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Evidence for separate receptors for melanophore stimulating hormone and catecholamine regulation of cyclic AMP in the control of melanophore responses

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Summary

1. Skins of the lizard *Anolis carolinensis* darken *in vitro* in response to melanophore stimulating hormone (MSH), a peptide hormone, as well as to catecholamines. These hormones darken *Anolis* skins by dispersing the subcellular organelle, the melanosome, out into the dendritic processes of the dermal melanophores.

2. Dibutyryl cyclic AMP and methylxanthines also darken skins. In addition, methylxanthines are synergistic with both catecholamines and MSH in causing skin darkening. These data suggest that the dispersion of melanosomes within melanophores in response to both MSH and catecholamines is mediated by cyclic AMP.

3. α-Adrenoceptor blocking agents inhibit MSH-induced darkening but potentiate catecholamine-induced darkening. β-Adrenoceptor blocking agents, in contrast, inhibit catecholamine-induced darkening but have no effect on MSH-induced darkening. This selective blockade of one receptor while the functional integrity of the other receptor is maintained suggests that MSH and catecholamines increase cyclic AMP levels through different receptors.

4. Catecholamines exert their action through β-adrenoceptors ; MSH darkens skins through what appears to be a component of the α-adrenoceptor. β-Adrenoceptor stimulation may stimulate adenyl cyclase to increase cyclic AMP levels whereas MSH may inhibit cyclic AMP phosphodiesterase thereby preventing cyclic AMP breakdown.

Introduction

Various tissues are regulated by both peptide or protein hormones as well as by catecholamines (such as adrenaline and noradrenaline). In adipose tissue, catecholamines and ACTH both stimulate lipolysis, and in the liver, glucagon as well as catecholamines regulate glycogenolysis. In these tissues, in the dog and cat at least, catecholamines apparently mediate their actions through β-adrenoceptors (Ellis, Kennedy, Eusebi & Vincent, 1967) possessed by the individual parenchymal cells. It has been suggested (Sutherland, Øye & Butcher, 1965 ; Sutherland & Robison, 1966) that catecholamines, as well as ACTH and glucagon, act as first

messengers to stimulate the formation of cyclic 3′,5′-adenosine monophosphate (cyclic AMP) which then acts as an intracellular second messenger to initiate either lipolysis or glycogenolysis. The purpose of this report is: (1) to provide data that both melanophore stimulating hormone (MSH, intermedin), a peptide, and catecholamines regulate melanosome dispersion within vertebrate melanophores and do so by apparently increasing the intracellular level of cyclic AMP which is then more directly responsible for stimulating melanosome dispersion; (2) to demonstrate that the receptors for MSH and catecholamines are separate, as evidenced by the selective blockade of one receptor while the functional integrity of the other receptor is maintained.

Methods

Male and female lizards, *Anolis carolinensis*, were obtained from the Snake Farm, Laplace, Louisiana. After decapitation, the back skins of the lizards were removed and rinsed in Ringer solution (pH 7·4). Each skin was then mounted on a metal ring and held in place thereon by an outer overlapping plastic ring (Shizume, Lerner & Fitzpatrick, 1954). These skins were then allowed to remain in Ringer solution for about 2 h to equilibrate.

The colour change of *Anolis* skins results from the intracellular movement of melanin granules (melanosomes) within melanophores. Dispersion of the melanosomes from a perinuclear position out into the melanophore processes results in darkening of the skins; conversely, melanosome aggregation results in lightening of the skins. These changes in response to hormonal or pharmacological stimulation are measured as changes in light reflectance from the outer epidermal surface of the skins using a Photovolt photoelectric reflection meter (Photovolt Corporation, New York, N.Y.) as originally described for the bioassay of MSH (Shizume *et al.*, 1954). Skin lightening results in an increase in reflectance whereas skin darkening decreases the reflectance. An initial reflectance value is obtained for each skin and these skins are then arranged so that the total average reflectance value for each group is approximately equal. The initial average reflectance value for each group of skins is given a value of 100%. Succeeding average values are compared with the initial (base) values and recorded as a percent of the initial value of 100%; for example, a value of 90% represents a 10% decrease in reflectance.

