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α-Melanocyte Stimulating Hormone Acts as a Selective Inducer of Secretory Functions in Human Mast Cells

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In the present study, we have investigated the pro-opiomelanocortin (POMC)-derived neuropeptide α-MSH for its ability to modulate activation of human mast cells. The *in vitro* ability of purified human skin mast cells to secrete various types of mast cell mediators was monitored in response to α-MSH at the mRNA and at the protein level. Picomolar concentrations of α-MSH induced a dose-dependent release of histamine from isolated human skin mast cells and from skin punch biopsies. However, no effect of α-MSH was seen regarding the expression of IL-1, IL-6, IL-8, TGF-β, and TNF-α. Melanocortin receptor MC-1 was identified at the transcriptional level by RT-PCR analysis but not at the protein level, whereas, in leukemic human mast cells (HMC-1), the mRNAs and the proteins for the MC-1 and MC-5 receptor were identified. These results suggest that α-MSH may selectively induce acute inflammary effects via secretion of histamine. © 2000 Academic Press

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Human skin is a rich source of the proopiomelanocortin (POMC) peptide α-MSH since this molecule is synthesised by keratinocytes and also by dermal fibroblasts (1, 2). α-MSH is involved in the regulation of epidermal melanin synthesis by melanocytes, and of a variety of inflammatory and immune reactions (2). Its anti-inflammatory effects are exerted either via a direct action on inflammatory cells (lymphocytes, neutrophils, macrophages) or—as has been shown in the brain—via those cells targeted by the inflammatory cells.

α-MSH actions are mediated by five different receptor subtypes named melanocortin receptor (MC) 1–5. These are all G-protein coupled and—as second messengers—activate either the adenylate cyclase-system, generating cAMP, or the JAK/STAT pathway (3, 4). The distribution of the receptor subtypes and their ratio differs from tissue to tissue. MC-1 receptors are expressed in melanocytes, endothelial cells, and fibroblasts, but not in neuronal tissue; MC-2 receptors are present in adrenocortical cells and were originally characterised as ACTH-receptors; MC-3 and MC-4 receptors are primarily found in certain brain regions, and MC-5 receptors have been described in skin and skeletal muscle (3).

Considering the high level of involvement of mast cells in inflammatory reactions, it is surprising that almost nothing is known about an interaction between POMC-peptides and human mast cells. The only data stem from studies of rat peritoneal and of cultured bone-marrow derived murine mast cells (5, 6).

In a first attempt to examine a putative stimulation of human mast cells by α-MSH, we were able to demonstrate the expression of MC-1 and MC-5 receptors on the human leukemic mast cell line 1 (HMC-1) (7). Since human mast cells might thus be potential target cells for α-MSH actions, the present investigation was aimed at clarifying whether α-MSH modulates or mediates the synthesis and release of cutaneous mast cell associated mediators and cytokines.

MATERIALS AND METHODS

HMC-1 cell culture, isolation, and stimulation of human skin mast cells. Human leukemic mast cells (HMC-1 cells) (kindly provided by Dr. Butterfield, Rochester, MN) which exhibit an immature phenotype (8, 9) were cultured in Iscove's medium (Biochrom, Berlin, Germany), supplemented with 10% FCS (Biochrom), 10 μM monothioglycerol (Sigma, Deisenhofen, Germany), and antibiotics (streptomycin and penicillin).

Human skin mast cells and punch biopsies (surface diameter of 4 mm) were prepared from juvenile foreskin obtained after circumcision. For isolation of mast cells, the epidermis was enzymatically detached by an overnight incubation at 4°C with dispase (Boehringer, Mannheim, FRG) at a concentration of 1 mg/ml. The remaining dermis was dispersed by an incubation with collagenase type IV (Worthington Biochem. Corp., Freehold, USA) for 1 h at 37°C. Thereafter, cells were washed and cultured overnight at 37°C and 5% CO_2/95% air in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Biochrom). Enrichment of mast cells was achieved

