Stimulation of Intracellular Free Calcium in GH3 Cells by γ3-Melanocyt-Stimulating Hormone. Involvement of a Novel Melanocortin Receptor?

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ABSTRACT

The melanocortin (MC) γ3MSH is a peptide that can be generated from the N-terminal domain of POMC and is believed to signal through the MC3 receptor. We recently showed that it induces a sustained rise in intracellular free calcium levels ([Ca\(^{2+}\)]\(_i\)) in a subpopulation of pituitary cells, particularly in the lactosomatotroph lineage. In the present study we report that γ3MSH and some analogs increase [Ca\(^{2+}\)]\(_i\) in the GH- and PRL-secreting GH3 cell line and evaluate on the basis of pharmacological experiments and gene expression studies which MC receptor may be involved.

A dose as low as 1 pm γ3MSH induced an oscillating [Ca\(^{2+}\)]\(_i\) increase in a significant percentage of GH3 cells. Increasing the dose recruited an increasing number of responding cells; a maximum was reached at 0.1 nm. γ2MSH, αMSH, and NDP-αMSH displayed a similar effect. SHU9119, an MC3 and MC4 receptor antagonist, and an MC5 receptor agonist, did not affect the number of cells showing a [Ca\(^{2+}\)]\(_i\) rise in response to γ3MSH. SHU9119 had also no effect when added alone. MTT, a potent synthetic agonist of the MC3, MC4, and MC5 receptor as well as an N-terminally extended recombinant analog of γ3MSH showed low potency in increasing [Ca\(^{2+}\)]\(_i\) in GH3 cells, but high potency in stimulating cAMP accumulation in HEK 293 cells stably transfected with the MC3 receptor. In contrast, a peptide corresponding to the γ2MSH sequence of POMC-A of Acipenser transmontanus increased [Ca\(^{2+}\)]\(_i\) in GH3 cells, but was about 50 times less potent than γ2- or γ3MSH in stimulating cAMP accumulation in the MC3 receptor expressing HEK 293 cells. By means of RT-PCR performed on a RNA extract from GH3 cells, the messenger RNA of the MC2, MC3, and MC4 receptor was undetectable, but messenger RNA of the MC5 receptor was clearly present.

These data suggest that the GH3 cell line does not mediate the effect of γ3MSH through the MC3 receptor. The involvement of the MC5 receptor is unlikely, but cannot definitely be excluded. The findings animate the hypothesis that there exists a second, hitherto unidentified, MC receptor that displays high affinity for γ3MSH. (Endocrinology 142: 257–266, 2001)

γ3MSH IS A peptide that can be generated from the N-terminal domain of POMC and further processed to γ2- and γ1MSH (1). In contrast to αMSH, the biological role of γMSH peptides is not clearly established, although several biological actions have been reported in the brain, adrenal gland, kidney, and the cardiovascular system (for review see Refs. 2 and 3). We recently showed that γ3MSH is biologically active in the anterior pituitary of immature rats, exerting a mitogenic effect on lactotrophs, somatotrophs, and thyrotrophs (4). γ3MSH also induces a sustained rise in the intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) in 15% of dispersed pituitary cells from immature rats (5). Within these responsive cells, 53% showed GH immunoreactivity (ir), 12% showed PRL ir, 2% showed TSH-β ir, 5% showed LH-β ir, and 10% showed ACTH ir, whereas 18% did not express any hormone to a detectable level (6).

MSH peptides act through specific membrane receptors known as melanocortin (MC) receptors, which are members of the family of G protein-coupled receptors, using elevation of cAMP as a signal transduction system (7, 8). Five MC receptor subtypes have been cloned. The MC1 receptor located in melanocytes and leukocytes has a role in pigmentation and inflammation (9). The MC2 receptor is the classical ACTH receptor of the adrenal gland (10). The MC3 and MC4 receptors are mainly expressed in the central nervous system (11). No clear-cut function of the MC3 receptor, which is also expressed in gut, heart, and placenta, has been defined yet, whereas the MC4 receptor is related to control of weight homeostasis (12–14). The MC5 receptor is expressed in various peripheral tissues (8, 15) and is believed to participate in excocrine gland regulation (16).

The only known receptor with high affinity for the γMSH peptide is the MC3 receptor (2, 7, 17). However, several observations on the action of γMSH peptides in the cardiovascular system suggest the existence of other γMSH receptors (18, 19). Recently, we showed the expression of MC3 receptor messenger RNA (mRNA) in the anterior pituitary of immature rats (5), but SHU9119, an MC3 receptor antagonist (13, 20), blocked the effect of γ3MSH on [Ca\(^{2+}\)]\(_i\), in less than 50% of the responsive cells (5).

In the present study we explored whether GH3 cells, a pituitary cell line representative for lactotrophs and somatotrophs, would display [Ca\(^{2+}\)]\(_i\) responses to γ3MSH, and if so, whether these [Ca\(^{2+}\)]\(_i\) responses would also be mediated by a receptor pharmacologically distinct from the MC3 receptor. Finally, it was tested by RT-PCR which MC receptors are expressed, at least at the mRNA level, in the GH3 cell line.
Materials and Methods

Peptides and pharmacological reagents

γ3MSH, γ2MSH, α-MSH (acylated), and [Nle<sup>4</sup>Phe<sup>7</sup>]αMSH were purchased from Peninsula Laboratories, Inc., Europe (Merseyside, UK). Ac-Nle<sup>4</sup>-cyclo-[Asp<sup>5</sup>(o-Nal<sup>3</sup>)]<sub>2</sub>Lys<sup>7</sup>]αMSH (4–10)-NH<sub>2</sub> (SHU11991) was obtained from Neossys Laboratories (Strasbourg, France), and Ac-Nle<sup>4</sup>-cyclo-[Asp<sup>5</sup>, Phe<sup>7</sup>]<sub>2</sub>Lys<sup>7</sup>]αMSH (4–10)-NH<sub>2</sub> (MTII) (21) was obtained from Bachem (Bubendorf, Switzerland). An N-terminal-extended form of γ3MSH, designated Ala<sup>1</sup>γ3MSH<sup>5–20,24</sup>POMC (1–74), in which the cysteines at positions 2, 8, 20, and 24 were mutated to Ala, thus eliminating the two naturally occurring cysteine bridges, was produced in our laboratory by means of the pGEX-4T-1 expression vector (Amersham Pharmacia Biotech, Uppsala, Sweden) in BL21 protease-deficient Escherichia coli cells, purified, and characterized as described previously (22). Mass spectrometry and N-terminal amino acid sequencing showed the authenticity of the product. A γ2MSH analog based on the POMC-A sequence of the white sturgeon Acipenser transmontanus (Tyr-Val-Met-Ser-His-Phe-His-Trp-Asn-Thr-Phe-Gly) was synthesized by Sigma-Genosys (Cambridge, UK). Peptides were prepared as stock solutions at a concentration of 0.1 mM in 0.1% crystalline BSA (Serva, Heidelberg, Germany). HBSS was purchased from Life Technologies, Inc., Europe (Paisley, UK), and pluronic F127 and fluo-3 acetoxymethyl ester (fluo-3/AM) were obtained from Molecular Probes, Inc. (Eugene, OR). All of these reagents were prepared as stock solutions and maintained at ~25°C. All reagents were analytical grade.

