A Synthetic Analog of the Calmodulin-binding Domain of Myosin Light Chain Kinase Inhibits Melanotropin Receptor Function and Activation of Adenylate Cyclase*

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In this study a synthetic analog of the calmodulin-binding domain of myosin light chain kinase, a 17-amino-acid peptide (M5) was used to examine the possible role of calmodulin in melanotropin receptor function. Binding of β-melanocyte-stimulating hormone to its membrane receptor and subsequent stimulation of adenylate cyclase (AC) were found to be specifically inhibited by M5 in a dose-dependent and noncompetitive manner, both in intact M2R melanoma cells and in a plasma membrane preparation derived thereof. Half-maximal inhibition of both hormone binding and melanotropin-sensitive AC activity was shown to occur at approximately 1 μM M5. In contrast, stimulation of AC by prostaglandin E_1, guanosine 5'-O-(3-thio)triphosphate, forskolin, and unstimulated enzyme activity were unaffected by the presence of M5, under the same assay conditions. Furthermore, addition of a molar excess of calmodulin to the AC assay completely abolished the inhibitory effects of the peptide on melanotropin-stimulated AC activity. Other peptides of similar size, which bind to calmodulin with low affinity (e.g. glucagon, somatostatin, and vasoactive intestinal peptide), were shown to be totally ineffective in inhibiting melanotropin-sensitive AC. These findings, along with those shown previously for other antagonists of calmodulin, suggest a role for an M5-binding protein, as of yet unidentified, involved in the regulation of the melanotropin receptor function.

Peptides corresponding to the carboxyl terminus of skeletal muscle myosin light chain kinase (MLCK)[1] have been shown to specifically inhibit activation of this enzyme by calmodulin (1) and are thought to constitute the calmodulin (CaM)-binding domain of MLCK (1, 2). Similarly, synthetic peptides corresponding to this region of MLCK bind calmodulin with

high affinity (3) and may be used as effective antagonists for a wide variety of calmodulin-dependent target enzymes, such as MLCK (1), cyclic nucleotide phosphodiesterase, calmodulin-sensitive brain adenylate cyclase (AC), phosphorylase B kinase, Bordetella pertussis AC, and others.[2] These results indicate that a common recognition site for calmodulin exists among calmodulin-dependent enzymes[2] and that peptides such as M13 (1, 3) and its truncated homologue, M5 (4), may be used in the characterization of calmodulin-dependent enzymes and processes.

Previously, we have shown that both the melanotropin (MSH) receptor and melanotropin-sensitive AC activity in the M2R mouse melanoma cell line (5) are selectively calcium-dependent (6, 7). Calcium acts to modulate the binding affinity of the receptor and thereby regulates calcium-dependent enzyme activation (6, 7). Calcium regulation of melanotropin receptor function is mediated by an unknown mechanism. We have previously addressed this question by examining the effects of calmodulin antagonists of differing chemical nature, e.g. fluphenazine and melittin, on melanotropin receptor function in M2R cell membranes. These antagonists were found to inhibit both melanotropin binding to its receptor and subsequent AC activation (8) at concentrations known to inhibit the activity of purified soluble calmodulin-dependent enzymes, such as cyclic nucleotide phosphodiesterase (9) and MLCK (10). Higher concentrations of both antagonists could inhibit both basal AC activity and that stimulated by prostaglandin E_1 (PGE$_1$), GTPγS, or forskolin. Similar concentrations of melittin were previously shown to inhibit thyrotropin-sensitive AC as well (11). These results indicated that while the rate-limiting step in melanotropin stimulation of AC, that of hormone binding, is highly sensitive to inhibition by these antagonists, various nonspecific effects of these agents could be demonstrated upon the subsequent steps leading to AC activation.

In this study, we examine the effects of M5 on melanotropin receptor function both in intact M2R cells and in plasma membrane preparations. The results indicate that M5 specifically inhibits both the binding of β-MSH and the subsequent stimulation of AC, in a dose-dependent and noncompetitive manner. In contrast to both melittin and fluphenazine, M5 does not inhibit the activity of unstimulated AC, nor enzyme stimulated by PGE$_1$, GTPγS, or forskolin. It appears then, that because of the high selectivity of this peptide in the inhibition of calmodulin-stimulated enzymes, that the melanotropin receptor may be regulated either by calmodulin or a related calcium-binding protein. Calmodulin regulation of melanotropin receptor function may impart the calcium sensitivity previously described by us (6, 7) for the association of the hormone with the receptor.

[1] The abbreviations used are: MLCK, myosin light chain kinase; M5, Lys-Arg-Arg-Trp-Lys-Lys-Asn-Phe-Ile-Ala-Val-Ser-Ala-Ala-Asn-Arg-Phe-Gly-NH$_2$; CaM, calmodulin; MSH, melanocyte-stimulating hormone or melanotropin; AC, adenylate cyclase; PGE$_1$, prostaglandin E$_1$; GTPγS, guanosine 5'-O-(3-thio)triphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

[2] D. K. Blumenthal and E. G. Krebs, personal communication.