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by positive magnetic selection using the MACS microbeads technology (Miltenyi Biotec, Bergisch Gladbach, Germany). For that purpose, dermal cells were incubated for 30 min at 4°C with the monoclonal antibody YB5.B8 which recognizes the c-kit receptor (CD117) (the antibody was kindly provided by Dr. L. Ashman, Adelaide, Australia). After washing with phosphate buffered saline (PBS) containing 1% BSA and 5 mM titriplex III (Merck, Darmstadt, Germany), a second incubation was done at 4°C for 30 min with a MACS-conjugated goat anti-mouse polyclonal antiserum (Miltenyi Biotec). Separation of labeled and unlabeled cells was achieved by passing cells over a large cell separation column (Miltenyi) that was placed in the magnetic field of a MiniMACS separator (Miltenyi). Cells were washed three times and, after removing the column from the separator, retained cells were eluted with 500 μl PBS containing 1% BSA and 5 mM titriplex III. An average of $2.3 \times 10^5 \pm 1.2 \times 10^5$ ($n = 10$) mast cells were isolated from one gram of tissue. Purity of mast cells was 95% ± 4%, as measured by flow cytometry (staining for CD117 and for the α-chain of the high affinity IgE-receptor, mAb 29C6, kindly provided by Dr. J. Hakimi, Hoffmann La Roche, USA) and toluidine blue staining at pH 0.5. Viability, as determined by staining the cells with trypan blue.

Mast cell mediator release assays (histamine, TNF-α, and IL-8). Isolated mast cells or skin punch biopsies were suspended in Tyrode's buffer (10 mM Hepes, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA) containing various concentrations of α-MSH (10 nM-0.01 pM, Sigma) or anti-IgE (Calbiochem-Novabiochem, Bad Soden, Germany) at a density of 5×10^5 cells per ml, and 100 μl were seeded per well in 96-well plates (Greiner, Frickenhausen, Germany). Skin punches were incubated in 200 μl per well in 96-well plates. Plates were incubated for 30 min at 37°C and 5% CO_2/95% air. Thereafter, cells were centrifuged, and the cell-free supernatants were stored at −80°C. The total histamine content of mast cells was determined by lysis in 2% perchloric acid. Histamine content was determined with a histamine-specific EIA kit (Coulter-Immunotech, Krefeld, Germany) or with a fluorescence-based histamine autoanalyzer II (Technicon, Dublin, Ireland). The cytokines IL-8 and TNF-α were quantified in the supernatants of cells stimulated for 24 h at 37°C and 5% CO_2/95% air, using cytokine-specific ELISA kits (Pharmingen, Hamburg, Germany and R&D Systems, Wiesbaden, Germany, respectively).

Isolation of RNA and RT-PCR. For RT-PCR and multiple PCR, total RNA was isolated from mast cells with RNeasy System (Qiagen, Hilden, Germany) and treated with RNAse-free DNAse (Boehringer Mannheim, Mannheim, Germany) to remove genomic DNA. After isopropanol precipitation, whole RNA was reversely transcribed with random primed M-MLV reverse transcriptase (Gibco, Eggenstein, Germany). The reverse transcriptase products from these samples were amplified by 35 cycles of PCR in 50 μl total reaction volumes. PCR conditions were standardized for each experiment by use of a master mixture containing 2.5 U Taq DNA-Polymerase, 1.5 mM $MgCl_2$, 0.2 mM nucleotide mix (dATP, dCTP, dGTP, dTTP), and 2 μl of cDNA per 50 μl of total PCR volume. The sequences of the upstream and downstream primers for melanocortin-receptors (MC-1, MC-2, MC-3, MC-4, MC-5) are as follows: MC-1R upstream 5′-GCC ATT TTC TTC CTC TGC TG-3′; downstream 5′-ATG TCA GCA CCT CCT TGA GC-3′; MC-2R upstream 5′-TGC TGG CTG TGT TCA AGAA-3′; downstream 5′-ATG GTC ACG ATG CTG TGG TA-3′; MC-3R upstream 5′-CCT CCA TCT GCA ACC TCCT-3′; downstream 5′-GTG CTG CTA TGC GCT TGA-3′; MC-4R upstream 5′-CAG CCT GCT TTC AAT TGCA-3′; downstream 5′-TCC TCT TAA TGT GAA GCC TG-3′; MC-5R upstream 5′-CAT TGC TGT GGA GGT GTT TCT-3′; downstream 5′-GTC ATG ATG TGG TGG TAG CG-3′. Detection of the expression of human inflammation related genes GM-CSF, TNF-α, IL-1β, IL-6, IL-8, and TGF-β and transcription factors NFκB, IκB, NF-AT, NF-ATc, and OCT-2 in human mast cells after treatment with α-MSH or phorbolesther (PMA) were performed with multiple PCR-kits (Maxim Biotech, San Francisco, CA). Differences in gene expression were determined by comparing the density of bands obtained from different samples against normalized GAPDH expression. Each sample was electrophoresed in 2% agarose gels. Quantification of ethidium bromide-stained gels was performed with GelWorks 1D software (Ultra-Violet Products Ltd, Cambridge, UK).

