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Dual Regulation of β-Melanotropin Receptor Function and Adenylate Cyclase by Calcium and Guanosine Nucleotides in the M2R Melanoma Cell Line

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SUMMARY

Binding of β-melanotropin (β-MSH) and subsequent activation of adenylate cyclase in the M2R mouse melanoma cell line is strongly dependent on the concentration of extracellular free calcium. This effect can be demonstrated both in the intact cell and in a plasma membrane preparation derived therefrom, using an EGTA buffer system. In contrast, stimulation of adenylate cyclase by prostaglandin E_1, forskolin, or guanosine 5′-*O*-(2-thiotriphosphate) is calcium insensitive. It is shown that calcium increases the binding affinity of β-MSH for its receptor by a factor of 20 (from 400 nM to 20 nM) without affecting maximal hormone binding. At supersaturating concentrations of β-MSH (>200 nM) binding gradually becomes calcium independent. Hormone-receptor complexes formed in the presence of calcium dissociated rapidly (≤2 min) and reversibly upon the elimination of calcium by excess EGTA. Among nine divalent metal cations tested, calcium was found to be the most effective in facilitating hormone binding. Whereas calcium promotes β-MSH binding, GTP and its stable analogs lead to a reduction in both maximal binding (65%) and affinity (2-fold). These effects are calcium independent, suggesting that the reciprocal control of β-MSH binding by calcium and guanosine nucleotides is mediated by two separate and independent mechanisms.

Calcium ions have been implicated in the actions of both α-MSH and β-MSH (1–4) as well as for ACTH (5–11). Originating from a common precursor protein, pro-opiomelanocortin (12), hormones of this type afford their regulatory functions by controlling adenylate cyclase activity in their respective target cells (5–7, 10, 13, 14). Previously, it has been shown that the activation of both adrenal cortical (5–7, 10) and fat cell (5) adenylate cyclase by ACTH requires extracellular calcium for maximal response. Likewise, an MSH-induced elevation of cAMP levels in melanocytes is thought to be calcium dependent, although the specific step(s) that require(s) calcium in the sequence of events that lead to hormonal activation of AC have not been adequately elucidated. Several reports have implied that extracellular calcium may be important for the interaction between hormone and receptor (3, 4, 11, 15). In a recent paper (11), ACTH binding, and subsequent corticosteroid synthesis, in rat adrenal cortical cells was demonstrated to require mM concentrations of extracellular calcium. A similar requirement of extracellular calcium has been described for the photoaffinity labeling of *Xenopus* melanophores using photoreactive α-MSH (3, 4). However, in these studies direct activation of AC was not determined; therefore, it was of importance to examine the calcium sensitivity of melanotropic hormone action at levels preceding and concurrent to AC stimulation.

Previously, we have demonstrated the specificity of the β-MSH receptor and β-MSH-sensitive AC in the M2R mouse melanoma cell line and in plasma membranes derived therefrom (16). In this study we demonstrate for the first time that the activation of mouse melanoma β-MSH-sensitive AC depends on two classes of calcium-pertinent sites which saturate at μM concentrations of calcium. Furthermore, using [^{125}I]-iodo-β-MSH, we clearly demonstrate that β-MSH binding to its receptor shows a similar requirement for calcium, thus forming the basis for the calcium-dependent action of β-MSH. Post-receptor events leading to the stimulation of AC were found to be calcium insensitive; thus, the role of calcium in the stimulation of AC by β-MSH is suggested to be confined to the regulation of hormone binding. This role of calcium contrasts the regulatory effects of guanosine nucleotides which closely resemble those described previously for other hormones and

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ABBREVIATIONS: α-MSH, α-melanotropin or α-melanocyte-stimulating hormone; β-MSH, β-melanotropin or β-melanocyte-stimulating hormone; AC, adenylate cyclase; ACTH, adrenocorticotropic hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)*N*,*N*′-tetraacetic acid; G_s, stimulatory guanosine nucleotide-binding protein; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GppNHp, guanosine 5′-(β,γ-imido)triphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); PBS, phosphate-buffered saline; PGE_1, prostaglandin E_1.

neurotransmitters (17). The unique interrelationship between the MSH receptor and these two regulatory agents is examined.

Experimental Procedures

Materials. ^{125}I-Iodine (carrier free) and [α-^{32}P]ATP (10–50 Ci/mmol) were supplied by Amersham. [2-^{3}H]Adenine (10.8 Ci/mmol) was supplied by Kamag, Israel. Native porcine β-MSH (LER-372) was purified and made available to us by Dr. Seymour Pomerantz. (This β-MSH preparation was obtained by Dr. Pomerantz from Dr. A. Lerner.) PGE_1 was donated by Dr. J. Pike of Upjohn. All other reagents were of analytical grade.

Culture of M2R mouse melanoma cells. The M2R melanoma cell line was obtained from Dr. J. Mather. Cells were cultured in a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium (DME) (Gibco) (18) supplemented with 10% horse serum (Biolab) in a humidified 8% CO_2 atmosphere at 37°. Cells were subcultured and plated as previously described (16).

Preparation of plasma membranes from M2R cells. The preparation of a plasma membrane-enriched fraction from M2R cells was performed as described previously (16). In brief, confluent M2R cell monolayers were scraped and washed in PBS, and further 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, as assayed by [^{125}I]-iodo-β-MSH binding or β-MSH-stimulated AC activity.

AC activity. AC activity in M2R plasma membranes was determined as previously described using [α-^{32}P]ATP as substrate (16). Assays were initiated by the addition of 5–16 μg of membrane protein and were incubated at 30° for 20 min. Termination of the assay and chromatographic separation of [α-^{32}P]cAMP were performed as described previously (19). One unit of enzyme was defined as the amount catalyzing the formation of 1 pmol of cAMP/min. Results were expressed as the mean ± standard error of triplicate determinations.

[2-^{3}H]cAMP accumulation in M2R cells. [2-^{3}H]cAMP accumulation in cell monolayers was performed by using the [^{3}H]adenine prelabeling method as previously described (16, 19). After 2 hr of prelabeling with [2-^{3}H]adenine (1–5 μCi/ml), cells were washed and further incubated (20 min, 37°) in culture medium (serum free) supplemented with 0.1 mM isobutylmethylxanthine, 0.1 mg/ml bovine serum albumin, and appropriate additions. Termination of the assay and chromatographic separation of [2-^{3}H]cAMP were performed as described previously (16). Accumulation of [2-^{3}H]cAMP was expressed as the percentage of total [2-^{3}H]adenine uptake, the mean ± standard error of triplicate determinations.

Preparation of [^{125}I]-iodo-β-MSH. Biologically active [^{125}I]-iodo-β-MSH was prepared as described previously (16). The specific radioactivity of [^{125}I]-iodo-β-MSH was calculated from a calibration curve in which AC was stimulated by the iodopeptide, relative to the stimulation obtained with a series of concentrations of authentic β-MSH.

