Calmodulin-Binding Peptides Interfere with Melanocyte-Stimulating Hormone Receptor Activity and Stimulate Adenosine 3′,5′-Monophosphate Production in M2R Mouse Melanoma Cells*

YOAV ESHEL AND YORAM SALOMON†

Department of Hormone Research, Weizmann Institute of Science, Rehovot 76100, Israel

ABSTRACT

The MSH receptor belongs to a unique class of G-protein-coupled receptors, in which calcium ions control the binding affinity of MSH by a yet unknown mechanism. Possible involvement of a calcium-binding protein [e.g. calmodulin (CaM)] in the regulation of MSH receptor activity has been studied in the M2R mouse melanoma cell line. In this study, we tested the inhibitory effects of a group of calmodulin-binding peptides (CBPs) on MSH receptor activities in intact M2R cells and membrane preparations derived from them. We also report here on stimulatory effects of CBPs on cAMP production in M2R cells that could not be produced in other cell lines lacking MSH receptors. This group of CBPs includes synthetic peptides comprising the CaM-binding domains of Ca2+/CaM-dependent enzymes, cytotoxic venom peptides, and peptide hormones that have been reported to directly interact with CaM. The results show that CBPs, at micromolar concentrations, inhibit MSH binding and consequent adenylate cyclase stimulation in a specific and concentration-dependent manner, but have no effect on adenylate cyclase stimulation by prostaglandin E2. On the other hand, when MSH was omitted and forskolin (0.5–1 μM) was added instead, CBPs had the opposite effect on cAMP production, stimulating it in M2R cells, but not in other cell types tested. Thus, these peptides can be considered as antagonists of MSH receptor and partial agonists of M2R adenylate cyclase. In contrast to MSH, the stimulatory effects of CBPs were unaffected by EGTA, suggesting a Ca2+-independent action of these peptides. Using phospholipid vesicles and M2R cells, we recently showed that CBP activity in M2R cells may include direct partition into the lipid bilayer of the cell membrane, permitting interaction with hydrophobic lipid-inserted domains of components of the signal transducing machinery. Based on these findings, we suggest that the mechanism of action of CBPs in the M2R cells includes two major components: 1) interaction with the cell surface membrane and penetration into the lipid milieu, and 2) interaction with exposed or lipid-embedded protein epitopes intrinsically associated with the MSH-receptor system, thereby affecting the MSH receptor cascade. (Endocrinology 134: 177–185, 1994)

HORMONES, neurotransmitters, and many drugs initiate their biological actions by interacting with receptor molecules on respective target cells. The ligand-receptor complex triggers a cascade of enzymatic reactions that involve intermediary guanine nucleotide-binding proteins (G-proteins). G-Proteins couple their receptors to specific effector enzymes to increase/decrease the level of second messengers (i.e. cAMP, inositol trisphosphate, Ca2+, etc.) (1).

The MSH receptor was reported by us as being unique among G-protein-regulating receptors in its requirement for extracellular calcium ions that control the binding affinity for MSH and, consequently, the activity of adenylate cyclase (AC) (2–4). We demonstrated that at physiological hormone concentrations, binding of βMSH and subsequent activation of AC in intact M2R cells and plasma membrane preparations were strictly dependent on extracellular calcium ions and involved a freely reversible mechanism for the binding and/or dissociation of the hormone (2). This effect of calcium was not only confined to the association of the receptor with βMSH, but was also seen with the highly potent αMSH analog, [Nle4,dPhe7]aMSH (5), in studies of melanocortin receptors in lacrimal tissue (6).

It was then hypothesized that the putative calcium regulatory site(s) involved in this phenomenon may reside on the receptor itself or be related to a putative Ca2+-binding protein that interacts with the MSH receptor. A suitable candidate for this purpose is calmodulin (CaM), a ubiquitous calcium receptor that regulates a host of calcium-mediated cellular and physiological phenomena in eukaryotic cells (7). To assess this theory, the effects of several CaM-binding peptides (CBPs), such as melittin (8), a bee venom-derived cytotoxic peptide (9), and the synthetic 17-amino acid peptide, M5 (10), comprising the C-terminal CaM-binding domain of myosin light chain kinase (MLCK) (11), on MSH receptor activity were tested in M2R cells (10) and lacrimal cell membranes (6). These CBPs were found to selectively inhibit MSH binding (IC50, 0.7 and 1.7 μM, respectively) to M2R mouse melanoma cells and consequent AC stimulation, presumably by interacting with a putative CaM-like site, thus interfering with MSH receptor functions (10).

