

Human immune cells express ppMCH mRNA and functional MCHR1 receptor

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Abstract Melanin-concentrating hormone (MCH) is highly expressed in the brain and modulates feeding behavior. It is also expressed in some peripheral tissues where its role remains unknown. We have investigated MCH function in human and mouse immune cells. RT-PCR analysis revealed a low expression of prepro-MCH and MCH receptor 1 (MCHR1) but not of MCHR2 transcript in tissular and peripheral blood immune cells. FACS and *in vitro* assay studies demonstrated that MCHR1 receptor expression on most cell types can trigger, in the presence of MCH, cAMP synthesis and calcium mobilization in peripheral blood mononuclear cells (PBMCs). Moreover, MCH treatment decreases the CD3-stimulated PBMC proliferation *in vitro*. Accordingly, our data indicate for the first time that MCH and MCHR1 may exert immunomodulatory functions. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Melanin concentrating hormone; Melanin concentrating hormone receptor; Immune cells; Proliferation; cAMP

1. Introduction

Melanin-concentrating hormone (MCH) is a cyclic peptide originally isolated from the salmon pituitary gland [1] that plays important roles in skin pigmentation or in some aspects of the stress response in fish. Later on, many studies have demonstrated that this peptide is ubiquitous throughout the vertebrate phylogeny [2]. In mammals, the highest MCH transcript level is observed in the brain, MCH expressing neurons being largely restricted to the lateral hypothalamus and the zona incerta whose fibers innervate a wide variety of brain and medullar nuclei [3]. This neuronal system is then well suited to coordinate goal-directed behaviors such as food intake, awakeness and sexual behavior. Many studies have now confirmed its importance in the field of nutrition [4–7]. Nevertheless, only few experiments have been focused on MCH production or on its effects in peripheral tissues; thus, at present, its role in these tissues remains largely unknown.

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Abbreviations: MCH, melanin-concentrating hormone; ppMCH, prepro-MCH; MCHR, MCH receptor; m, mouse; h, human; PBMC, peripheral blood mononuclear cell

Only recently MCH was shown to stimulate insulin release in CRI-G1 and RINm5F insulinoma cells lines, a model of Langerhans islets cells [8], and to regulate leptin synthesis and secretion in rat adipocytes [6]. Using RT-PCR, Hervieu and Nahon detected prepro-MCH (ppMCH) messenger in the stomach, testes, intestine and spleen of both the rat and the mouse [9,10]. By *in situ* hybridization on rat spleen sections, MCH mRNAs were revealed in scattered cells of unknown phenotype [9]. In mouse spleen extracts however, significant amounts of MCH immunoreactive material corresponding to an intermediate cleavage form were detected but no immunocytochemical experiments have been performed to localize it at the cell level [11]. On another hand, it had been demonstrated by RT-PCR that the mouse and human MCH receptor 1 (m- and hMCHR1) are expressed, also at low levels, in the spleen and thymus [12]. Moreover, by analysis of the 5'-upstream region of the mMCHR1 gene we have revealed the presence of many putative binding sites for MZF1 and Ikaros transcription factors [13]. Finally, a gamma-interferon responsive element has been found in the MCH gene [14]. These data led us to examine if MCH may play a functional part in the immune system.

2. Materials and methods

2.1. Cell lines and culture conditions

Chinese hamster ovary (CHO) cell line expressing the hMCHR1 receptor was kindly provided by E. Burgeon (Euroscreen, Brussels, Belgium). Human peripheral blood mononuclear cell (PBMC) were obtained from buffy coats of healthy donors (Transfusion Center, Liège, Belgium). C6 and SKN-BE cell lines were generous gifts from B. Rogister (CNCM, University of Liège, Liège, Belgium).