EXPERIMENTAL PROCEDURES

Materials

[^{125}I]Iodine (carrier free) was supplied by Amersham (Aylesbury, United Kingdom) and [α-^{32}P]ATP (10–50 Ci/mmol) by Kamag (Dimona, Israel). Native porcine β-MSH (LER-372) was purified and made available to us by Dr. S. Pomerantz (University of Maryland, Baltimore, MD). PGE$_1$ was donated by Dr. J. Pike of UpJohn (Kalamazoo, MI). Purified synthetic M5 peptide (Lys-Arg-Arg-Trp-Lys-Lys-Asn-Phe-Ile-Ala-Val-Ser-Ala-Ala-Asn-Arg-Phe-Gly-NH$_2$) was kindly provided by Drs. P. J. Kennelly and E. Krebs (Howard Hughes Medical Institute at Seattle, University of Washington, Seattle, WA) (4). Partially purified *B. pertussis* adenylate cyclase was kindly provided by Dr. E. Hanski (Department of Hormone Research, The Weizmann Institute of Science, Rehovot, Israel). Highly purified testicular calmodulin was kindly provided by Drs. Y. Shiloach and C. Klee (National Institutes of Health, Bethesda, MD). All other reagents used were of analytic grade.

Culture of M2R Mouse Melanoma Cells

The M2R melanoma cell line was cultured as previously described (5).

Preparation of Plasma Membranes from M2R Cells

The preparation of a plasma membrane-enriched fraction from M2R cells was performed as described previously (5). In brief, confluent M2R cell monolayers were scraped and washed in phosphate-buffered saline, lysed, and homogenized in ice-cold buffer consisting of 1 mM NaHCO$_3$, 1 mM dithiothreitol, 0.2 mM magnesium acetate, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and pancreatic deoxyribonuclease 1 (1500 units/ml of packed cells). Cell membrane fractions, essentially melanosome-free, were obtained following two noncontinuous sucrose gradient centrifugation steps. This method routinely yields a 3–5-fold purification with 50% recovery, using both [^{125}I]iodo-β-MSH binding and β-MSH-stimulated AC activity as plasma membrane markers.

Adenylate Cyclase Activity

AC activity in M2R plasma membranes was determined as previously described using [α-^{32}P]ATP as substrate (5). Assay mixtures contained 25 mM Tris acetate, pH 7.5, 5 mM magnesium acetate, 0.5 mM [α-^{32}P]ATP (1–2 × 10^6 cpm/assay; 40–80 cpm/pmol), 1 mM dithiothreitol, 0.1 mM isobutylmethylxanthine, 5 mM phosphoenolpyruvate, 10 μg/ml phosphoenolypyruvate kinase, 50 μM cAMP, 10 μM GTP, 50 μg/ml bovine serum albumin, and the appropriate additions (including M5) in a final volume of 50 μl. Assays were initiated by the addition of 5–10 μg of membrane protein and were incubated at 30 °C for 20 min. Termination of the assay and chromatographic separation of [α-^{32}P]cAMP were performed as described previously (12). One unit of enzyme is defined as the amount catalyzing the formation of 1 pmol of cAMP/min. Results are expressed as the mean ± standard error of the mean of triplicate determinations. Linear conversion of data was done using standard linear regression analysis.

cAMP Accumulation in Intact M2R Cells

[2-^{3}H]cAMP accumulation in M2R cell monolayers was performed by the [^{3}H]adenine prelabeling method, as previously described (5). Accumulation of [2-^{3}H]cAMP is expressed as a percentage of total [2-^{3}H]adenine uptake, the mean ± S.E. of triplicate determinations.

Preparation of [^{125}I]Iodo-β-MSH

Biologically active [^{125}I]iodo-β-MSH was prepared as described previously (5). The specific radioactivity of [^{125}I]iodo-β-MSH was calculated from a calibration curve in which AC activity in M2R cell membranes was stimulated by the [^{125}I]iodopeptide, relative to AC stimulation obtained with a series of concentrations of authentic β-MSH.

[^{125}I]Iodo-β-MSH Binding Studies

Binding of [^{125}I]Iodo-β-MSH to Plasma Membranes—Binding of [^{125}I]iodo-β-MSH to plasma membranes derived from M2R cell monolayers was carried out for 45 min at 30 °C and terminated by filtration on cellulose acetate filters as previously described (6). Binding was determined in the absence of phosphate using a Tris 25 mM, pH 7.2,

saline (150 mM NaCl) solution containing bovine serum albumin (1 mg/ml), leupeptin (10 μg/ml), and the appropriate additions (including M5) in a final volume of 100 μl. The concentration of free calcium in the binding assays was fixed at 0.5 mM using a calcium-EGTA buffer, unless otherwise described. Binding was initiated by the addition of 50–100 μg of membrane protein to the assay mixture. Assays were terminated by dilution (1:20) in ice-cold phosphate-buffered saline. The membrane suspension was filtered on Millipore EG25 filters (2.5-cm diameter) presoaked in bovine serum albumin (10 mg/ml) and was washed twice with 2 ml of phosphate-buffered saline. Filters were counted in an autogamma spectrometer, and specific binding was determined as described previously (5). Specific binding was calculated in terms of femtomoles of β-MSH bound per milligram of membrane protein and was expressed as a mean ± S.E. of triplicate determinations, unless otherwise indicated. Linear conversion of data was done using standard linear regression analysis.