The established common denominator of these peptides is their ability to be recognized by and interact with CaM (12). This is certainly the case for those peptides that function as CaM-binding domains in Ca2+/CaM-dependent enzymes, e.g. MLCK (11), human erythrocyte Ca2+-ATPase (13), and...
Bordetella pertussis AC (14). These peptides retain their CaM-binding capacity when free in solution. Other members of this group include naturally occurring peptides, such as hormones, or cytotoxic peptides empirically found and reported to interact with CaM (15). Some of these peptides have been tested in this study (Table 1). Interactions of these peptides (e.g. M5) with CaM were established either by direct binding measurements (16, 17) or indirectly by their ability to interfere with the stimulation of CaM-dependent activation of other Ca²⁺/CaM-regulated enzymes (18). Assuming that the enzyme-derived CBPs are the most specific amino acid sequences selected by evolution for recognition by CaM, we thought that they may serve as potential probes of putative CaM-dependent steps in pathways of higher complexity (e.g. the MSH receptor system). Being aware of the limitations of this approach, we do not advocate unequivocally earmarking the activities of CBPs described in this or previous studies to the presence or participation of CaM in the test system described. However, we found it intriguing that this group of peptides, in modest concentrations, afforded rather selective effects on the MSH-responsive signaling pathway in intact M2R mouse melanoma cells, and we wished to explore its basis.

Based on their secondary structural properties, M5 (16), the synthetic 28-amino acid peptide C28W (representing the CaM-binding domain of the human erythrocyte plasma membrane calcium ATPase) (19), and other CBPs (12) were proposed to assume a predominantly α-helical conformation (>50%) upon contact with the hydrophobic core of CaM. Similar considerations may apply with respect to the interactions of mastoparan (20), C20W (a truncated C28W) (21), C28W, and M5 (22) with phospholipid bilayers. To further examine the mode of action of CBPs in a cellular system, we employed biophysical methods and showed that M5 and C28W can interact with artificial phospholipid bilayers and with the lipid component of native M2R melanoma cells and cell membranes (22). Furthermore, we showed that CBPs actually partition into lipid bilayers (partition coefficients, 10⁵–10⁶ m⁻¹) (22). It was further proposed that this property allows for association of CBPs with lipid-inserted domains of membrane proteins, e.g. components of the MSH receptor system. Such an interaction may influence the activity of these proteins and be crucial for the observed effects of CBPs on the cellular responses reported here and previously (6, 8, 10, 23).

In this study we tested the inhibitory effects of a group of CBPs, including a number of structural analogs of M5 and C28W (Table 1), on MSH binding and activity in M2R cell monolayers and membrane preparations derived from them to gain information concerning the structural requirements of their relevant site(s) of interaction. In addition, we describe here a novel independent effect of CBPs that led to enhanced cAMP production in M2R cells, but not in other cells that do not contain MSH receptors. The results suggest that, on the one hand, characteristic inhibition by CBPs of MSH binding in M2R cells is not confined to a specific primary sequence of CBPs, as reported for CBP-CaM interaction (12). On the other hand, analogs of M5 and C28W exhibit different inhibition efficacy, which emphasizes the relevance of specific residues (e.g. Trp) in the peptide primary sequence. These phenomena will be interpreted in relation to the mechanism by which CBPs appear to affect MSH receptor activity. A model integrating the information gained in this and previous studies concerning the MSH receptor system is presented.

### Materials and Methods

#### Materials

**Peptides.** Melittin, mastoparan, porcine βMSH, and the synthetic analog of αMSH, [Nle⁴,0Phe⁷]αMSH, were obtained from Sigma. A synthetic analog of ACTH-(1-17) [Synchrodyn-(1-17)] was kindly provided by Dr. W. von Rechenberg (Hoechst AG, Frankfurt, Germany). A synthetic 20-amino acid peptide representing part of the calmodulin-binding

