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THE ASSOCIATION BETWEEN THE MELANOCYTE-STIMULATING HORMONE RECEPTOR AND THE α_2-ADRENOCEPTOR ON THE *Anolis* MELANOPHORE

R.J. CARTER & S. SHUSTER

Department of Dermatology, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 4LP

1 The primary effect of catecholamines was to lighten *Anolis* skin previously darkened by α-melanocyte-stimulating hormone (α-MSH). In concentrations above 10^{-7} M noradrenaline, 10^{-6} M adrenaline and 10^{-5} M dopamine, darkening of subpopulations of melanophores occurred. Subsequent experiments were concerned with the effect of low catecholamine concentrations on α-MSH action.

2 The relationship between MSH receptors and α-adrenoceptors on the *Anolis* melanophore was studied by a kinetic approach using the *rate* bioassay method and by use of α-adrenoceptor agonists and antagonists.

3 α-MSH dose-response curves were shifted, in parallel, to the right in the presence of the catecholamines, noradrenaline, adrenaline and dopamine, and Lineweaver-Burke plots and Arunlakshana-Schild plots indicated that the catecholamines antagonized MSH action by a competitive mechanism.

4 Phentolamine had an inhibitory effect on the action of adrenaline but not on the action of MSH. Therefore MSH and catecholamine actions were mediated by separate receptors.

5 The classical kinetics of competition are not confined to competition at a single receptor.

6 The α-adrenoceptor was defined as the α_2-subtype since (a) the α_2-selective agonist, clonidine, was found to mimic catecholamine action. (b) The α_2-selective antagonist, yohimbine, blocked the actions of clonidine and adrenaline. (c) The α_1-selective antagonist, prazosin, had negligible blocking effects on adrenaline and clonidine.

7 We conclude that a close association exists between the separate MSH receptor and α_2-adrenoceptor on the *Anolis* melanophore. The competition that takes place between MSH and catecholamines must occur after hormone-receptor interaction, possibly through a common adenylate cyclase moiety oppositely controlled by the two receptors involved.

Introduction

Catecholamines antagonize melanocyte-stimulating hormone (MSH)-induced darkening of the *Anolis* skin (Sand, 1935; Kleinholz, 1938; Horowitz, 1958; Lerner, 1959; Lerner & Case, 1959; Goldman & Hadley, 1969; Tilders, van Delft & Smelik, 1975) and their action is thought to be mediated by α-adrenoceptors on the melanophore (Abe, Butcher, Nicholson, Baird, Liddle & Liddle, 1969; Hadley & Goldman, 1969; 1970; Bagnara & Hadley, 1973; Pettinger, 1977). In the present paper, we have examined the interaction of MSH and catecholamines on the *Anolis* melanophore. By using the *rate* bioassay, we were able to apply a kinetic approach to a study of the mechanism and by using selective α-adrenoceptor agonist and antagonist drugs we were able to define the α-adrenoceptor subtype. Some of these results have been reported to a joint meeting of the Medical Research Society and Physiological Society (Carter & Shuster, 1981).

Methods

Lizards

The lizards (*Anolis carolinensis*) were obtained from De Natuurvriend, Donkerregaard, Utrecht and were housed in a terrarium with 14 h light and 10 h dark. A temperature gradient of 20°–35°C was maintained across the terrarium during the light hours while the minimal temperature attained at night was 18°C. The lizards were fed with *Tenebrio* larvae and flies and they drank water off the vegetation which was sprayed twice daily.

Bioassay

The *rate* method of MSH bioassay (Carter & Shuster, 1978a) was used in these experiments. Uniformly green 2 mm square skin fragments were dissected

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from the trunk of a green adult lizard and used for the assay after 1–2 h equilibration in the assay medium. A series of identical two fold dilutions of α-MSH were prepared, in duplicate, in columns in a plastic agglutination dish, each dilution containing 0.1 ml. To the first pair of columns, 0.1 ml of assay medium alone was added to each dilution. To the following pairs of columns, 0.1 ml of a concentration of adrenoceptor agonist drug, antagonist drug or combination of agonist and antagonist was added. The drugs were diluted and added to the appropriate dilutions immediately before bioassay in order to avoid their possible breakdown in solution. Green *Anolis* skin fragments were then added to each dilution and the time was recorded for each skin fragment to change colour to the specific and uniform brown-green colour as described previously (Carter & Shuster, 1978a).

