HORMONAL CONTROL OF MELANOCYTES

MSH-Sensitive Adenyl Cyclase in the Cloudman Melanoma

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Cyclic AMP occupies a position of central importance in hormone biochemistry (1, 2). Of especial interest is its possible function in mediating hormone regulation of pigment cell metabolism. It is known to affect cell membrane properties producing changes in sodium transport (3), water permeability (4), and hormone release (5), the last process having some analogies to melanosome dispersion in amphibian dermal melanocytes. The fine molecular details underlying these cyclic AMP-mediated changes in membrane function are as little understood as the ultimate molecular details of the role of cyclic AMP in pigment cells.

Our interest in the Cloudman mouse melanoma was provoked by the results of studies done with the melanocyte system in the skin of *Rana pipiens*. We found that cyclic AMP mimics the effects of melanocyte-stimulating hormone (MSH) on frog dermal melanocytes [a dispersion of melanosomes especially into the peripheral dendritic processes of the melanocyte] (6). This finding and the fact that methyl xanthines (which inhibit cyclic AMP phosphodiesterase) could darken amphibian skin were taken as partial support for the hypothesis that the regulation of melanocyte metabolism by MSH was mediated by the adenyl-cyclase system. Additional support for this idea was provided by Butcher and coworkers in a study which showed that MSH could increase the cyclic AMP content of pigmented dorsal *R. pipiens* skin while having no effect on the cyclic AMP content of the unpigmented abdominal skin (7). An element still lacking in the proof of the MSH–cyclase hypothesis was the demonstration that MSH could selectively activate adenyl cyclase in the appropriate broken-cell preparations, especially membrane particles derived from homogeneous collections of melanocytes. Initial attempts to pursue this question in amphibian melanocytes were complicated by the mechanical problems encountered in preparing membrane fragments from dermal melanocytes (associated with the abundance of collagen) and the fact that the population of dermal melanocytes is widely dispersed among other cell types, none of which respond to MSH peptides, but most of which contain cyclase molecules exhibiting basal cyclase activity in the absence of added hormones. These difficulties were avoided by doing these experiments on the effect of MSH in broken cells from the Cloudman mouse melanoma, an essentially homogeneous population of mam-

malian melanocytes which exhibited a vigorous and selective stimulation of adenyl cyclase activity by MSH. Early studies with this system including the profile of hormonal sensitivity which characterized the cyclase, have been described in detail (8). The primary features of the system include responsiveness to both alpha and beta MSH as well as to MSH peptides which have been racemized by exposure to tenth-normal sodium hydroxide. There is the expected cross reactivity to ACTH, no response to or inhibition by melatonin and minimal activation by catecholamines. The latter observation (with melatonin and norepinephrine) are perhaps contrary to what might have been expected in frog skin (9).

Additional interest in the melanoma from the point of view of the cyclase system and the regulation of gene expression has derived from the recent finding by Pastan (10) that the levels of the enzyme tyrosinase in the melanoma appear to be regulated by cyclic AMP. We have examined the adenyl cyclase system from both melanotic and relatively amelanotic varieties of the melanoma and in both instances an excellent cyclase response to the MSH peptides in broken-cell preparations was observed. This finding of an intact cyclase response in amelanotic cells suggests that the complex sequence of reactions regulating pigment synthesis in the mammalian melanocyte, or at least the melanoma melanocyte, can be lesioned distal to the locus of action of cyclic AMP.

In the present study, we should like to discuss some additional aspects of the regulation and characteristics of the Cloudman melanoma adenyl cyclase system. We will emphasize the selectivity and efficacy of prostaglandins as regulators of this cyclase system in broken-cell preparations. The interaction of calcium with the MSH and prostaglandin activations of adenyl cyclase in mammalian and amphibian melanocytes, and the failure of MSH to affect prostaglandin synthetase activity, will be discussed. In this context we will consider the hypothesis (11) that prostaglandins are obligatory intermediates in the activation of any cyclase system by tissue-specific hormones. We will also consider the distribution of melanoma cyclase in the various centrifugal fractions derived from homogenates of melanoma, and finally the effects of various regulators on the cyclic AMP content of melanoma slices in which cellular architecture is relatively preserved. These observations are briefly discussed in the context of current understanding of cyclic nucleotide biochemistry as it might bear on the biochemistry of pigment cells.