Flow cytometry. HMC-1 cells and primary skin mast cells were analyzed with receptor-specific polyclonal antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) for the expression of MC-1 and MC-5 receptors. Briefly, cells were prefixed with 4% freshly prepared paraformaldehyde and were then incubated with saturating concentrations of the polyclonal antiserum or a control serum (Dianova, Hamburg, Germany) for 30 min at 4°C. After washing, cells were labeled with an FITC-conjugated $F(ab')_2$ fragment of donkey anti-goat IgG (Dianova) for 30 min at 4°C. Specificity of staining was additionally checked by preincubating the antiserum with specific blocking peptides (Santa Cruz Biotechnology) for at least 2 h at 4°C. In addition, binding experiments were performed with the fluorescein-labeled α-MSH analogue [Nle^4, D-Phe^7]-α-MSH (Molecular Probes, Leiden, The Netherlands) in the presence and absence of a 100-fold molar excess of unlabeled α-MSH. At least 5000 cells were analyzed by an Epics XL flow cytometer (Coulter, Krefeld, Germany).

Measurement of cAMP. For cAMP measurements, 1×10^5 mast cells were stimulated in 200 μl Tyrode's buffer containing increasing concentrations of α-MSH (10 nM-1 pM). Cells were incubated for 30 min at 37°C. Fifty μM forskolin (Calbiochem-Novabiochem) were used as a positive control. After incubation, total cellular cAMP was measured by a specific EIA system, according to the manufacturer's instructions (Amersham Pharmacia Biotech, Freiburg, Germany).

RESULTS

α-MSH-induced histamine release in human skin mast cells. In order to demonstrate α-MSH-dependent effects on the secretory behavior of mast cells, human cutaneous mast cells were immunomagnetically enriched up to 95% from normal human foreskin by positive selection using an anti-CD117 monoclonal antibody and a MiniMACS separation device. Cells isolated in this fashion showed a viability of >95% and were able to release histamine in response to anti-IgE or substance P (Fig. 1). After treatment of mast cells with α-MSH, a dose-dependent release of histamine was detectable (Fig. 1). The dose-response curve was bell-shaped over a concentration range from 0.01 to 10 pM, with a maximal response at 1 pM. In comparison to the anti-IgE-induced histamine release, the response evoked by α-MSH was 30–60% lower. The kinetics of histamine release induced by α-MSH and anti-IgE showed also conspicuous differences (Fig. 2) since, whereas the anti-IgE-induced effect was fast and most of the histamine was released in less than 7.5 min, α-MSH induced a significantly slower release, with detectable amounts only at 15 min after stimulation (Fig. 2).

In addition to the experiments done with isolated skin mast cells, we also stimulated skin punch biopsies in the same manner as described above. Under these conditions, α-MSH also induced a dose-dependent histamine release which was maximal at 100 pM (Fig. 3).

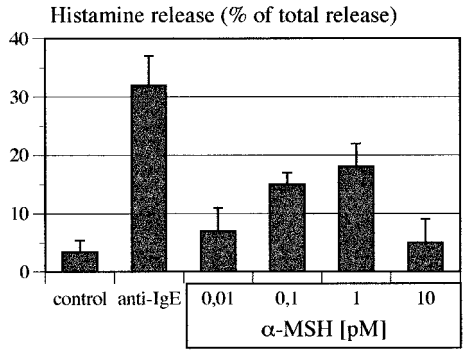

FIG. 1. Anti-IgE and α-MSH-induced histamine release in isolated human skin mast cells. Cells were suspended in Tyrode's buffer containing various concentrations of α-MSH or anti-IgE at a density of 5×10^5 cells per ml, and 100 μl were seeded per well in 96-well plates. Plates were incubated for 30 min at 37°C and 5% CO_2/95% air. Thereafter, cells were centrifuged, and the cell-free supernatants were stored at −80°C. The total histamine content of mast cells was determined by lysis in 2% perchloric acid. Histamine content was determined with a histamine-specific EIA kit or with a fluorescence-based histamine auto analyzer II. Histamine release was expressed as percent of total release. Shown are means ± SD of three separate experiments.