[^{125}I]-iodo-β-MSH binding studies. *Intact cells.* Binding of [^{125}I]-iodo-β-MSH to intact M2R cell monolayers was carried out for 45 min at 37°, as described previously (16). Nonspecific binding was determined by including excess (1 μM) unlabeled β-MSH in the incubation medium. Specific binding was obtained by subtracting nonspecific binding values from the total bound radioactivity and was expressed as the mean ± standard error of triplicate determinations. Cell number was determined by counting in a hemocytometer monolayers disintegrated by 0.05% trypsin treatment.

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° and terminated by centrifugation as described previously (16) or by filtration on cellulose acetate filters. Binding was initiated by the addition of 30–100 μg of membrane protein to the assay mixture. Assays were terminated by dilution (1:20) in ice-cold PBS lacking either calcium or magnesium (PBS^-). The membrane suspension was filtered on Millipore EG25 filters (2.5 cm diameter) presoaked in bovine serum albumin (10 mg/ml) and was washed once with 3 ml of PBS^-. Filters were counted in an autogamma spectrometer and specific binding was determined as described previously (16). Specific binding was calculated in terms of fmol of β-MSH bound/mg of membrane protein and was expressed as a mean ± standard error of triplicate determinations, unless otherwise indicated.

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, M2R plasma membranes were preincubated with the radiolabeled hormone for 45 min, as described above for binding studies. In the second step, various additions (e.g., GTP, EGTA, and PBS) 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°. Dilution of the assay mixture after the various additions was ≤1%, unless otherwise indicated. Termination of the assay and filtration on cellulose acetate filters was as described above for binding studies.

Protein determination. Protein was determined according to the method of Bradford (20), using bovine serum albumin (Sigma) as a standard.

Free Ca^{2+} ion concentrations. Free Ca^{2+} ion concentrations were set by appropriate Ca-EGTA buffers as described by Bartfai (21) and by Tsien and Rink (22). Free calcium concentrations used in AC assays were calculated with respect to both ATP, Mg-ATP, and Ca-ATP concentrations (21). For binding and cAMP accumulation studies, free calcium concentrations were calculated with the aid of a computer program [constants derived from Tsien and Rink, (22)], kindly made available to us by Dr. T. Abrams.

Results

Calcium ion-dependent stimulation of cAMP accumulation by β-MSH in M2R melanoma cells. β-MSH (0.1 μM) strongly stimulates cAMP production in cultured M2R mouse melanoma cells grown in DME-F12 culture medium. This cellular response to the hormone was completely dependent on the concentration of extracellular free calcium (Fig. 1). At calcium concentrations ≤50 nM there was essentially no cellular response to the addition of β-MSH. Upon a gradual increase in extracellular free calcium (achieved by calculated reduction in EGTA concentrations), a dose-dependent increase in β-MSH-dependent intracellular accumulation of cAMP was observed. This calcium dependency for β-MSH action was observed at two ranges of free calcium concentrations: one saturated below 10 μM and a second saturated above 100 μM free calcium. In contrast to these findings, no such calcium dependency was seen when cAMP accumulation was determined in response to stimulation with PGE_1 (10 μM) or in the absence of stimulant (basal).

Assuming that the two hormones operate through different receptor systems but utilize a common GTP-regulatory protein and AC pool, it was postulated that the clear difference in the calcium ion dependency between β-MSH and PGE_1 must reside at a step preceding G_S protein activation.

Calcium-dependent binding of [^{125}I]-iodo-β-MSH to M2R melanoma cells. In an attempt to identify the basis for this calcium-dependent action of β-MSH, we have prepared biologically active [^{125}I]-iodo-β-MSH. Using such iodinated β-MSH, it was found that hormone binding to cultured M2R cells shows a strong correlation with ambient free Ca^{2+} ion concentrations (as shown in a representative experiment, Fig. 2). Furthermore, the calcium concentration dependency of [^{125}I]-

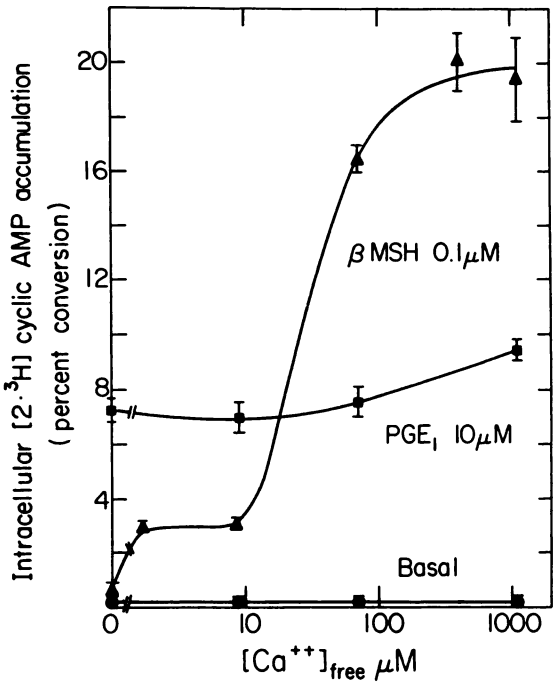

Fig. 1. Calcium-dependent β-MSH stimulation of cAMP production in M2R cell monolayers. M2R cell monolayers, prelabeled with [2-^{3}H] adenine, were incubated in the absence (basal) or presence of β-MSH (0.1 μM) or PGE_1 (10 μM), and cAMP accumulation was determined at various extracellular free calcium concentrations. Free calcium ion concentrations (≤50 nM–1.05 mM) were varied by the addition of decreasing concentrations of EGTA-Tris (pH 7.5) buffer (2.0–0 mM), respectively. Cell number was 1.9×10^5 cells/well. All other details were described under Experimental Procedures.

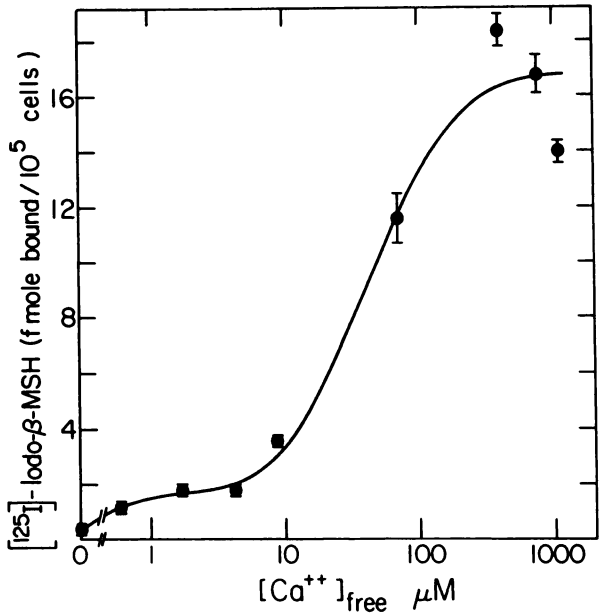

Fig. 2. Calcium-dependent binding of β-MSH to M2R cell monolayers. M2R cell monolayers were incubated with [^{125}I]-iodo-β-MSH (20 nM) at varying concentrations of free calcium, set by varying EGTA concentrations as described in the legend to Fig. 1. [^{125}I]-iodo-β-MSH binding was determined as described under Experimental Procedures.

iodo-β-MSH binding is essentially identical to that observed for the stimulation of cAMP production in these cells. Two separate ranges of calcium concentrations that facilitate hormone binding have been observed: one saturated below 10 μM free calcium and one saturated above 100 μM free calcium. The results described thus far strongly imply that calcium ions regulate β-MSH stimulation of AC by modulating the interaction of β-MSH with its receptor.