MATERIALS AND METHODS

Both melanotic and relatively amelanotic strains of the S-91 Cloudman melanoma were obtained from Dr. Harry Demopoulos of the New York University School of Medicine. The tumors were maintained in male mice of the strain DBA/2J (Jackson Memorial Labs.) and were transplanted under aseptic conditions by intraperitoneal injection of finely minced tissue suspended in 0.15 M saline. Tumors grew as encapsulated solid masses with adhesions to omentum and peritoneum. The intraperitoneal location produced growth which was more homogeneous and less necrotic than the subcutaneous location. Time for appearance of a 1-cm tumor was about $2\frac{1}{2}$ weeks.

Tissue was homogenized with a motor-driven Potter–Elvejhem (12 strokes, Teflon on glass, 4°) in 0.12 M potassium acetate buffer, pH 7.4, which contained 0.03 M KCL. Washed particles were prepared from the above homogenates (8). Tissue slices (0.2-mm thickness) were made with a McElwain chopper (Ivan

Sorvall Co.) at 4°C. Slices were incubated in oxygenated Krebs-Ringer bicarbonate for 10 min at 37°C, and incubations were terminated by freezing the samples in liquid nitrogen, grinding with a mortar and pestle, and thawing into 50% acetic acid which contained [8-^{3}H] cyclic AMP (Schwartz–Mann) to permit estimation of recovery.

ACTH and MSH were provided by Drs. Lande and Lerner of Yale University. The prostaglandins and 7-oxa-13-prostynoic acid were provided by Drs. Caldwell and Speroff of Yale University. Indomethacin was a gift from Merck & Co. Prostaglandins were stored at −20°C. in absolute ethanol. Immediately before use they were dried and dissolved in the reaction buffer. Indomethacin and 7-oxa-13-prostynoic acid were dissolved in methanol and diluted 1:40 into the broken-cell preparations.

Cyclase activity was assayed as previously described (12). [8-^{14}C] ATP was used as the substrate. Labeled cyclic AMP with added cold cyclic AMP for visualization was purified by descending thin-layer chromatography at 31°C on polyethyleneimine–cellulose for 8 hr with 1 M ammonium acetate–methanol (2:5). The origin was then removed by shaving just behind the cyclic AMP spots, the plates were dried, rewicked, and rechromatographed for 12 hr with 1-butanol–acetic acid–water (2:1:1). The purified cyclic AMP still on PEI cellulose was shaved directly into scintillation vials, dispersed by sonication, and counted with toluene PPO cocktail in the presence of Cabosil in a Beckman LS 200 liquid scintillation spectrometer. Assay of adenyl cyclase activity was carried out using a 3-min incubation period at 37°C, in the presence of 1.6 mM [8-^{14}C] ATP (45 Ci/mole, Schwartz–Mann), 7 mM aminophylline, and an ATP regenerating system (38 mM phosphocreatine and 80 μg/ml creatine phosphokinase) in buffer 1 (3 mM MgSO$_4$, 0.4 mM EDTA, and 32 mM glycylglycine, pH 7.4). Assay of 3'5' nucleotide phosphodiesterase was carried out by incubating in the presence of 10^{-4} M [8-^{14}C] cAMP (39 Ci/mole, Schwartz–Mann) in buffer 1 and measuring the disappearance of substrate in 1 min. Protein kinase was assayed by the method of Kuo and Greengard (13). Protein concentrations were determined by the method of Lowry (14). Cyclic AMP levels of tissue slices or skin were determined by the radioimmunoassay method of Steiner (15) with minor modifications (16).

Prostaglandin synthetase was assayed by measuring conversion of labeled arachidonic acid to labeled prostaglandins [5,6,8,9,11,12,14,15-^{3}H] arachidonic acid (5000 Ci/mole, New England Nuclear) was purified just prior to use by column chromatography (17). Samples were incubated in a total volume of 0.1 ml containing 32 mM glycylglycine buffer, pH 7.4, 10^{-7} M labeled and 1.6×10^{-5} M unlabeled arachidonic acid, with or without 2×10^{-5} M MSH. The incubation was carried out at 37°C in a shaking incubator under air for 20 min. The samples were acidified to pH 2.5 with 1 N HCl, and extracted twice with 8 ml of ethyl acetate. The combined ethyl acetate extracts were dried under nitrogen. Samples were purified by column chromatography (17) followed by thin-layer chromatography (18), and counted on a Packard Tri-Carb Liquid Scintillation Counter (Model 3375). Counts were found both in the E fraction (PGE$_2$) and in the F fraction (PGF$_2$). To further confirm that the counts in the E fraction were prostaglandin, it was converted to prostaglandin B[19], rechromatographed, and the label was found in the PGB fraction.

