

Expression of the Melanin-Concentrating Hormone Receptor in Porcine and Human Ciliary Epithelial Cells

Edith Hintermann,¹ Carl Erb,² Christiane Talke-Messerer,¹ Rong Liu,² Heidi Tanner,¹ Josef Flammer,² and Alex N. Eberle¹

PURPOSE. To evaluate whether the receptors for melanin-concentrating hormone (MCH) and its functional antagonist α -melanocyte-stimulating hormone (α -MSH) are expressed in the ciliary epithelium. Furthermore, to examine whether MCH, a neuropeptide involved in fluid and electrolyte homeostasis, may influence ion flux mediated by Na,K (adenosine triphosphatase)-ATPase in a ciliary epithelial cell line.

METHODS. Expression of MCH receptors (MCH-R) and α -MSH receptors (MSH-R) on primary porcine ciliary pigmented epithelial (PE) cells and on a human nonpigmented ciliary epithelial (NPE) cell line, ODM-2 was investigated by radioligand binding studies and reverse transcription-polymerase chain reaction (RT-PCR). The MCH-R was further characterized by photocrosslinking. Influence of MCH on Na,K-ATPase activity was evaluated by an Rb⁺ transport assay.

RESULTS. MCH-R expression was observed at both the mRNA and protein levels in PE and NPE cells. In contrast, MSH-Rs were not detectable. At the mRNA level, expression of *slc-1* was shown and with crosslinking, a 44-kDa protein was labeled. MCH showed no effect on Na,K-ATPase activity of NPE cells.

CONCLUSIONS. The presence of MCH-R in ciliary epithelial cells of both human and porcine origin but the absence of MSH-Rs indicates that in these cells, MCH and α -MSH do not form a functionally antagonistic hormonal pair as they do in several other systems. Although effects of MCH on intestinal water and ion transport have been documented, a direct control of Na,K-ATPase activity was not detected in human NPE cells *in vitro*. (*Invest Ophthalmol Vis Sci.* 2001;42:206–209)

MCH is a neuropeptide mainly synthesized in the hypothalamus with projections reaching most regions of the brain.¹ Transcripts of the MCH precursor have also been detected in the periphery.¹ Its widespread distribution suggests that MCH influences a number of central and peripheral activities, including processes associated with emotional responses, cognition, general arousal, stress, and food intake.¹ In most of these studies, MCH was shown to act as a functional antagonist of α -melanocyte-stimulating hormone (α -MSH). Recently, the orphan G-protein-coupled-receptor SLC-1 was shown to be activated by MCH. This receptor is mainly expressed in the brain but shows moderate expression in the eye.² Because MCH is also involved in the control of sodium, potassium, and water transport in other systems^{3,4} and because proper regu-

lation of water and salt balance is a prerequisite for a constant flow of aqueous humor through the eye, we sought to elucidate whether receptors for MCH are expressed by ciliary epithelial cells and whether MCH may influence Na⁺,K⁺-adenosine triphosphatase (ATPase) activity in these cells.

MATERIALS AND METHODS

Cell Culture

Primary cultures of porcine ciliary pigmented epithelial (PE) cells were established according to Helbig et al.⁵ Ciliary processes from six eyes were dissected, transferred into phosphate-buffered saline (PBS), washed, and incubated overnight at 4°C in PBS containing 0.025% trypsin and 0.01% EDTA. The ciliary epithelium was dissociated by incubation at 37°C for 15 minutes. Isolated cells were pipetted off, and ciliary processes were reincubated with fresh PBS-trypsin-EDTA. This step was repeated, the three cell suspensions were pooled and centrifuged, and the cells were seeded in Dulbecco's modified Eagle's medium (Gibco, Paisley, UK) containing 4.5 g/l glucose, supplemented with 10% fetal calf serum (Amimed, Basel, Switzerland), 2 mM L-glutamine (Gibco), penicillin (100 U/ml; Gibco), and streptomycin (100 μ g/ml; Gibco). The simian virus (SV)-40-transformed cell clone ODM-2 derived from human nonpigmented ciliary epithelium (NPE), originally established and described by Martin-Vasallo et al.,⁶ was a kind gift of Martin B. Wax, Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, Missouri, and was cultured under the same conditions as the PE cells.