Cell culture

The GH3 cell line was obtained from American Type Culture Collection (CCL 82.1; Manassas, VA). At the start of this study, cells were cultured in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.). In later experiments it was found that certain serum batches attenuated the effect of γ3MSH, which was avoided when cells were cultured in defined culture medium (mixture of DMEM and F12 medium; Life Technologies, Inc.) as described previously (23) supplemented with 1% FCS. Cultures were maintained in a water-saturated incubator (1.5% CO<sub>2</sub>-98.5% air) at 37°C. Cultures were washed with HBSS every 3 days. Twenty-four hours before an experiment, cells were trypsinized and seeded on a Lab-Tek chambered slide (1.8 cm<sup>2</sup>/chamber; 250,000 cells/chamber; Nunc, Roskilde, Denmark), coated with polyornithine (0.004%; Sigma-Genosys) and maintained in defined culture medium without serum supplement.

Intracellular free calcium measurements

[Ca<sup>2+</sup>], changes in response to the different peptides were tested 24 h after trypsinization. [Ca<sup>2+</sup>], was recorded by a video imaging system allowing the simultaneous study of 30–40 cells/field. Imaging and measurement of [Ca<sup>2+</sup>] were performed by means of the fluorescent dye fluo-3 (24) as previously described (25). Briefly, cells were incubated for 15 min at 37°C with 10 μM fluo-3/AM and 0.02% pluronic F127 in HBSS. After loading, cells were rinsed once with and kept in HBSS (bath medium). Peptides were directly diluted in bath medium at the concentration indicated in the experiments. Recording of the fluorescence was performed at room temperature using a Meridian Insight confocal microscope (Okemse, MI) based on an Olympus Corp. IMT2 inverted microscope (Melville, NY) with a D-plan APO objective for high magnification (1.25). By using an argon ion laser (332 argon ion laser, Coherent, Palo Alto, CA), and the emitted light was recorded at 530 nm. The fluorescence was amplified using an image intensifier (Dage MTI, Michigan City, IN) and collected by a cooled charge-coupled device camera (Meridian Instruments, Lansing, MI). The video image obtained was recorded on high-quality VHS videotape, equipped with a digital noise reduction board and a RS232 computer interface. From the VCR, the recorded image was digitized up to the video rate (25 images/sec). The video images were digitized at (8-bit accuracy to yield 256 gray levels, with 768 x 512 pixels maximal spatial resolution) with a frame grabber board (Vision-EZ, Data Translation, Marlboro, MA) and transferred to the computer hard disk. A maximum of 200 images of up to 768 x 512 pixels were used per analysis. For a total recording time of 4 min, we collected one image every 1.18 sec. Subsequent image analysis was performed using in-house software.

To obtain a continuous trace of mean fluorescence as a function of time, polygons were drawn over each recorded cell on the light-optical control picture. The average fluorescence value in each polygon was then calculated by adding all pixel fluorescence values (8 bits/pixel) and subsequently dividing this total amount by the number of pixels in the polygon, yielding a value between 0 and 255. Fluor-3 was chosen as Ca<sup>2+</sup> indicator because of its large optical signal, which allowed a very good signal to noise ratio in a single frame. The lack of emission or excitation spectral shift of fluo-3 on Ca<sup>2+</sup> binding makes it difficult to calibrate fluorescence signals in terms of precise and absolute values of free [Ca<sup>2+</sup>]. Therefore, fluorescence signals are expressed as (F–F<sub>0</sub>)<sup>2</sup>/F<sub>0</sub> ratios, with F being the fluorescence during response to a peptide, and F<sub>0</sub> the minimal resting fluorescence before stimulation. Changes in normalized fluorescence are therefore indicative of changes in [Ca<sup>2+</sup>] values.

Part of the GH3 cells displayed spontaneous Ca<sup>2+</sup> oscillations with variable frequency and amplitude. The mean amplitude of nonscillating cells was 0.11. Spontaneous oscillations were defined as oscillations with amplitude greater then 0.25. In nonscillating cells, a positive Ca<sup>2+</sup> response was defined by the appearance of fluo-3 fluorescence oscillations with amplitude exceeding 0.25. In spontaneously oscillating cells, a positive Ca<sup>2+</sup> response was defined by the appearance of fluorescence oscillations with an integrated fluorescence (expressed per min) exceeding the mean basal integrated fluorescence (expressed per min) and/or by the appearance of oscillations displaying an augmentation in frequency (Fig. 1). Integrated fluorescence, delay in the onset of response,
and oscillation frequency of [Ca\(^{2+}\)], were determined for each responsive cell.