α-MSH-induced cytokine-release in human skin mast cells. In order to gain insights into the types of cytokines potentially released on exposure of mast cells to α-MSH, we examined the expression of various proinflammatory cytokines at the mRNA-level by a specially designed multiplex-PCR system. In this way, we were able to show that mast cells constitutively express mRNA for IL-6, IL-8, and TGF-β. Cells treated for 4 h

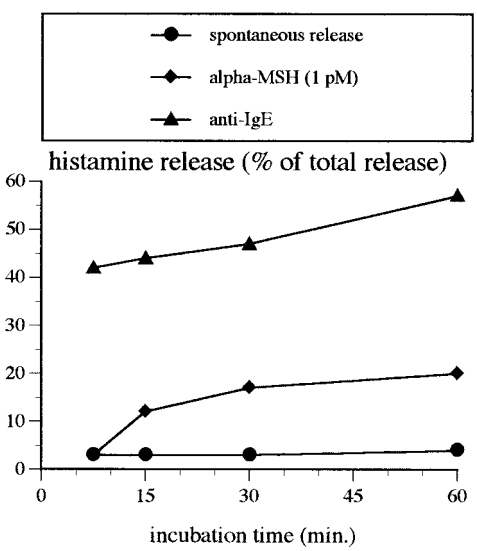

FIG. 2. Time-dependent release of histamine in response to anti-IgE or α-MSH (1 pM). Histamine content was determined with a histamine-specific EIA kit or with a fluorescence-based histamine autoanalyzer II. Histamine release was expressed as percent of total release. Shown are data from one representative experiment.

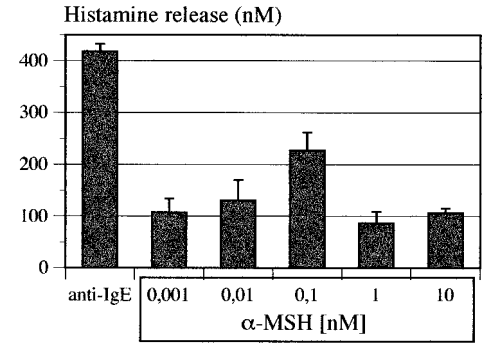

FIG. 3. Anti-IgE and α-MSH-induced histamine release in skin punch biopsies (surface diameter of 4 mm) prepared from juvenile foreskin. The tissue were suspended in 200 μl Tyrode's buffer per well in 96-well plates containing various concentrations of α-MSH or anti-IgE. Plates were incubated for 30 min at 37°C and 5% CO_2/95% air. Thereafter, the cell-free supernatants were stored at −80°C. Histamine released was expressed as absolute amounts (nM). Shown are means ± SD of three separate experiments.

with 1 pM α-MSH exhibited the same expression pattern, whereas stimulation of mast cells with PMA kept under the same conditions resulted in a *de novo* expression of the mRNA's for TNF-α and IL-1 (Fig. 4). Since IL-8 and TNF-α are performed mediators that are stored in unstimulated mast cells (10, 11) and released on stimulation with PMA and via FcεRI-mediated mechanisms, we examined their release after stimulation of human skin mast cells with α-MSH using cytokine-specific ELISA-systems. Under these conditions, we were however unable to detect any release of these molecules, although cells were incubated for 24 h with a broad range of α-MSH concentrations (0.1 to 10 nM) (data not shown).

Expression of MC-receptors at the mRNA-level. The bell-shaped dose-response curve and the high sensitivity of the α-MSH-induced histamine release suggest a receptor-mediated effect. We therefore looked for the expression of specific MC-receptors at the mRNA level by RT-PCR analyses. For this purpose, we examined total mRNA of skin mast cells and of HMC-1 cells for comparison. In HMC-1 cells, transcripts for the MC-1 and MC-5 (not shown) receptor were detectable, while in purified skin mast cells, only the message for the MC-1 receptor was identified (Fig. 5). Transcripts for the other melanocortin receptors (MC-2, 3, and 4) were not detectable in mast cells by RT-PCR analysis (not shown).