Receptor Binding Assays and Photoaffinity Labeling

Peptides were from Bachem (Bubendorf, Switzerland), except [D-Phe¹³, L-Tyr¹⁹]-MCH⁷ and variant primate MCH_v,¹ which were synthesized in our laboratory. The radioligands [¹²⁵I]-[D-Phe¹³, Tyr¹⁹]-MCH, [¹²⁵I]-[D-Bpa¹³, Tyr¹⁹]-MCH, which contained a photoreactive D-(*p*-benzoyl) phenylalanine (Bpa) residue, and [¹²⁵I]-[Nle⁴, D-Phe⁷]- α -MSH were obtained by radioiodination with solid phase-bound glucose oxidase-lactoperoxidase⁷ and are abbreviated, respectively, [¹²⁵I]MCH, [¹²⁵I]Bpa-MCH, and [¹²⁵I] α -MSH in this text.

Binding assays⁷ were started by adding 50 μ l (0.05 picomoles) of either [¹²⁵I]MCH or [¹²⁵I] α -MSH to a 0.5-ml cell suspension (1×10^6 cells) and 50 μ l competitor peptide in a 1:3-dilution series (highest concentration 1.7 μ M). In saturation experiments, the competitor peptide concentration was 1.7 μ M and 0.005 to 0.4 picomoles of radioligand was added. Cells were incubated for 2 hours at 10°C. Triplicate aliquots (150 μ l) were layered onto 150 μ l ice-cold silicon oil of a density of 1013 kg/cm³. Unbound radioactivity was removed by a 2-minute centrifugation at 4°C and 12,000g. Binding data were analyzed by computer (Prism; GraphPad Software, San Diego, CA) and are presented as means \pm SEM. K_i values were calculated from median inhibitory (IC₅₀) values using the corrected Cheng-Prusoff equation of Munson and Rodbard.⁸

For photoaffinity labeling,⁹ the binding reaction contained 5×10^6 cells, 0.2 picomoles [¹²⁵I]Bpa-MCH and 2 μ M MCH (where indicated). The suspension was incubated at 10°C for 2 hours, followed by UV-irradiation on ice for 30 minutes, using a spectrum of more than 310 nm and an irradiation intensity of 180 mW/cm². Cells were washed in

From the ¹Laboratory of Endocrinology, Department of Research (ZLF), and ²University Eye Clinic, University Hospital and University Children's Hospital, Basel, Switzerland.

Supported by Swiss National Science Foundation and Millennium Pharmaceuticals, Cambridge, Massachusetts.

Submitted for publication December 3, 1999; revised June 5 and September 14, 2000; accepted September 28, 2000.

Commercial relationships policy: N.

Corresponding author: Alex N. Eberle, Department of Research (ZLF), University Hospital, Hebelstrasse 20, CH-4031 Basel, Switzerland. alex-n.eberle@unibas.ch

cold PBS and lysed, and the membrane fraction was prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.⁹

Detection of *slc-1* mRNA by RT-PCR

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted from PE and NPE cells with a kit (RNeasy; Qiagen, Chatsworth, CA) including DNase I digestion, according to the instructions of the manufacturer. First-strand cDNA was produced by M-MLV reverse transcriptase (Promega, Madison, WI) using 1 μ g of RNA and 0.2 μ g oligo (dT)₁₅. Each RNA preparation was controlled for the presence of genomic DNA and subjected to digestion (RNase ONE; Promega) to show specific amplification from cDNA. PCR was performed with cDNA corresponding to 50 ng total RNA, 200 μ M dNTP, 1 μ M of each primer, 1.5 mM MgCl₂, and 2 U *Taq*-DNA-polymerase (Promega) in a total volume of 40 μ l with 40 cycles of 94°C (45 seconds), 59°C (50 seconds), and 72°C (45 seconds). Primer sequences were 5'-ACCATGTGCACCTCATCAC-3' for both human and rat *slc-1* sense primers and 5'-TCTCACAGAGCAGCATGTAC-3' and 5'-TCTCACAGAGCACTATGTAC-3' for human and rat *slc-1* antisense primers, respectively. Because the *slc-1* sequence is very highly conserved between species, the rat *slc-1* primers could be used for detection of *slc-1* transcripts in porcine PE cells. PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