**Stimulation of cAMP accumulation in the human embryonic kidney (HEK) 293-cell line stably transfected with the MC3 receptor and in the Chinese hamster ovary (CHO) cell line stably transfected with the MC5 receptor**

HEK 293 cells stably transfected with the rat MC3 receptor were a gift from Dr Ulrike Sahm (University of Bath, Bath, UK) with permission of Dr. R. D. Cone (Vollum Institute, Oregon Health Sciences University, Portland, OR). CHO cells stably transfected with the rat MC5 receptor were a gift from Dr. J. C. Schwartz (Unité de Neurobiologie et Pharmacologie de l’INSERM, Center Paul Broca, Paris, France). Cells were seeded in 24-well plates at a density of 5 x 10^4 cells/well and cultured in DMEM (Life Technologies, Inc.) with 10% FCS (Life Technologies, Inc.) in a humidified air-CO\(_2\) (1.5%) incubator at 37°C. On day 2 or 4 of each experiment, cells were washed once with DMEM containing 0.5 mM isobutylmethylxanthine (Aldrich, Steinheim, Germany) and 1% BSA. Test peptides diluted in DMEM containing 0.5 mM isobutylmethylxanthine and 1% BSA were added to the cells during 1 h (vehicle for controls). cAMP content was quantified using an enzymatic immunoassay kit distributed by Amersham Pharmacia Biotech (Uppsala, Sweden). Extraction was performed in 400 μl lysis buffer provided with the kit. In each experiment γ3MSH served as a positive control, and substances were tested in quadruplicate.

**RT-PCR**

Total RNA from the GH3 cell line, pituitary cells and brain of 14-day-old rats, adrenal gland from adult rats, and HEK293 cells stably transfected with the MC3 receptor were isolated by the guanidium thiocyanate-phenol-chloroform extraction procedure (26). After a deoxyribonuclease I (1 U/10 μl, 1 h at 25°C; Life Technologies, Inc.) treatment, to eliminate genomic DNA, 25 μg total RNA were reverse transcribed into cDNA at 42°C using random hexamer primers (Perkin-Elmer Corp., Branchburg, NJ) and Moloney leukemia virus reverse transcriptase (Perkin-Elmer Corp.) in a 20-μl final volume, followed by PCR, both as described below. To check for possible artifacts generated by amplification of remnants of genomic DNA, the RT-PCRs performed for detecting mRNA of MC receptors (MC receptor genes lack introns) (27, 28) were also performed on a nonreverse transcribed RNA sample for detecting mRNA of MC receptors (MC receptor genes lack introns) by amplification of remnants of genomic DNA, the RT-PCRs performed with established GenBank sequences with commercially available software (Genejockey II, Biosoft, Cambridge, UK). Primers were synthesized by Pharmacia Biotech. The forward and backward primers for GH cDNA were: 5′-GCTGCAACTCT-CAGACTCTTGG-3′ (nucleotides 464–470 and 653–672) and 5′-GTCTGAGAACAGAACGACA-3′ (nucleotides 1608–1687). The forward and backward primers for PRL cDNA were: 5′-ACCATGAACGACGCTGTCG-3′ (nucleotides 474–494) and 5′-CTTGTCCTCAGAGTACCTG-3′ (nucleotides 453–434) and yielded a product of 291 bp. The forward and backward primers for MC3 receptor cDNA (GenBank accession no. J00760) were: 5′-ACCATGAAACGACGCTGTCG-3′ (nucleotides 747–750). This set yielded a product of 226 bp. The forward and backward primers for MC4 receptor cDNA (GenBank accession no. U70667) were: 5′-CAATTGCTGAGTTGCTGCTAA-3′ (nucleotides 549–570) and 5′-CTAACGTCATGCTGCTGTAAC-3′ (nucleotides 774–770). This set yielded a product of 226 bp. The forward and backward primers for MC5 receptor cDNA (GenBank accession no. U67863) were: 5′-TTTCTCCTTGGCAGCCATG-3′ (nucleotides 389–408) and 5′-GAAGGCGGCTGCGGTGCTC-3′ (nucleotides 694–672). This set yielded a product of 305 bp. The forward and backward primers for MC2 receptor cDNA (GenBank accession no. L27081) were: 5′-TTCCCTGACGAGCTG-3′ (nucleotides 469–487) and 5′-CAGGGCCTGAAGATGGTGTGAC-3′ (nucleotides 696–672). This set yielded a product of 228 bp. As the sequence of rat MC2 receptor has not yet been determined, we choose the primers for MC2 receptor DNA based on sequence homologies between MC2 receptor DNA from mouse and hamster. The forward and backward primers for MC2 receptor cDNA (mouse, GenBank accession no. D31952; hamster, GenBank accession no. U71279) were: 5′-CAATTGCAAGAAAATAAGAAGCATAT-3′ (mouse, nucleotides 137–159; hamster, nucleotides 46–67) and 5′-AT- GTACCTGTCTGGTTTCTTCC-3′ (mouse, nucleotides 462–443; hamster, nucleotides 370–351), yielding a product of 325 bp.

PCR of the RT-generated cDNA was performed using a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer Corp.). The PCR mixture contained 1 μl reverse transcriptase template added to a mixture of (final concentrations) 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 4.5 mM MgCl\(_2\), 200 μM of each deoxy-NTP, sense and antisense primer (1 μM of each), and 1 U AmpliTaq Gold (Perkin-Elmer Corp.) in a final volume of 25 μl. All experiments were repeated at least three times. Values were expressed as the mean ± SEM of several independent experiments and compared using ANOVA (NCSS, Statistical Solutions Ltd, Cork, Ireland). Parameters of [Ca\(^{2+}\)], oscillations induced by the peptides (integrated fluorescence, frequency of oscillations, and delay) were compared with those recorded in resting conditions. Statistical analysis was performed using one- or two-way ANOVA. The Tukey-Kramer test was used whenever the ANOVA test indicated a significant difference. Curve fitting and EC\(_{50}\) value calculation, if applicable, were performed with the statistical software packages Curve Expert 1.3 or Prism (GraphPad Software, Inc., San Diego, CA). Differences were considered statistically significant at P < 0.05.

**Results**

**Characterization of [Ca\(^{2+}\)]\(_i\) in GH3 cells**

To study the basal [Ca\(^{2+}\)]\(_i\) activities in GH3 cells, more than 1000 cells were recorded in resting conditions. Sixty percent of the cells showed spontaneous basal [Ca\(^{2+}\)]\(_i\), whereas the remainder did not show any oscillation over a total recording time of 4 min. A small number of cells (~10%) that were spontaneously inactive during the 1 min of recording of basal activity started to show spontaneous [Ca\(^{2+}\)]\(_i\), oscillations at a particular time point during the next 3 min of recording. Therefore, in each experiment a control well with cells was recorded during these 3 min without adding the peptide under study. The data obtained were corrected for these false positive responders. There was no effect on [Ca\(^{2+}\)]\(_i\) after application of the vehicle only (609 cells were tested in a number of randomly chosen experiments).

