogenous MCH receptors and to determine a novel coupling to G proteins and their signaling pathways.

Although the physiological relevance of MCH inhibition of forskolin-stimulated cAMP accumulation and MAPK activation in SK-MEL-37 cells is not clear at present, MAPK is known to be important in the mitogenic signaling of a range of polypeptides. We did not detect any proliferative response to MCH in SK-MEL-37 cells. Thus, the precise roles of the activation of signaling pathways by MCH, and, in particular, its relation to the effect of MCH on tumor progression on melanoma cells, remain to be elucidated.

In conclusion, we have shown the functional presence of MCH receptor SLC-1 coupled to PTX-sensitive $G\alpha_i/G\alpha_o$ in the human melanoma cell line SK-MEL-37, leading to inhibition of forskolin-stimulated cAMP accumulation and induction of MAPK activity. The presence of well-characterized MCH receptors in SK-MEL-37 cells allows us to analyze the molecular details of the signaling pathways induced by MCH in a physiological cellular environment.

ACKNOWLEDGMENTS

We thank Drs. Y. Yajima and H. Nagasaki for valuable discussion and comments. This work was supported by the Ministry of Education, Science, Culture, and Sports of Japan.

REFERENCES

1. Kawauchi, H., Kawazoe, I., Tsubokawa, M., Kishida, M., and Baker, B. I. (1983) Characterization of melanin-concentrating hormone in chum salmon pituitaries. *Nature* **305,** 321–323.
2. Vaughan, J. M., Fisher, W. H., Hoeger, C., and Vale, W. (1989) Characterization of melanin-concentrating hormone from rat hypothalamus. *Endocrinology* **125,** 1660–1665.
3. Bittencourt, J. C., Presse, F., Arias, C., Peto, C., Vaughan, J., Nahon, J. L., Vale, W., and Sawchenko, P. E. (1992) The melanin-concentrating hormone system of the rat brain: An immuno- and hybridization histochemical characterization. *J. Comp. Neurol.* **319,** 218–245.
4. Qu, D., Ludwig, D. S., Gammeltoft, S., Piper, M., Peleymounter, M. A., Cullen, M. J., Mathes, W. F., Przypek, J., Kanarek, R., and Maratos-Flier, E. (1996) A role for melanin-concentrating hormone in the central regulation of feeding behavior. *Nature* **380,** 243–247.