#### Table 1. Primary sequences of CBPs used in this study

<table>
<thead>
<tr>
<th>Designation</th>
<th>Peptide sequence</th>
<th>Group</th>
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<tr>
<td>CBPs derived from MLCK</td>
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<td>CBPs derived from Ca²⁺-ATPase</td>
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* An ACTH-(1-17) analog.
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domain of the B. pertussis AC (BPAC) was a present from Dr. D. K. Blumenthal (University of Utah, Salt Lake City, UT). C20W, C20A, and C28Y were provided by Drs. T. Vorherr (Hoffman LaRuche, Basel, Switzerland) and E. Carafoli (ETH, Zurich, Switzerland). GnRH and vasoactive intestinal peptide (VIP) were supplied by Drs. Doron Leibovitz and Matt Fridkin of the Weizmann Institute of Science. C20W, C5, and Tyn25S were synthesized and purified as previously described for M2 (22). The pure peptides were shown to be homogeneous (95-99%) by analytical HPLC and amino acid analysis. Peptide identity was further confirmed by amino acid sequence analysis performed at the core facility of the Biological Services of the Weizmann Institute.

Methods

Cells and cell membranes. Culture of M2R mouse melanoma cells: M2R mouse melanoma cells were cultured in Ham's F-12 and Dulbecco's Modified Eagle's Medium (DMEM, 1:1), supplemented with 10% heat-inactivated horse serum, as previously described (24).

Culture of other cell lines: Baby hamster kidney (BHK), rat osteosarcoma (ROS), Rati, Hela, NIH-3T3, and neuroblastoma × glioma (NG108-15) cells were cultured in DMEM (ROS were cultured in 1:1 Ham's F-12:DMEM supplemented with 10% heat-inactivated fetal calf serum. Neuroblastoma × glioma hybrid cells were additionally supplemented with HAT medium [final concentrations, 10^-4 M hypoxanthine, 4 x 10^-5 M aminopterin, and 1.6 x 10^-5 M thymidine]. BHK were additionally supplemented with penicillin (200 IU/ml) and streptomycin (100 mg/ml). Cells were maintained in a humidified 8% CO2 atmosphere at 37°C and subcultured every 5-7 days.

Preparation of a plasma membrane-enriched fraction from M2R mouse melanoma cells: Plasma membrane fractions were prepared from cultured M2R cells by differential centrifugation, as previously described (24). Cell membrane fractions, essentially melanosome free, in 1 mm bicarbonate buffer (pH 7.0) were divided into aliquots (0.5 ml/tube) and kept frozen in liquid nitrogen until used.

\[ ^{125}\text{I} \text{iodination of MSH:} \]

\[ ^{125}\text{I} \text{iodination of} \ \text{[Nle}^6, \text{OPhe}^7\text{]}\text{MSH was performed by the chloramine-T method, as previously described (6).} \]

\[ ^{125}\text{I} \text{iodination of} \ \text{[Nle}^6, \text{OPhe}^7\text{]}\text{MSH and/or} ^{125}\text{I} \text{MSH to M2R cell membrane preparations:} \]

MSH binding was determined at 30°C in a final volume of 0.1 ml PBS containing 1 mg/ml BSA, 10 \mu g/ml leupeptin, 10 \mu g/ml soybean trypsin inhibitor, 1 mm para-aminobenzamidine, and 50-100 \mu g/ml membrane protein. In hinders where \text{Nle}^6, \text{OPhe}^7\text{]MSH (0-100 \mu M) were added to the incubation medium and kept on ice for 15 min before the binding reaction started. Binding was initiated by the addition of \text{[Nle}^6, \text{OPhe}^7\text{]}\text{MSH and/or} ^{125}\text{I} \text{MSH (10^5 cpm/tube; 1 mm).} \]

The incubation was terminated after 20 min, when binding reached equilibrium, by the addition of 2 ml ice-cold 1 mg/ml BSA in PBS, and tubes were placed on ice. The membrane suspensions were then filtered on nitrocellulose filters, presoaked in 1 mg/ml BSA-PBS, and washed twice with 2 ml of the same buffer, as described previously (24). Filters were counted in an autogamma spectrometer. Specific \text{[Nle}^6, \text{OPhe}^7\text{]}\text{MSH binding was calculated by subtracting the values of nonspecific binding obtained in the presence of excess (1 \mu M) unlabeled \text{MSH, from the total radioactivity bound.} \]

Results

Effect of CBPs on \( ^{125}\text{I} \text{MSH binding} \)