**Effects of γ3MSH on [Ca\(^{2+}\)]\(_i\) in GH3 cells**

Addition of γ3MSH induced a sustained rise in [Ca\(^{2+}\)]\(_i\), in spontaneously nonoscillating as well as in oscillating cells from picomolar doses. An example is shown in Fig. 1. At all doses used, about half of the responsive cells were nonoscillating. As shown in Table 1 there was a significant rise in the integrated fluorescence. In spontaneously oscillating cells the frequency of [Ca\(^{2+}\)]\(_i\) oscillations also increased. There was no consistent relation observed between the γ3MSH concentrations and the delay in the [Ca\(^{2+}\)]\(_i\) responses, the integrated...
21.2% of the cells were responsive at 0.1 and 1 nM compared with vehicle. Values obtained with vehicle only (false positive responders) were subtracted from the values obtained after addition of peptide for each concentration tested (P < 0.001). No statistical differences were found between the different concentrations of peptide tested. Delay of [Ca\textsuperscript{2+}] response (sec).

<table>
<thead>
<tr>
<th>Integrated fluorescence</th>
<th>Basal values</th>
<th>0.1 pm</th>
<th>1 pm</th>
<th>10 pm</th>
<th>100 pm</th>
<th>1 nM</th>
<th>10 nM</th>
<th>100 nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>n1 - 27</td>
<td>n2 - 50</td>
<td>n1 - 45</td>
<td>n1 - 92</td>
<td>n1 - 54</td>
<td>n1 - 98</td>
<td>n1 - 40</td>
<td></td>
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</tr>
<tr>
<td>T - 340</td>
<td>T - 364</td>
<td>T - 300</td>
<td>T - 610</td>
<td>T - 473</td>
<td>T - 1088</td>
<td>T - 575</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spontaneously oscillating cells</td>
<td>9.2 ± 0.4</td>
<td>19.0 ± 0.8</td>
<td>21.7 ± 1.1</td>
<td>20.8 ± 1.2</td>
<td>22.5 ± 0.8</td>
<td>26.5 ± 1.1</td>
<td>19.9 ± 0.8</td>
<td>15.2 ± 0.6</td>
</tr>
<tr>
<td>Nonoscillating cells</td>
<td>5.7 ± 0.3</td>
<td>15.1 ± 1.5</td>
<td>17.9 ± 1.4</td>
<td>23.0 ± 1.5</td>
<td>21.6 ± 0.6</td>
<td>19.9 ± 0.8</td>
<td>15.2 ± 0.6</td>
<td>18.5 ± 0.9</td>
</tr>
<tr>
<td>Delay of Ca\textsuperscript{2+} response (sec)</td>
<td>5.7 ± 0.3</td>
<td>15.1 ± 1.5</td>
<td>17.9 ± 1.4</td>
<td>23.0 ± 1.5</td>
<td>21.6 ± 0.6</td>
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</tr>
</tbody>
</table>

Data are the mean ± SE. [Ca\textsuperscript{2+}] responses recorded in nonoscillating and spontaneously oscillating cells were separately analyzed. n1, Number of responding spontaneously oscillating cells; n2, number of responding nonoscillating cells; T, total number of cells tested. The three parameters of oscillation is statistically different from the frequency of oscillation after the addition of peptide for each concentration tested (P < 0.001). No statistical differences were found between the different concentrations of peptide tested. Delay of [Ca\textsuperscript{2+}] response (sec). No statistical differences were found between the different concentrations of peptide tested.

Effects of various agonists and an antagonist of the MC3 receptor on [Ca\textsuperscript{2+}] in GH3 cells

In the first set of experiments, the melanocortin peptides αMSH and NDP-αMSH were tested. The MC3, MC4, and MC5 receptor are equally activated by αMSH at 1 nM (for EC\textsubscript{50} values, see Ref. 13), but only the MC3 receptor is activated by γ2- and γ3MSH with high affinity (EC\textsubscript{50} ~ 4 nM (11)). As shown in Fig. 3A, αMSH induced a significant increase in [Ca\textsuperscript{2+}] in 8% and 14% of the GH3 cells at doses of 0.1 and 1 nM, respectively. NDP-αMSH caused an increase in 19.1% of the GH3 cells at 1 nM (Fig. 3A). These values were not significantly different from those obtained with γ3MSH.

We also tested the activity of Ala\textsubscript{2,8,20,24}-POMC-(1–74). This peptide was chosen to determine whether a long N-terminal extension of γ3MSH would differentially affect biological response in GH3 cells compared with the MC3 receptor-mediated response in HEK 293 cells (see below). At the doses of 0.01, 1, 10, and 100 nM, Ala\textsubscript{2,8,20,24}-POMC-(1–74) did not significantly change [Ca\textsuperscript{2+}] in GH3 cells (Fig. 3A).

In a second experimental set-up, the effect of MTII (13, 20, 29), a potent agonist of the MC3 (EC\textsubscript{50} 0.19–0.27 nM) (13, 20) and MC4 receptor (EC\textsubscript{50} 0.019–0.057 nM) (13, 20) and a somewhat weaker agonist of the MC5 receptor (EC\textsubscript{50} 1.36 nM) (13), was studied. MTII had no significant effect on [Ca\textsuperscript{2+}] at doses of 0.1, 1, 10, and 100 nM (Fig. 3B).

The Acipenser transmontanus γ2MSH sequence Tyr-Val-Met-Ser-His-Phe-His-Trp-Asn-Thr-Phe-Gly was tested because chondrostean fish display a degenerated γ2MSH sequence, including a mutation in the His-Phe-Arg-Trp core sequence (30), anticipating a low potency of the Acipenser peptide in MC3 receptor-mediated responses (31). Acipenser...
γ2MSH was compared with mammalian γ2MSH and γ3MSH. *Acipenser* γ2MSH was ineffective at 0.01 nM, but was significantly effective at 0.1 and 1 nM. At the latter doses it was only slightly less active (although not statistically significant) than the same doses of γ2MSH tested in the same experiment (Fig. 3B). It is noteworthy that a maximal effect was reached at the same dose as that of γ2MSH (or γ3MSH compared with data in Fig. 2).