Darkening of dorsal frog skin (*Rana pipiens*) was measured by the procedure previously described by Bitensky and Burstein (6).

RESULTS

The sensitivity of melanoma cyclase to different prostaglandins (PG) is shown in Fig. 1. Activation by MSH is included for comparison. The magnitude of activation by PGE_1 is $1\frac{1}{2}$ to 2 times greater than that seen with MSH, but other prostaglandins are less effective than MSH. In the presence of saturating amounts of PGE_1, PGE_2, and $PGF_{1\alpha}$, MSH produces additional activation of melanoma cyclase (Table 1). MSH activation is also additive with the activation produced by saturating amounts of fluoride (Table 1). The activation of cyclase by PGE_1 appears to reach half maximal saturation at a concentration of 1 $\mu g/ml$. (Fig. 2). Activation of cyclase by MSH is lowered by ethyleneglycol bis-(β aminoethyl ether) N, N, N',N' tetra-acetic acid (EGTA) and restored by calcium. Although fluoride activation is blocked by EGTA this inhibition is not prevented or reversed by cal-
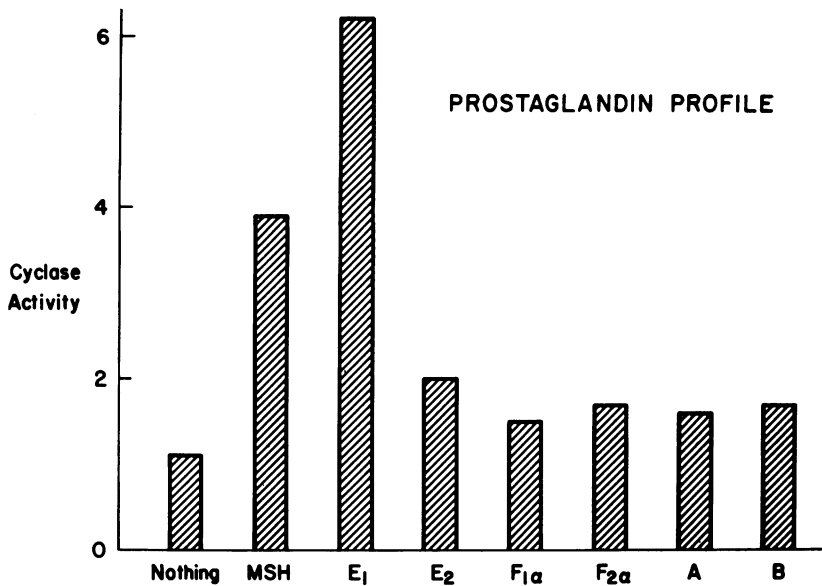

FIG. 1. Activation of melanoma adenyl cyclase by MSH and by various prostaglandins. Cyclase activity is nmoles cAMP/min/mg protein. MASH concentration is 50 $\mu g/ml$. Prostaglandin concentration is 10 $\mu g/ml$.

TABLE 1

ADDITIVE EFFECTS OF MSH, PROSTAGLANDINS, AND FLUORIDE

	Adenyl cyclase activity[a]	
Regulator	Without MSH	With MSH
Control	1.7	5.9
MSH	5.9	5.9
PGE_1	8.5	11.0
PGE_2	2.6	6.8
PGF_1	2.3	6.5
Fluoride	7.5	10.0

[a] Activities are expressed as nmoles cAMP/10 min/mg of protein. Regulator concentrations are as follows: MSH 50 $\mu g/ml$, PGE_1, PGE_2, and PGF_1 10 $\mu g/ml$, and fluoride 10 mM. Experiments were carried out with whole homogenate preparations.
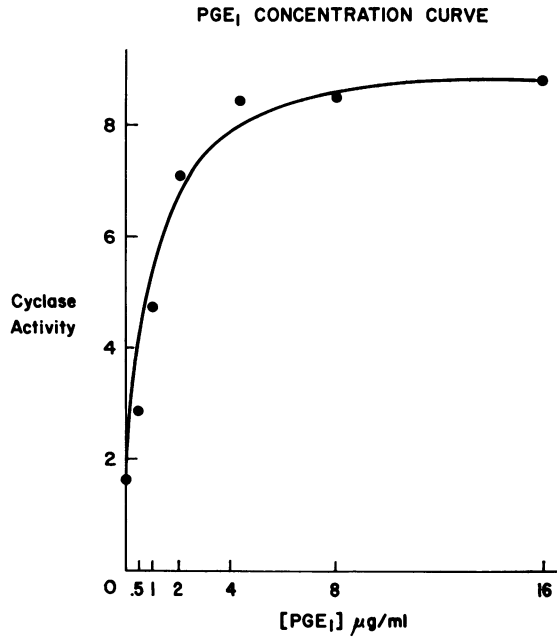
PGE₁ CONCENTRATION CURVE

Fig. 2. Prostaglandin E₁ concentration curve for activation of melanoma adenyl cyclase. Cyclase activity is nmoles cAMP/min/mg protein.