Na,K-ATPase Activity Assay

ODM-2 cells cultured in 12-well plates were incubated for 30 minutes at 37°C in 1.5 ml of KCl-Earle's balanced salt solution (KCl-EBSS) containing (in millimolar) 116.3 NaCl, 26.2 NaHCO₃, 1 NaH₂PO₄, 0.8 MgSO₄, 1.8 CaCl₂, and 0.2 KCl. Rb⁺ uptake was initiated by incubating the cells for 15 minutes with 1.5 ml EBSS containing RbCl instead of KCl (RbCl-EBSS) and 10 nM, 100 nM, or 1 μ M MCH or vehicle. The cells were then washed three times with 1.5 ml cold 0.1 M MgCl₂. Then, 0.1 ml EtOH was added, and the cells were allowed to dry. Intracellular Rb⁺ was extracted by incubating the cells for 1 hour in 1 ml 3 mM CsCl. Rb⁺ content was determined by emission flame photometry, using an atomic absorption spectrophotometer (model 460; Perkin-Elmer, Norwalk, CT) with a wavelength of 780 nm and a slit of 0.2 nm. Monolayers were assayed for protein content by Bradford assay. Ouabain-sensitive Rb⁺ transport was calculated as total transport minus transport in the presence of 3 mM ouabain. Statistical analysis was performed with the unpaired *t*-test, and *P* < 0.05 was considered significant.

RESULTS

Binding Studies with [¹²⁵I] α -MSH and [¹²⁵I]MCH

A series of radioligand-binding experiments with [¹²⁵I]MCH and [¹²⁵I] α -MSH showed that optimal binding conditions for PE and NPE cells were the same as those established for mouse melanoma cells⁷ (data not shown). Whereas [¹²⁵I] α -MSH did not bind specifically to PE or NPE cells, [¹²⁵I]MCH displayed specific binding to both cell types, with PE exhibiting approximately 2.7-fold higher binding than NPE cells (Fig. 1). Independent of the radioligand used, nonspecific binding to PE cells was always higher than to NPE cells.

Competition- and Saturation-Binding Experiments with [¹²⁵I]MCH

Competition-binding studies were performed using unlabeled ligand in the concentration range from 2 μ M to 0.26 nM. With PE cells, MCH displayed a *K*_i of 62.3 ± 3.6 nM, whereas MCH_v showed almost no binding (*K*_i > 1 μ M). With NPE cells, both MCH and MCH_v inhibited binding of [¹²⁵I]MCH with a *K*_i of 14.8 ± 2.1 nM and 180.4 ± 57.5 nM, respectively. Saturation-binding experiments showed that the MCH-R expressed by

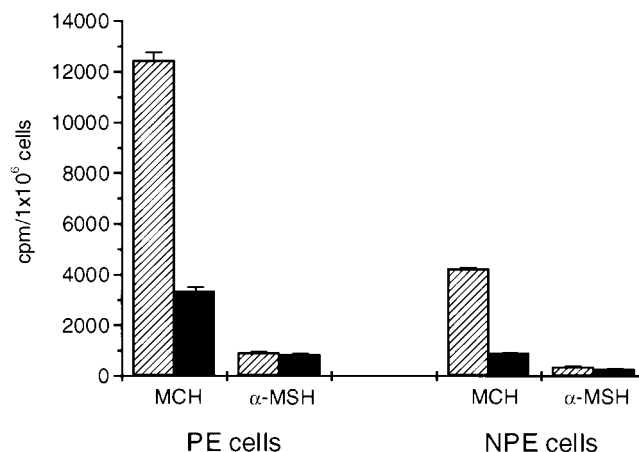


FIGURE 1. Total (*hatched*) and nonspecific (*black*) binding of [¹²⁵I]MCH and [¹²⁵I] α -MSH radioligand to PE cells and a NPE cell line. Radioligand binding to 1×10^6 cells per sample was investigated; nonradioactive competitors were added at a concentration of 2 μ M. PE cells were primary cells of porcine origin, and NPE cells were derived from an SV-40 immortalized human cell clone ODM-2. The values are the mean of *n* = 3 experiments, each of which consisted of 3 samples per group.

NPE cells had a slightly higher affinity with the MCH radioligand (Fig. 2B) compared with the MCH-R of PE cells (Fig. 2A). The *K*_D of the representative experiment shown in Figure 2 was 0.15 ± 0.04 nM for NPE cells and 0.26 ± 0.05 nM for PE cells (*n* = 3; for further details see legend to Fig. 2). In contrast, the receptor number on PE cells (6600 ± 650 binding sites per cell) was approximately 2.3-fold higher than that on NPE cells (2820 ± 340 binding sites per cell; *n* = 3).