Expression of MC-receptors at the protein-level. For studies on MC-receptor protein expression, HMC-1 and skin mast cells were stained with MC-1- and MC-5-specific polyclonal antisera and analyzed by flow cytometry. Sixty to eighty percent of HMC-1 cells stained positively for the MC-5 receptor and less than 10% of cells expressed the MC-1 receptor (Fig. 6). In skin mast cells, a specific signal was only detectable for the MC-5

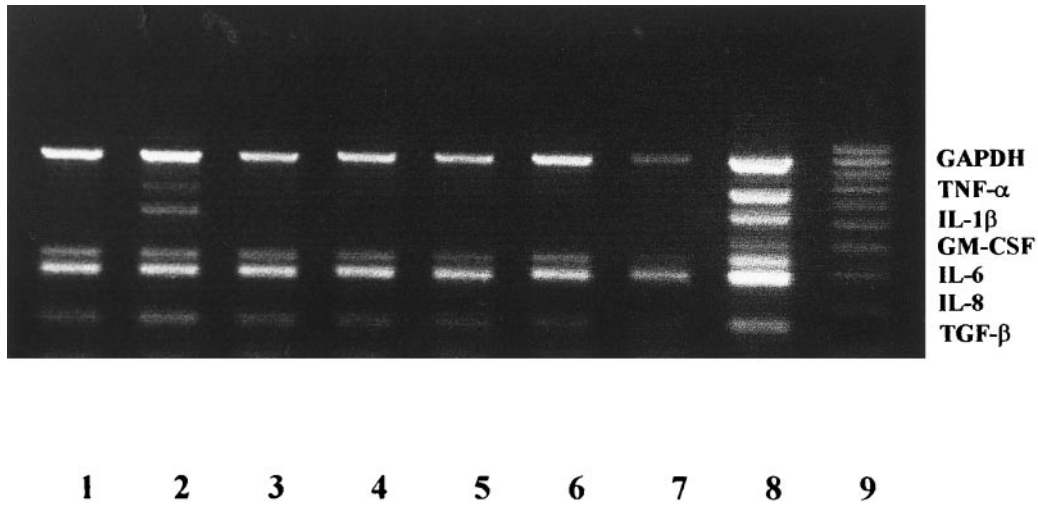

FIG. 4. Multiplex RT-PCR of GAPDH, TNFα, IL-1β, GMCSF, IL-6, IL-8, and TGFβ in α-MSH- and PMA-stimulated skin mast cells. Lane 1, unstimulated skin mast cells; lane 2, PMA stimulated; lanes 3–7, α-MSH stimulated (10^{-6} to 10^{-10} M); lane 8, pos. control; lane 9, DNA ladder (100 bp).

receptor (Fig. 6). The specificity of staining was checked by preadsorption of the antisera with antisera-specific blocking peptides.

α-MSH-induced activation of cAMP and transcription factors. In order to examine whether the observed α-MSH effects are mediated by the G-protein coupled MC-1 receptor, we measured the generation of cAMP in HMC-1 cells and skin derived mast cells. For this purpose, cells were treated with α-MSH (1 pM-10 nM) for 30 min. Total cAMP was quantified by a specific EIA. Although forskolin, an activator of adenylate cyclase, induced a significant increase in cAMP, α-MSH showed no effect (data not shown).

Additionally, we examined whether mast cells treated for 4 h with 1 pM α-MSH showed a different expression pattern of transcription factors in comparison to untreated cells. This was done by a multiplex PCR-system. We were however unable to detect any difference in the expression of NFκB, NFAT, NFATc, OCT-2, or IκB, as compared to untreated cells (Fig. 7).

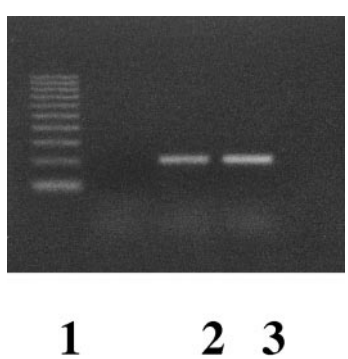

FIG. 5. Expression of MC-1 receptor at the mRNA-level in isolated skin mast cells and HMC-1 cells. RT-PCR of MC-1 receptor in skin mast cells. Lane 1, DNA ladder (100 bp); lane 2, mast cells; lane 3, HMC-1 cells.