Dissociation of Bound [^{125}I]Iodo-β-MSH—Dissociation of bound [^{125}I]iodo-β-MSH from its receptor was determined in two steps. First, for hormone binding, M2R plasma membranes were preincubated with the radiolabeled hormone for 45 min under standard assay conditions, as described above. In the second step, various additions (e.g. M5, melittin, or Tris-saline solution) were introduced to the assay mixture, and the amount of residual bound hormone was determined at various times thereafter. Incubation during the second step was carried out at 30 °C. Dilution of the assay mixture due to the various additions was ≦1%, unless otherwise indicated. Termination of the assay and filtration on cellulose acetate filters was as described above for binding.

Binding of [^{125}I]Iodo-β-MSH to Intact M2R Cell Monolayers—Binding of [^{125}I]iodo-β-MSH to intact cells was determined as previously described (5). Binding was expressed as femtomoles of β-MSH bound/100,000 cells, the mean ± S.E. of triplicate determinations.

Free Calcium Concentrations

Free Ca^{2+} ion concentrations were set by appropriate calcium-EGTA buffers as described previously (6).

Protein Determination

Protein was determined according to Bradford (13), using bovine serum albumin as a standard.

RESULTS

Inhibition by M5 of Adenylate Cyclase Activity in M2R Cell Membranes—Adenylate cyclase in a plasma membrane-enriched fraction from M2R cells, when incubated under standard assay conditions, was highly responsive to stimulation by β-MSH, PGE$_1$, and GTPγS (Fig. 1). Upon addition of increasing concentrations of M5 to the assay, dose-dependent inhibition of melanotropin-stimulated AC activity is observed (Fig. 1). Half-maximal inhibition (IC$_{50}$) of β-MSH-stimulated AC activity occurs at 0.93 ± 0.03 μM M5 (n = 3), while complete inhibition is observed at 10 μM M5. In contrast, stimulation of the enzyme by PGE$_1$, GTPγS, forskolin (not shown), and the activity of the unstimulated enzyme (basal) are not affected by the presence of M5 over the concentration range tested. It is apparent then that inhibition of the enzyme is specific to melanotropin stimulation of AC alone. Because of this selective effect it was of interest to examine whether M5 affects AC stimulation by β-MSH by direct competition with the hormone for the MSH-binding domain of the receptor. We, therefore, examined the inhibitory effects of M5 on AC stimulation at increasing concentrations of β-MSH (Fig. 2). It can be seen that inhibition by M5 (1 μM) of the response of AC to MSH was independent of hormone concentration and that M5 inhibits MSH-stimulated AC in a noncompetitive manner. In a series of experiments, it was found that the V_{max} of melanotropin-stimulated AC activity was decreased by 45 ± 4% (n = 3) in the presence of 1 μM M5, while no significant change in the half-maximally effective dose of MSH was observed (60 ± 8 and 47 ± 1 nM in the presence and absence of M5, respectively (n = 3), as determined from
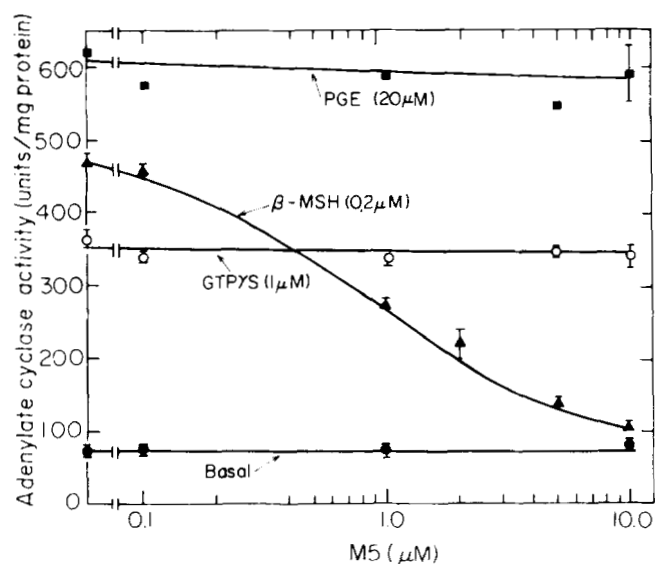
FIG. 1. **Inhibition by M5 of adenylate cyclase activity in M2R cell membranes.** M2R cell membranes (10 μg/assay) were incubated with increasing concentrations of the M5 peptide (0–10 μM) in the presence of β-MSH (0.2 μM), PGE$_1$ (20 μM), GTPγS (1 μM), or in the absence of stimulant (*basal*). All other details were as described under "Experimental Procedures."
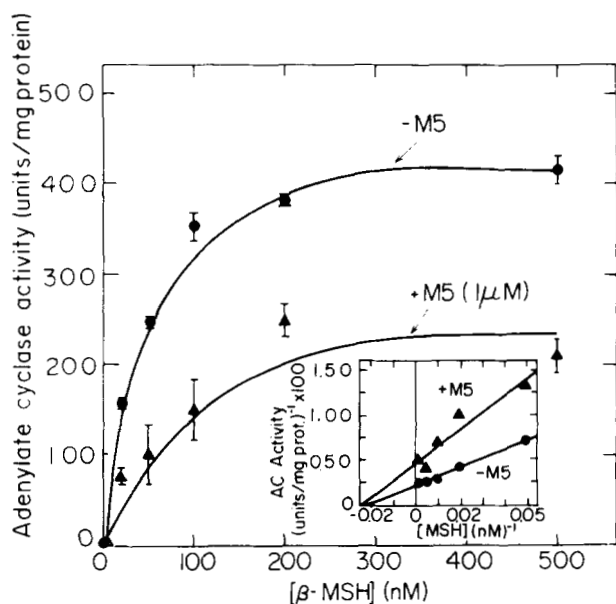