In these experiments extracellular free calcium ion concentrations were manipulated by using Ca-EGTA buffers. However, the membrane of the intact cell may pose a barrier to the intracellular equilibration of buffered calcium concentrations. Furthermore, in the intact cell it may be difficult to measure the direct response of AC by various receptor-independent stimulants. Consequently, it was of interest to examine the phenomenon of calcium-dependent β-MSH action under cell-free conditions which permit free access of calcium to both faces of the plasma membrane.

Effect of calcium on the activation of AC in M2R cell membranes by β-MSH. AC in a plasma membrane-enriched fraction from M2R cells, when incubated under the standard assay conditions, was highly responsive to β-MSH, PGE_1, forskolin, and GTPγS, as shown by us previously (16). In order to test the effect of calcium on the stimulation of AC by these stimulants, we performed the assay in either the absence (≤5 nM) or presence (10 μM) of added calcium (Table 1). The results indicate that the addition of calcium increased the response of AC to β-MSH by a factor of 3. In contrast, no stimulatory effect on the response of AC to PGE_1 was evident. Likewise, the addition of calcium did not augment the response of AC to either forskolin or GTPγS and did not affect the activity of unstimulated enzyme. The slight inhibition of enzyme activity, observed in the presence of calcium, is probably due to a general inhibitory effect of this cation on the activity of AC. In order to assess the effect of calcium on β-MSH-dependent stimulation of the enzyme, we next varied β-MSH concentrations in the assay (0–3000 nM) and determined AC activity in the absence or presence of 50 μM free Ca^{2+} (Fig. 3). As can be seen from the results of this experiment, calcium appears to increase the affinity of β-MSH to its receptor by shifting the apparent half-maximally effective concentration of the hormone by a factor of 6, from 370 nM to 60 nM in a representative experiment (Fig. 3). For a series of experiments the half-maximal concentration of β-MSH required for AC stimulation in the absence or presence of calcium is shifted from 369 ± 8 nM to 75 ± 6 nM ($n = 3$), respectively (calculated from the net increment only). Interestingly, calcium seems to affect AC stimulation by β-MSH with an optimum attained at approximately 200 μM. Thus, calcium-dependent activation of the β-MSH-sensitive

TABLE 1

The effect of calcium on AC activity in M2R cell membranes

M2R plasma membranes were incubated with various stimulants (e.g., β-MSH, PGE_1, forskolin, and GTPγS) in the presence or absence of calcium. The assay mixture included 0.1 mM EGTA-Tris, pH 7.5, and either calcium chloride, to yield a final free calcium concentration of 10 μM, or H_2O, to yield a free calcium concentration of ≤5 nM. The assay was initiated with the addition of membrane protein (3 μg/assay). All other details were as described under Experimental Procedures.

Stimulant	AC activity				Net Change
	$[Ca^{2+}] \leq 5$ nM		$[Ca^{2+}] = 10$ μM		
	Total	Net	Total	Net	
	units/mg of protein				%
None	204 ± 5	0	193 ± 13	0	
β-MSH (0.2 μM)	336 ± 16	132	562 ± 5	369	+280
PGE_1 (1 μM)	547 ± 1	343	455 ± 12	262	−24
Forskolin (1 μM)	1041 ± 24	837	974 ± 44	781	−7
GTPγS (1 μM)	605 ± 3	401	537 ± 2	344	−14

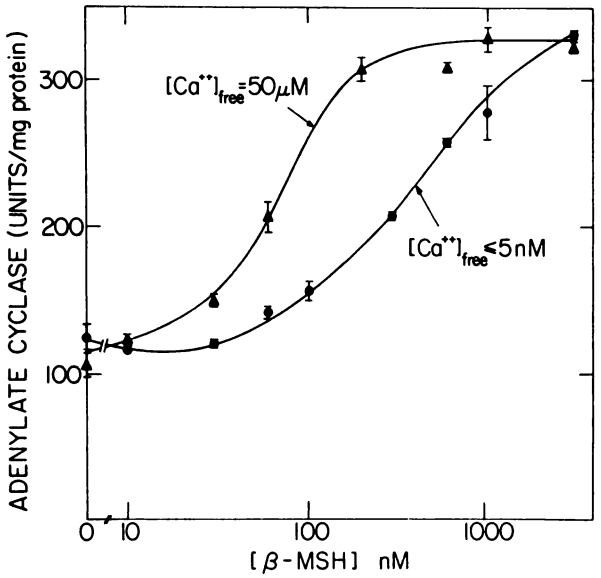

Fig. 3. Calcium-dependent activation of AC in M2R plasma membranes by β-MSH. M2R plasma membranes were washed by centrifugation once in a 6 mM EGTA-Tris (pH 7.5), 45 mM Tris-acetate buffer (pH 7.5) and three times in 45 mM Tris buffer (pH 7.5) alone. AC activity (16 μg of membrane protein/assay) was determined in an assay cocktail containing 0.1 mM EGTA with varying concentrations of β-MSH (0–3000 nM) in the presence or absence of 50 μM free calcium. All other details were as described under Experimental Procedures.

AC is only apparent below subsaturating concentrations of β-MSH.

β-MSH-stimulated cAMP production in the intact cell showed a biphasic dependency on calcium ion concentrations (Fig. 1). However, using the M2R plasma membrane preparation, it was not possible to examine enzyme stimulation at calcium concentrations higher than 50 μM since any further increment in calcium caused a marked inhibition of AC activity (data not shown).

Effect of calcium on β-MSH binding to M2R cell membrane preparations. After demonstrating the calcium dependence specific to β-MSH stimulation of AC, it was now of interest to examine the effect of calcium on β-MSH binding to M2R plasma membranes. Binding of [^{125}I]-iodo-β-MSH, at varying hormone concentrations (5–750 nM), was performed in the presence or absence of calcium (100 μM) (Fig. 4). The results of dose-dependent β-MSH binding under these conditions indicate that calcium raises the apparent binding affinity of β-MSH by a factor of 23 (shifting the K_d from 475 nM to 21 nM in a representative experiment, Fig. 4). However, maximal binding of β-MSH was essentially identical under both incubation conditions (1130 and 1065 fmol/mg of protein, respectively, in the presence and absence of calcium). In similar experiments the apparent binding affinity of β-MSH was shifted from 418 ± 40 nM (n = 3) in the absence of calcium to 23 ± 3 (n = 7) in its presence.