TABLE 2

ROLE OF CALCIUM IN MELANOMA CYCLASE ACTIVATION[a]

	Control	MSH	E₁	Fluoride
Control	2.0	5.5	8.0	4.5
EGTA	1.7	3.7	7.9	2.5
EGTA + Ca²⁺	2.0	6.0	7.8	2.3
EGTA + Mg²⁺	1.8	4.0	8.3	2.9

[a] Activities are expressed as a nmole cAMP/10 min/mg of protein. Reagent concentrations are as follows: MSH 50 μg/ml, PGE₁ 10 μg/ml, fluoride 10 mM, EGTA 7 mM, Ca and Mg⁺⁺ 1 mM. This concentration of cations is in addition to the 5 mM Mg⁺⁺ from the reaction mixture. Whole homogenate preparations were used throughout.

cium or magnesium. PGE₁ activation appears to occur without the calcium dependence found for activation by MSH (Table 2).

Activation by MSH is partially blocked by 7-oxa-13-prostynoic acid (a prostaglandin antagonist) and by indomethacin (an inhibitor of prostaglandin synthetase). The kinetics of inhibition by 7-oxa-13-prostynoic acid appear to be competitive with MSH, (Fig. 3) as well as with PGE₁ (data not shown). The concentrations required for both inhibitors, however, is rather high and raised questions concerning the specificity and locus of the inhibition. The 70% inhibition of MSH effects by indomethacin (Table 3) is seen only at concentrations of inhibitor which are 100 times as large as those required for the inhibition of prostaglandin synthetase (20).

The effects of MSH on prostaglandin synthesis were examined in melanoma. The tritiated prostaglandin precursor (arachidonic acid) was readily converted to PGE₂ and PGF₂α by melanoma homogenates. The homogenate produced 2.6 pmoles PGE₂/min/mg protein and 1.0 pmoles PGF₂α/min/mg protein. The rates

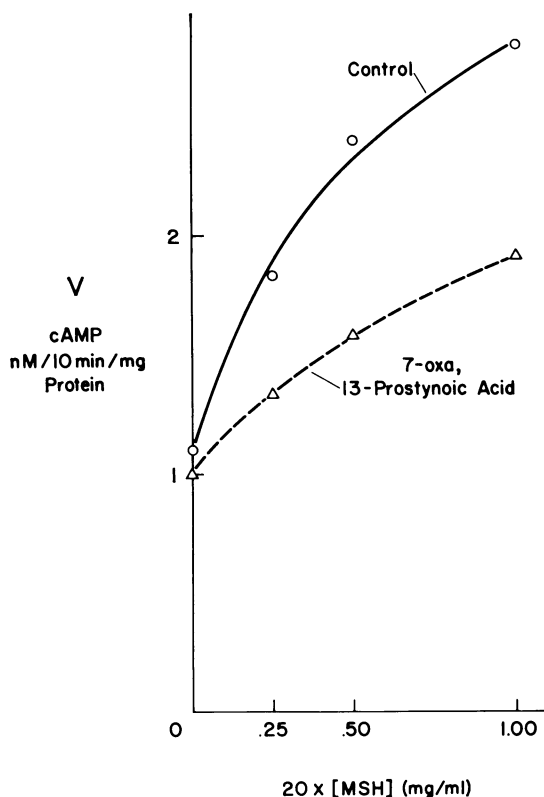FIG. 3. Inhibition of MSH activation of melanoma adenyl cyclase by 7-oxa-13-prostynoic acid (75 µg/ml). Cyclase activity is nmoles cAMP/min/mg protein.