We have previously shown that synthetic \text{MSH inhibits} \( ^{125}\text{I} \text{MSH binding to M2R cell monolayers and membranes derived from them in a dose-dependent manner (10). To test the generality of these observations and the possible relationship of these findings to the CaM-binding properties of these peptides, we first examined the ability of other CBPs (Table 1) to interfere with \( ^{125}\text{I} \text{MSH binding to M2R cell monolayers (Fig. 1A). As can be seen, the inhibitory effect observed for all CBPs tested was dose dependent and restricted for each tested CBP to a narrow concentration range. Specifically, C20W inhibited \text{MSH binding IC}_{50} 2.9 \pm 0.45 (\pm \text{SEM}) \mu M; \text{n = 4} \) in a fashion similar to \text{MSH (IC}_{50} 3.8 \pm 0.85 \mu M; \text{n = 5). Melittin was slightly less effective (IC}_{50} 6.4 \pm 1.1 \mu M; \text{n = 4), and mastoparan (26) was much less effective in this regard (IC}_{50} 67 \pm 12 \mu M; \text{n = 3). In contrast, we found that GnRH, which is not known as a CBP and was used here as a nonrelevant control, was ineffective even at concentrations as high as 100 \mu M (IC}_{50} >100 \mu M).} \]

In experiments with plasma membrane preparations, the tested peptides could, in principle, interact with both cytoplasmic and extracellular faces of the membrane. We, therefore, examined the effect of the CBPs on \( ^{125}\text{I} \text{MSH binding to M2R cell monolayers, where their access to the membrane is restricted to the extracellular face only. Nevertheless, an almost identical pattern of inhibition of} ^{125}\text{I} \text{MSH binding was observed with respect to C20W and M5 compared with the results obtained with cell membranes (Fig. 1B). M5 was slightly less effective (IC}_{50} 6.2 \pm 0.93 \mu M; \text{n = 3) than reported above. VIP, a known CBP (27), had a strong inhibitory effect on MSH binding (IC}_{50} 7.5 \pm 1.3 \mu M; \text{n = 3) at a concentration range about 1000-fold higher than that described for its actions as a hormone in respective target organs (28). Another synthetic CBP tested represents part of a CaM-binding domain of the BPAC (14). The inhibitory effect of this peptide (IC}_{50} 59 \pm 9.5 \mu M; \text{n = 3) assigns it, together with mastoparan, to a second, less effective group of CBPs that inhibit the binding of MSH in the submillimolar range. GnRH, as shown above (Fig. 1A), had no significant effect on MSH binding (Fig. 1B).} \]
Inhibition of \([\text{Nle}^4,\text{D-Phe}^7]\alpha\text{MSH}\) binding to M2R cell membranes by C2OW, C28W, and M5, and their respective analogs C20A, C28Y, Ac-Met-M5, Tyr-M5, and M5AA

Most of the CBPs contain two or three aromatic residues in their primary sequence (one of which is tryptophan; see Table 1). O'Neil et al. (29) pointed out that the tryptophanyl residues may play an important role in the mechanism of interaction between CaM and its target enzymes.

Based on these considerations, we tested whether the tryptophanyl residue in the tested CBPs also plays a significant role in the inhibition of MSH binding. We compared the abilities of the Trp-containing CBPs, representing the CaM-binding domain of the \(\text{Ca}^{2+}\)-pump, C20W and C28W with those of their respective analogs C20A (Trp→Ala) and C28Y (Trp→Tyr) to inhibit \([\text{Nle}^4,\text{D-Phe}^7]\alpha\text{MSH}\) binding to M2R cell membranes. Although we found only a slight decrease in the IC_{50} of C20A (7.5 ± 1.25 (±SEM) \(\mu\text{M}\); \(n = 3\)) compared to that of C20W (3.2 ± 0.56 \(\mu\text{M}\); \(n = 3\)), the Trp→Tyr substitution in C28W reduced the efficacy of this peptide more profoundly by about 8-fold (from 5.5 ± 0.78 SEM \(\mu\text{M}\) (\(n = 3\)) in C28Y to 40 ± 4.6 \(\mu\text{M}\) (\(n = 3\)) in C28Y, respectively; Fig. 2A). This finding is of particular interest, because the substitution in this case is between similar aromatic residues.