When cells were pretreated for 5 min with the MC3 receptor antagonist SHU9119 (0.1 and 1 μM) and then tested for 

\[ \text{[Ca}^{2+}] \text{]i} \]

responses to 0.1 and 10 nM γ3MSH, in the presence of the same amount of the antagonist, the percentage of cells showing 

\[ \text{[Ca}^{2+}] \text{]i} \]

responses did not alter (Fig. 4). Treatment with 0.01, 1, or 10 nM SHU9119 (three independent experiments; data not shown) or with 1 μM SHU9119 alone (three independent experiments; Fig. 4) did not affect 

\[ \text{[Ca}^{2+}] \text{]i} \]

in GH3 cells (tested immediately after application of the peptide).

**Effect of TRH on \[Ca^{2+}\]i in GH3 cells**

As a positive control, the effect of TRH was tested. TRH stimulates acute release of both PRL and GH, reaching a maximum at 100 nM (32), and stimulation of GH3 cells with TRH is well known to raise 

\[ \text{[Ca}^{2+}] \text{]i} \]

At a dose of 100 nM, TRH caused a 

\[ \text{[Ca}^{2+}] \text{]i} \]

rise in 20% of the GH3 cells, which was comparable to the number of cells responding to 1 nM γ3MSH tested in the same experiment (data not shown).

**Effect of γ3MSH and analogs on cAMP levels in HEK 293 cells expressing the MC3 receptor and in CHO cells expressing the MC5 receptor**

In the HEK 293 cells transfected with the MC3 receptor, 1 nM MTII caused a 10-fold increase in cAMP levels, as expected (Table 2). *Acipenser* γ2MSH (0.01 and 1 nM) and γ3MSH (0.01 and 1 nM). In each experiment at least two concentrations were tested together and compared with vehicle. Values obtained with vehicle only (false positive responders) were subtracted from the values obtained after addition of peptides. Each point is expressed as the mean ± SEM of the total number of cells responding (spontaneously oscillating and non-oscillating cells combined). γ3MSH was always tested together with the other analogs. Number of experiments: γ3MSH, eight; NDP-αMSH, four; MTII, seven; and *Acipenser* γ2MSH, 0.01 and 1 nM were tested three times together with γ2MSH; 0.01 and 100 nM were tested four times together with γ3MSH. The total number of cells tested for each dose level ranged between 203 and 563. **, P < 0.05; ***, P < 0.01 (vs. control). αMSH (1 nM) is different from both 0.01 and 100 nM at P < 0.05. *Acipenser* γ2MSH (0.01 nM) is different from both 1 and 100 nM at P < 0.05. *Acipenser* γ2MSH (0.1 nM) is different from *Acipenser* γ2MSH (0.01, 1, and 100 nM). No statistically significant effects were found for MTII and *Acipenser* γ2MSH (0.01, 1, and 100 nM).

![Figure 3. A](https://academic.oup.com/endo/article-abstract/142/1/257/2988854)

**Fig. 3.** A, Effects of αMSH, NDP-αMSH, and *Acipenser* γ2,8,20,24-POMC-(1–74) on [Ca2⁺]i in GH3 cells, compared with that of γ3MSH. B, Effect of MTII and *Acipenser* γ2MSH on [Ca2⁺]i in GH3 cells compared with that of γ2MSH (0.1 and 1 nM) and γ3MSH (0.01 and 1 nM). In each experiment at least two concentrations were tested together and compared with vehicle. Values obtained with vehicle only (false positive responders) were subtracted from the values obtained after addition of peptides. Each point is expressed as the mean ± SEM of the total number of cells responding (spontaneously oscillating and non-oscillating cells combined). γ3MSH was always tested together with the other analogs. Number of experiments: αMSH, eight; NDP-αMSH, four; MTII, seven; and *Acipenser* γ2MSH, 0.01 and 1 nM were tested three times together with γ2MSH; 0.01 and 100 nM were tested four times together with γ3MSH. The total number of cells tested for each dose level ranged between 203 and 563. **, P < 0.05; ***, P < 0.01 (vs. control). αMSH (1 nM) is different from both 0.01 and 100 nM at P < 0.05. *Acipenser* γ2MSH (0.01 nM) is different from both 1 and 100 nM at P < 0.05. *Acipenser* γ2MSH (0.1 nM) is different from *Acipenser* γ2MSH (0.01, 1, and 100 nM). No statistically significant effects were found for MTII and *Acipenser* γ2MSH (0.01, 1, and 100 nM).

![Figure 4.](https://academic.oup.com/endo/article-abstract/142/1/257/2988854)

**Fig. 4.** Effect of the MC3 receptor antagonist SHU9119 on the [Ca2⁺]i responses induced by γ3MSH. Values obtained with vehicle only (false positive responders) were subtracted from the values obtained after addition of peptides. Data are the mean ± SEM. A dose of 0.1 nM γ3MSH with and without 100 nM SHU9119 was tested in three different experiments; a dose of 10 nM γ3MSH with and without 1 μM SHU9119 was tested in four different experiments. The total number of cells tested for each dose level ranged between 214 and 421. No significant differences between [Ca2⁺]i responses in the presence and absence of SHU9119 were found.
TABLE 2. Effects of γ3MSH, γ3MSH in the presence of a 100-fold excess of SHU9119, MTII, and Ala^{2,8,20,24}-POMC-(1–74) on cAMP accumulation in HEK 293 cells stably transfected with the MC3 receptor

<table>
<thead>
<tr>
<th>Exp</th>
<th>Peptide conc. (nM)</th>
<th>0</th>
<th>1</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>0.43 ± 0.03</td>
<td>3.96 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.13 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ3MSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MTII</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>7.04 ± 2.85</td>
<td>55.86 ± 2.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>57.03 ± 5.02&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ3MSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ala^{2,8,20,24}-POMC-(1–74)</td>
<td>11.7 ± 1.04</td>
<td>59.2 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>13.57 ± 5.6</td>
<td>132.83 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>37 ± 5.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ3MSH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ3MSH + 1 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SHU9119</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells were tested 1 day (Exp 1) or 3 days (Exp 2 and 3) after replating in the 24-well plate. Data are expressed as picomoles of intracellular cAMP per dish and are the mean ± SEM of one representative experiment of two or three independent experiments. EC_{50} values for Ala^{2,8,20,24}-POMC-(1–74) were 5.1 nM for the presented values and 1.7 nM for a second experiment.