TABLE 3

INHIBITION OF CYCLASE ACTIVATION BY INDOMETHACIN

MSH concentrations (µg/ml)	→	Adenyl cyclase activity			
		0	6.25	12.5	25
Indomethacin (50 µg/ml)		0.7	0.9	1.0	1.2
Indomethacin (5 µg/ml)		1.2	1.4	2.1	2.9
Control		1.2	1.5	2.1	3.0

[a] Activities are expressed as nmole cAMP/10 min/mg of protein. Whole homogenate preparations were used throughout.

of synthesis were not influenced by those concentrations of MSH which profoundly activate melanoma cyclase in the same homogenate preparations.

The distribution of cyclase in melanoma homogenates was somewhat atypical in that there was a measurable (25%) amount of the MSH-responsive cyclase activity in a 200,000 g supernatant fraction. There is some loss of PGE_1 stimulation of this fraction compared to other fractions. The bulk of the cyclase activity, however, did sediment with the heavy particulate fraction (Table 4).

The melanoma homogenates also exhibit phosphodiesterase and protein kinase activities. Both of these cyclase-related enzymes appear primarily in the soluble

TABLE 4

SEDIMENTATION CHARACTERISTICS OF CYCLASE SYSTEM IN MELANOMA HOMOGENATES[a]

Tissue (g) sedimented	Time of centri- fugation	Basal activity	MSH stimulated	PGE$_1$ stimulated
1,000	½ hr	1.5 (33%)	3.1 (46%)	6.5 (54%)
10,000	½ hr	0.9 (20%)	1.0 (15%)	2.3 (19%)
50,000	1 hr	0.7 (16%)	0.5 (7%)	1.4 (12%)
200,000	1 hr	0.5 (11%)	0.5 (7%)	0.7 (6%)
200,000 Supernatant	1 hr	0.9 (20%)	1.7 (25%)	1.1 (9%)
Homogenate		1.0 (100%)	1.2 (100%)	4.3 (100%)

[a] Specific activities are expressed as nmoles/10 min/mg of protein. Numbers in parentheses are the percentages of total basal, and hormone- or PGE$_1$-stimulated activity. Sedimented particulate fractions were suspended in an equal volume of reaction buffer. [MSH] was 50 μg/ml and [PGE$_1$] was 10 μg/ml.

fraction. The phosphodiesterase hydrolyzed 2.6 nmoles of cAMP/mg protein and showed the usual sensitivity to methyl xanthines. The protein kinase incorporated 7.2 pmole of acid-precipitable PO_4 into histone/min/mg protein in response to 10^{-6} M cAMP.

The melanoma cyclase was also evaluated as a slice preparation. Basal levels of cyclic AMP (1.4 pmole/mg of tissue) were markedly increased by MSH (5.3 pmoles) and PGE$_1$ (6.1 pmoles). Attempts were made to detect ectopic receptors with parathormone, vasopressin, glucagon, TSH, and FSH. The melanoma slices showed hormone sensitivity which was confined to PGE$_1$, MSH, and related (ACTH) peptides. Half-maximal activation of melanoma-homogenate cyclase is seen at 5×10^{-7} M MSH. The slice preparation showed half-maximal activation at 2×10^{-7} M MSH, a concentration somewhat higher than would be expected to function *in vivo*.

In view of the effect of PGE$_1$ on mammalian melanocytes, its efficacy in amphibian melanocytes was also examined. Preliminary experiments with pigmented dorsal frog skin indicate that prostaglandin E$_1$ at 10μg/ml darkens the skin to nearly the same final optical density as MSH and with a comparable rate of darkening. Also, PGE$_1$ raises levels of cyclic AMP in dorsal frog skin. In the absence of added stimulators the cyclic AMP levels are 0.13 pmoles cAMP/mg of protein, with 50 μg/ml of MSH 0.67 pmoles cAMP/mg protein, and with 10 μg/ml of PGE$_1$ 0.43 pmoles cAMP/mg of protein.

DISCUSSION

Regulation of the adenyl cyclase system in the mouse Cloudman melanoma is quite complex, and appears to involve prostaglandins and calcium as well as MSH. The cyclase of the melanoma shows a marked and selective response to PGE$_1$ even in broken-cell preparations. Prostaglandin activation of broken-cell adenyl cyclase of this magnitude has not been observed in other tissues. The stimulation of cyclase by PGE$_1$ is significantly greater than the stimulation produced by porcine MSH. Since the amino acid sequence of mouse MSH is not known it is conceivable, though unlikely, that this apparent greater efficacy of PGE$_1$ rests in the preference of the tissue for murine rather than porcine MSH. Such a preference for murine

MSH could not be the result of greater binding affinity since both the prosta-
glandins and the peptides are being used at the saturating concentrations. Further,
there is no species variation for all of the αMSH peptides (five species) which
have thus far been sequenced. It seems rather that the potential for activation
is greater for the prostaglandins than for the peptides, and that prostaglandins are
important regulators of the melanoma cyclase.