Cells were kept in RPMI 1640 or DMEM (BioWhitaker, Verviers, Belgium) supplemented with 10% fetal calf serum (BioWhitaker), 100 IU/ml penicillin/streptomycin (BioWhitaker). This study protocol was approved by the Ethics Committee of the University Hospital of Liège

2.2. Lymphocytes isolation

Tonsils from 1–8-year-old children were surgically removed and placed in a cold physiological solution containing 0.4% BSA (Sigma, St. Louis, MO, USA). Lymphocyte populations were prepared by gently teasing the tonsils. For human PBMCs isolation, human blood was diluted 1:1 in RPMI 1640 (BioWhitaker), and centrifuged on a Ficoll (AP Biotech, Uppsala, Sweden) gradient for 25 min at 830 × g. Interface cells were harvested and washed three times with PBS [15].

2.3. RNA isolation and RT-PCR

Total RNAs were extracted from human PBMC, human tonsils, spleens and thymuses of C57BL6 mice using Instapure (Eurogentec, Seraing, Belgium) according to the manufacturer's protocol. 5 µg of total RNAs was reverse transcribed by MMLV (Invitrogen Life Tech-

nologies, Carlsbad, CA, USA) with a random hexamer (AP Biotech) for GAPDH and MCHR analysis or with a specific oligonucleotide hybridizing in the 3'-untranslated region for ppMCH analysis.

2 μ l of cDNA was amplified in a 100- μ l volume of final reaction mix (2.5 U of Taq DNA polymerase (AP Biotech); 1.5 mM MgCl₂; 200 μ M dNTPs (AP Biotech)) with 50 pmol of specific primers: hppMCH forward 5'-GTTTCAAAGAACACAGGCTCC-3' (exons 1 and 2), reverse 5'-TATCAGACTTGCCAACAAGGTC-3' (exon 3); hMCHR1 forward 5'-CCCGATAACCTCACTTCGG-3' (exon 1), reverse 5'-GACTATTGGCATCCATGGC-3' (exon 2); hMCHR2 forward 5'-AACTGCCAGTGTGGTGGATACAG-3' (exon 2), reverse 5'-CTGTCTCCAACGTGTCAAGTC-3' (exon 4); mppMCH forward 5'-GGTATCAGACTTGCCAACATGG-3' (exons 1 and 2), reverse 5'-AAGAATTCAAAGAACACAGGCTCC-3' (exon 3); mMCHR1 forward 5'-CCAGGATAATTTACATTGGCG-3' (exon 1), reverse 5'-CCCAAAGTCCAGACACCAT-3' (exon 2); GAPDH forward 5'-ACCACAGTCCATGCCATCAC-3'; reverse 5'-TCCACACCCTGTTGCTGTA-3'. PCR conditions were as follows: 1 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C, and a final extension of 10 min at 72°C. 10- μ l aliquots of amplified products were analyzed on agarose gels.

2.4. Phenotypic analysis

MCHR1 expression was carried out with a rabbit polyclonal antibody, raised against C-terminal part (kindly provided by G. Hervieu), and revealed by a swine anti-rabbit FITC antibody (DAKO, Denmark, Glostrup). The double-staining phenotype was performed on 2×10^6 cells incubated with an anti-CD3 (PerCP), anti-CD14 (PE) or anti-CD-19 (PerCP) antibody (B-D Bioscience, Erembodegem, Belgium) during 30 min at 4°C, washed with PBS, and treated with a FACS[®] permeabilizing solution (B-D Bioscience). After incubation with the MCHR1 primary antibody for 1 h at room temperature, the cells were washed in order to remove the excess of primary antibody. The secondary antibody was then added to the cells for 30 min at room temperature and the cells were washed and harvested with a FACSCalibur (B-D Bioscience). The data were analyzed with the CellQuest software.

2.5. Ca²⁺ measurement

Ten million cells were incubated in 1 ml of HBSS without Ca²⁺ containing 6 μ l of a 10% pluronic acid solution and 2 μ l of Fluo3/AM 2 mM (Molecular Probes, Leiden, The Netherlands) for 20 min at 20°C. Four volumes of HBSS without Ca²⁺ were then added and the incubation was continued for 40 min at 37°C. Cells were washed three times by HBSS without Ca²⁺, resuspended in HBSS, with or without Ca²⁺ and directly harvested with a FACSCalibur as described above.

