neas soaked in serum for 72 hours are the most satisfactory for penetrating keratoplasty. The exact mechanism by which our soaking regimen decreases the apparent antigenicity of the donor material is not clear, and our results suggest that more than one mechanism may be operable.

The demonstration of the effectiveness of the treatment of corneal buttons with ALS in modifying the homograft reaction should be immediately applicable in man in those cases where graft rejection is expected to occur, that is, in patients who have already had graft reaction, highly vascularized corneal tissue, and so forth.

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## Calcium Requirement for Melanophore-Stimulating Hormone Action on Melanophores

Abstract. *The calcium ion is specifically required for the action of melanophore-stimulating hormone on melanosome dispersion within lizard* (Anolis carolinensis) *melanophores in vitro. The response to this hormone is directly related to the concentration of the $Ca^{2+}$ ion. Lithium, choline, rubidium, and cesium will replace the sodium and potassium of Ringer solution if $Ca^{2+}$ is present. Calcium ions are not required for melanosome dispersion itself, since theophylline or dibutyryl cyclic adenosine monophosphate reversibly darkens lizard skins in the absence of calcium.*

Melanophore-stimulating hormone (MSH) initiates the movement (dispersion) of melanosomes from a perinuclear (aggregated) position out into the dendritic processes of vertebrate melanophores (*1*). This leads to a rapid darkening of the skin of most poikilotherms and may precede and be prerequisite for melanin synthesis (*2*). A number of in vitro studies have been directed toward understanding the possible ionic requirements for MSH action. It has been reported that there is an "absolute" requirement for the sodium ion in the action of MSH on frog (*Rana pipiens*) melanophores (*3*). Not only was the calcium ion reported not to be required for melanophore responses to MSH, but, indeed, the response was said to be enhanced in a $Ca^{2+}$-free medium (*3, 3a*). Others (*4*) have suggested that melanosome dispersion within frog melanophores results from an increase in the intracellular level of calcium ions and a simultaneous decrease in intracellular potassium ions. Fingerman has implicated a role for $Ca^{2+}$ ions in the hormonal control of crustacean chromatophores (*5*). Horowitz (*6*) showed that melanosome aggregation within melanophores of the lizard *Anolis carolinensis* was facilitated by the removal of $Ca^{2+}$ ions from Ringer solution. Novales (*3, 3a*), on the other hand, reported that there is a sodium requirement for MSH action on melanophores of this lizard.

In the present report we demonstrate a specific $Ca^{2+}$ ion requirement for MSH action on melanophores of the lizard *Anolis carolinensis*. We also show that neither sodium nor potassium ions are necessary for MSH action and, in addition, that the $Ca^{2+}$ ion requirement is for an early event in MSH action, since this divalent cation is not necessary for melanosome movements per se. The in vitro response of the integumental melanophores of *Anolis* to MSH or other agents under varying ionic conditions was studied by photometric reflectance methods as described originally for frog skin (*1*) and modified slightly for *Anolis* skin (*7*). Melanosome dispersion in response to MSH results in a change from a bright green to a dark brown color and this change can be measured as a decrease in reflectance from the outer (epidermal) surface of isolated skins. Conversely, melanosome reaggregation resulting in a lightening of skins is correlated with an increase in reflectance.

As reported previously (*3, 3a*), MSH darkens *Anolis* skins immersed in Ringer (NaCl, 111 m*M*; $NaHCO_3$, 2 m*M*; KCl, 2 m*M*; $CaCl_2$, 1 m*M*) solution (Fig. 1). Skins residing solely in sodium chloride (120 m*M*) or in an equivalent amount of NaCl plus KCl (2 m*M*) are almost totally unresponsive to MSH. The addition of the $Ca^{2+}$ ion (1 m*M*) to the NaCl solution permits a response equal to that of the Ringer control group of skins (Fig. 1). The degree of darkening of lizard skins to MSH is clearly directly related to the concentration of the $Ca^{2+}$ ion present (Fig. 2).

These results indicated that either the $Ca^{2+}$ ion is needed directly for MSH action or else its role is permissive to the action of the $Na^+$ ion. Other monovalent cations were substituted for sodium to determine whether the $Na^+$ ion is specifically required in addition to the $Ca^{2+}$ ion for MSH action. Melanophore-stimulating hormone failed to darken skins in isotonic solutions (120 m*M*) of either lithium or choline chloride. The presence of $Na^+$ (1 m*M*) or $K^+$ (1 m*M*) ions in combination with either $Li^+$ or choline ions failed to reestablish a response of the melanophores to MSH. There was, however, a near