DISCUSSION

In the present study, we have shown for the first time that isolated human skin mast cells are able to mount a secretory response in the presence of picomolar concentrations of α-MSH, resulting in the release of histamine in a concentration-dependent manner. Our previous studies (7) and the present data showing MC-1 and MC-5 receptors in human leukemic mast cells (HMC-1) and the MC-1 receptor in primary skin mast cells at the mRNA level and at the protein level, suggest that the α-MSH effects observed are MC-mediated. This assumption is further supported by the high efficiency of α-MSH-induced histamine release from isolated skin mast cells since effects were seen at picomolar concentrations of α-MSH.

Although the MC-receptors are known to couple with G-proteins, however, we could not detect an activation of adenylate cyclase after treatment of cells with α-MSH. A rapid degradation of cAMP by an enhanced activation of phosphodiesterases was excluded since in the presence of the phosphodiesterase inhibitor IBMX, the same results were observed. As an alternative explanation, MC-receptors might be expressed at only low levels in mast cells or the histamine releasing effect of α-MSH could be linked to a non-cAMP mediated signal transduction pathway. Thus, the activation of various post-receptor signaling systems such as those involving inositol triphosphate (IP_3), mobilization of intracellular calcium or the JAK/STAT pathway have been described in the past (4, 12–14). Analysis of possibly involved transcription factors in unstimulated

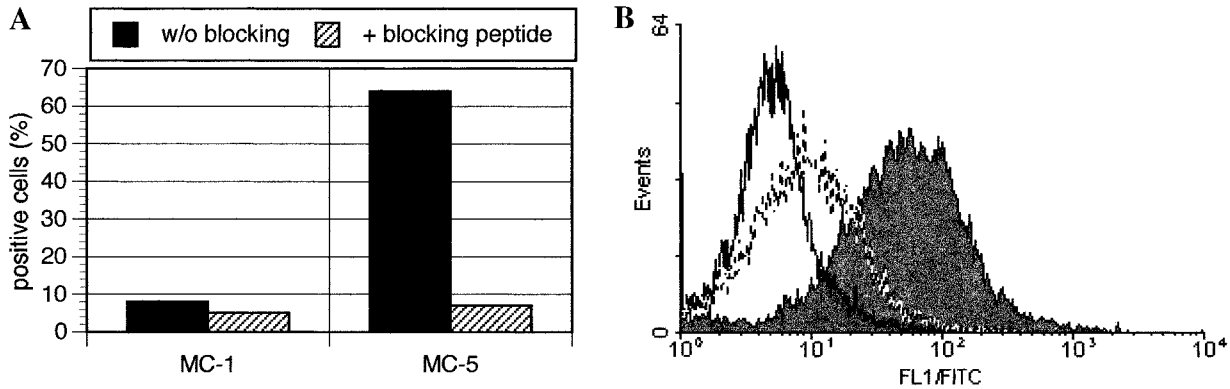

FIG. 6. Expression of MC-receptors at the protein-level in isolated HMC-1 cells (A) and skin mast cells (B). Cells were stained with MC-1- and MC-5-specific polyclonal antisera and analyzed by flow cytometry. Specificity of staining was checked by preadsorption of the antisera with antisera-specific blocking peptides. In A, data are expressed as percent of positively stained cells in comparison to cells stained with purified goat control immunoglobulins. In B, staining for the MC-5 receptor in skin mast cells is shown. The filled curve represents the signal for cells stained with the MC-5-specific antiserum in the absence of the corresponding blocking peptide. The dotted curve demonstrates the specificity of staining with a preadsorbed anti-MC-5 serum, and the curve with the closed line represents the negative staining with purified goat control immunoglobulins.

and α-MSH-treated cells gave, however, also no indication for α-MSH-induced postreceptor signaling events.

Thus, IκB, NF-AT and NF-ATc were found to be constitutively expressed, NFκB and OCT-2 messages were undetectable, and results were unaltered in α-MSH-treated skin mast cells. In agreement with our data, NF-AT-like transcription factors have been identified in the past in human and mouse mast cells (15). These molecules were shown to be involved in the anti-IgE- and PMA-induced transcriptional activation of various cytokine genes (15).