FIG. 2. **Inhibition by M5 of β-MSH-stimulated adenylate cyclase activity.** M2R cell membranes (10 μg/assay) were incubated with increasing concentrations of β-MSH (0–500 nM) in the presence or absence of M5 (1 μM). The *inset* shows the double-reciprocal plot of MSH-stimulated AC activity (as determined from the net increment over basal activity) *versus* the amount of added hormone. The half-maximal concentration of β-MSH required for stimulation of AC in the absence or presence of M5 (1 μM) was 42 and 46 nM, respectively. All other details were as described under "Experimental Procedures."

the net increment over basal activity). Therefore, it appears that M5 does not exert its actions upon melanotropin-stimulated AC activity by occupation of the MSH-binding domain but rather by interacting at a regulatory site distal to hormone binding.

To examine whether the inhibitory effect of M5 is indeed associated with its ability to interact with endogenous CaM in the membranes, we attempted to neutralize its effect on

melanotropin-stimulated AC by the addition of a molar excess of authentic CaM to the assay (Table I). When enzyme activity is measured in the presence of M5 (5 μM) melanotropin-stimulated AC activity is inhibited 87%, while neither PGE$_1$-stimulated AC nor unstimulated AC is significantly affected, similar to that described in Fig. 1. Because M5 presumably interacts with calmodulin at high affinity, we next examined the effect of added exogenous CaM to the AC assay. When measured in the presence of a 2-fold molar excess of purified testicular CaM (10 μM) both hormone-stimulated and basal activities are slightly enhanced (approximately 30%). A similar effect could also be demonstrated for forskolin-stimulated AC (not shown). It appears that CaM augmentation of both intrinsic and agonist-stimulated AC results from a general stimulatory effect upon the catalytic subunit of AC itself. When a molar excess of CaM (10 μM) is added in the presence of M5 (5 μM) no inhibitory effects of M5 on β-MSH-stimulated AC can be observed. Clearly, added exogenous CaM can prevent specific M5 inhibition of the enzyme. Conversely, the presence of M5 and CaM together appears to reduce the ability of CaM to augment catalytic AC activity.

As a comparison, we also have examined the effects of high concentrations of several nonrelated peptides that have been reported (1) to bind to CaM with low affinity (*e.g.* vasoactive intestinal peptide (5 μM), somatostatin (10 μM), and glucagon (10 μM)) and found that none of these peptides could inhibit melanotropin-stimulated AC. Hence, inhibition of melanotropin-stimulated AC activity is specific to particular peptides, such as M5 and melittin (8), that bind to calmodulin with high affinity.

Inhibition by M5 of β-MSH Binding to M2R Cell Membranes—Because the rate-limiting and calcium-sensitive step in the stimulation of AC is that of hormone binding, it was of interest to examine whether the effects of M5 on MSH-stimulated AC activity may result from inhibition of the binding of [^{125}I]iodo-β-MSH to its cell membrane receptor. When M2R cell membranes are incubated with increasing concentrations of M5 (0–10 μM) in the presence of [^{125}I]iodo-β-MSH (5 nM) dose-dependent inhibition of hormone binding is observed (Fig. 3). As can be seen, M5 induces half-maximal inhibition of hormone binding at approximately 1 μM M5 and complete inhibition at 10 μM, similar to that observed for the inhibition of β-MSH-stimulated AC (Fig. 1). In a series of three experiments, the IC$_{50}$ for M5 inhibition of hormone binding was 1.67 ± 0.27 μM M5.

To assess the nature of M5 inhibition of hormone binding, we next examined dose-dependent binding of melanotropin in the presence or absence of 1 μM M5 (Fig. 4). Binding in the presence of M5 (1 μM) results in a 40% decrease in maximal hormone binding, while the apparent binding affinity (K_d) for the hormone remains unchanged (*i.e.* 11 nM β-

TABLE I

Effects of CaM and M5 on adenylate cyclase activity in M2R cell membranes

Basal and agonist-stimulated AC activity were measured in the presence or absence of added calmodulin (10 μM) and/or M5 (5 μM). Enzyme activity was measured using 10 μg of membrane protein/assay, as described under "Experimental Procedures."