The reciprocal of darkening time, darkening speed, was plotted against log dose of α-MSH for each dose-response in the presence or absence of added catecholamine. Double-reciprocal plots (Lineweaver-Burke, 1934) were drawn in which darkening time was plotted against reciprocal dose in the presence or absence of catecholamine as well as Arunlakshana-Schild plots (1959) (log dose-ratio −1 against log dose of catecholamine). Least square regression and analysis of variance was used for comparison of dose-response curves and calculation of potencies and 95% fiducial limits of potencies (Bliss, 1952).

Results

In preliminary experiments on skin samples, catecholamines, in concentrations of $>10^{-7}$ M noradrenaline, $>10^{-6}$ M adrenaline or $>10^{-5}$ M dopamine, were found to produce localized dark green-black spots of about 1 mm diameter. This speckling effect ('excitation darkening', Sand, 1935) is only produced by high concentrations of catecholamines and is quite unlike the uniform browning response of the skin to MSH peptides. It is though to be due to stimulation of β-adrenoceptors present only in a sub-population of melanophores (Hadley & Goldman, 1969; 1970; Bagnara & Hadley, 1973). This atypical effect of catecholamines was not studied further. When added in lower concentrations, the catecholamines had no effect on green *Anolis* skin but they were found to lighten skin fragments previously darkened by MSH (Figure 1). Since the major action of the catecholamines appeared to be by opposition to MSH activity, in subsequent experiments the effect of adrenergic control was therefore studied on α-MSH dose-response curves.