Table 1. Response (change in reflectance) of *Anolis* skins to MSH ($6 \times 10^{-9}$ g/ml), theophylline (10 m*M*), or dibutyryl cyclic AMP (DcAMP, 10 m*M*), in the presence or absence of calcium ion (1 m*M*). Each value represents the maximal mean percent reflectance change, ± standard error, of the skins comprising each experimental group. (Number of skins in parentheses.)

| | |
|---|---|
| NaCl, EDTA (5 m*M*), MSH (6) | 0 ± 0.67 |
| NaCl, $Ca^{2+}$, MSH (6) | 39 ± 3.69 |
| NaCl, EDTA (5 m*M*), theophylline (6) | 58 ± 2.31 |
| NaCl, $Ca^{2+}$, theophylline (6) | 58 ± 3.58 |
| NaCl, EDTA (5 m*M*), DcAMP (8) | 49 ± 2.54 |
| NaCl, $Ca^{2+}$, DcAMP (8) | 52 ± 1.44 |

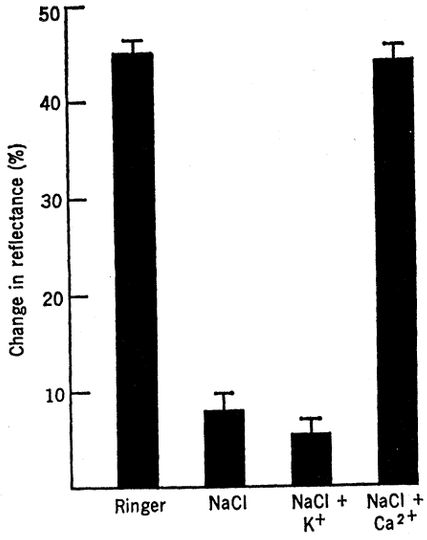


Fig. 1. Response (percent of change in reflectance) of *Anolis* skins to MSH ($4 \times 10^{-9}$ g/ml) in Ringer solution, or in isotonic NaCl (120 m*M*), or isotonic NaCl containing either 2 mmole of $K^+$ or 1 mmole of $Ca^{2+}$. Values represent the maximal responses, ± standard errors of the means, of the 16 skins representing each experimental group.

maximal response of the skins to MSH when $Ca^{2+}$ (1 m*M*) was added to either lithium or choline chloride isotonic (120 m*M*) solutions. Similar results were obtained with rubidium or cesium ions in place of the sodium ion. Only the $Ca^{2+}$ ion (1 m*M*) added to isotonic solutions of these monovalent cations reestablished the response of

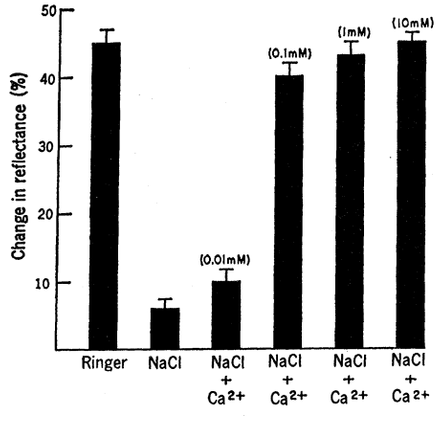


Fig. 2. Calcium-dependent dose response (percent of change in reflectance) of *Anolis* skins to MSH ($4 \times 10^{-9}$ g/ml). MSH was added to skins immersed in Ringer solution, isotonic NaCl (120 m*M*), or isotonic NaCl containing $Ca^{2+}$ ion at 0.01 m*M*, 0.1 m*M*, 1 m*M*, or 10 m*M* concentration. Values represent the maximal responses, ± standard errors of the means, of the 20 skins comprising each experimental group.

melanophores to MSH. The reported inhibition of MSH in a sodium-free potassium Ringer solution and the implication, therefore, of a sodium requirement for MSH action on *Anolis* melanophores as suggested previously (*3, 3a*), can now be explained simply as a further demonstration of a $Ca^{2+}$ requirement for MSH action.

Other divalent ions were substituted for $Ca^{2+}$ to determine the specificity of the $Ca^{2+}$ requirement for MSH action. Strontium, barium, or beryllium ions (1 m*M*) could replace $Ca^{2+}$ and permit melanosome dispersion in response to MSH. These other ions, however, are of limited significance, being generally unavailable under normal physiological conditions. The other divalent cations ($Mg^{2+}$, $Zn^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Cu^{2+}$) studied either failed to substitute for $Ca^{2+}$ or were apparently lethal to the melanophores. Either phosphate or bicarbonate, but not sulfate, anions could replace the chloride ion of Ringer solution and allow full MSH expression, but only if $Ca^{2+}$ was present. Thus the $Ca^{2+}$ ion is the only ion normally present within Ringer solution which is specifically required for MSH action. High concentrations of $CaCl_2$ (120 m*M*) mimic the action of MSH by dispersing melanosomes and darkening *Anolis* skin.