The hormonal and pharmacological agents were obtained, prepared, and utilized as previously described (Goldman & Hadley, 1969a; Goldman & Hadley, 1969b). Statistical comparisons of mean values were made using Student's *t* test.

Results

Melanophore stimulating hormone, a peptide hormone, is the most potent darkening agent of *Anolis carolinensis* skin (Fig. 1A). Catecholamines such as isoprenaline also disperse melanosomes thereby darkening *Anolis* skins, but to a lesser extent (Fig. 1B). Catecholamines and MSH play a normal physiological role in melanosome dispersion in amphibians (Goldman & Hadley, 1969b), as well as in the lizard, *Anolis* (Hadley & Goldman, 1969). β-Adrenoceptors regulate this catecholamine-induced darkening of *Anolis* skins (Goldman & Hadley, 1969a). As for many other tissues, *Anolis* melanophores also possess α-adrenoceptors which mediate melanosome aggregation resulting in skin lightening, a response which is

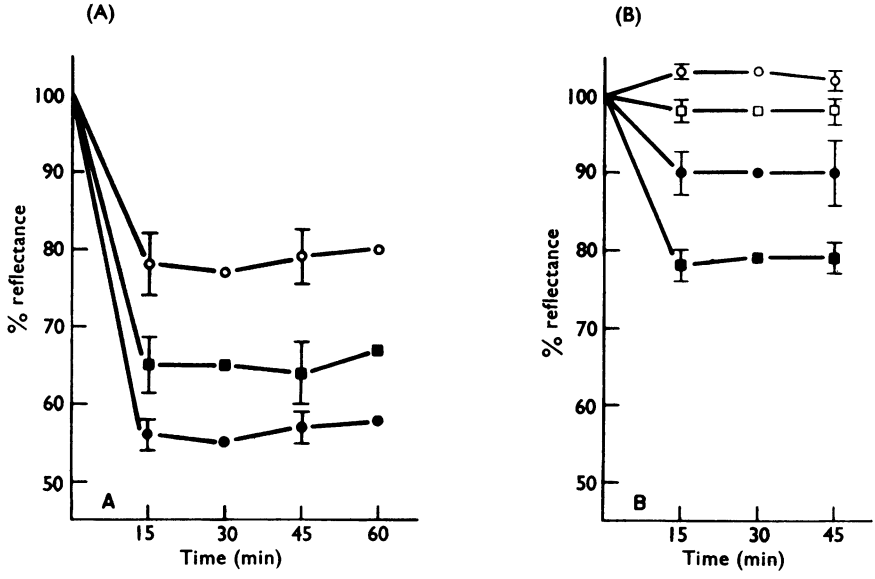

FIG. 1. Comparative darkening of *Anolis carolinensis* skins by MSH and isoprenaline. A, Three groups of seven skins each were treated with different concentrations of MSH (○, 1×10^{-9} g/ml ; ■, 3×10^{-9} g/ml ; ●, 6×10^{-9} g/ml). B, Three groups of eight skins each were treated with different concentrations of isoprenaline (□, 1×10^{-9}M ; ●, 1×10^{-8}M ; ■, 1×10^{-7}M) while one group remained untreated as a Ringer control (○). In both graphs each point represents the mean reflectance measurement from the seven or eight skins comprising the group. Vertical bars represent the standard error of the mean.