<sup>a</sup> P < 0.001 vs. control.

<sup>b</sup> P < 0.001 vs. 1 nM.

<sup>c</sup> P < 0.001 vs. γ3MSH.

TABLE 3. Effect of Acipenser transmontanus γ2MSH compared to that of mammalian γ3MSH on cAMP accumulation in HEK 293 cells stably transfected with the MC3 receptor

<table>
<thead>
<tr>
<th>Peptide conc. (nM)</th>
<th>0.1</th>
<th>0.3</th>
<th>1</th>
<th>10</th>
<th>30</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.29 ± 0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ3MSH</td>
<td>0.94 ± 0.07</td>
<td>4.63 ± 0.61</td>
<td>9.40 ± 0.66</td>
<td>16.04 ± 0.48</td>
<td>16.67 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>Acipenser γ2MSH</td>
<td>0.39 ± 0.08</td>
<td>3.07 ± 0.24</td>
<td>7.84 ± 0.90</td>
<td>10.02 ± 0.21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cells were tested 1 day after replating in the 24-well plate. Data are expressed as picomoles of intracellular cAMP per dish and are the mean ± SEM of one representative experiment performed in quadruplicate dishes for each data point. All values are statistically different from control and from each other, except for 10 vs. 100 nM γ3MSH (at least P < 0.05). EC_{50} values for γ3MSH and A. transmontanus γ2MSH were 1 and 17 nM, respectively. In another experiment γ3MSH and Acipenser γ2MSH were tested at 1 nM, and in a third experiment γ3MSH and Acipenser γ2MSH were tested at 0.01, 1, and 100 nM (estimated EC_{50}, 0.07 nM for γ3MSH and 6.8 nM for Acipenser γ2MSH).

TABLE 4. Effect of Ala^{2,8,20,24}-POMC-(1–74) compared to that of γ3MSH on cAMP accumulation in CHO cells stably transfected with the MC5 receptor

<table>
<thead>
<tr>
<th>Peptide conc. (nM)</th>
<th>1</th>
<th>3</th>
<th>10</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.12 ± 0.26</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ3MSH</td>
<td>3.34 ± 0.53</td>
<td>13.63 ± 4.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.95 ± 3.42&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.38 ± 14.89&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ala^{2,8,20,24}-POMC-(1–74)</td>
<td>1.87 ± 0.53</td>
<td>1.64 ± 0.64</td>
<td>1.12 ± 0.11</td>
<td>8.11 ± 1.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Cells were tested 1 day after replating in the 24-well plate. Data are expressed as picomoles of intracellular cAMP per dish and are the mean ± SEM of one representative experiment (a total of three independent experiments) performed in quadruplicate dishes for each data point. EC_{50} values for γ3MSH and Ala^{2,8,20,24}-POMC-(1–74) were 11.3 and 143 nM, respectively. In a prior experiment γ3MSH was tested at 1 nM, and Ala^{2,8,20,24}-POMC-(1–74) was tested at 1, 10, and 100 nM. In a second experiment γ3MSH was tested at 100 nM, and Ala^{2,8,20,24}-POMC-(1–74) at 1, 10, 100, and 10 μM (estimated EC_{50}, 590 nM).

<sup>a</sup> Values are statistically different from control and from each other, except for 100 vs. 1000 nM γ3MSH (at least P < 0.05).

100-fold excess of SHU9119 over γ3MSH almost completely blocked the effect of γ3MSH (Table 2). This corresponds with data reported by others (20).

As shown in Table 4, both γ3MSH and Ala^{2,8,20,24}-POMC-(1–74) caused an increase in cAMP in CHO cells transfected with the MC5 receptor. γ3MSH showed activity starting from a 3-nM dose, whereas Ala^{2,8,20,24}-POMC-(1–74) was more than 10 times less potent.

**Melanocortin receptor expression studies**

The expression of the MC2, MC3, MC4, and MC5 receptors in GH3 cells was studied at the mRNA level. Figure 5 shows the results of RT-PCR analysis of total RNA extracts from GH3 cells compared with that from rat pituitary, rat brain, rat adrenal gland, and the MC3 receptor expressing HEK 293 cells (positive controls). The primers designed for the MC receptor cDNAs amplified the fragments of the expected sizes for mRNA of MC2 receptor (325 bp), MC3 receptor (226 bp), MC4 receptor (305 bp), and MC5 receptor (228 bp). GH3 cells expressed neither MC2, MC3, nor MC4 receptor mRNA. As expected, rat brain contained MC3, MC4, and MC5 receptor mRNA. Pituitary expressed MC3, MC4, and MC5 receptor. Adrenal gland was positive for MC2 receptor mRNA.
As shown in Fig. 6, rat-specific primers for GH and PRL cDNA amplified PCR-generated fragments of the expected sizes of 242 bp (GH) and 291 bp (PRL). The latter data confirm the mammosomatotropic phenotype of the GH3 cell line used in our study.

**Discussion**

The present investigation shows that the GH- and PRL-producing GH3 cell line is responsive to γ3MSH in terms of an increase in \([\text{Ca}^{2+}]_i\). Increasing doses of the peptide recruited an increasing number of responding cells. The mediating receptor appears to have high affinity for the peptide, as a dose as low as 1 pM was effective, and a maximum was reached at 0.1 nM.

The data obtained from experiments with various agonists and antagonists of MC receptors strongly plead against mediation of the effect of γ3MSH by the MC3 receptor. First, γ3MSH induced an increase in \([\text{Ca}^{2+}]_i\) at doses several orders of magnitude beneath those reported in the classical cAMP stimulation test in cell lines stably transfected with the MC3 receptor (reviewed in Ref. 34 and also in the present study). The minimal dose effective on \([\text{Ca}^{2+}]_i\) in GH3 cells was as low as 1 pM, and the maximal effect was seen at 0.1 nM.