Another clue concerning the structural requirements for the inhibitory effect of CBP on MSH receptor binding came from experiments with M5 analogs (Fig. 2B). The addition of acetylated methionine to the N-terminus in Ac-Met-M5 did not affect its inhibitory ability and may even have enhanced it slightly compared to that of M5 (IC_{50}, 1.85 ± 0.37 and 4.1 ± 0.72 \(\mu\text{M}\), respectively; \(n = 3\)). On the other hand, the addition of Tyr to the N-terminus of M5 (Tyr-M5) decreased the inhibitory potential of this analog by about 5-fold (IC_{50}, 19.6 ± 2.7 \(\mu\text{M}\); \(n = 3\)). Most profound were the double Asn→Ala substitutions in positions 7 and 15, M5AA (30), in which the inhibitory effect of this analog on \([\text{Nle}^4,\text{D-Phe}^7]\alpha\text{MSH}\) binding was essentially lost (Fig. 2B).

**Inhibition of MSH-stimulated cAMP production in M2R mouse melanoma cell monolayers by CBPs**

Gerst and Salomon (10) showed that M5 inhibits \(\beta\text{MSH}\) stimulation of cAMP production in intact M2R cells. The effect of M5 on MSH stimulation appears to concur well with the ability of this CBP to inhibit MSH binding, as described above (Figs. 1 and 2) and by Gerst and Salomon (10). On the basis of those observations, we examined the modulatory activity of several CBPs (Table 1) on cAMP production in M2R mouse melanoma cells. We chose C28W and M5 as representative peptides, because other CBPs (C20W, Ac-Met-M5, Tyr-M5, and VIP) gave similar results (data not shown). We found that C28W and M5 (10 \(\mu\text{M}\) each) profoundly inhibited \(\beta\text{MSH}\) stimulation of AC in M2R cell monolayers (89% and 87% inhibition, respectively; Fig. 3A).
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16.0

A

%Conversion

B

CAMP

Basal β-MSH M5 C28W M5 

[3H]cAMP %Conversion

Basal β-MSH PGE1 C28W C28W 

Forskolin: 

FIG. 3. Inhibition of MSH-stimulated cAMP production in cultured M2R cell monolayer by C28W and M5. M2R cell monolayers, prelabeled with [2-3H]adenine for 2 h, were incubated for 20 min at 37°C without (basal) or with the indicated additions. βMSH concentration was 10 nM; all other test substances were used at 10 μM. A, Effects of C28W and M5 on βMSH stimulation. B, Effect of C28W on PGE1 stimulation. [3H]cAMP production was determined as described in Materials and Methods (n = 3–6). Values are the mean of triplicate determinations ± SD.

In contrast, stimulation of cAMP production in these cells by PGE1 was not affected by C28W (Fig. 3B), which is in full agreement with a previous observation made with M5 (10), suggesting that these inhibitory effects of CBPs are confined to the MSH receptor cascade. GnRH, which served as a control, did not inhibit cAMP production stimulated by βMSH in these cells (Fig. 3A). CBPs, on their own, did not stimulate [2-3H]cAMP production above basal levels to any significant extent (Fig. 3).

Stimulation of cAMP production in M2R cell monolayers by CBPs

Preliminary experiments indicated that M5 in the presence of low concentrations of forskolin (1 μM) can act as a partial agonist and stimulate cAMP production in M2R cells (Gerst, J. E., and Y. Salomon, unpublished data). This observation provided us with an additional and independent way to assess the interactions of CBPs with M2R mouse melanoma cells. As shown in Fig. 4, C28W and M5, in combination with forskolin, induced a synergistic and significant stimulation of cAMP levels of about 6-fold over forskolin-stimulated control values. Glucagon, another CBP (27), although a much weaker CBP compared to M5 and C28W, served as a negative control in this assay. It should be noted that the low forskolin concentrations used in these experiments, when added alone (Figs. 4 and 5) caused a very small increase in cAMP levels compared to βMSH (Fig. 3).