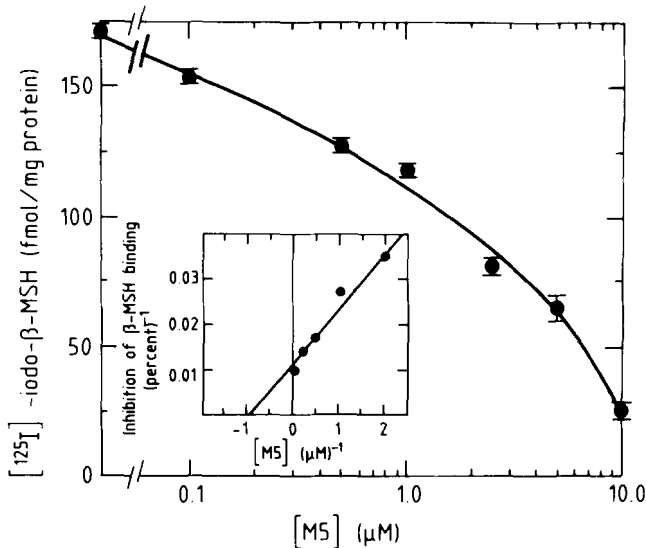| Additions	Adenylate cyclase		
	None	β-MSH (0.2 μM)	PGE$_1$ (20 μM)
	units/mg protein		
None	193 ± 5	597 ± 10	538 ± 2
M5 (5 μM)	209 ± 4	261 ± 2	539 ± 14
CaM (10 μM)	269 ± 6	763 ± 7	684 ± 4
M5 + CaM	251 ± 2	678 ± 9	636 ± 20

FIG. 3. **Dose-dependent inhibition of β-MSH binding to M2R cell membranes by M5.** M2R cell membranes (65 μg/assay) were incubated with [^{125}I]iodo-β-MSH (3 nM) and increasing concentrations of M5 (0–10 μM), under standard binding assay conditions. The *inset* shows the double-reciprocal plot of the inhibition of β-MSH binding *versus* the concentration of added M5. The IC$_{50}$ for M5 inhibition of hormone binding is 1.2 μM. All other details were as described under "Experimental Procedures."
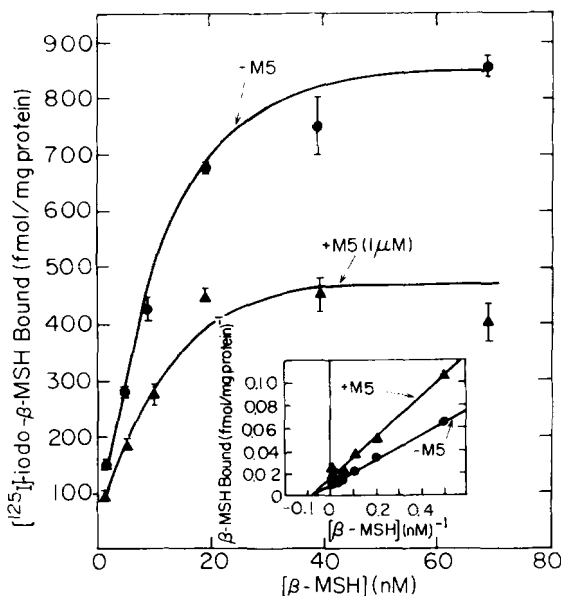

FIG. 4. **Inhibition by M5 of β-MSH binding to M2R cell membranes.** Binding of [^{125}I]iodo-β-MSH to M2R cell membranes (65 μg/assay) was determined in an assay mixture containing increasing concentrations of β-MSH, up to 70 nM, in the presence or absence of M5 (1 μM). The *inset* shows the double-reciprocal plot of β-MSH bound *versus* the amount of added hormone. The binding affinity of receptor for β-MSH was approximately 12 nM in either the absence or presence of M5. All other details were as described under "Experimental Procedures."

MSH and 12 nM β-MSH in the presence or absence of M5, respectively). For a series of similar experiments, it was found that maximal hormone binding is inhibited by 41.5 ± 1.7% (n = 3) by 1 μM M5, while the binding affinity remains unaffected (14 ± 4 and 11 ± 3 nM β-MSH (n = 3) in the presence or absence of M5, respectively). These results are similar to what we have previously observed for both fluphenazine and melittin (8) and indicate that M5 inhibits melanotropin binding

in a noncompetitive manner and does not compete with the hormone for the binding domain of the receptor. This result is in full agreement with the noncompetitive nature of the M5 inhibitory effect on melanotropin-stimulated AC (Fig. 2).

We should like to emphasize that the apparent lower affinity shown for AC activation (Fig. 2), as compared to the binding of β-MSH (Fig. 4), results primarily from the presence of GTP in the AC assay mixture. Guanosine nucleotides, which are essential for the activation of AC, are known to decrease the affinity of various hormones and neurotransmitters to their respective receptors, such as described previously for the melanotropin receptor (6, 7).