This experiment shows that the primary role of calcium is to modulate the affinity of the receptor-hormone complex. However, β-MSH binding becomes calcium independent at hormone concentrations in excess of 100 nM. At this high hormone concentration range the receptor becomes fully saturated, even in the absence of calcium.

In the previous experiment (Fig. 3), the effect of calcium on stimulation of AC by MSH showed only a 6-fold shift in the apparent affinity. The difference in magnitude of the calcium-dependent effect between these two experiments (Figs. 3 and 4) appears to result from the presence of GTP in the AC assay. Guanosine nucleotides are generally known to decrease the affinity of certain hormones and neurotransmitters to their specific receptors (17). We therefore examined the effect of added GTP on β-MSH binding in the presence of calcium (Fig. 4). As can be seen, the addition of GTP (25 μM) shifted the apparent binding affinity of β-MSH by a factor of 2.1, from 23 ± 3 nM (n = 7) to 49 ± 4 nM (n = 3), and lowered the maximal binding of β-MSH by 63 ± 4% (n = 3). When compared, the apparent affinities obtained for AC activation (Fig. 3) and β-MSH binding in the presence of GTP were found to be 75 ± 6 nM (n = 3) and 49 ± 4 nM (n = 3), respectively.

Metal ion specificity of [^{125}I]-iodo-β-MSH binding. To test the specificity of the metal ion requirement involved in β-MSH binding, we next compared binding of the hormone to its receptor with various divalent metal cations substituting for calcium (Table 2). The assay mixture included 0.5 mM EGTA-Tris, pH 7.5, and a 1.5 mM concentration of the respective metal cation, yielding a minimum free metal cation concentration of 1 mM. In addition, this experiment was performed at a β-MSH concentration (10 nM) which displayed strong calcium-dependent binding, as shown previously (Fig. 4).

It can be seen that calcium best supports β-MSH binding. The order of effectivity of the various cations tested was: Ca^{2+} > Sr^{2+} ≥ Ni^{2+} > Ba^{2+} > Mn^{2+} ≥ Cd^{2+} > Co^{2+} > Cu^{2+} > Mg^{2+} ≥ no ion added. Binding in the absence of added metal cations represented approximately 5% of the calcium-induced binding. Both Sr^{2+} and Ni^{2+} are adequate substitutes for calcium under these binding conditions; however, physiologically, neither metal is present in sufficient concentrations to support binding. It is therefore suggested that, among this group of divalent

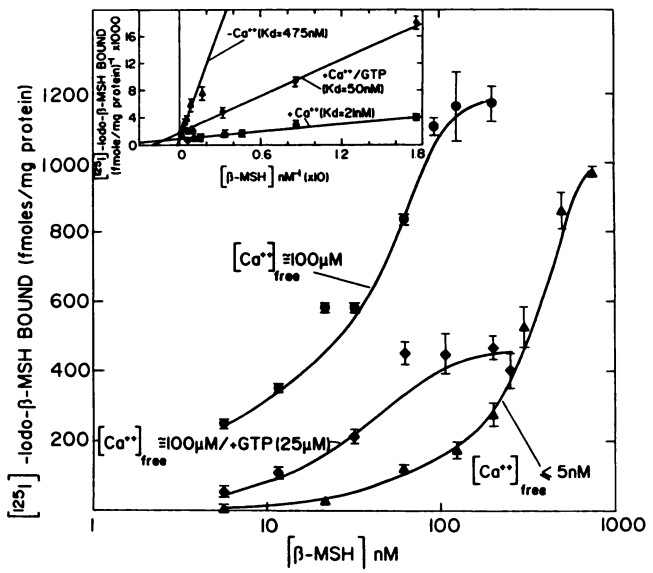

Fig. 4. Calcium-dependent binding of β-MSH to M2R plasma membranes. Binding of [^{125}I]-iodo-β-MSH was determined in an assay mixture cocktail containing 0.5 mM EGTA-Tris (pH 7.5) with varying concentrations of β-MSH (5–750 nM) in the presence or absence of 100 μM free calcium, using 50 μg of plasma membrane protein/assay. In the presence of calcium (100 μM), binding was also determined with GTP (25 μM). The *inset* shows the double reciprocal plots of β-MSH bound versus the concentration of β-MSH used. All other details were as described under Experimental Procedures.

metal cations, calcium alone best fulfills the cationic requirement for β-MSH binding.

Reversability of [^{125}I]-iodo-β-MSH binding. It was shown above that calcium modulates the apparent binding affinity of β-MSH to its receptor (Fig. 4). To test whether calcium is also required for maintaining the stability of the receptor-hormone complex, β-MSH (25–350 nM) was prebound (40 min) to M2R plasma membranes under standard assay conditions in the presence of calcium (1 mM). Excess EGTA (10 mM) was then added to the assay to reduce free calcium concentrations to ≤15 nM, and the amount of residual bound hormone was then determined at various times thereafter (Fig. 5). It can be seen that, at all hormone concentrations tested, prebound β-MSH is maximally dissociated within 2–3 min after the addition of EGTA. At subsaturating concentrations of β-MSH (≤75 nM), no apparent rebinding occurs, as expected from the absolute calcium dependency seen for hormone binding at these concentrations in Fig. 4. In contrast, at higher concentrations of β-MSH (≥150 nM), rebinding of the hormone in the absence of calcium is evident in a time- and concentration-dependent manner, as previously described in Fig. 4. This receptor-hormone complex formed in the absence of calcium was insensitive to further addition of EGTA (not shown). Upon addition of calcium (at 30 min) to the assay mixtures, in order to reestablish the free calcium concentration at 1 mM, a rapid increase in β-MSH binding was seen at hormone concentrations <350 nM. Hormone binding upon the reestablishment of the initial free calcium concentrations equilibrated in a time-dependent manner and attained levels similar to those seen prior to the addition of EGTA within 30 min.

These experiments (Figs. 4 and 5) clearly demonstrate the existence of at least two separate receptor-hormone complexes that are formed upon β-MSH binding. The first complex represents the calcium-dependent high affinity state formed in the presence of calcium, whereas the second complex represents a calcium-independent low affinity state formed in the absence of calcium. In addition, binding of the hormone appears to be freely reversible between these states upon a shift from calcium-containing to calcium-depleted conditions and vice versa.

Guanosine nucleotide effects on the binding of β-MSH. In light of the distinct relationship between the β-MSH receptor-hormone complex and both calcium and GTP (Fig. 4), we further investigated the effect of guanosine nucleotides on hormone binding and dissociation.

In agreement with the results obtained with GTP (Fig. 4), we also observed that GppNHp (10 μM) lowered the binding affinity of β-MSH by a factor of 2.4 ± 0.1 (n = 3).