Gerst et al. (2) showed that the activation of the AC in M2R cells by MSH, but not by PGE1, is calcium dependent. It was of special interest to test whether the stimulatory effect of the CBPs on cAMP production is also calcium dependent. The activity of most of these CBPs in their native biological role as CaM targets is strictly dependent on the interaction of calcium ions with CaM (31). To our surprise, we found that the stimulation of cAMP production in M2R cells by C28W in the presence of forskolin was not affected by the presence of 5 mM EGTA in the incubation medium (Fig. 5).
It can clearly be seen that under the same conditions, stimulation of cAMP by β-MSH, but not by PGE₁, was almost completely abolished by the addition of EGTA. The same calcium-independent stimulatory effect observed with C28W was also demonstrated for other tested CBPs (M5, C20W, C28Y, C28A, and VIP; data not shown). None of the tested CBPs stimulated cAMP production in M2R cells by itself, which is in agreement with the results using C28W and M5 (Fig. 3). In comparison with the stimulatory effects of M5 and C28W in the presence of forskolin in M2R cells (5- to 6-fold), we found that other CBPs stimulated cAMP production to various degrees (C28Y, 1-fold; C20W, 2- to 3-fold; VIP, 12- to 13-fold above the respective forskolin-stimulated cAMP levels; data not shown). In contrast, M5AA was totally inactive in this regard, in agreement with its low inhibitory effects on MSH binding (Fig. 2B). GnRH, as mentioned above, was ineffective in stimulating cAMP levels in M2R cells in the presence of forskolin (data not shown).

To determine whether the effects of the CBPs on cAMP production are cell specific, i.e., confined to melanoma cells that bear MSH receptors, we tested their ability to raise cAMP levels in other cell types that are not known to have MSH receptors. Results of experiments performed using six arbitrarily selected cell lines are summarized in Table 2. It can be seen that except for two cases, one in HeLa cells, in which VIP plus forskolin stimulated cAMP production, and the second in ROS cells, in which [Nle⁴,nPhe⁷]αMSH plus forskolin stimulated cAMP production, no other effect was elicited by CBPs alone or in combination with forskolin in any of the cell lines tested (Table 2). As will be discussed later, we attribute these stimulatory effects by VIP and MSH to the presence of intrinsic receptors for these hormones in the respective cell types.

Discussion

Using independent experimental approaches, we demonstrated in this study that several CBPs from natural and synthetic sources affect the activity of the MSH receptor and the level of cAMP in M2R mouse melanoma cells. Two major effects of CBPs were described: 1) interference with MSH actions at the binding and AC stimulation levels, and 2) intrinsic stimulatory activity of cAMP levels. All of the cAMP production studies were performed in the presence of saturating isobutylmethylxanthine concentrations, conditions in which the activity of cAMP phosphodiesterase, a CaM-dependent enzyme (32, 33) is largely inhibited. We, therefore, excluded the possible involvement of cAMP phosphodiesterase in the mechanism of action of CBPs.

In spite of considerable variability in primary sequence and chain length, the structural requirements for CBPs in this system resemble certain aspects in the structure-function relationship described for their interaction with CaM (34). For instance, the higher efficacy of C20W and C28W on M2R cells compared to that of their respective Trp-deficient analogs C20A and C28Y demonstrates similarity between the structural requirements for interaction of these peptides with CaM, on the one hand, and with the site mediating their inhibition of MSH binding, on the other. It was reported that the tryptophanyl residues might be important for the interaction between CaM and synthetic enzyme-derived CBPs (35–37), hormonal (27) and cytotoxic peptides such as mastoparan-X (38), and melittin (9). The results reported herein concur well with the above reports and emphasize the importance of the Trp residue in the interaction of CBPs (excluding VIP and mastoparan) with M2R cells. The results shown in Fig. 2B suggest that the positively charged amino acids in M5 are more important for the inhibitory effect than the hydrophobic residues. The loss of activity of MSAA emphasizes the importance of the two asparagine groups in positions 7 and 15, probably as points of attachments between the peptide and its binding site(s) on M2R cells. In addition, the decrease in the activity of Tyr-M5 of about 5-fold relative to that of M5 suggests the importance of the positively charged cluster at the N-terminus modified by the Tyr addition.

The rather steep concentration-dependent inhibition curves obtained with the various CBPs, affording the full range of activity within a 10-fold concentration range, suggest a relatively high degree of specificity. Together, these features suggest that the putative CBP-binding site(s) that mediates the effects in the M2R cells is not likely to result from nonspecific interactions of CBPs with membrane components. Rather, we can cautiously consider its behavior to resemble that of a protein-associated site, even though we have no direct evidence of its exact molecular nature.