Effect of M5 on the Dissociation of β-MSH Receptor-Hormone Complexes in M2R Cell Membranes—We have previously shown that the stability of preformed receptor-hormone complexes is profoundly affected by agents such as EGTA and guanosine nucleotides (6, 7). Similarly, melittin, but not fluphenazine, was found to cause a rapid dissociation of bound hormone (8), indicating that the calmodulin antagonists may have distinct mechanistic differences in their ability to inhibit melanotropin receptor function. To characterize the effects of M5 on the stability of preformed receptor-hormone complexes, we examined the ability of M5 to dissociate β-MSH bound to M2R cell membranes. [^{125}I]Iodo-β-MSH was bound to M2R cell membranes under standard assay conditions and following formation of receptor-hormone complexes, M5 was added to a final concentration of 10 μM. Residual bound hormone was determined up to 30 min thereafter (Fig. 5). As can be seen, M5 (10 μM) is found to have little ability to dissociate preformed receptor-hormone complexes. In contrast, melittin (10 μM) strongly affects the stability of such complexes and causes a rapid dissociation of up to 80% of the bound hormone. Dissociation of bound hormone in the absence of either melittin or M5 was negligible.

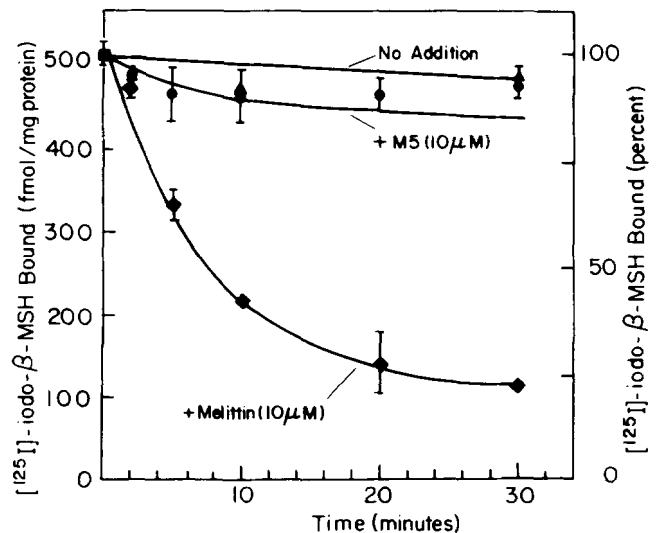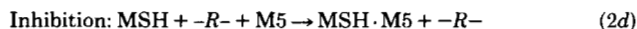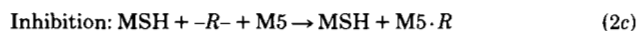*Effect of M5 on cAMP Accumulation and Binding of β-MSH in Intact M2R Cells*—We next determined whether M5 pos-

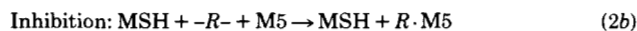FIG. 5. **Time-dependent dissociation of bound β-MSH from M2R cell membranes by M5 and melittin.** [^{125}I]Iodo-β-MSH (13 nM) was preincubated with 1300 μg of M2R membrane protein in 1.3 ml, under standard assay conditions, for each incubation condition. At the end of the preincubation, one set of triplicate samples from each condition was removed to determine the initial amount of bound hormone. Subsequently, either M5 (10 μM final concentration), melittin (10 μM final concentration), or an equivalent volume of tris (25 mM) saline was introduced and samples (100 μg of membrane protein/sample) were removed at various times thereafter to determine the amount of residual bound hormone. All other details were as described under "Experimental Procedures."

sesses the ability to interfere with melanotropin receptor activity in intact cultured M2R cells, as shown in a representative experiment (Fig. 6). [2-^{3}H]Adenine-loaded cells show little intrinsic (basal) accumulation of [2-^{3}H]cAMP ($\leqq$0.3% of the total adenine uptake) either in the presence or absence of M5 (5 μM). In contrast, β-MSH (10 nM) significantly elevates intracellular [2-^{3}H]cAMP levels by more than 35-fold (10.6% of the total adenine uptake), an effect which is almost completely blocked (84 $\pm$ 2% inhibition, $n = 3$) in the presence of 5 μM M5. M5 inhibition of the cellular response to β-MSH is dose-dependent and half-maximal at 1.1 $\pm$ 0.1 μM ($n = 2$), similar to that previously demonstrated for M2R plasma membrane preparations. Conversely, the binding of β-MSH (10 nM) to M2R cell monolayers, when measured in the presence of M5, is similarly inhibited IC$_{50}$ = 2.0 $\pm$ 0.7 μM ($n = 3$). Inhibition of β-MSH binding by M5 (5 μM) was 85 $\pm$ 3% in a series of three experiments.

In contrast, the effect of PGE$_1$ (5 μM) on the intracellular accumulation of cAMP was only slightly inhibited (20 $\pm$ 9%, $n = 3$) by the presence of M5 (5 μM), indicating that M5 inhibition of the cellular response to β-MSH is selective even in the intact melanocyte.