2.6. Cyclic AMP measurement

Two million PBMCs per well stimulated by purified anti-CD3 antibody (10 ng/ml) (B-D Biosciences), were cultured with or without 100 nM MCH (Bachem, Bubendorf, Switzerland). Following 24, 48 or 72 h of culture, the cells were pelleted and washed twice with PBS. The cAMP was then extracted by sonication in ethanol 95%/HCl 0.01 N. The cellular suspensions were dried in a speed-Vac apparatus and stored at -80°C. The cAMP level was measured using the Enzy-melImmunoAssay kit (AP Biotech) according to the manufacturer's protocol.

2.7. T-cell proliferation

PBMCs stimulated by purified anti-CD3 antibody (1 or 10 ng/ml) were cultured at a density of 10⁵ cells/well, with or without different concentrations of MCH (1, 10, 100 and 1000 nM). During the last 4 h of culture, the cells were incubated with 1 μ Ci of [³H]thymidine (6.7 Ci/mmol, AP Biotech). Following the culture (24, 48 or 72 h), cells were harvested on fiberglass filters and isotope incorporation was measured by liquid scintillation counting.

2.8. Statistical methods

For comparison of two groups, the non-parametric Mann-Whitney test was used using Instat Mac software packages (Graph Pad Software, San Diego, CA, USA).

Results of proliferation were expressed as mean \pm standard deviation. The relationship between [³H]thymidine incorporation levels and MCH dose, culture time and anti-CD3 concentrations was analyzed by means of the general linear mixed model (GLMM). Linear and

quadratic effects for MCH dose were tested. Results were considered to be significant at the 5% critical level ($P < 0.05$). Calculations were carried out using SAS (version 6.12 for Windows).

3. Results

3.1. Expression of ppMCH and MCHR mRNA in immune cells

Some previous data showed an expression of ppMCH and MCHR1 mRNA at least in the spleen [9] but no study had ever demonstrated the presence of these transcripts in isolated immune cells. Therefore, RT-PCR analysis was performed to evaluate their expression in mouse splenocytes and thymocytes. For ppMCH mRNA analysis, we have used primers allowing to selectively amplify 'authentic' ppMCH but not its variant or MGOP [17]. After electrophoresis, faint specific bands for ppMCH and MCHR1 mRNA were obtained in mouse splenocytes and thymocytes (Fig. 1A) and the expected sequences were confirmed by sequencing. The study was extended to human immune cells. The ppMCH and MCHR1 mRNA were detected in all tonsillar, PBMC and granulocytes preparations. On the contrary, MCHR2 receptor mRNA was never detected in the tonsillar and PBMC samples (Fig. 1B). This confirms its more restricted tissue distribution [12]. Our data indicate that immune cells express mRNA encoding ppMCH and the MCHR1 receptor.

3.2. Expression of functional MCHR1 receptor

To verify that the MCHR1 receptor is effectively translated we have used a rabbit antibody raised against the C-terminal part of the receptor, validated for immunocytochemistry studies [8,16]. It was first tested for FACS analysis. CHO cells stably expressing the human receptor and C6 glioma cells, not expressing it, were analyzed. According to the RT-PCR results (not shown), a strong fluorescent signal was observed in the CHO cells whereas no fluorescence was detected in the C6 glioma cells. Additional control carried out with non-permeabilized PBMC was also negative (Fig. 2A). This demonstrate that the antibody recognizes an intracellular epitope only present in MCHR1 expressing cells and therefore demonstrate a specific immunoreactivity to MCHR1. The phenotypic analysis revealed an average of 95% MCHR1-positive cells among the PBMC and 50% in the tonsillar cell population. Using double labellings with anti-CD3 PerCP or anti-CD19 PerCP antibodies, we measured that more than 98% of the T-lymphocytes and more than 97% of the B-lymphocytes expressed MCHR1. Within the monocyte population (CD14+), we also observed a high percentage (>97%) of positive MCHR1 cells (Fig. 2B).