The inhibitory effect of guanosine nucleotides on β-MSH binding was found to be concentration dependent (Fig. 6). In this experiment M2R membranes were incubated with [^{125}I]-iodo-β-MSH (5 nM) in the presence of GTP concentrations varying from 0.01 μM to 75 μM. The concentration of GTP required for half-maximal inhibition of β-MSH binding was 0.77 μM in this representative experiment and was 0.9 ± 0.2 μM in three experiments.

We next examined the effects of various guanosine nucleotides on the stability of preformed receptor-hormone complex. As can be seen (Fig. 7), saturating concentrations of GTP (25 μM) enhance the dissociation of bound β-MSH in a time-dependent manner. The half-life of the receptor-hormone complex was 3.5 min in the presence of GTP, whereas dissociation of the complex was negligible in its absence. The extent of dissociation did not exceed 70% at the end of measurement (15 min). Under similar conditions, we tested for the ability of other guanosine nucleotides to dissociate bound β-MSH (Table

TABLE 2

Selectivity of metal cation-dependent binding of β-MSH to M2R cell membranes

Incubation of M2R plasma membranes was performed in the presence of 0.5 mM EGTA-Tris, pH 7.5, and 1.5 mM concentrations of various metal ions. [^{125}I]-iodo-β-MSH (10 nM) was added and binding was determined under standard assay conditions for 45 min using 45 μg of plasma membrane protein/assay. All other details were as described under Experimental Procedures.

Metal ion additions	[^{125}I]-iodo-β-MSH bound	Percentage of total binding
	fmol/mg of protein	
$CaCl_2$	393 ± 15	100
$SrCl_2$	293 ± 22	75
$NiCl_2$	292 ± 8	75
$BaCl_2$	220 ± 15	56
$Mn(C_2H_3O_2)_2$	167 ± 11	43
$CdCl_2$	148 ± 37	38
$Co(C_2H_3O_2)_2$	117 ± 12	30
$Cu(C_2H_3O_2)_2$	82 ± 8	21
$Mg(C_2H_3O_2)_2$	25 ± 5	6
None	19 ± 7	5

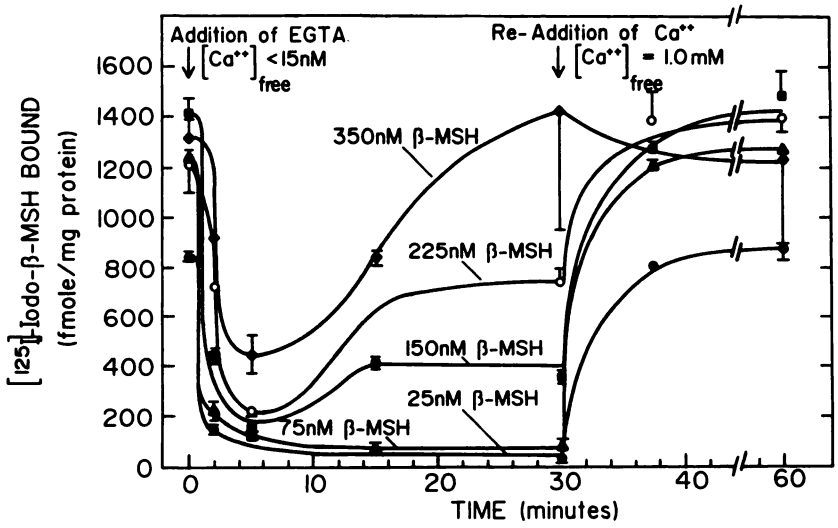

Fig. 5. Reversibility of [^{125}I]-iodo-β-MSH binding to M2R cell membranes. [^{125}I]-iodo-β-MSH (25–350 nM) was incubated with 1600 μg of M2R membrane protein in 1.6 ml under standard assay conditions in the presence of 0.5 mM EGTA-Tris (pH 7.5) and 1.5 mM calcium. At the end of incubation one set of triplicate samples from each incubation condition was removed to determine the initial amount of bound hormone. EGTA-Tris buffer (0.1 M), pH 7.5 (150 μl), was then added to the remainder to yield a final EGTA concentration of 10 mM. Triplicate samples of 100 μg of membrane protein each were then removed at the indicated times to determine the amount of residual [^{125}I]-iodo-β-MSH bound. Subsequently, $CaCl_2$ was added to restore the free calcium concentration at 1 mM and duplicate samples were taken at the indicated times for an additional 30 min. All samples taken contained 100 μg of membrane protein. All other details were as described in Experimental Procedures.

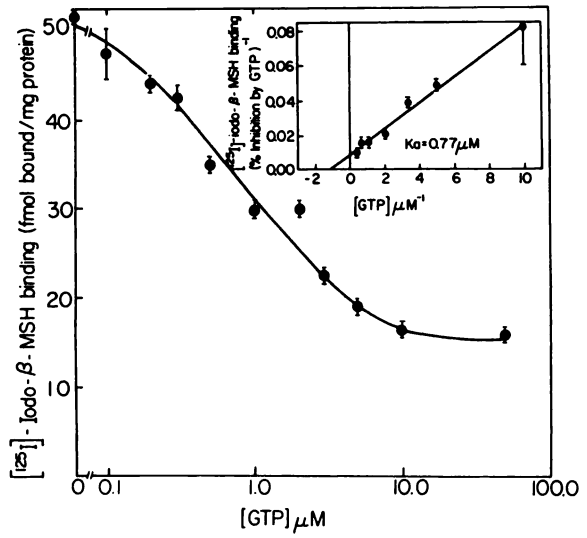

Fig. 6. Inhibition of [^{125}I]-iodo-β-MSH binding by GTP. [^{125}I]-iodo-β-MSH (5 nM) was incubated with 50 μg of M2R plasma membrane protein under standard binding conditions, in the absence or presence of GTP (0.01–75 μM). The GTP concentration required for half-maximal inhibition was calculated from the double reciprocal plot of percentage inhibition versus GTP concentration (*inset*). All other details were as described under Experimental Procedures.

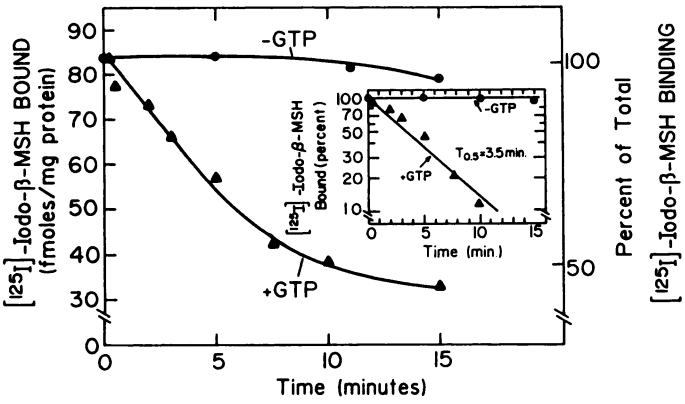

Fig. 7. Time-dependent GTP-induced dissociation of bound [^{125}I]-iodo-β-MSH. [^{125}I]-iodo-β-MSH (25 nM) was incubated with 650 μg of M2R cell membrane protein in 1.3 ml under standard assay conditions, in the presence of 0.5 mM EGTA-Tris (pH 7.5) and 1.5 mM calcium. At the end of incubation one set of triplicate samples from each system was removed to determine the initial amount of bound hormone. Subsequently, either GTP (25 μM final concentration) or an equivalent volume of PBS was introduced and samples were removed at the indicated times to determine the amount of residual bound hormone. Each sample contained 50 μg of membrane protein. The half-life ($T_{0.5}$) of the receptor-hormone complex was determined from a semilogarithmic plot of the percentage of residual bound hormone versus time (*inset*). The 100% value of β-MSH bound represents the total amount of hormone which can be dissociated by GTP. All other details are as described under Experimental Procedures.