Taken together, these results suggest a highly specific, but MC-receptor-independent effect of α-MSH on mast cell histamine release. The effects described in this report for human skin mast cells resemble those described previously for the mast cell degranulating peptide (MCDP) (16, 17). This peptide exerts histamine releasing capacities at low concentrations (10–100 nM) and anti-inflammatory effects at micromolar concentrations. They have been shown to depend on a short sequence of basic amino acids, as also described for other peptides containing clusters of basic and hydrophobic amino acids which are able to induce an IgE-independent degranulation of mast cells (18). We can not exclude the possibility that the sequence -His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11} of the α-MSH molecule is primarily responsible for the histamine releasing effect observed in human mast cells, although we were able to detect MC-receptors in these cells.

Besides the prestored and rapidly released vasoactive mediator histamine, we also examined whether α-MSH might modulate the cytokine profile of mast cells. At the mRNA-level, the constitutive expression of IL-8, IL-6, and TGF-β was detectable, but it remained uninfluenced by α-MSH. Similarly, other cytokines in-

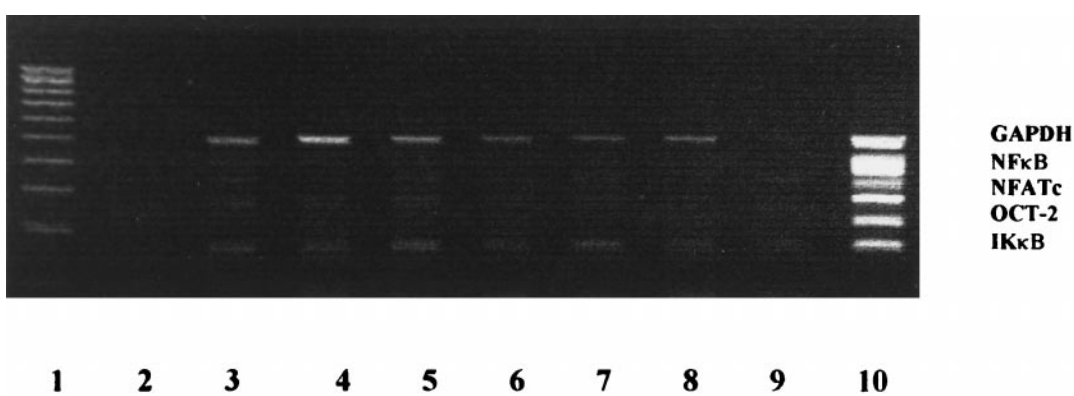

FIG. 7. Multiplex RT-PCR of GAPDH, NFκB, NFAT, NFATc, OCT-2, and IκB in α-MSH- and PMA-stimulated skin mast cells. Lane 1, DNA ladder (100 bp); lane 3, unstimulated mast cells; lane 4, PMA stimulated; lanes 5–9, α-MSH stimulated (10^{-6} to 10^{-10} M); lane 10, pos. control.

ducible by PMA, like GM-CSF, IL-1β and TNF-α, were not altered by α-MSH. These results were confirmed for IL-8 and TNF-α secretion at the protein level.

Until now, only one recent publication has demonstrated histamine release from α-MSH and β-endorphin-stimulated human foreskin slices, in accordance with our data, since cutaneous mast cells were the only likely source of the histamine (19). Our present findings confirm this and provide final proof that mast cells are indeed the direct target of α-MSH-induced histamine release.

While these data on the secretory response suggest that the α-MSH-effects on human mast cells, as described here, induce primarily proinflammatory activities, other data published so far suggest the opposite, although they derive almost exclusively from murine mast cells (20). Thus, PMA-activated cultured murine bone marrow derived mast cells (BMCMC) have been shown to express the MC-1 receptor and to respond to α-MSH with a downregulation of histamine release from antigen-stimulated cells (5). The same group of workers also ascertained a transcriptional downregulation of IL-1β, TNF-α, and lymphotactin in α-MSH-stimulated BMCMC (5). Since all these effects were only observed in prestimulated cells, they might have been due to stimulation-dependent modulation of MC-receptor expression. In our cell system, prestimulation with PMA or anti-IgE failed, however, to alter the expression of MC receptors (unpublished).

In summary, our results provide evidence that in vitro, human mast cells respond to picomolar concentrations of α-MSH with a liberation of histamine. The stimulatory mechanisms involved remain unclear from the present experiments, although indications for the expression of MC-receptors are evident. Furthermore, this study once again underlines the fact that fundamental phenotypic differences exist between human mast cells, as examined here, and data obtained with and published previously for rodent mast cells.

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