Characterization of MCH-R by Photocrosslinking and RT-PCR

Binding of the photoreactive [¹²⁵I]Bpa-MCH radioligand to PE and NPE cells and subsequent UV-crosslinking revealed a specifically labeled membrane protein with an apparent molecular weight of 44 kDa on SDS-PAGE. The labeling was prevented by the addition of 2 μ M MCH to the binding reaction (Fig. 3A). The covalently linked receptor-ligand complex could be solubilized from membrane fractions only with strong detergents (data not shown). In addition, the presence of *slc-1* transcripts was shown in both cell types by RT-PCR (Fig. 3B). DNA sequencing of the transcript from NPE cells showed that it corresponded to the human *slc-1* sequence.

Na,K-ATPase Assays with NPE Cells

Na,K-ATPase-dependent ion flux was measured by Rb⁺ transport, and as positive control, the selective β -adrenergic receptor agonist isoproterenol was used, which is a known stimulator of the ouabain-sensitive Na,K-ATPase enzyme.⁵ As shown in Figure 4, ouabain-sensitive Na,K-ATPase activity in NPE cells revealed a significant concentration-dependent increase on isoproterenol stimulation. In contrast, MCH did not influence ouabain-sensitive Na,K-ATPase activity under these conditions.

DISCUSSION

Little is known about the expression of MCH receptors in peripheral tissues and their physiological role in water and ion transport. To date, the highest specific binding of MCH to peripheral cells was found on cells isolated from the skin.^{7,9,10} In this report, we present the first evidence of the expression of MCH-R in nonpigmented and pigmented cells of the ciliary

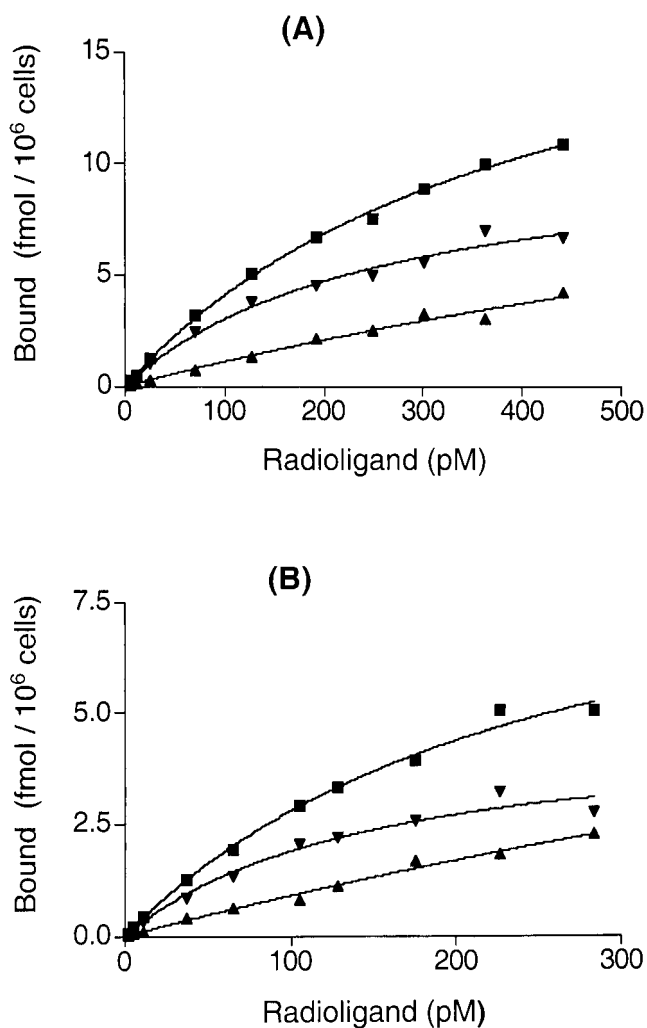


FIGURE 2. Saturation-binding studies with [125 I]MCH tracer. One of three saturation-binding experiments, performed with PE (A) and NPE (B) cells and natural MCH as unlabeled peptide, is shown. The mean of total (\blacksquare), nonspecific (\blacktriangle), and specific (\blacktriangledown) binding yielded a K_D of 0.26 ± 0.05 nM for PE cells with a B_{max} of 6600 ± 650 binding sites per cell ($n = 3$; 95% CI = 0.14–0.38 nM; 5040–8120 sites per cell) and, respectively, a K_D of 0.15 ± 0.04 nM for NPE cells with a B_{max} of 2820 ± 340 binding sites per cell ($n = 3$; 95% CI = 0.06–0.23 nM; 2030–3600 sites per cell). The mean K_D from three independent experiments ($n = 9$) was 0.42 ± 0.25 nM for PE cells and 0.28 ± 0.19 nM for NPE cells.