3). As can be seen, both GTPγS and GDPβS were similarly effective in inducing the dissociation of bound β-MSH both in the time and extent of dissociation.

It was now of importance to determine whether GTP-induced dissociation of the receptor-MSH complex is also calcium sensitive. Therefore, we compared the ability of GTP to dissociate β-MSH prebound either in calcium-containing or calcium-depleted conditions. M2R membranes were preincubated with saturating concentrations of β-MSH in either the presence (100 μM) or absence (≤5 nM) of calcium for 45 min (Fig. 8). At the

TABLE 3

Effect of guanosine nucleotides on β-MSH binding

[^{125}I]-iodo-β-MSH (10–25 nM) was prebound to M2R plasma membranes under standard assay conditions. Subsequently, GTP, GTPγS, GDPβS (25 μM), or PBS$^-$ (control) was added and residual bound hormone was determined up to 15 min as described in Fig. 7. All other details were as described under Experimental Procedures.

Guanosine nucleotide added	$T_{0.5}$	Dissociation of prebound [^{125}I]-iodo-β-MSH
	min	%
GTP	4.0 ± 0.3 (n = 4)	69.0 ± 3.5 (n = 8)
GTPγS	3.5 ± 0.2 (n = 2)	64.0 ± 5.7 (n = 3)
GDPβS	4.2 ± 0.1 (n = 2)	79.7 ± 5.2 (n = 3)

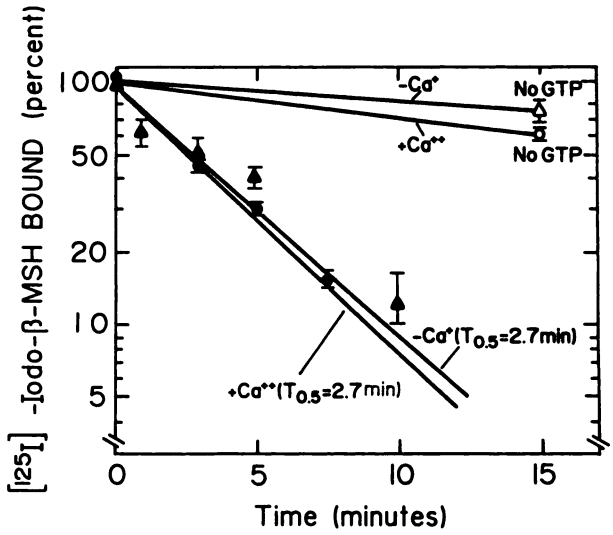

Fig. 8. GTP-induced dissociation of β-MSH prebound in the presence or absence of calcium. M2R plasma membrane protein (1575 μg) was preincubated with [^{125}I]-iodo-β-MSH for 45 min. One system contained 100 μM free calcium and 200 nM [^{125}I]-iodo-β-MSH, whereas the other (calcium-free system) contained ≤5 nM free calcium and 600 nM [^{125}I]-iodo-β-MSH. At the end of the preincubation step the membrane suspensions (1.8 ml each) were layered on a 2.5-ml 10% sucrose cushion prepared with either PBS or PBS$^-$, respectively. After centrifugation (15 min; 17,000 × g) the washed membrane pellets were resuspended to their original volumes in binding medium lacking both hormone and leupeptin. Following the addition of GTP to a final concentration of 25 μM or an equivalent volume of the respective PBS, samples (100 μl) were removed at the times indicated and residual bound hormone was determined as previously described under Experimental Procedures. Parallel systems containing 2 μM unlabeled β-MSH were treated similarly and served in the calculation of nonspecific binding. The 100% bound value is the total amount of preformed complex which can be dissociated by GTP, which represents 65% of the total specific binding in this experiment.

end of the incubation period the membranes were washed free of unbound hormone. Under these conditions, significant levels of both calcium-containing and calcium-free receptor-hormone complexes are formed, e.g., 700 and 300 fmol/mg of protein, respectively. These two forms of bound β-MSH were then challenged with GTP (25 μM) in a manner described in the experiment shown in Fig. 7. As can be seen (Fig. 8), GTP induced identical rates of dissociation of β-MSH prebound in either the presence or absence of calcium. In a series of experiments the half-life of the receptor-hormone complex was 2.6 ± 0.2 min (n = 3) in the presence of calcium and 2.5 ± 0.4 min (n = 3) in its absence. It appears that these rates of dissociation are slightly faster than those measured in the continual presence of free hormone (Fig. 7), in which rebinding of dissociated

hormone may occur. Dissociation of the preformed complexes was negligible in the absence of GTP. The extent of dissociation induced by GTP was 65 ± 3% ($n = 3$) and 65 ± 6% ($n = 3$) of the initial prebound complexes in either the presence or absence of calcium, respectively. These results clearly show that the effect of GTP on the dissociation of bound β-MSH is independent of the conditions under which the receptor-hormone complex is formed, i.e., in the presence or absence of calcium. Therefore, it appears that the effects of both calcium and guanosine nucleotides on β-MSH action are independent of each other and are likely to operate through separate mechanisms.

Discussion

As demonstrated in this study, the activation of melanoma AC by β-MSH selectively requires calcium. Our results imply that the calcium-requiring step in enzyme activation occurs at the level of receptor-hormone interaction, i.e., β-MSH binding, as deduced from the parallel calcium concentration dependency of both processes (Figs. 1 and 2). This calcium-dependent control of AC activity by hormone differs from the direct regulation by calcium-calmodulin of the catalytic subunit of AC in porcine brain (23) and abalone sperm (24), which appears to be hormone independent. In addition, it appears that calcium is not involved in subsequent steps that lead to AC activation, since stimulation of the enzyme by PGE_1 was calcium insensitive (Fig. 1). Furthermore, activation of AC by GTPγS or forskolin, which are known to stimulate AC via G_S (17) and the catalytic subunit of AC (25), respectively, was also calcium insensitive (Table 1). Our observations therefore define the role of calcium as facilitating the formation of the receptor-hormone complex.