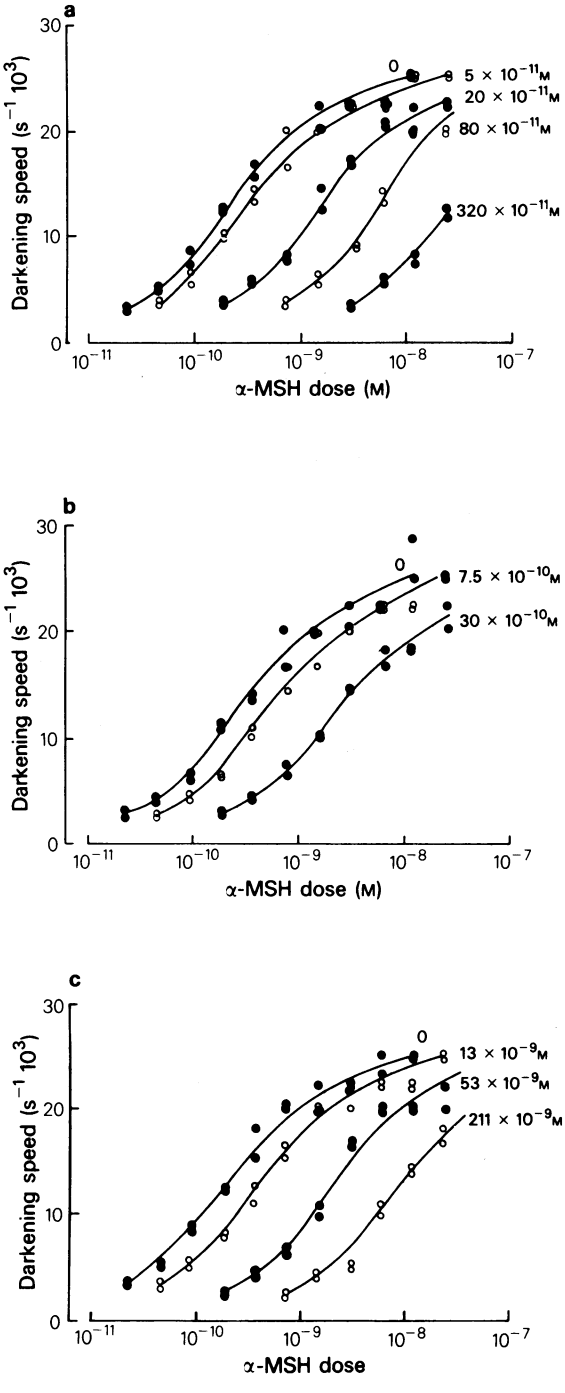

Figure 1 The effect of (−)-noradrenaline bitartrate (a), (−)-adrenaline bitartrate (b) and dopamine (c) on the α-MSH dose-response curve. Dose-response curves were obtained to α-MSH alone and in the presence of various concentrations of the catecholamines.

Figures 1a, b and c show the sigmoid dose-response curves to α-MSH in the presence or absence of noradrenaline, adrenaline and dopamine respectively. In each case the slopes of the α-MSH dose-response curves in the presence of the catecholamines did not differ significantly from the slope of the α-MSH dose-response curve on its own ($P > 0.05$). Increasing concentrations of the catecholamines caused a progressive shift of the α-MSH dose-response curves to the right and the shifts were significant ($P < 0.01$) with (−)-noradrenaline 4.9×10^{-11} M, adrenaline 7.5×10^{-10} M and dopamine 1.3×10^{-8} M. The slopes of the α-MSH curves in the double reciprocal plots (Figures 2a, b and c) increased progressively as the concentration of catecholamine increased while the intercept on the y-axis (darkening time) remained constant in each case.