DISCUSSION

Two principal models for receptor activation by MSH and consequent inhibition by M5 may be considered:

Activation: MSH + -R- + $X \rightarrow$ MSH·R·X (1a)

Inhibition: MSH + -R- + X + M5 $\rightarrow$ MSH + -R- + X·M5   (1b)

Activation: MSH + -R- $\rightarrow$ MSH·R (2a)

Inhibition: MSH + -R- + M5 $\rightarrow$ MSH + R·M5   (2b)

Inhibition: MSH + -R- + M5 $\rightarrow$ MSH + M5·R (2c)

Inhibition: MSH + -R- + M5 $\rightarrow$ MSH·M5 + -R-   (2d)
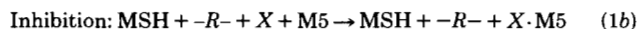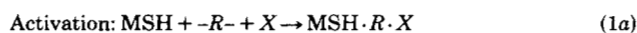
-R-, receptor (R) denoted with an MSH-binding site (left hand side, -R) and a single X (Equation 1) or M5 (Equation 2) binding domain (right hand side, R-). X, a receptor-binding protein required for receptor activation by MSH.

In the first model (Equation 1a), hormone binding and receptor activation require receptor association with a protein

FIG. 6. **Inhibition of β-MSH binding and cAMP accumulation in intact M2R cell monolayers.** For β-MSH binding studies M2R cell monolayers were incubated with [^{125}I]iodo-β-MSH (10 nM) in the presence or absence of M5 (5 μM) for 45 min, under standard assay conditions. For cAMP accumulation studies, M2R cell monolayers, prelabeled with [2-^{3}H]adenine for 3 h, were incubated (20 min) with β-MSH (10 nM), PGE$_1$ (5 μM), or in the absence of stimulant (basal), in the presence or absence of M5 (5 μM). All other details were described under "Experimental Procedures."

X (by itself, capable of binding M5). Binding of M5 to X prevents the association of R and X, and consequently inhibits R (Equation 1b) in a manner described for the inhibition of CaM-regulated enzymes by M5 or M13 (1, 3, 4). This model is compatible with the noncompetitive inhibition by M5 shown for both MSH binding and AC activation (Figs. 2 and 4). It also suggests that a sequence homologous to M5 is expected to be present on the receptor at the X-binding site.

In the second model (Equation 2a), MSH activates the receptor by binding to the MSH-binding domain, but not if R is also occupied by M5 at another site (Equation 2b). This model (Equation 2b), like Equation 1b, is also compatible with noncompetitive inhibition by M5 (Figs. 2 and 4). Models 2c and 2d can be excluded, since they would be strictly compatible with competitive-type inhibition of MSH action by M5. Both models for M5 inhibition of melanotropin receptor function (Equations 1b and 2b) are equally supported by our results and a distinction between them cannot be made as of yet.

Inhibition of β-MSH binding by M5 is phenotypically similar to that described by us previously for other CaM antagonists (melittin and fluphenazine), which also inhibit hormone binding in a noncompetitive manner (8). However, the inhibitory effects of M5 on the responsiveness of AC differ from those seen with melittin and fluphenazine in several respects. First, these antagonists were found to inhibit AC in a differential manner. At low antagonist concentrations, the responsiveness of AC to β-MSH was specifically inhibited, whereas the response of AC to PGE$_1$, GTPγS, and forskolin, as well as basal enzyme activity were unaffected (8). However, at higher concentrations of these antagonists (*i.e.* melittin (>5 μM) and fluphenazine (>100 μM)) AC activity was dose-dependently inhibited in a nonselective manner. In contrast, M5 inhibition was absolutely specific for the response of AC to β-MSH throughout the concentration range tested (Fig. 1), an effect that was not mimicked by unrelated peptides such as glucagon, somatostatin, or vasoactive intestinal peptide. Second, both melittin and fluphenazine were found to inhibit β-MSH-stimulated AC in a competitive manner (8), while inhibition by M5 is clearly noncompetitive in nature (Fig. 2). This difference may result from the interaction of either melittin or fluphenazine at more than one site in the pathway leading to AC activation. In addition, another notable difference between the effects of M5 and melittin is the lack of ability of M5 to induce dissociation of bound hormone (Fig. 5). It seems to us that the essential sites of action for both melittin and M5 are identical, and through alternative sites of association melittin yields its differential effects.

Based on the known amphipathic qualities of both melittin (14) and mastoparans (15) and their strong similarity to M13, in terms of both general structure (although not sequence) and anti-CaM activity (1), M5 may be presumed to act in a similar manner. Because of the lipophilic nature of these peptides both melittin and mastoparans (which are also somewhat effective in this system (data not shown)) may partition into the lipid bilayer of the plasma membrane in order to elicit their effects, some of which may be directly related to their anti-CaM activity. Thus, it was surprising to find that over the concentration range tested, M5 inhibition of AC was specific and confined to the melanotropin-stimulated enzyme, as opposed to melittin, which has a general nonselective inhibitory effect on AC activity at high (>5 μM) concentrations (8). This quantitative difference between the actions of these two peptides may relate to their differential ability to partition into the lipid bilayer, for which no data concerning M5 is presently available.