The calcium dependency shown for β-MSH binding and stimulation of AC is observed at two separate ranges of free calcium concentrations: one saturated below 10 μM free calcium and a second saturated above 100 μM free calcium (Figs. 1 and 2). If the location of these calcium-pertinent sites is extracellular, then it can be presumed that these sites operate under nonlimiting calcium conditions, since levels of extracellular free calcium are in the mM range. However, regulation of the binding of β-MSH by these sites may be particularly relevant in situations where extracellular levels of free calcium may vary transiently. Furthermore, it must be taken into account that manipulation of extracellular calcium, by the use of EGTA, may also indirectly influence intracellular free calcium concentrations, as shown by Streb and Shultz (26). Hence, manifest changes in external free calcium may influence internal calcium-binding sites relevant for β-MSH action, which may be restricted to intramembranal and/or intracellular domains otherwise cryptic to extracellular EGTA. As intracellular free calcium concentrations are generally in the range of 100 nM (26), it is logical to presume that these putative calcium-binding sites are of significant regulatory value only if they are internal and thus exist divorced from extracellular free calcium. Consequently, local changes in intramembranal and/or intracellular calcium concentrations may then influence cellular responsiveness to MSH, without necessarily affecting the actions of other hormones.

Conversely, the observation that both epinephrine (27, 28) and PGE_1 (2) are potentiators of melanophore dispersion in amphibia via a cAMP-dependent mechanism may support the possibility that these agonists may regulate β-MSH action by affecting calcium levels at the relevant sites. In addition, a distinct possibility exists that MSH may be able to regulate its own calcium-dependent action, by directly modulating calcium fluxes across the plasma membrane. Recently, Kojima *et al.* (29) demonstrated for ACTH that aldosterone secretion from adrenal glomerulosa cells may result from the synergistic effects of cAMP and calcium, and that ACTH may directly increase calcium entry into the cell.

It appears that, under calcium-limiting conditions, saturating β-MSH concentrations form a stable receptor-hormone complex in the presence of EGTA. This calcium-independent complex contrasts the strongly calcium-dependent state seen for β-MSH binding and AC activation at subsaturating (physiological) concentrations of hormone. Although it may be presumed that the low affinity calcium-independent complex is artifactual under physiological conditions, it nevertheless indicates that a stable cation-independent receptor-hormone complex can be formed and that the cationic requirement for β-MSH binding is not absolute (Figs. 3–5).

The 20-fold increase in hormone binding affinity, as seen in the presence of calcium, appears to be the major regulatory mechanism of β-MSH binding and stimulation of AC. In contrast, maximal receptor occupancy was found to be identical (approximately 1100 fmol/mg of membrane protein) in either the presence or absence of calcium (Fig. 4). This result suggests that the same population of receptors participates in both calcium-dependent and calcium-independent hormone binding. Of consequence, we have found that only the calcium-dependent form of the receptor-hormone complex is EGTA sensitive. The transition between these two states, induced by the removal of calcium, involves prior dissociation of the hormone and subsequent calcium-independent reassociation (Fig. 5). This transient dissociation by the hormone suggests that the receptor-hormone complex formed under these conditions represents two different states of bound β-MSH, varying in affinity. Furthermore, the transition between the two states cannot be achieved by simple dissociation of calcium from the calcium-pertinent sites.

Despite the supposed differences, both states of the receptor-hormone complex stimulate AC activity equally well, when measured at saturating hormone concentrations (Fig. 3). Thus, the involvement of calcium in the actions of β-MSH is relevant only at subsaturating concentrations of hormone. Circulating levels of MSH in humans, for example, are in the nM range (30), well within concentrations shown to be physiologically effective for both tyrosinase activation in Cloudman mouse melanoma cells (31) and melanosomal dispersion in amphibia (32).

The point made in this study, that calcium is required for β-MSH action under physiological conditions, is further supported by the findings that calcium alone best fulfills the metal cationic requirement for hormone binding (Table 2). Among other cations tested Sr^{2+}, Ni^{2+}, and Ba^{2+} facilitated β-MSH binding; however, their concentrations under physiological conditions are not likely to suffice for hormone binding *in vivo*.

It should be noted that high basal rates of AC activity (Fig. 3) tend to obscure the calcium sensitivity of β-MSH action defined at low hormone concentrations, which is easily observed in the intact cell (Figs. 1 and 2) and in binding assays using plasma membranes (Fig. 4). In addition, the calcium sensitivity seen under these conditions may also become less apparent due to the affinity change induced by GTP (Figs. 3 and 4). This guanosine nucleotide-induced shift in the affinity of hormone

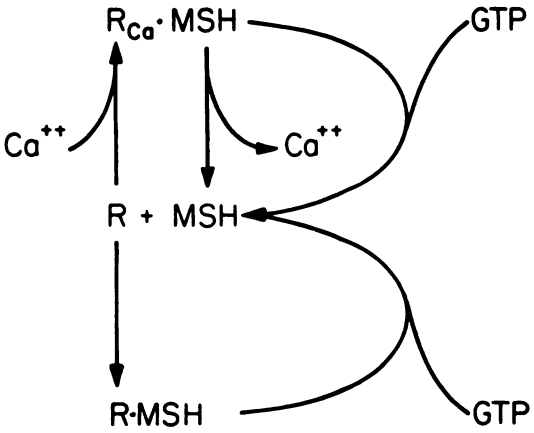

Fig. 9. A model for the dual regulation by calcium and guanosine nucleotides of MSH binding. Binding of MSH to its receptor (*R*) may occur either in the presence or absence of calcium (Ca^{2+}), leading to the formation of a high affinity EGTA-sensitive calcium-containing complex (R_{Ca}·MSH) or a low affinity EGTA-insensitive, calcium-depleted complex (*R*·MSH). Interconversion between R_{Ca}·MSH and *R*·MSH requires prior dissociation of the receptor-hormone complex and subsequent rebinding. Formation of either complex leads to the direct stimulation of AC. In addition, both complexes are sensitive to guanosine nucleotides (e.g., GTP), which decrease stability of the complexes leading to enhanced dissociation of MSH from *R*.

binding has been well documented for a large number of hormones (17).

The finding that guanosine nucleotides lower the affinity of β-MSH for its receptor contrasts with the increase in binding affinity as seen by the addition of calcium. We have seen, however, that the kinetics of GTP-induced dissociation of β-MSH are identical whether the receptor-hormone complex is formed in the presence or absence of calcium. This indicates that the guanosine nucleotide-sensitive site, presumably located on the α subunit of the guanosine nucleotide-regulatory protein, whose existence in this cell line we have previously demonstrated (16), is probably calcium unrelated in nature. Together, both calcium and guanosine nucleotides exert their effects on β-MSH regulation of AC by directly affecting both receptor affinity and occupancy. The reciprocal nature of this regulation of MSH binding and AC activity in M2R melanoma cells may exemplify a mechanism for receptor fine-tuning, where levels of both intracellular guanosine nucleotides and intramembranal/intracellular calcium feed back to regulate the extent of β-MSH stimulation. A schematic representation of calcium and guanosine nucleotide regulation of β-MSH binding is depicted in Fig. 9, although the exact mechanisms by which these agents regulate MSH receptor function and the location(s) of the calcium-pertinent sites have yet to be explored.