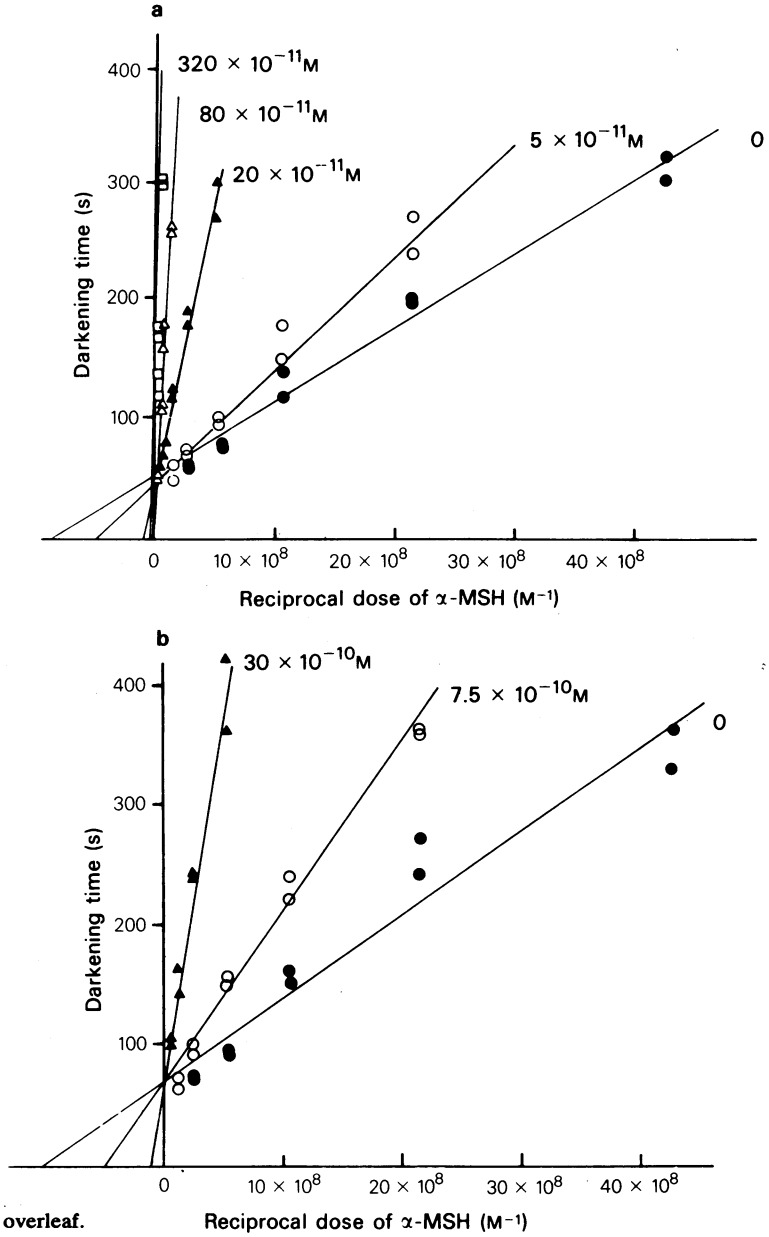

Figure 2 See legend overleaf.

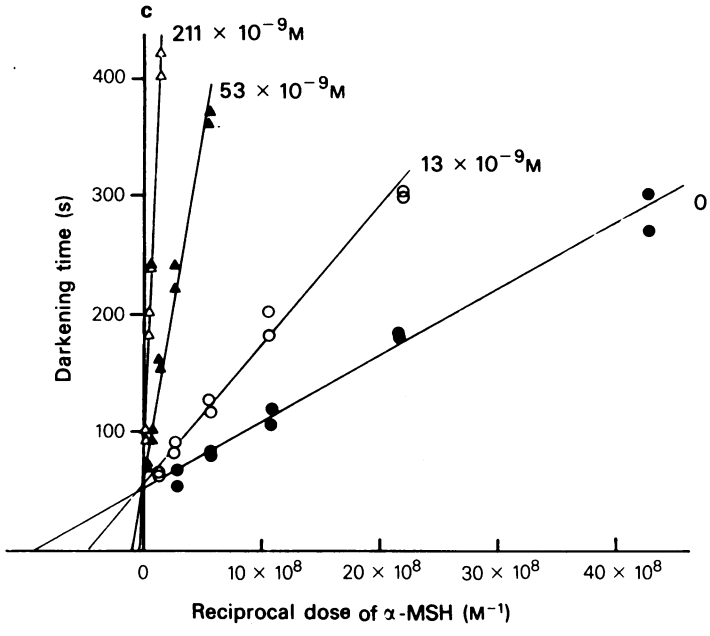

Figure 2 The effect of (−)-noradrenaline bitartrate (a), (−)-adrenaline bitartrate (b) and dopamine (c) on the double-reciprocal plot for α-MSH (Lineweaver-Burke). In each case darkening time (reciprocal of rate of darkening) was plotted against reciprocal of dose of α-MSH.

Significant linear slopes ($P<0.01$) were obtained for the catecholamines in the Arunlakshana-Schild plots (Figure 3) and the slopes did not deviate significantly from unity ($P>0.05$). Calculated pA_2 values from the Arunlakshana-Schild plot were noradrenaline, 10.2; adrenaline, 9.06 and dopamine, 7.96.

These findings suggested that catecholamines antagonized MSH action by a competitive mechanism. Therefore a possible, but unlikely, explanation for these findings was that the catecholamines and MSH shared a common receptor. To exclude this, we studied the effect of the α-adrenoceptor antagonist, phentolamine, on the action of MSH and on the inhibitory effect of adrenaline on MSH activity. Figure 4 shows that α-MSH potency was not altered by phentolamine whereas much of the inhibitory effect of adrenaline 3×10^{-9} M on the MSH response (Figure 4) was blocked by phentolamine 5×10^{-7} M. Thus MSH and catecholamines act on independent receptors and the competition between them occurs at a site other than on a common receptor.