To assess the functional activity of the MCHR1 receptor, we have analyzed the MCH effect on the cAMP concentration. An inhibitory effect on forskolin-stimulated cAMP accumulation was obtained similar to the results of previous studies on CHO transfected cells [18]. We obtained equivalent results with neuroblastoma SKN-BE cells (data not shown). However, the addition of 100 nM and 1000 nM MCH during 1 day on activated PBMC produced a very significant increment in the total content of cAMP ($P < 0.01$) (Fig. 3A). After 48 or 72 h of culture, the values did not significantly differ from the control values (Fig. 3B).

To extend the MCHR1 functional activity, we have measured the calcium response of PBMC after addition of MCH.

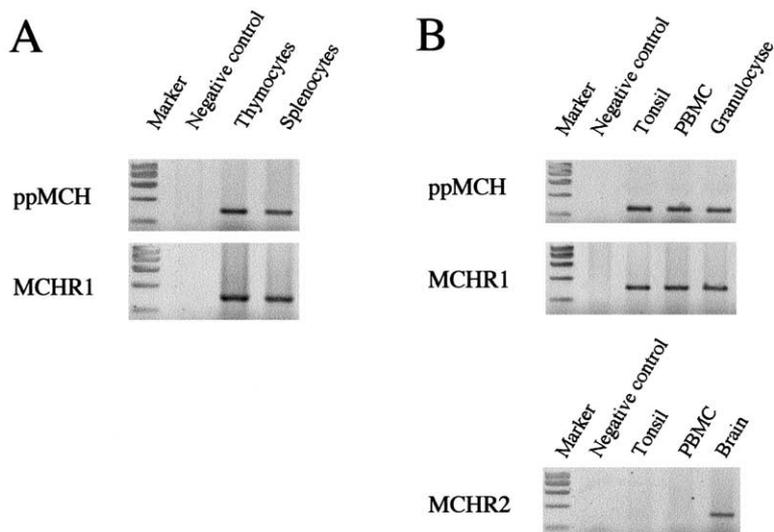


Fig. 1. RT-PCR analysis of ppMCH, MCHR1 and MCHR2 mRNA expression in mouse (A) and human (B) immune cells. In negative control, the MMLV reverse transcriptase was omitted. For hMCHR2, a brain mRNA sample was used as positive control

Flow cytometric method by using the calcium probe fluo-3 was applied in the analysis of intracellular calcium mobilization upon *in vitro* stimulation with 1 μ M MCH (Fig. 3C). A fast mobilization was observed following MCH addition meaning a fast release of calcium from the endoplasmic reticulum into the cytoplasm. Using calcium containing medium, we found that intracellular calcium mobilization reached higher levels and stayed longer as expected for Ca^{2+} influx phenomenon meaning an extracellular calcium entry in addition to

the calcium release from the endoplasmic reticulum into the cytoplasm (Fig. 3C).

3.3. Inhibition of *in vitro* lymphocyte proliferation by MCH

To evaluate a possible modulation effect of MCH on immune cell functions, we tested its action on proliferation using the [3 H]thymidine incorporation method. Cultured human PBMC were stimulated with two concentrations of a purified anti-CD3 antibody (1 and 10 ng/ml) and treated with different