Acknowledgments

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References

1. Vesely, D. L., and M. E. Hadley. Calcium requirement for melanophore-stimulating hormone action on melanophores. *Science* (*Wash. D. C.*) **173**:923–925 (1971).
2. Van de Veerdonk, F. C. G., and E. Brouwer. Role of calcium and prostaglandin (PGE_1) in the MSH-induced activation of adenylate cyclase in *Xenopus laevis*. *Biochem. Biophys. Res. Commun.* **52**:130–136 (1973).
3. De Graan, P. N. E., A. N. Eberle, and F. C. G. Van de Veerdonk. Calcium requirement for α-MSH action on tail fin melanophores of *Xenopus* tadpoles. *Mol. Cell. Endocr.* **26**:327–329 (1982).
4. De Graan, P. N. E., C. J. Van Dorp, and F. C. G. Van de Veerdonk. Calcium sites in MSH stimulation of *Xenopus* melanophores: studies with photoreactive α-MSH. *Mol. Cell. Endocr.* **26**:315–326 (1982).
5. Bar, H. P., and O. Hechter. Adenyl cyclase and hormone action. I. Effects of adrenocorticotropic hormone, glucagon, and epinephrine on the plasma membrane of rat fat cells. *Proc. Natl. Acad. Sci. USA* **63**:350–355 (1969).
6. Lefkowitz, R. J., J. Roth, and I. Pastan. Effects of calcium on ACTH stimulation of the adrenal: separation of hormone binding from adenyl cyclase stimulation. *Nature* (*Lond.*) **228**:864–866 (1970).
7. Sayers, G., R. J. Beall, and S. Seele. Isolated adrenal cells: adrenalcorticotropic hormone, calcium, steroidogenesis, and cyclic adenosine monophosphate. *Science* (*Wash. D. C.*) **175**:1131–1133 (1972).
8. Nakamura, M., and P. F. Hall. Inhibition of steroidogenic response to corticotropin in mouse adrenal tumor cells (Y-1) by the ionophore A23187. *Biochim. Biophys. Acta* **542**:330–339 (1978).
9. Perchellet, J. P., and R. Sharma. Mediatory role of calcium and guanosine 3′,5′-monophosphate in adrenocorticotropin-induced steroidogenesis by adrenal cells. *Science* (*Wash. D. C.*) **203**:1259–1261 (1979).
10. Schimmer, B. P. The adrenocortical tumor cell line, Y1, in *Functionally Differentiated Cell Lines* (G. Sato, ed.). Alan R. Liss, Inc., New York, 61–92 (1981).
11. Cheitlin, R., D. I. Buckley, and J. Ramachandran. The role of extracellular calcium in corticotropin-stimulated steroidogenesis. *J. Biol. Chem.* **260**:5323–5327 (1985).
12. Mains, R. E., B. A. Eiper, and S. Ling. Common precursor to corticotropins and endorphins. *Proc. Natl. Acad. Sci. USA* **74**:3014–3018 (1978).
13. Wong, G., and J. Pawelek. Control of phenotypic expression of cultured melanoma cells by melanocyte-stimulating hormones. *Nature New Biol.* **241**:213–215 (1973).
14. Varga, J. M., A. De Pasquale, J. Pawelek, J. S. McGuire, and A. B. Lerner. Regulation of melanocyte stimulating hormone action at the receptor level: discontinuous binding of hormone to synchronized mouse melanoma cells during the cell cycle. *Proc. Natl. Acad. Sci. USA* **71**:1590–1593 (1974).
15. Tsubokawa, M., K. Muramoto, and J. Ramachandran. Characterization of ACTH receptors in bovine adrenal membranes. *Fed. Proc.* **42**:2090 (1983).
16. Gerst, J. E., J. Sole, J. Mather, and Y. Salomon. Regulation of adenylate cyclase by β-melanotropin in the M2R melanoma cell line. *Mol. Cell. Endocr.* **46**:137–147 (1986).
17. Rodbell, M. The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* (*Lond.*) **284**:17–22 (1980).
18. Mather, J. P., and G. H. Sato. The growth of mouse melanoma cells in hormone-supplemented, serum-free medium. *Exp. Cell Res.* **120**:191–200 (1979).
19. Salomon, Y. Adenylate cyclase assay. *Adv. Cyclic Nucleotide Res.* **10**:35–57 (1979).
20. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
21. Bartfai, T. Preparation of metal-chelate complexes and design of steady-state kinetic experiments involving metal nucleotide complexes. *Adv. Cyclic Nucleotide Res.* **10**:219–242 (1979).
22. Tsien, R. Y., and T. J. Rink. Neutral carrier ion-selective microelectrodes for measurement of intracellular free calcium. *Biochim. Biophys. Acta* **599**:623–638 (1980).
23. Brostrom, C. O., Y. C. Huang, B. M. Breckinridge, and D. J. Wolff. Identification of a calcium-binding protein as a calcium-dependent regulator of brain adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **72**:64–68 (1975).
24. Kopf, G. S., and U. D. Vacquier. Characterization of a CaM-stimulated adenylate cyclase frm abalone spermatozoa. *J. Biol. Chem.* **259**:7590–7596 (1984).
25. Seamon, K., and J. W. Daly. Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. *J. Biol. Chem.* **256**:9799–9801 (1981).
26. Streb, H., and I. Schultz. Regulation of cytosolic free Ca^{2+} concentration in acinar cells of rat pancreas. *Am. J. Physiol.* **245**:G347–G357 (1983).
27. Van de Veerdonk, F. C. G., and T. M. Konijn. The role of adenosine 3′,5′-cyclic monophosphate and catecholamines in the pigment migration process in *Xenopus laevis*. *Acta Endocrinol.* **64**:364–376 (1970).
28. Brouwer, E., and F. C. G. Van de Veerdonk. Possible involvement of α- and β-receptors in the natural colour change and the MSH-induced dispersion in *Xenopus laevis in vivo*. *Eur. J. Pharmacol.* **17**:234–239 (1972).
29. Kojima, I., K. Kojima, and H. Rasmussen. Role of calcium and cAMP in the action of adrenocorticotropin on aldosterone secretion. *J. Biol. Chem.* **260**:4248–4256 (1985).
30. Kutsky, R. J. *Handbook of Vitamins, Minerals and Hormones*, Ed. 2. Van Nostrand Reinhold Inc., New York, 348–353 (1981).
31. Lambert, D. T., C. Stachelek, J. M. Varga, and A. B. Lerner. Iodination of β-melanotropin. *J. Biol. Chem.* **257**:8211–8215 (1982).
32. De Graan, P. N. E., A. J. Van de Kamp, D. R. W. Hup, W. H. Gispen, and F. C. G. Van de Veerdonk. Calcium requirement for α-MSH action on melanophores: studies with forskolin. *J. Recept. Res.* **4**:521–536 (1984).

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