We emphasize that when melanocyte peptides are used in combination with the
prostaglandins they provide additional stimulation of cyclase. This additivity sug-
gests that hormones and prostaglandins activate cyclase through distinct pathways
although it might instead be due to contributions of other cell types such as capil-
laries (16). The additivity, coupled with the fact that MSH has no effect on prosta-
glandin synthetase, suggests that prostaglandins are not obligatory intermediates
in the cyclase-activation sequence. Further, while indomethacin, which is a known
inhibitor of prostaglandin synthetase (20), does, in fact, inhibit the activation of
melanoma cyclase by MSH, it does so at concentrations 100 times greater than
those required to inhibit prostaglandin synthetase. Hence, the inhibitor may act
at the hormone receptor or some other locus and have little if anything to do with
prostaglandin synthesis in the homogenate or washed-particle preparations in which
cyclase is being assayed. On the other hand, activation by MSH as well as PGE_1
is antagonized in a seemingly competitive manner by 7-oxa-13-prostynoic acid.
Also, our data do not exclude the possibility that the hormone (MSH) promotes
release of sequestered endogenous prostaglandins.

The requirement for calcium in the activation of melanoma cyclase by MSH
is analogous to the calcium requirement for activation of adrenal cortical cyclase
by ACTH (21). The lack of a calcium requirement in the activation of melanoma
cyclase by prostaglandins suggests that MSH and prostaglandins insert into the
cyclase-activation sequence at different points. Certainly the profound structural
dissimilarities between the two classes of activators is compatible with the concept
that they possess independent binding sites for activation of cyclase.

The presence of a cyclic AMP-activated protein kinase in the melanoma is not
surprising, since such an enzyme has been demonstrated or implicated as a member
of the sequence by which cyclic AMP promotes the elaboration of hormone-
directed specialized cell function in many tissues. Melanoma protein kinase may
mediate the cyclic AMP-directed induction of tyrosinase, and thus participate in
the regulation of pigment synthesis. The fact that in at least some amelanotic
melanomas the cyclase system is intact up to and including the protein kinase,
suggests that the lesion in melanin synthesis in such tumors is probably distal to
the protein kinase.

Although the cyclase apparatus has been unequivocably implicated in the essen-
tial regulation of pigment metabolism, more data are needed in the definition of
the components actually involved in pigment synthesis and movement of pigment
granules; that is to say the targets of the cyclic AMP-activated protein kinase re-
main unknown. Recent observations with cytochalasin suggest the possibility that
microfilaments may participate in the cyclic AMP-directed darkening of melano-
cytes (22, 23).

SUMMARY

Adenyl cyclase has been examined in broken-cell and slice preparations in a
transplantable Cloudman melanoma. Activation by α or β MSH (melanocyte-stim-

ulating hormone) of melanoma adenyl cyclase requires the presence of millimolar calcium and is prevented by EGTA. (Activity increases from 3 to 10 nmoles of cAMP/10 min/mg.) Prostaglandin E_1 activates melanoma adenyl cyclase 1.5–2 times as much as MSH. Prostaglandin E_2 is half as effective as MSH and prostaglandins $F_{1\alpha}$, $F_{2\alpha}$, A, and B have very slight activity. Calcium is not required for activation of melanoma cyclase by the prostaglandins. 7-oxa-13-prostynoic acid and indomethacin interfere with activation by MSH. In the case of indomethacin the levels required for inhibition are two orders of magnitude higher than those which inhibit prostaglandin synthetase. MSH does not stimulate prostaglandin synthesis (accumulation of E_2 or $F_{2\alpha}$) in broken-cell preparations of the melanoma. There is a partial additivity between prostaglandin E_1 and MSH stimulation of melanoma cyclase. The data support the existence of two separate cyclase receptors in the melanoma, one responsive to prostaglandin E_1 and the second to MSH. MSH does not appear to stimulate cyclase by stimulating prostaglandin synthetase. Release of stored prostaglandins, however, or stimulation of the synthesis of prostaglandin precursors by MSH cannot be excluded at present. Prostaglandin E_1 has also been found to mimic MSH in frog skin causing both increases in cyclic-AMP levels and darkening.

ACKNOWLEDGMENTS

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