5. Shimada, M., Tritos, N. A., Lowell, B. B., Flier, J. S., and Maratos-Flier, E. (1998) Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature* **396,** 670–674.
6. Bachner, D., Kreienkamp, H-J., Weise, C., Buck, F., and Richter, D. (1999) Identification of melanin-concentrating hormone (MCH) as the natural ligand for the orphan somatostatin-like receptor 1 (SLC-1). *FEBS Lett.* **457,** 522–524.
7. Chambers, J., Ames, R. S., Bergsma, D., Muir, A., Fitgerals, L. R., Hervieu, G., Dytko, G. M., Foley, J. J., Martin, J., Liu, W-S., Park, J., Ellis, C., Ganguly, S., Konchar, S., Cluderray, J., Leslie, R., Wilson, S., and Sarau, H. M. (1999) Melanin-concentrating hormone is the cognate ligand for the orphan G-protein-coupled receptor SLC-1. *Nature* **400,** 261–265.
8. Lembo, P. M. C., Grazzini, E., Cao, J., Hubatsch, D. A., Pelletier, M., Hoffert, C., St-Onge, S., Pou, C., Labrecque, J., Groblewski, T., O'Donnell, D., Payza, K., Ahmad, S., and Walker, P. (1999) The receptor for the orexigenic peptide melanin-concentrating hormone is a G-protein-coupled receptor. *Nat. Cell Biol.* **1,** 267–271.
9. Saito, Y., Nothacker, H-P, Wang, Z., Lin, S, H-S., Leslie, F., and Civelli, O. (1999) Molecular characterization of the melanin-concentrating-hormone receptor. *Nature* **400,** 265–269.
10. Shimomura, Y., Mori, M., Sugo, T., Ishibashi, Y., Abe, M., Kurokawa, T., Onda, H., Nishimura, O., Sumino, Y., and Fujino, M. (1999) Isolation and identification of melanin-concentrating hormone as the endogenous ligand of the SLC-1. *Biochem. Biophys. Res. Commun.* **261,** 622–626.
11. Hervieu, G., Cluderay, J. E., Harrison, D., Meakin, J., Nasir, S., and Leslie, R. A. (2000) The distribution of the mRNA and protein products of the melanin-concentrating hormone (MCH) receptor gene, slc-1, in the central nervous system. *Eur. J. Neurosci.* **12,** 1194–1216.
12. Saito, Y., Cheng, M., Leslie, F. M., and Civelli, O. (2001) Expression of the melanin-concentrating hormone (MCH) receptor mRNA in the rat brain. *J. Comp. Neurol.* **435,** 26–40.
13. Hawes, B. E., Kil, E., Green, B., O'Neill, K., Fried, S., and Graziano, M. P. (2000) The melanin-concentrating hormone receptor couples to multiple G proteins to activate diverse intracellular signaling pathway. *J. Neurochem.* **141,** 4524–4532.
14. Saito, Y., Nothacker, H-P., and Civelli, O. (2000) Melanin-concentrating hormone receptor: An orphan receptor fits the key. *Trends Endocrinol. Metab.* **11,** 299–303.
15. An, S., Cutler, G., Zhao, J. J., Huang, S. G., Tian, H., Li, W., Liang, L., Rich, M., Bakleh, A., Du, J., Chen, J. L., and Dai, K. (2001) Identification and characterization of a melanin-concentrating hormone receptor. *Proc. Natl. Acad. Sci. USA* **98,** 7576–7581.
16. Hill, J., Duckworth, M., Murdock, P., Rennie, G., Sabido-David, C., Ames, R. S., Szekeres, P., Wilson, S., Bergsma, D. J., Gloger, I. S., Levy, D. S., Chambers, J. K., and Muir, A. I. (2001) Molecular cloning and functional characterization of MCH_2, a novel human MCH receptor. *J. Biol. Chem.* **276,** 20125–20129.
17. Mori, M., Harada, M., Terao, Y., Sugo, T., Watanabe, T., Shimomura, Y., Abe, M., Shintani, Y., Onda, H., Nishimura, O., and Fujino, M. (2001) Cloning of a novel G protein-coupled receptor, MCHR2, a subtype of the melanin-concentrating hormone receptor. *Biochem. Biophys. Res. Commun.* **283,** 1013–1018.
18. Sailer, A. W., Sano, H., Zeng, Z., McDonald, T. P., Pan, J., Pong, S. S., Feighner, S. D., Tan, C. P., Fukami, T., Iwaasa, H., Hreniuk, D. L., Morin, N. R., Sadowski, S. J., Ito, M., Ito, M., Bansal, A., Ky, B., Figueroa, D. J., Jiang, Q., Austin, C. P., MacNeil, D. J., Ishihara, A., Ihara, M., Kanatani, A., Van der Ploeg, L. H., Howard, A. D., and Liu Q. (2001) Identification and characterization of a second melanin-concentrating hormone receptor, MCH-2R. *Proc. Natl. Acad. Sci. USA* **98,** 7564–7579.
19. Wang, S., Bihan, J., O'Neil, K., Weig, B., Fried, S., Laz, T., Bayne, M., Gustafson, E., and Hawes, B. E. (2001) Identification and pharmacological characterization of a novel human melanin-concentrating hormone receptor, MCH-R2. *J. Biol. Chem.*, in press.
20. Baker, B. I. (1993) The role of melanin-concentrating hormone in color change. *Ann. N.Y. Acad. Sci.* **680,** 279–289.
21. Drozdz, R., Siegris, W., Baker, B. I., Chluba-da, T., and Eberle, A. N. (1995) Melanin-concentrating hormone binding to mouse melanoma cells *in vitro*. *FEBS Lett.* **359,** 199–202.
22. Burgaud, J. L., Poosti, R., Fehrentz, J. A., Martinez, J., and Nahon, J. L. (1997) Melanin-concentrating hormone binding sites in human SVK14 kelatinocytes. *Biochem. Biophys. Res. Commun.* **241,** 622–629.
23. Ludwig, D. S., Mountjoy, K. G., Tatro, J. B., Gillette, J. A., Frederich, R. C., Flier, J. S., and Maratos-Flier, E. (1998) Melanin-concentrating hormone: A functional melanocortin antagonist in the hypothalamus. *Am. J. Physiol.* **274,** E627–E633.
24. Bradley, R. L., Kokkotou, E. G., Maratos-Flier, E., and Cheatham, B. (2000) Melanin-concentrating hormone regulates leptin synthesis and secretion in rat adipocytes. *Diabetes* **49,** 1073–1077.
25. Tadayyon, M., Welters, H. J., Haynes, A. C., Cluderay, J. E., and Hervieu, G. (2000) Expression of melanin-concentrating hormone receptors in insulin-producing cells: MCH stimulates insulin release in RINm5F and CRI-G1 cell lines. *Biochem. Biophys. Res. Commun.* **275,** 709–712.
26. Hintermann, E., Erb, C., Talke-Messerer, C., Liu, R., Tanner, H., Flammer, J., and Eberle, A. N. (2001) Expression of the melanin-concentrating hormone receptor in porcine and human ciliary epithelial cells. *Invest. Ophthamol. Vis. Sci.* **42,** 206–209.
27. Chen, Y-T., Gure, A. O., Tsang, S., Stockert, E., Jager, E., Knuth, A., and Old, L. J. (1998) Identification of multiple cancer-testis antigens by allogeneic antibody screening of a melanoma cell line library. *Proc. Natl. Acad. Sci. USA* **95,** 6919–6923.
28. Sato, M., Kataoka, R., Dingus, J., Wilcox, M., Hildebrandt, J. D., and Lanier, S. (1995) Factors determining specificity of signal transduction by G-protein-coupled receptors. *J. Biol. Chem.* **270,** 15269–15276.
29. Shibata, T., Suzuki, C., Ohnishi, J., and Miyazaki, H. (1996) Identification of regions in the human angiotensin II receptor type 1 responsible for G_i and G_q coupling by mutagenesis study. *Biochem. Biophys. Res. Commun.* **218,** 383–389.
30. Wintersgill, H. P., Warburton, P., Bryson, S. E., Ball, S. G., and Balmforth, A. J. (1992) Characterization of the angiotensis II receptor expressed by the human hepatoma cell line, PLC-PRF-5. *Eur. J. Pharmacol.* **227,** 283–291.
31. Macrez-Lepretre, N., Kallkbranner, F., Morel, J-L., Schhultz, G., and Mironneau, J. (1997) G protein heterotrimer $G\alpha_{13}\beta_1\gamma_3$ couples the angiotensin AT_{1A} receptor to increases in cytoplasmic Ca^{2+} in rat portal vein myocytes. *J. Biol. Chem.* **272,** 10095–10102.