epithelium. The affinity of MCH with MCH-R expressed by PE or NPE cells was comparable to that found with cloned SLC-1 or MCH binding sites on mouse and human melanoma cells.⁹ The variant MCH_v peptide whose mRNA was exclusively detected in the primate brain¹ showed low affinity in the micromolar range when tested with human NPE cells. Saturation-binding experiments revealed that MCH-Rs of PE and NPE cells have a higher affinity with radiolabeled MCH than with the natural sequence, as previously found for both melanoma cells and keratinocytes.^{7,10} The K_D calculated for NPE cells (0.15 ± 0.04 nM) is similar to that found for the MCH-binding sites in human (0.09 ± 0.01 nM) or mouse melanoma cells⁹ (0.12 ± 0.01 nM). The K_D of PE cells was slightly higher (0.26 ± 0.05 nM)—i.e., similar to that published for keratinocyte MCH-R (0.7 nM).¹⁰

Photocrosslinking studies revealed a specifically labeled membrane protein with an apparent molecular weight of 44 kDa that corresponds to the size of a G-protein-coupled receptor. Comparable molecular weights have been determined

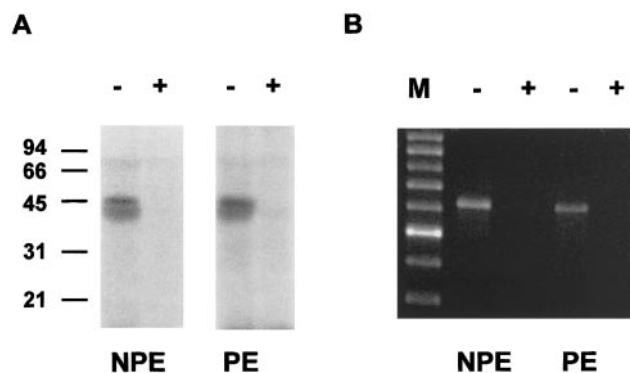


FIGURE 3. Expression of MCH-R. (A) Covalent photolabeling of MCH-R with photoreactive [125 I]Bpa-MCH. NPE and PE cells were incubated with radioligand in the presence (+) or absence (-) of 2μ M MCH as competitor and were subsequently UV-irradiated. Membrane fractions of the lysed cells were analyzed by SDS-PAGE on a 12% polyacrylamide gel. Radioactive bands were detected by autoradiography. Numbers on the left indicate molecular weight in kDa. (B) RT-PCR analysis of *slc-1* expression in PE and NPE cells. One microgram total RNA was used for cDNA synthesis (-) or treated with RNase I (+) before cDNA synthesis. PCR with *slc-1*-specific primers was performed with cDNA corresponding to 50 ng total RNA. The expected size of the PCR product is 610 bp. Marker (M) sizes are as follows: 1, 0.9, 0.8, 0.7, 0.6, 0.5, 0.4, and 0.3 kB.

for MCH-R expressed on mouse skin melanoma cells⁹ and on keratinocytes.¹⁰ Because we found *slc-1* transcripts in both PE and NPE cells, it can be concluded that the labeled protein in these cells is the melanin-concentrating hormone receptor SLC-1.²

The absence of expression of MSH-R in both pigmented and nonpigmented cells of the ciliary epithelium indicates that in these cells the hormonal pair α -MSH and MCH, which are functional antagonists in many other systems, play no role in the control of pigmentation and do not appear to exert receptor-mediated functional antagonism. It seems therefore that PE cells are more closely related to retinal pigment epithelial cells, which do not respond to α -MSH treatment,¹¹ than to skin melanocytes, in which melanogenesis is regulated by α -MSH.

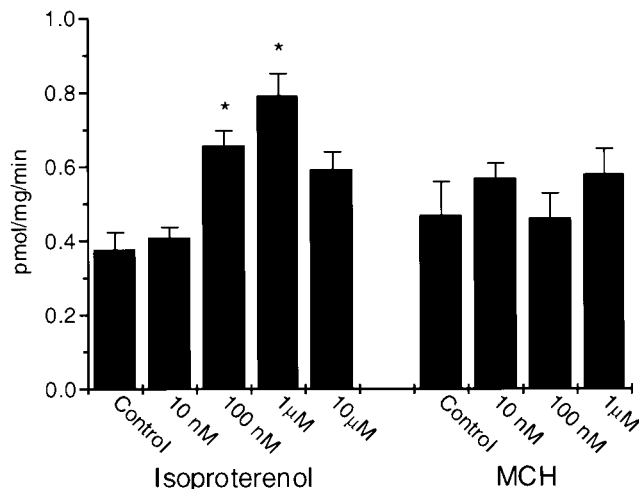


FIGURE 4. Effect of isoproterenol and MCH on Na,K-ATPase activity of NPE cells. Na,K-ATPase-dependent Rb^+ transport was calculated from total transport minus transport in the presence of 3 mM ouabain, a specific Na,K-ATPase inhibitor. The value is the mean \pm SEM from eight experiments. *Significantly different from control, $P < 0.05$; unpaired *t*-test.