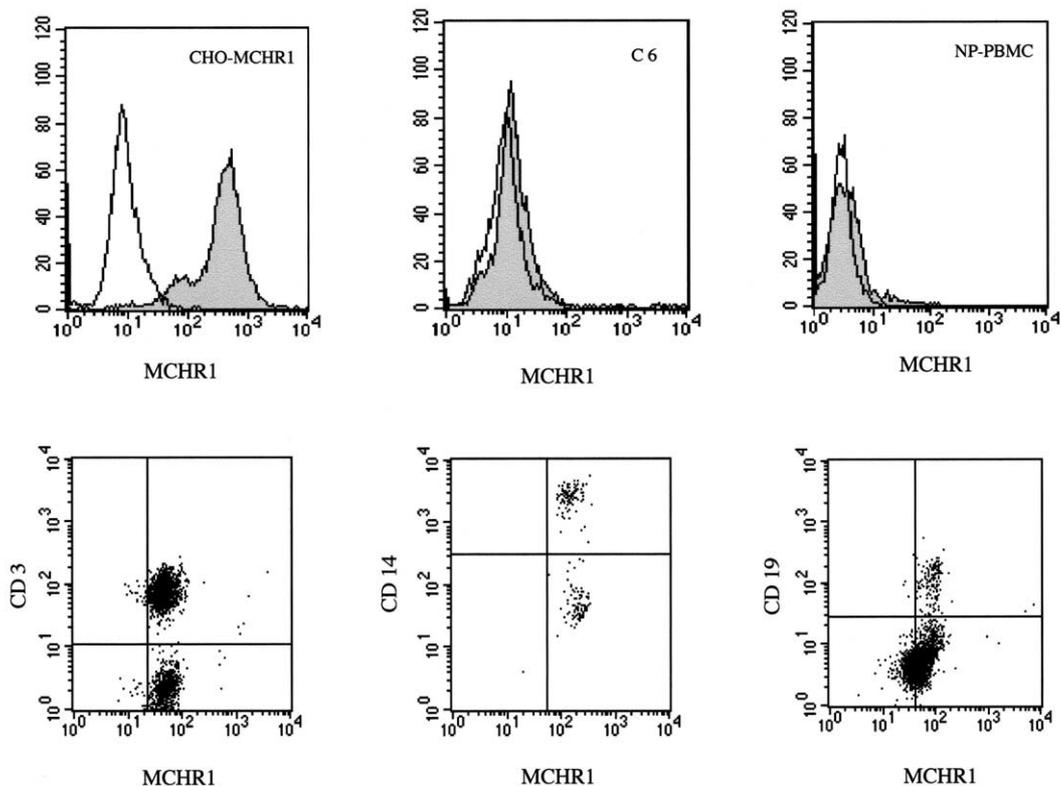


Fig. 2. Flow cytometry analysis of MCHR1 receptor. A: Validation of the anti-MCHR1 antibody for FACS. CHO cells stably expressing MCHR1, C6 glioma cells and non-permeabilized (NP) PBMC stained with the rabbit anti-MCHR1 antibody (shaded area) as compared to control incubated only with the secondary antibody (light area). B: Representative dot plot analysis of human PBMC double-stained with the anti-MCHR1 antibody and with anti-CD3 (PerCP), or anti-CD14 (PE) or anti-CD19 (PerCP) antibodies.