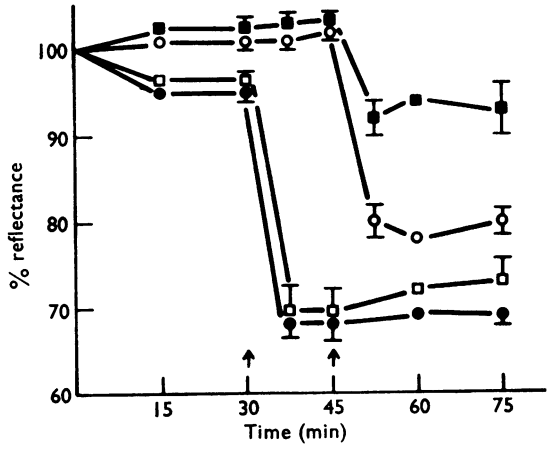

FIG. 2. Enhancement of catecholamine-induced darkening of *A. carolinensis* skin by phentolamine. Two groups (□ and ●) of eight skins each were preincubated in phentolamine (10^{-4}M) while two other groups (■ and ○) of eight skins each remained in Ringer solution. At 30 min (arrow) isoprenaline (10^{-6}M) was added to one of the groups (●) containing phentolamine, while adrenaline (10^{-6}M) was added to the other group (□). At 45 min (arrow) the same concentrations of isoprenaline (○) and adrenaline (■) were each added to one of the groups of skins in Ringer solution. Each point on the graph represents the mean reflectance value from the eight skins per group. Vertical bars indicate the standard error of the mean.

antagonistic to β-adrenoceptor stimulation (Goldman & Hadley, 1969a). Therefore, to obtain a maximal darkening by catecholamines, the presence of an α-adrenoceptor blocking agent is required. Phentolamine, an α-adrenoceptor blocking agent, inhibits this antagonistic α-adrenoceptor activity thereby allowing a more complete stimulation of the β-adrenoceptors by either adrenaline or isoprenaline (Fig. 2). Similar results have been obtained using the α-adrenoceptor blocking agent, Dibenamine (Goldman & Hadley, 1969a).

Dibutyryl cyclic AMP, a potent derivative of cyclic AMP, causes a maximal darkening of *Anolis* skins (Table 1), suggesting that the normal physiological darkening of skins by MSH and catecholamines may be mediated through cyclic AMP. The methylxanthines, caffeine and theophylline, inhibit cyclic AMP phosphodiesterase (which degrades cyclic AMP) thereby increasing intracellular levels of cyclic AMP (Sutherland & Rall, 1958; Butcher & Sutherland, 1962). Methylxanthines not only darken *Anolis* skins (Hadley & Goldman, 1969) but are also synergistic with the darkening produced by MSH (Table 1). Catecholamines lighten skins previously darkened by MSH by stimulating α-adrenoceptors, but when added to methylxanthine-darkened skins they greatly increase the darkening (Table 2). These data indicate that catecholamines and MSH both cause melanosome dispersion within melanophores by mechanisms which in both cases appear to involve cyclic AMP.

TABLE 1. *Darkening of* Anolis carolinensis *skin by MSH, dibutyryl cyclic 3′,5′-AMP and methylxanthines*

Experiment	Number of Lizards	Treatment	% Change in reflectance	*P*
A	12	1. DbcAMP	−45±2·52	1 and 2 >0·5
	12	2. MSH	−44±2·10	1 and 3 <0·001
	12	3. Ringer control	+ 1±1·36	2 and 3 <0·001
B	8	1. Theo	−21±1·47	1 and 2 >0·5
	8	2. MSH	−20±1·45	1 and 3 <0·001
	8	3. Theo+MSH	−48±2·08	2 and 3 <0·001
	8	4. Ringer control	− 1±0·94	2 and 4 <0·001

Abbreviations and concentrations: DbcAMP, dibutyryl cyclic 3′,5′-AMP (1×10^{-2}M); MSH, melanophore stimulating hormone (5×10^{-9} g/ml in exp. *A*; 3×10^{-9} g/ml in exp. *B*); Theo, theophylline (1×10^{-3}M). Values for % change in reflectance are means ± S.E. A plus sign (+) indicates an increase in reflectance (skin lightening) whereas a minus sign (−) indicates a decrease in reflectance (skin darkening). Means represent the maximal response within 60 min after addition of the agents. The differences between the groups (*P* value) were evaluated with Student's *t* test. In experiment *A* the difference between the DbcAMP group (group 1) and the MSH group (group 2) was not significant ($P>0.5$). However, the difference between the DbcAMP group (group 1) and the Ringer group (group 3) and the difference between the MSH group (group 2) and the Ringer group (group 3) were highly significant ($P<0.001$). Similar comparisons are made in experiment *B*.