Although we do not presently have direct evidence to support this notion, the putative site of interaction may be the MSH receptor molecule itself. Three lines of evidence support this idea. Firstly, CBPs inhibit MSH binding to M2R cells, as shown here and previously (8, 10), and to lacrimal cell membranes that contain high levels of MSH receptor (6). Secondly, CBPs inhibit cAMP stimulation by MSH, but not by PGE₁, in the same cells (10). Thirdly, CBPs demonstrate intrinsic stimulatory activity on cAMP stimulation in M2R cells, but not in a number of MSH receptor-deficient cell types.

This interaction of CBPs with the MSH receptor may take one of the following forms: 1) association of the CBP with
the MSH receptor as a ligand in the classical sense, i.e. with an external component of the receptor. In this case, our data would predict that CBPs and MSH do not compete for the same binding site due to the noncompetitive relationship of the two ligands reported previously (8, 10); and 2) interaction with lipid-embedded regions of the MSH receptor; this option is based on the ability of CBPs to interact with and insert into lipid bilayers, including M2R cell membranes, as recently shown (22). This property, which appears essential for the effects of CBPs on M2R cells, may also include interaction of CBPs with portions of other membrane proteins, including components of the MSH receptor cascade within the lipid milieu, and affect their function. This novel aspect of peptide-cell membrane interactions is of general interest and will have to be explored further in the future. In addition, the possibility that CBPs affect M2R cells by changing ion permeability and membrane potential (22) should be considered. However, the selective effect of these peptides on the response of the cells to MSH vs. PGE, makes it less likely. Considering the two possibilities mentioned above, we suggest that the MSH receptor molecule may contain an intrinsic CBP-binding site. Alternatively, CBPs may compete with the MSH receptor for a CaM-like protein or for other proteins, e.g. G-protein, that are essential for MSH receptor activity. The interaction of mastoparan with G-proteins and the effect on their function in a reconstituted system have been ascribed to its amphiphilic nature and its ability to interact with a portion of the G, subunit that is normally recognized by the receptor molecule (39-42). Consequently, in a situation of cells in culture, as described here, one can visualize how CBPs may demonstrate their inhibitory effects, at the level of MSH binding and stimulation of cAMP production, by interfering with receptor-G-protein interactions. However, the mild nature of the enzyme-derived CBPs induces the above mentioned cellular effects without causing cell lysis, as is often the case for the cytotoxic venom peptides. The phenotypic effect of the CBP will also depend on the type of G-protein involved. For instance, CBPs may reduce the apparent affinity of the receptor for MSH by preventing the normal course of the GTPase cycle (43-45). This effect is expected to interfere with MSH binding and AC stimulation by MSH. The interaction of CBPs with G, may enhance cAMP production and be synergized by forskolin to appreciable levels. This MSH receptor-independent mechanism may explain the intrinsic stimulatory effect of CBPs in M2R cells and be compatible with the calcium-independent mode of this response to CBPs. At this point, it is worth mentioning that the question of whether calcium ions are involved in the inhibition of MSH binding and AC stimulation by CBPs could not be probed due to the essential role of calcium in the MSH-binding process in M2R cells (2).

Direct interaction of CBPs with the MSH receptor may still be viewed in the simple-minded, partial agonist manner, producing a low effector response. Finally, as a partial agonist acting via the MSH receptor, but at a site distinct from the MSH-binding domain, the action of the CBP's may be calcium independent. Under all circumstances, the interactions of MSH and CBP with the MSH receptor, according to our model, are mutually exclusive (Fig. 6). It should be noted that inhibition by melittin and M5 of MSH binding and consequent adenylate cyclase activation was previously determined to be noncompetitive with respect to MSH, using plasma membrane preparations derived from M2R cells (8, 10). Determination of the exact type of inhibition afforded by the various CBPs in intact cells, as reported here, will await additional studies in which MSH concentrations will also be varied. Other circumstances, i.e. the absence of MSH receptor, different lipid compositions, and the identity of G-proteins or CaM-like proteins, may explain why CBPs had no effect on cAMP levels in any of the other cell types tested.