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lines \([\text{EC}_{50} \text{ for cAMP accumulation, 0.19, 0.27, and 1.2 nm, depending on the report (13, 20, 35)}]\), failed to induce an increase in \([\text{Ca}^{2+}]\), in GH3 cells at doses up to 100 nm, which is 3 orders of magnitude above the dose inducing a maximal effect of \(\gamma\text{MSH}\) in these cells. Fourth, Ala2,8,20,24-POMC-(1–74), an analog of \(\gamma\text{MSH}\) N-terminally extended with more than 50 amino acid residues \(\text{i.e.} \text{POMC-(1–74)}\), in which the cysteines at positions 2, 8, 20, and 24 are mutated to Ala was highly effective in stimulating the MC3 receptor stably transfected in HEK 293 cells, but had no effect on \([\text{Ca}^{2+}]\), in GH3 cells, even at a dose 1000-fold higher than the maximally effective dose of \(\gamma\text{MSH}\) in the latter cells. In contrast, \textit{Acipenser} \(\gamma\text{MSH}\) was a weak agonist in the cAMP assay, but was fairly effective on \([\text{Ca}^{2+}]\), in GH3 cells. Finally, by means of RT-PCR, no mRNA of the MC3 receptor was detectable in GH3 cells, although this mRNA was detected in brain and normal pituitary, as reported previously (5).

Does another known MC receptor mediate the \([\text{Ca}^{2+}]\), response to \(\gamma\text{MSH}\)? It is most unlikely that the MC4 receptor is involved. First, SHU9119, an even more potent antagonist of this receptor (20), was ineffective on \(\gamma\text{MSH}\)-induced \([\text{Ca}^{2+}]\), changes in GH3 cells, and MTII, also a potent agonist of the MC4 receptor \(\text{EC}_{50} 0.019, 0.057, \text{or 0.17 nm, depending on the reported study (13, 20, 35)}\), was ineffective up to doses several orders of magnitude higher than the effective doses of \(\gamma\text{MSH}\). Second, mRNA of the MC4 receptor could not be detected in GH3 cells. In contrast, MC5 receptor mRNA was found in GH3 cells (this study). The \(\text{EC}_{50}\) for the effects of \(\gamma\text{MSH}\) peptides on cAMP levels through the MC5 receptor transfected in cell lines is in the middle nanomolar range (8, 28, 36, 37). In our hands, as tested for accumulation of cAMP in CHO cells stably transfected with the rat MC5 receptor, the \(\text{EC}_{50}\) of \(\gamma\text{MSH}\) was 11.3 nm. Thus, it is reasonable to reflect on the hypothesis that the MC5 receptor may be involved in the \(\gamma\text{MSH}\)-induced \([\text{Ca}^{2+}]\), responses. However, several findings do not support this hypothesis. First, although the \(\text{EC}_{50}\) value for the \(\gamma\text{MSH}\) effect in GH3 cells could not be exactly determined because of the bell-shaped dose-response curve, it appears to be about 3 orders of magnitude lower than that for \(\gamma\text{MSH}\) in the CHO cell line. Second, SHU9119 is a full agonist of the MC5 receptor \(\text{EC}_{50} 0.43–2.31 \text{nm} (20, 38)\), but it did not cause \([\text{Ca}^{2+}]\), responses in GH3 cells at concentrations up to 1 \(\mu\text{M}\). Third, MTII (21) is also a very potent agonist of the MC5 receptor \(\text{EC}_{50} 1.4–2.3 \text{nm} (20, 35, 38)\), but it did not induce a significant increase in \([\text{Ca}^{2+}]\), at a dose up to 100 nm. Finally, Ala2,8,20,24-POMC-(1–74) was 10 times less potent than \(\gamma\text{MSH}\) in the CHO MC5 receptor cell line, but was at least 1000 times less potent than \(\gamma\text{MSH}\) in GH3 cells (ineffective at 100 nm, whereas \(\gamma\text{MSH}\) was maximally effective at 0.1 nm). Because of its high potency on \([\text{Ca}^{2+}]\), \(\gamma\text{MSH}\) is also unlikely to act through an MC1 or MC2 receptor (34). Furthermore, there was no evidence found for expression of the MC2 receptor in GH3 cells, and the inactivity of MTII in GH3 cells is not in accordance with the reported \(\text{EC}_{50}\) value of MTII in (the subnanomolar range) at the MC1 receptor (20).

It should be realized, however, that the interaction of the MC5 receptor with agonists and antagonists has mainly been studied in cell lines overexpressing the receptor after transfection. One should bear in mind that the orders of potency of these agonists and antagonists at the MC5 receptor expressed in its natural microenvironment, such as in GH3 cells, may be different compared with those at the receptor transfected in heterologous cell lines. Therefore, we cannot exclude with certainty that \(\gamma\text{MSH}\) in GH3 cells acts on \([\text{Ca}^{2+}]\), through the MC5 receptor. However, if this were the case, the receptor would acquire a gain of function of some 3 orders of magnitude with respect to the natural agonist \(\gamma\text{MSH}\) and a loss of function of a similar magnitude with respect to several chemically distinct synthetic MC5 receptor agonists [MTII, SHU9119, and Ala2,8,20,24-POMC-(1–74)]. These data are difficult to reconcile with the hypothesis that the \([\text{Ca}^{2+}]\), response to \(\gamma\text{MSH}\) in GH3 cells is mediated by the MC5 receptor. It is reasonable, therefore, to propose the hypothesis that there may exist a hitherto uncharacterized receptor with high affinity for \(\gamma\text{MSH}\) peptides.

It is interesting to note that the high pharmacological potency of \(\gamma\text{MSH}\) on this hypothetical novel receptor is not associated with selectivity, as \(\alpha\text{MSH}\) and \(\text{NDP-\alpha\text{MSH}}\) were also effective. The pharmacological characteristics of the \(\gamma\text{MSH}\) effect in GH3 cells are therefore different from those reported in the cardiovascular system, in which an increase in heart rate has been noticed with \(\gamma\text{MSH}\), but not with \(\gamma\text{MSH}\) or \(\alpha\text{MSH}\) (39). In contrast, MTII and SHU9119, which both have a structurally constrained cyclic MSH core sequence (20, 21), act, respectively, as potent agonist and antagonist at the MC3 receptor in transfected HEK 293 cells, but not at the receptor mediating \([\text{Ca}^{2+}]\), changes in GH3 cells. Thus, constraining the MSH core sequence by cyclization does not affect interaction with the MC3 receptor, but seems to impair interaction with the hypothetical receptor for \(\gamma\text{MSH}\) in GH3 cells. A differential influence of structural restraint was also seen by N-terminal extension of \(\gamma\text{MSH}\) in Ala2,8,20,24-POMC-(1–74). Such extension was deleterious for the action at the hypothetical novel \(\gamma\text{MSH}\) receptor, but had minimal influence on the action at the MC3 receptor.