TABLE 2. *Effect of adrenaline on MSH- and theophylline-induced skin darkening*

Number of Lizards	Treatment	% Change in reflectance	*P*
8	1. Theo	−20±2·80	
8	2. MSH	−20±1·45	
8	3. A after Theo	−19±3·17	3 and 4 <0·001
8	4. A after MSH	+20±3·83	3 and 5 <0·01
8	5. A Ringer control	− 6±2·80	4 and 5 <0·001

See Table 1 for abbreviations and explanations. Theo (1×10^{-3}M); MSH (3×10^{-9} g/ml); A, adrenaline (1×10^{-5}M). Skins were incubated in theophylline, MSH or Ringer for 30–60 min, after which adrenaline was added. In groups 3–5, values represent only the change after the addition of adrenaline, not any change caused by theophylline or MSH alone.

If, indeed, cyclic AMP mediates the action of both catecholamines and MSH on melanophores, the question remains as to whether these hormones act through the same receptor mechanism. As demonstrated in Fig. 2, α-adrenoceptor blockade enhances catecholamine darkening by unmasking the β-adrenoceptors present, but this α-adrenoceptor blockade also completely blocks MSH darkening (Fig. 3). Although Dibenamine blocks MSH action, the addition of noradrenaline causes these same skins to darken. This results from β-adrenoceptor stimulation which is greatly enhanced by Dibenamine over that of control skins (Fig. 3). This darkening by noradrenaline after Dibenamine blockade of the α-adrenoceptor and MSH, demonstrates that the concentration of Dibenamine used is not toxic and that the skins are responding in a normal manner. In contrast, dichloroisoprenaline, a β-adrenoceptor blocking agent, blocks the catecholamine-induced darkening but does not inhibit the response to MSH (Table 3). These results, then, clearly establish a preferential blockade of one receptor while the functional activity of the other receptor is maintained.

TABLE 3. *Effect of β-adrenoceptor blockade on catecholamine- and MSH-induced darkening*

Number of lizards	Treatment	% Change in reflectance	*P*
8	1. MSH	−48±3·55	1 and 2 >0·5
8	2. DCI+MSH	−50±2·92	
8	3. Iso	−16±2·65	3 and 4 <0·001
8	4. DCI+Iso	− 2±1·98	

MSH, (3×10^{-9} g/ml); DCI, dichloroisoprenaline (2×10^{-5}M); Iso, isoprenaline (1×10^{-5}M). Skins were preincubated in DCI for 30 min before the addition of MSH or isoprenaline. Values represent the maximal response within 30 minutes after the addition of MSH or isoprenaline.

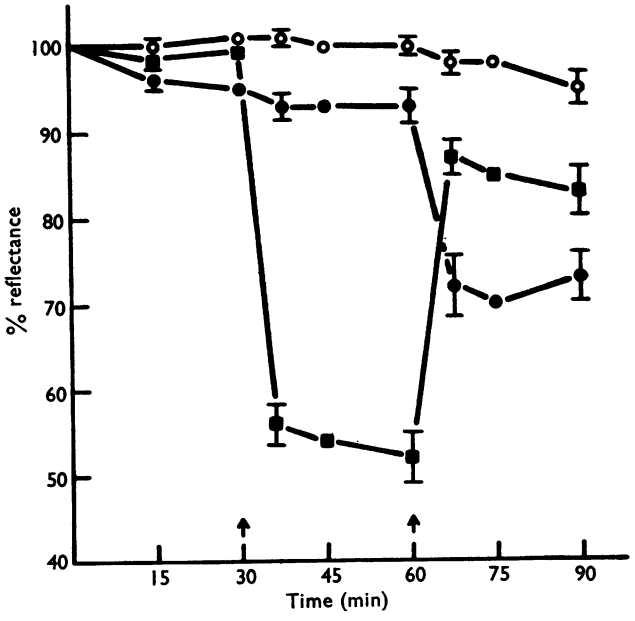

FIG. 3. Inhibition of MSH-induced darkening of *Anolis* skins by Dibenamine. One group of eight skins (●) was preincubated in Dibenamine (10^{-5}M) while two other groups of eight skins each (○ and ■) remained in Ringer solution. At 30 min (arrow) MSH (3×10^{-9} g/ml) was added to the group of skins in Dibenamine (●) and to one Ringer group (■). At 60 min (arrow) noradrenaline (10^{-5}M) was added to all the skins. Each point in the figure represents the average reflectance value for the eight skins in the group. Standard error of the mean is indicated by vertical bars.