To the best of our knowledge, this is the first demonstration of AC activation in any system by synthetic peptides of this kind. The ability to demonstrate this phenomenon in the presence of forskolin, specifically in M2R cells, may relate to another unique property of these cells. Treatment of M2R cells with MSH and forskolin, as described in this study, stimulates cAMP to phenomenally high levels (>30% conversion). As reported by us previously, nearly 50% of the total adenine nucleotide pool can be consumed (24), depleting ATP levels by about the same extent (46, 47). Consequently, interaction with CBPs, even if they represent very poor partial agonists, eliciting no activity on their own, may be amplified beyond proportion, specifically in these cells. The positive response of HeLa cells to VIP may be ascribed to specific receptors for VIP, as reported recently (48). The cAMP response to MSH in ROS cells can be explained by the low MSH receptor content that has been found by us (data not shown). Yet, no other CBP was found to be active in these or any of the other cells tested in the presence of forskolin.

The relative effectiveness of C28W and C20W, as reported in this study, was basically similar and within the low 2-6 μM concentration range. C20W was about 2-fold more efficient than C28W when acting as an MSH inhibitor, but about 2-fold less efficient than C28W as a cAMP stimulant (data

**Fig. 6.** Activation of the MSH receptor system; involvement of calcium ions, MSH, and CBPs: a model. The MSH receptor (R) contains three putative binding sites: 1) a calcium-binding site, 2) a MSH-binding site, and 3) a CBP-binding site. The inactive MSH receptor binds calcium and subsequently interacts with MSH to give an active complex (MSH receptor-MSH). Upon addition of EGTA (5 mm), the MSH receptor-MSH complex dissociates to give an inactive receptor state. Alternatively, the MSH receptor can bind a CBP molecule in a calcium-independent manner and produce a partially active state.
not shown). A similar ratio, in the same concentration range, was reported (13) for the inhibitory effects of these peptides on the CaM-independent activity of a proteolyzed Ca\(^{2+}\) pump preparation. Here the peptides competed for the endogenous CBP acceptor site of the pump where C28W was about 2-fold more effective than C20W (Kd \(= 3\) and 7 \(\mu\)M, respectively). However, when tested as CaM antagonists in the same system, the peptides acted in the nanomolar concentration range, where C28W was 5-fold more efficient than C20W (Kd \(= 8\) vs. 40 nM, respectively). The dissociation constants for CaM-peptide complexes were also in the nanomolar range, but varied largely between the peptides (Kd of C28W, 0.1 nM; Kd of C20W, 50 nM) (13). Other CBPs have been described to interact with CaM in the nanomolar range (12). In avian MLCK, however, the affinity for CaM is a lot lower (49). The comparison, therefore, of the efficiency of these CBPs is complex and dependent on the type of activity studied and possibly the site and mechanism by which the peptide affords its effect, including the case of M2R cells described here. Furthermore, partition of CBPs into the lipid bilayer of the membrane, which may precede their interaction with membrane proteins, as proposed recently by us (22), will make it even more difficult to accurately estimate and compare such affinities.

The occurrence of CaM-like binding sites in several enzymes has previously been suggested (50). The catalytic domain of MLCK, which is recognized and autoregulated by M5, its C-terminal peptide (11), can, therefore, be considered such an example. C28W, the CaM-binding domain of the CaM-regulated Ca\(^{2+}\) pump, has also been described to bind with a defined internal sequence of this enzyme (13, 51). Likewise, the 4–5 intradiscal loop of visual rhodopsin has been proposed to contain homology with CaM itself (52). Most recently, the involvement of intrinsic CaM-like domains in the autoregulation of plant protein kinases has been reported (53).

Based on the experimental observations described in this study and previous reports (8, 10) and considerations raised in the discussion, we propose a model for MSH receptor activation by calcium ions, MSH, and CBPs (Fig. 6). In this model, it is assumed that the MSH receptor contains separate binding sites for MSH, Ca\(^{2+}\), and CBP. Binding of MSH to the receptor-Ca\(^{2+}\) complex activates the receptor. Chelation of Ca\(^{2+}\) with EGTA will reverse this step (2). On the other hand, upon CBP binding in the absence of calcium, the MSH receptor becomes partially active, even in the absence of MSH. The bound CBPs, acting as weak partial agonists, may synergize with forskolin and stimulate cAMP production in M2R cells.

The recent cloning of the MSH receptor (54–56) will enable future experiments to identify a putative CBP-binding domain that appears to mediate the antagonistic/partial agonistic action of the CBPs in MSH receptor-containing cells.

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