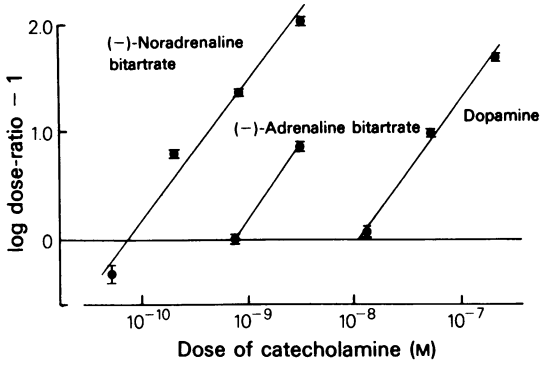

Figure 3 Arunlakshana-Schild plot for the inhibitory effect of the catecholamines on α-MSH activity. Dose-ratios were calculated from the data shown in Figure 1 and vertical bars represent 95% confidence limits of the estimated dose-ratios.

Phentolamine blocks both the α_1- and α_2-adrenoceptor subtypes and adrenaline is both an α_1- and α_2-agonist. The following experiments were therefore done to define which of these α-adrenoceptor subtypes was present in the *Anolis* melanophore. Clonidine, which is an α_2 selective agonist, shifted the α-MSH dose-response curve to the right and in parallel when given in concentrations of between 2×10^{-8} M and 3×10^{-7} M, and its effect was therefore identical to that of the catecholamines, adrenaline, noradrenaline and dopamine. Moreover, the Arunlakshana-Schild plot of its activity was linear with a slope that did not deviate significantly from unity ($P>0.05$) and its calculated pA_2 value was 7.73. This suggested that catecholamine action could be mediated by α_2-adrenoceptors on the melanophore.

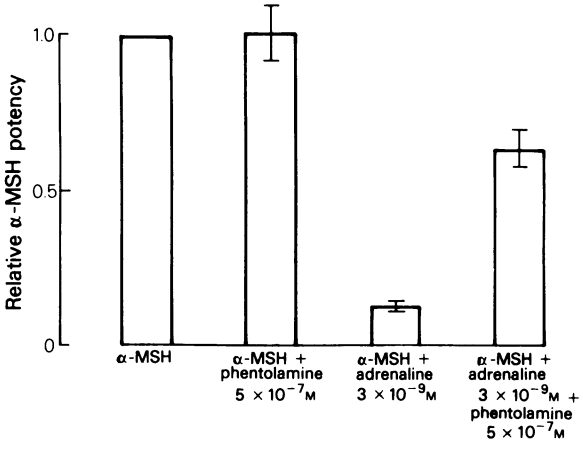

Figure 4 Potencies of α-MSH (calculated from the dose-response curves) in the presence of phentolamine, adrenaline or phentolamine + adrenaline calculated relative to that of α-MSH on its own. Vertical bars represent 95% fiducial limits of the estimated potencies.

To test the specificity of the melanophore response to clonidine further, we used yohimbine which is a selective α_2-antagonist. The results (Figure 5) show that the inhibitory action of clonidine 10^{-7} M on the α-MSH response was blocked by yohimbine 5×10^{-7} M providing further evidence of an α_2-adrenoceptor. If the competitive action of catecholamines on MSH was due solely to α_2-adrenoceptor activity then it would be expected that the effect of adrenaline, which has α_1- and α_2-agonist properties, could be blocked by an α_2, but not by an α_1-antagonist. Figure 5 shows that the inhibitory action of adrenaline 5×10^{-9} M was blocked by yohimbine 5×10^{-7} M. Furthermore we could find no evidence of α_1-adrenoceptor activity using prazosin which is an α_1-selective antagonist. Thus, while adrenaline 3×10^{-9} M reduced α-MSH potency to 0.20, with prazosin 10^{-7} M in addition, the α-MSH potency was still 0.20. Higher concentrations of prazosin, 10^{-6} and 10^{-5} M, increased α-MSH potency in the presence of adrenaline (3×10^{-9} M) to 0.31 and 0.45 respectively, but we considered these concentrations too high to be used as evidence for the presence of an α_1-adrenoceptor. Neither 10^{-7} nor 10^{-6} M prazosin altered the potency of α-MSH in the presence of clonidine (10^{-7} M).