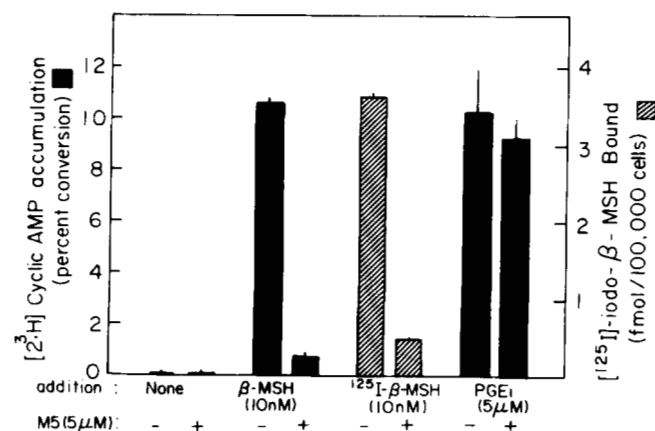Our finding that M5 acts both on intact cells and plasma

M5 Inhibition of Melanotropin Receptor Function

membrane preparations derived thereof, with equal efficacy, requires further consideration. If M5 acts from the exofacial side of the plasma membrane, and is unable to penetrate the bilayer, then it can be expected that the peptide interacts with relevant calmodulin or calmodulin-like protein(s) present on the extracellular surface of the cell. A putative CaM or CaM-like domain, with which M5 may specifically associate, could be an integral component of the melanotropin receptor itself (Equation 2a), a model which offers a basis for the specificity of the effects of M5. A precedent for such a putative CaM-like-binding domain has been suggested for the four to five intra-discal loop of visual rhodopsin (16, 17), a presumed archetype of membrane receptors that associate with a GTP regulatory (G) protein, including the melanotropin receptor. Alternatively, M5 may reach its site of action by penetrating into the membrane, and thus, may interact with low affinity to CaM or CaM-like proteins at sites embedded within the lipid bilayer or at sites associated with the endofacial side of the membrane. Such a model has been recently proposed for the interaction of mastoparan with G-proteins in mast cells (18).

Using the *B. pertussis* AC assay for the determination of CaM (19), we could demonstrate that M2R membrane preparations contain significant quantities of CaM (no less than 157 ± 32 pmol/mg membrane protein ($n = 3$)). Such quantities of CaM exceed melanotropin receptor content by no less than a factor of 75 (5). In the same light, the addition of excess highly purified CaM could overcome the inhibitory effects of M5, suggesting that the activity of the peptide may be related to its ability to bind to CaM, although this result, by itself, cannot prove that the M5-binding site associated with MSH receptor function is identical to CaM (Table I).

Under the assay conditions used, the quantities of endogenous CaM alone are approximately 1.6 and 10 pmol/assay, for the AC and MSH binding assays, respectively. Hence, at M5 concentrations of 1 μM no less than 50–100 pmol of peptide are present in the assay mixture and thus, M5 concentrations cannot be expected to be limiting. Therefore, levels of endogenous CaM may provide only a partial explanation for the higher concentration required for half-maximal inhibition of melanotropin receptor function by M5, as compared to M13 inhibition of highly purified, presumably CaM-free, MLCK (1) (the apparent binding affinities of M13 and M5 for CaM are similar, ≤ 1 nM (4)). Alternatively, the apparent low affinity of M5 could be explained by possible degradation of the peptide during incubation. However, we have ruled out the possibility of extensive proteolysis of M5 in our assays, as prolonged preincubation (up to 45 min) of M5 (2 μM) with M2R cell membranes does not reduce its efficacy (not shown).

A more plausible explanation for the higher concentrations of M5 required for the inhibition of melanotropin receptor activity may relate to the idea that the putative M5-binding site resides on a relevant protein that is not identical with CaM. Conversely such a protein may have a lower intrinsic affinity for M5. An analogous situation has been recently described (20) for the low affinity recognition of an anti-M13 antibody for avian MLCK, implying that CaM-binding domains of CaM-regulated proteins may differ intrinsically. Alternatively, the M5-binding protein, by itself, may be an enzyme that recognizes an M5-like sequence on the MSH receptor, in a manner reminiscent to that described for the recognition of myosin P light chain by MLCK (4). Prelimi-

nary results made using detergent-solubilized preparations of M2R cell membranes indicate that the calcium sensitivity of hormone binding is retained upon detergent extraction (0.5% cholate) (data not shown). This may lend credence to the idea that the melanotropin receptor itself contains either an integral or tightly associated calcium-binding domain essential for its function under physiological conditions.

The melanotropin receptor is the only receptor so far demonstrated to require calcium for the regulation of hormone binding affinity and to be sensitive to various antagonists of CaM. Because of the similarity between the melanotropins and ACTH, and the calcium requirement involved in ACTH receptor function (21), it would be interesting to see if the ACTH receptor is also sensitive to M5.

So far, the unique and unequivocal demonstration that melanotropin binding is both calcium-dependent and sensitive to antagonists of CaM is not proof enough to implicate CaM as the calcium pertinent site(s) involved in melanotropin receptor function. Further studies are still required to link these observations.

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