In sheep, prolonged treatment with intracerebroventricularly injected MCH produced a significant increase in urine volume and urinary Na^+ and K^+ excretion without any influence on water intake.³ In addition, MCH mRNA abundance changed in response to salt loading or dehydration in rat.¹ These results suggest that MCH is involved in the central control of salt and water homeostasis. During the search for a suitable in vitro model to study peripheral MCH effects on water and salt balance, our interests focused on the control of aqueous humor formation in the ciliary body of the eye. The bulk of the ion transporting capacity is thought to be provided by the NPE cells, containing two to three times more Na,K-ATPase enzymatic activity than the PE cells. In addition, PE cells express only one Na,K-ATPase isoform.⁵ These findings and the fact that MCH-R expressed on NPE cells showed higher affinity with MCH than MCH-R expressed on PE cells led us to test the former cells for an MCH-induced change of Na,K-ATPase activity. Because neither of the three different hormone concentrations tested had any effect on ouabain-sensitive Rb^+ transport, it is likely that the Na,K-ATPase enzyme is not the target protein of the MCH response or that MCH does not directly influence the Na,K-ATPase but regulates another effector which in turn influences K^+ transport. Further studies are needed to test whether the MCH binding sites found in the ciliary epithelium mediate a physiological response.

Acknowledgments

The authors thank Roma Drozd, Institute of Physiology, Basel, for help with the Na,K-ATPase assay, and Kerstin Wunderlich, University Eye Clinic, Basel, for help with the PE cells.

References

1. Nahon JL. The melanin-concentrating hormone: from the peptide to the gene. *Crit Rev Neurobiol.* 1994;8:221-262.
2. Saito Y, Nothacker H, Wang Z, Lin SHS, Leslie F, Civelli O. Molecular characterization of the melanin-concentrating hormone receptor. *Nature.* 1999;400:265-269.
3. Parkes DG. Diuretic and natriuretic actions of melanin-concentrating hormone in conscious sheep. *J Neuroendocrinol.* 1996;8:57-63.
4. Hervieu G, Volant K, Grishina O, Descroix-Vagne M, Nahon JL. Similarities in cellular expression and functions of melanin-concentrating hormone and atrial natriuretic factor in the rat digestive tract. *Endocrinology.* 1995;137:1-9.
5. Helbig H, Korbmacher C, Wiederholt M. K^+ -conductance and electrogenic Na^+/K^+ transport of bovine pigmented ciliary epithelium. *J Membr Biol.* 1987;99:173-177.
6. Martin-Vasallo P, Ghosh S, Coca-Prados M. Expression of Na,K-ATPase alpha subunit isoforms in the human ciliary body and cultured ciliary epithelial cells. *J Cellular Physiol.* 1989;141:243-252.
7. Hintermann E, Drozd R, Tanner H, Eberle AN. Synthesis and characterization of new radioligands for the mammalian melanin-concentrating hormone (MCH) receptor. *J Recept Signal Transduct Res.* 1999;19:411-422.
8. Munson PJ, Rodbard D. An exact correction to the "Cheng-Prusoff" correction. *J Recept Res.* 1988;8:533-546.
9. Drozd R, Hintermann E, Eberle AN. Characterization of the receptor for melanin-concentrating hormone (MCH) on melanoma cells by photocrosslinking. *Ann NY Acad Sci.* 1998;839:210-212.
10. Burgaud JL, Poosti R, Fehrentz JA, Martinez J, Nahon JL. Melanin-concentrating hormone binding sites in human SVK14 keratinocytes. *Biochem Biophys Res Commun.* 1997;241:622-629.
11. Goodall T, Buffey JA, Rennie IG, et al. Effect of melanocyte stimulating hormone on human cultured choroidal melanocytes, uveal melanoma cells, and retinal epithelial cells. *Invest Ophthalmol Vis Sci.* 1994;35:826-837.