Discussion

Skin colour in *Anolis* is influenced both by MSH and catecholamines. Although relatively high concentrations of catecholamines stimulate a localized subpopulation of melanophores the primary action is to lighten pigmented skin. The present studies suggest that one mechanism by which this takes place is an interaction with MSH activity and although we used only α-MSH, we have found the same effect of catecholamines on the action of other MSH peptides

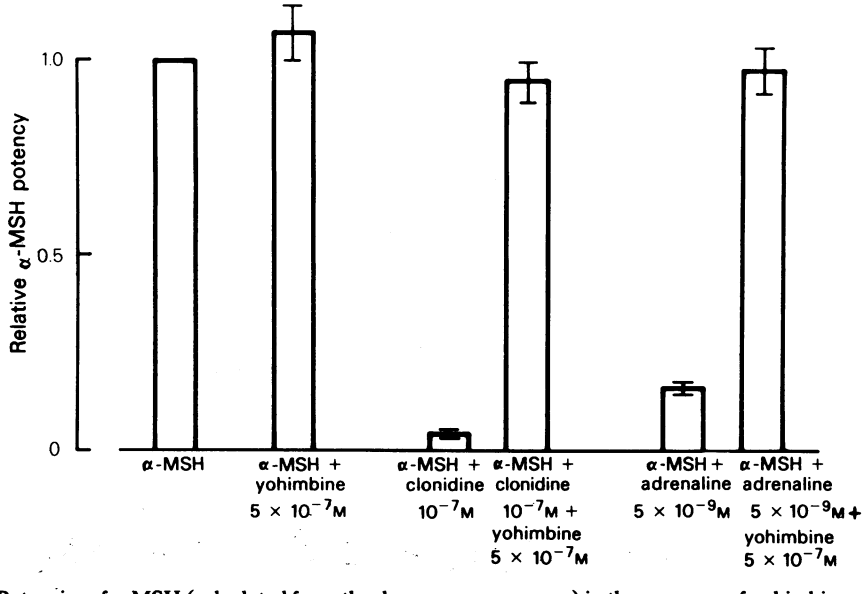

Figure 5 Potencies of α-MSH (calculated from the dose-response curves) in the presence of yohimbine, clonidine, (−)-adrenaline bitartrate, clonidine plus yohimbine or (−)-adrenaline bitartrate plus yohimbine, calculated relative to that of α-MSH on its own. Vertical bars represent 95% fiducial limits of the estimated potencies.

(unpublished). We found clear cut kinetic evidence of competitive interaction despite the pharmacological evidence of separate receptors. The classical studies in which the kinetics of competitive interaction were elucidated (e.g. Arunlakshana & Schild, 1959) were concerned with competition at a single receptor. Our evidence is that identical kinetic behaviour may also be characteristic of substances with separate receptors where competition takes place at some other site. For this reason, kinetic evidence cannot be used to predict the site of competitive interaction without independent evidence, e.g. pharmacological, as in the present study, or receptor binding techniques.

Our pharmacological evidence of two separate receptors, the MSH receptor and an α-adrenoceptor, suggested that the competition between MSH and catecholamines must occur at a point after stimulation of their respective receptors. Elucidation of the α-adrenoceptor as an α_2 subtype suggested a possible site for this competition since it has recently been shown that α_2-adrenoceptor stimulation leads to inhibition of adenylate cyclase activity (Rodbell, 1980) and it is well established that MSH receptor stimulation activates adenylate cyclase (Abe *et al.*, 1969). Thus the point at which competition takes place could be an adenylate cyclase moiety common to the two discrete receptors (Figure 6).