Chondrostean fish, like \textit{Acipenser transmontanus} (white sturgeon), have a duplicated POMC gene (POMC A and POMC B) (40). POMC B has a conserved \(\gamma\text{MSH}\) core sequence, His-Phe-Arg-Trp, which has never been suggested that the \(\gamma\text{MSH}\) sequence is degenerated in chondrostean fish (30). It is well known that single amino acid substitutions in the core sequence of MSH peptides cause a dramatic decrease in affinity and activity of these peptides at the MC receptors (31, 41). \textit{Acipenser} \(\gamma\text{MSH}\) was indeed about 50 times less potent at the MC3 receptor in HEK 293 cells compared with mammalian \(\gamma\text{MSH}\). However, the finding of a fairly effective action of \textit{Acipenser} \(\gamma\text{MSH}\) in GH3 cells (maximal effect reached at the same dose as that of \(\gamma\text{MSH}\) or \(\gamma\text{MSH}\)) suggests that the core sequence for the effect on \([\text{Ca}^{2+}]\), is different from that of MSH peptides at the known MC receptors. Moreover, these data suggest that the \(\gamma\text{MSH}\) sequence from the POMC A gene of \textit{Acipenser} may not be corrupt for all its biological actions.

It is surprising that only about 20% of the GH3 cells responded to \(\gamma\text{MSH}\). It is known that GH3 cells are functionally heterogeneous (42, 43). This functional heterogeneity could be translated in heterogeneity in receptor expression or action. Moreover, electrophysiological experiments have
shown that only about 50% of cultured GH3 cells are excit-
able, and that during certain cell cycle phases cells are elec-
trically inactive and retain secretion rates at their basal level after treatment with TRH (44). As during standard culture the GH3 cell population is a mixture of cells in different stages of the cell cycle, it can be expected that [Ca²⁺], changes in response to γδMSH may not occur in all cells. In fact, TRH induced a [Ca²⁺] response in only about 20% of the cells.

An intriguing finding was that the dose-response curve of γδMSH was biphasic. Increasing doses in the picomolar range recruited an increasing number of cells displaying a [Ca²⁺] rise, whereas above 1 nM the number of responding cells markedly declined. It is possible that γδMSH activates two signal transduction pathways with opposite effects, together creating a bell-shaped dose-response curve. Such a phenomenon was reported in Hepa cells transfected with the human MC3 receptor (45). Bell-shaped dose response relations are also characteristic of trophic effects of melanocortins on cultured neurons in vitro as well as on neurite outgrowth in vivo (46–48). Although the significance and mechanism of the bell-shaped dose response remain unclear, it can be spec-
ulated that the [Ca²⁺] response under physiological condi-
tions occurs only within a certain window of γδMSH con-
centrations. Such a response characteristic is reminiscent of that of morphogen gradients that induce different cellular responses and cell fates at different concentrations (49). Thus, in a physiological environment γδMSH may act on [Ca²⁺] dependent processes not in cells in the immediate vicinity of the site of peptide secretion, but on remote cells where the concentration has fallen to lower levels as a consequence of diffusion.

In conclusion, the present data strongly suggest that the action of γδMSH peptides on [Ca²⁺] in the GH3 lactosomatotroph cell line is not mediated by the MC3 receptor. Al-
though the MC5 receptor cannot definitely be excluded as the responsible receptor, mediation of the [Ca²⁺] response by this receptor seems unlikely. The data encourage further testing of the hypothesis that a hitherto unidentified MC receptor, displaying high affinity for the [Ca²⁺], response to γδMSH may exist. We previously reported that SHU9119 failed to block the [Ca²⁺] response to γδMSH in GH and PRL cells in normal rat pituitary (6). Possibly, normal lactotrophs and somatotrophs are selectively inactive and retain secretion rates at their basal level, and that during certain cell cycle phases cells are elec-
trically inactive and retain secretion rates at their basal level. This hypothesis is supported by the observation that only about 50% of cultured GH3 cells are excit-
able, and that during certain cell cycle phases cells are elec-
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An intriguing finding was that the dose-response curve of γδMSH was biphasic. Increasing doses in the picomolar range recruited an increasing number of cells displaying a [Ca²⁺] rise, whereas above 1 nM the number of responding cells markedly declined. It is possible that γδMSH activates two signal transduction pathways with opposite effects, together creating a bell-shaped dose-response curve. Such a phenomenon was reported in Hepa cells transfected with the human MC3 receptor (45). Bell-shaped dose response relations are also characteristic of trophic effects of melanocortins on cultured neurons in vitro as well as on neurite outgrowth in vivo (46–48). Although the significance and mechanism of the bell-shaped dose response remain unclear, it can be spec-
ulated that the [Ca²⁺] response under physiological condi-
tions occurs only within a certain window of γδMSH con-
centrations. Such a response characteristic is reminiscent of that of morphogen gradients that induce different cellular responses and cell fates at different concentrations (49). Thus, in a physiological environment γδMSH may act on [Ca²⁺] dependent processes not in cells in the immediate vicinity of the site of peptide secretion, but on remote cells where the concentration has fallen to lower levels as a consequence of diffusion.

In conclusion, the present data strongly suggest that the action of γδMSH peptides on [Ca²⁺] in the GH3 lactosomatotroph cell line is not mediated by the MC3 receptor. Al-
though the MC5 receptor cannot definitely be excluded as the responsible receptor, mediation of the [Ca²⁺] response by this receptor seems unlikely. The data encourage further testing of the hypothesis that a hitherto unidentified MC receptor, displaying high affinity for the [Ca²⁺], response to γδMSH may exist. We previously reported that SHU9119 failed to block the [Ca²⁺] response to γδMSH in GH and PRL cells in normal rat pituitary (6). Possibly, normal lactotrophs and somatotrophs are selectively inactive and retain secretion rates at their basal level, and that during certain cell cycle phases cells are elec-
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