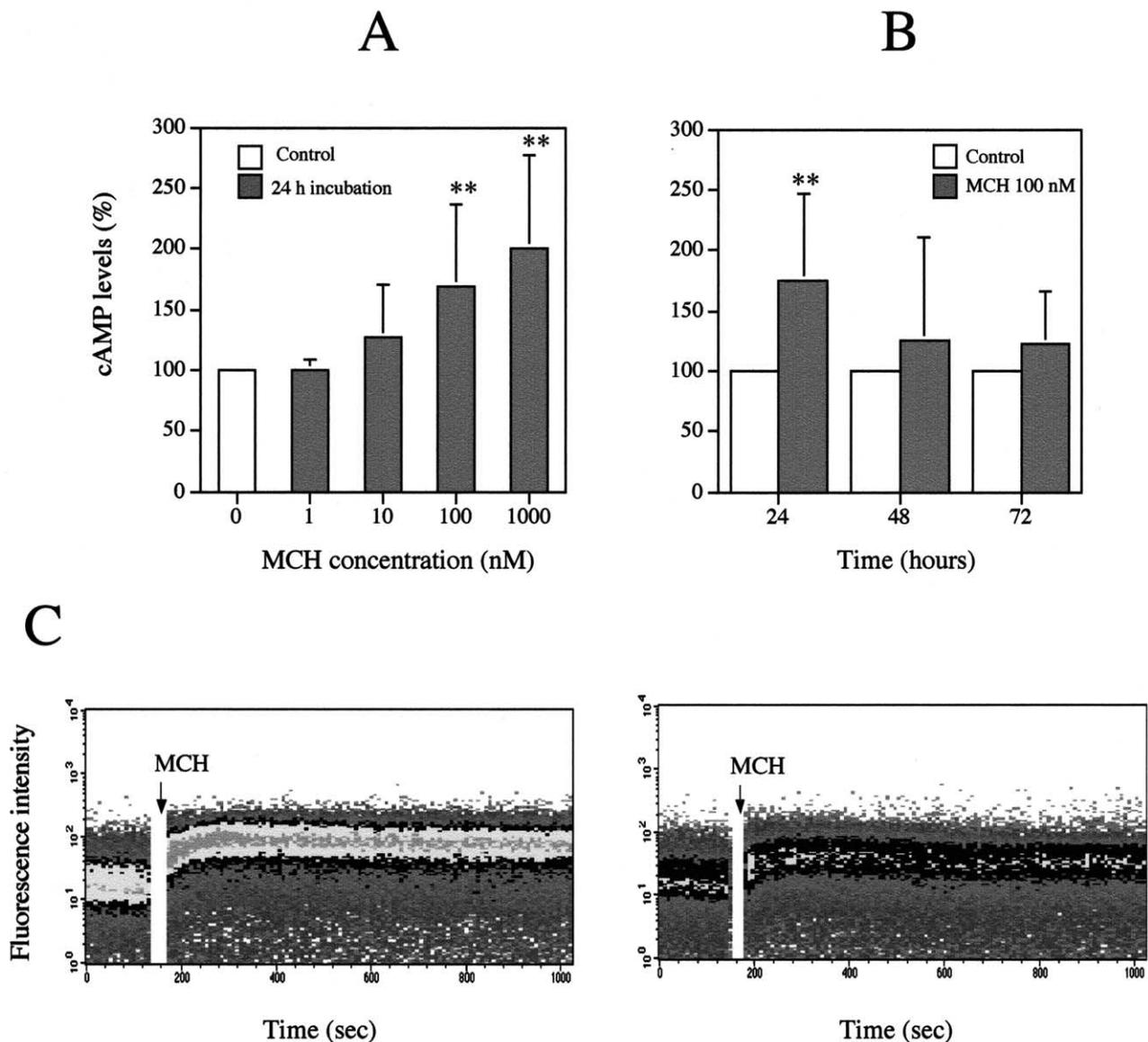


Fig. 3. Effect of MCH on cAMP concentrations (A, B) and calcium mobilization (C). A: Dose response of human PBMC incubated for 24 h with 10 ng/ml anti-CD3 and with different concentrations of MCH. B: Influence of incubation time on cAMP level of human PBMC cultured in the presence of anti-CD3 10 ng/ml and with (shaded bars) or without (light bars) addition of 100 nM MCH. Data are expressed as a percentage of the cAMP produced and values are the mean \pm S.E.M. from eight or ten different samples performed in triplicate. C: Intracellular calcium mobilization was induced in human PBMC following addition of 1 μ M MCH in a physiological solution containing (left panel) or not calcium (right panel).

concentrations of MCH (0, 1, 10, 100 and 1000 nM) for 48 and 72 h (Fig. 4). By applying a GLMM to the [³H]thymidine incorporation data, we found a significant linear ($P < 0.0001$) and quadratic ($P < 0.0001$) effect of MCH dose as well as a significant effect of culture time ($P < 0.0001$) (Table 1). The

[³H]thymidine incorporation levels were significantly higher following 72 h than following 48 h of culture. The anti-CD3 concentration did not affect [³H]thymidine incorporation levels ($P = 0.11$). As a result these data indicate that MCH reduces the human PBMC proliferative activity.

Table 1
Results of the GLMM application to [³H]thymidine levels

Effect	Regression coefficient	Standard error	P value
Intercept	20 234	7426.8	–
Dose	–84.46	19.78	< 0.0001
Dose ²	0.0820	0.0194	< 0.0001
Time	505.7	85.50	< 0.0001
Anti-CD3	–271.6	169.9	0.11

$n = 171$ observations.