Discussion

Both MSH and catecholamines darken skins of the lizard, *Anolis carolinensis,* by dispersing melanosomes within melanophores. Methylxanthines as well as dibutyryl cyclic 3′,5′-adenosine monophosphate also darken *Anolis* skins and it has been suggested (Hadley & Goldman, 1969) that both catecholamines and MSH darken *Anolis* skins by increasing intracellular concentrations of cyclic AMP within melanophores. Our demonstration that both MSH and catecholamines are synergistic with theophylline in causing darkening substantiates a role for cyclic AMP in MSH- as well as catecholamine-induced melanosome dispersion.

There is strong evidence from many other studies that stimulation of β-adrenoceptors leads to an increase in cyclic AMP (Robison, Butcher & Sutherland, 1967). Although it has been suggested that " it seems likely that in most and perhaps all tissues the *beta* receptor and adenyl cyclase are the same " (Robison *et al.,* 1967), more recently it was suggested that " in those tissues where adrenergic *beta* receptors occur, these receptors are closely associated with (if not an integral component of) the adenyl cyclase system " (Sutherland, Robison & Butcher, 1968). Nevertheless, the receptor through which MSH would stimulate adenyl cyclase to form cyclic AMP which would then cause melanosome dispersion has not been defined.

Bitensky, Russell & Robertson (1968) have recently suggested that in the rat liver two separate adenyl cyclase systems exist: one system would be stimulated by glucagon and the other by adrenaline. Other data obtained from studies on lipolysis in fat cells have suggested (Stock & Westermann, 1966 ; Lech & Calvert, 1967 ; Fain, 1967 ; Wenke, Lincova, Cepelik, Cernohorsky & Hynie, 1967) that different receptors or sites of action exist for catecholamines and ACTH. Similarly, Levey & Epstein (1969), working on glucagon stimulation of the heart, have stated that " the heart appears to contain more than one receptor site responsible for activation of adenyl cyclase ". It is reasonable, therefore, to suggest that catecholamines and MSH stimulate separate receptors which in turn then stimulate adenyl cyclase in *Anolis* skins.

As Moran (1966) has pointed out, the interaction of a hormone or pharmacological agent with the receptor " represents the first step of a multistep sequential reaction which leads to the response of the cell ". Along this line of thinking, two or more different receptors in one tissue can be stimulated to give a common effect (Moran, 1966). Applying this to *Anolis* melanophores, catecholamines could stimulate adenyl cyclase through one receptor and MSH through a different receptor. The final result would, nevertheless, be the same—namely, melanosome dispersion.

The inhibition of MSH darkening by Dibenamine, an α-adrenoceptor blocking agent, suggests that MSH may be working through a component of the α-adrenoceptor. Pauk & Reddy (cited in LaRaia, Craig & Reddy, 1968) have similarly observed that " the glycogenolytic effect of glucagon on the rat liver has been blocked by α-adrenergic agents ". Fain, Galton & Kovacev (1966) have also found that phenoxybenzamine, an α-adrenoceptor blocking agent similar to Dibenamine, reduces the lipolytic activity of growth hormone but has little effect on the lipolytic effect of catecholamines. Most workers have probably assumed that MSH increases cyclic AMP by a direct activation of adenyl cyclase. However, it seems reasonable to suggest that MSH might, in fact, be acting through an action on phosphodiesterase, an enzyme which destroys cyclic AMP. If MSH were to inhibit

the activity of phosphodiesterase, this should, theoretically, lead to an intrinsic intracellular increase in cyclic AMP. Such an interpretation shifts the emphasis of hormone action from a direct and active stimulation of adenyl cyclase to an indirect one involving phosphodiesterase inhibition. This hypothesis is consistent with the data presented in this communication.

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