The α_2-receptor has mostly been associated with the presynaptic neuronal membrane (Starke, Endo & Taube, 1975; Langer, 1974; 1976; Berthelsen & Pettinger, 1977; Rodbell, 1980), although recent work suggests it is not confined to it (Grant & Scrutton, 1979). Our present finding of an α_2-

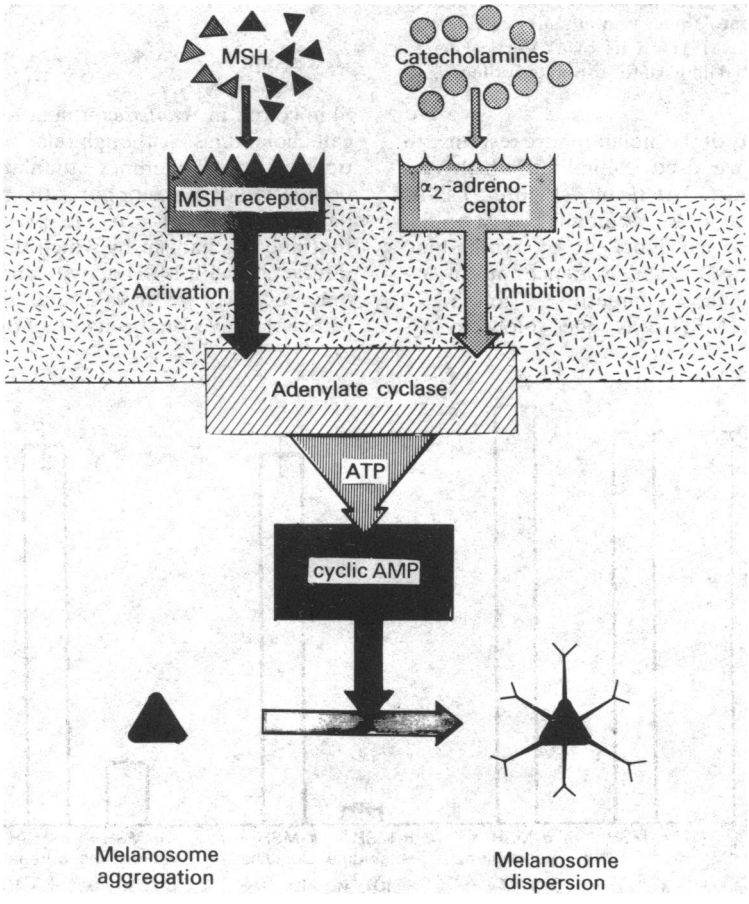

Figure 6 Diagrammatic representation of the possible association between the MSH receptor and α_2-adrenoceptor on the *Anolis* melanophore. If a common adenylate cyclase moiety is linked to both the MSH receptor and α_2-adrenoceptor stimulation of the MSH receptor would activate the enzyme, while α_2-adrenoceptor stimulation would inhibit it. Competitive interaction between MSH peptides and the catecholamines would therefore occur at the common adenylate cyclase moiety.

adrenoceptor on the *Anolis* melanophore is therefore consistent with our earlier finding (Carter & Shuster, 1978b) that pigment cells have similarities to brain cells, a phenomenon perhaps to be explained by the common neural crest origin of both cell types. Thus, as well as acting on cutaneous pigment cells, the MSH peptides have been found in and act upon neural tissues (Kastin, Miller, Nockton, Sandman, Schally & Stratton, 1973; Garrud, Gray & De Wied, 1974; Leonard, Kafoe, Thody & Shuster, 1976; Ashton, Millman, Telford, Thompson, Davies, Hall, Shuster, Thody, Coy & Kastin, 1977; De Wied, 1977; Shuster, Smith, Plummer, Thody & Clark, 1977; Shuster, Carter, Thody, Smith, Fisher & Cook, 1978; O'Donohue, Miller & Jacobowitz, 1978; Oliver & Porter, 1978; Eskay, Giraud, Oliver & Brownstein, 1979; Thody, 1980). In the light of the present findings on the *Anolis* melanophore, the possible interaction between the MSH peptides and α_2-adrenoceptors taking place in other tissues, particularly the CNS, must also now be considered. At the same time, the activity of the cutaneous pigment cell may itself serve as a model for the physiology and pharmacology of less accessible tissues such as the brain (Carter & Shuster, 1978b; Shuster, 1981).

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