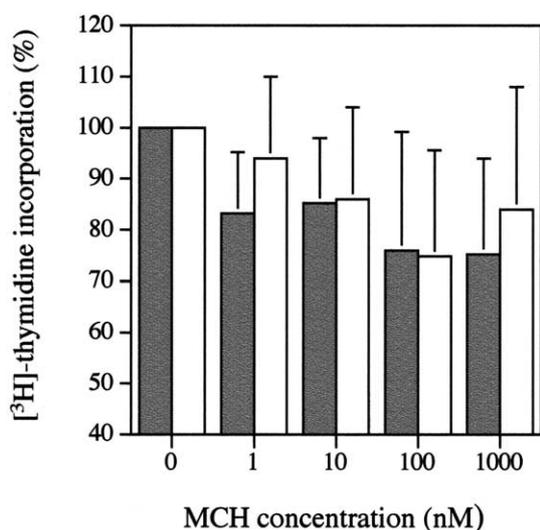


Fig. 4. MCH action on human anti-CD3-stimulated PBMC proliferation following 48 h (shaded bars) and 72 h (light bars) of culture. Data are expressed as percentages of [^3H]thymidine incorporation measured in the presence of different MCH concentrations. For each MCH concentration, values represent means \pm S.E.M. from ten determinations obtained in 13 (48 h) and nine (72 h) different samples.

4. Discussion

The involvement of MCH and its receptor(s) in the immune system still remain unexplored. To our knowledge, we present here the first study reporting data demonstrating that MCH and its receptor MCHR1 can act as modulators of the immune system. Previously, the ppMCH and MCHR1 transcripts were observed in tissues like spleen or thymus but not in specific immune cells [9,10]. On base of RT-PCR analysis we showed the presence of the ppMCH and MCHR1 mRNA not only in mouse splenocytes and thymocytes but also in human tonsillar and blood cells. The absence of MCHR2 expression in human tonsils and PBMC is in agreement with its restricted tissue distribution described in previous studies [13,19,20].

Our work shows also that immune cells express functional MCHR1 receptors at their surface. By flow cytometry, we observed that a high percentage of the T- and B-lymphocytes from PBMC or tonsil express MCHR1. To verify if the receptor is functionally coupled to G proteins, MCH effect on the cAMP levels and Ca^{2+} signalling pathways was analyzed. The calcium mobilization was increased in PBMC following addition of MCH. Interestingly, the MCH peptide stimulated cAMP production in human PBMC whereas it decreased cAMP levels in CHO-hMCHR1, SK-N-BE or melanoma SK-MEL-37 cell lines [21]. Indeed, the concentration of G proteins, receptor density, compartmentalization of signal transduction machinery and accessibility of G proteins may change according to the cell type, leading to different interactions [22]. For example, MCH increases the forskolin-stimulated insulin release when applied on CRI-GI and RINm5F cell lines naturally producing MCHR1 mRNA, suggesting that the peptide increases cAMP levels [8]. In accordance to our results, MCHR1 appears functional in PBMC and probably coupled to a Gs protein. However, the observed effect could also be due to the presence of another, yet still uncharacterized, MCHR coupled mainly to Gs.

In vitro proliferation assay highlights a possible modulatory function on lymphocyte by MCH. Indeed, the MCH peptide significantly decreased CD3-stimulated PBMC proliferation. This reduction of T-cell proliferation might be correlated with an increase of cAMP production [23]; indeed, the observed inhibition of PBMC proliferation, following 48 and 72 h of culture is probably correlated with the cAMP level increase observed following 24 h of culture. It would be of interest to determine the T-cell subtypes sensitive to MCH and the cytokines induced by this peptide. The phenotype of immune cells able to secrete MCH containing peptide (i.e. preMCH) or mature MCH peptide must be further analyzed as well as the factors controlling this production.

In conclusion, in the current stage of our knowledge, this study is the first to demonstrate the production of ppMCH and functional expression of MCHR1 receptor by mouse and human immune cells. An immunomodulatory role for MCH and MCHR1 receptor can thus be proposed for both the B- and T-cell populations.

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