The immediacy of the $Ca^{2+}$ ion requirement for MSH action is clearly shown in Fig. 3. Skins only darken minimally in the absence of the $Ca^{2+}$ ion. Addition of $Ca^{2+}$ (1 m*M*) to the $Ca^{2+}$-free Ringer causes a rapid and maximal darkening of skins. Transfer of maximally darkened skins to $Ca^{2+}$-free Ringer (but still containing MSH, as before) results in a complete reversal (or lightening) of the darkened skins. The minimal darkening response of lizard skins to MSH in isotonic NaCl (Fig. 1) or $Ca^{2+}$-free Ringer (Fig. 3) could be totally inhibited or reversed by adding EDTA (ethylenediaminetetraacetic acid, 5 m*M*) to the skins (Table 1). Ouabain (0.01 to 0.5 m*M*) did not inhibit the response of *Anolis* skins to MSH.

Melanophore-stimulating hormone stimulates an increase in cyclic adenosine 3,5′-monophosphate (cyclic AMP) within dorsal frog (*Rana pipiens*) skin (*8*). This intracellular second messenger (*9*), or its dibutyryl derivative, darkens frog (*10, 11*) and lizard (*12, 13*) skins directly, suggesting that this nucleotide may play a normal role in melanophore regulation. Methylxan-

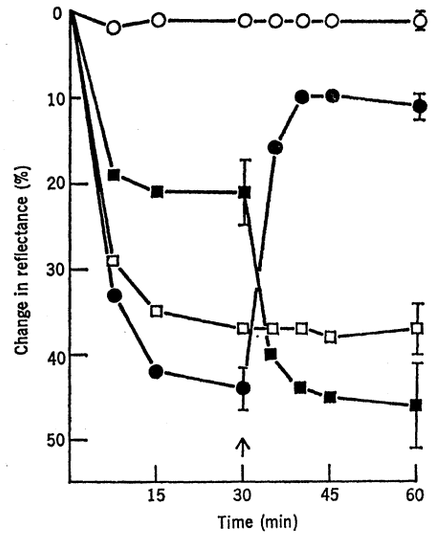


Fig. 3. In vitro demonstration of the immediacy of the darkening response (melanosome dispersion) of MSH-treated *Anolis* skins to added $Ca^{2+}$. MSH ($4 \times 10^{-9}$ g/ml) was added to skins immersed in Ringer solution (● + □) or in a $Ca^{2+}$-free Ringer solution (■). One group of skins (○) was maintained as an unstimulated Ringer control. At 30 minutes (arrow), $Ca^{2+}$ (1 m*M*) was added to the $Ca^{2+}$-free Ringer skins (■) and at the same time one group of Ringer MSH-darkened skins (●) was transferred to $Ca^{2+}$-free Ringer (containing an identical concentration of MSH, as before). Each point on the graph is the mean of 16 measurements of reflectance. Vertical lines indicate the standard errors.

thines such as caffeine and theophylline darken frog (*3, 11*) and lizard (*12*) skins, apparently by increasing intracellular levels of cyclic AMP through inhibition of cyclic AMP phosphodiesterase (*14*).

Although the present results clearly establish a $Ca^{2+}$ requirement for MSH action, the $Ca^{2+}$ ion is not required for melanosome dispersion per se, since theophylline darkens lizard skins in $Ca^{2+}$-free isotonic (120 m*M*) solutions of NaCl, LiCl, RbCl, CsCl, or isotonic (240 m*M*) sucrose. In addition, either theophylline or dibutyryl cyclic AMP will reversibly darken *Anolis* skins (Table 1) even when $Ca^{2+}$ ions are removed and EDTA (5 m*M*) is added, thus ruling out the possibility that these agents mediate their actions by releasing $Ca^{2+}$ from bound intracellular pools.

It would appear then that the $Ca^{2+}$ requirement for MSH action is related to an increase in cyclic AMP which is then responsible for melanosome dispersion. It is not known how MSH increases melanophore cyclic AMP

levels. It could do so by stimulation of adenyl cyclase activity, or, as suggested elsewhere (*15*), by an inhibition of cyclic AMP phosphodiesterase activity. It would appear that $Ca^{2+}$ must play a role in regulating the activity of one of these two enzymes, since $Ca^{2+}$ is not required for cyclic AMP–stimulated melanosome dispersion. The $Ca^{2+}$ requirement for MSH action on melanophores is consistent with a similar cation requirement for the lipolytic action of adrenocorticotropic hormone (ACTH) on isolated fat cells (*16*) or the stimulation of adenyl cyclase activity in adrenal cortical microsomal fractions (*17*). The similarity in structure of these two hormones might suggest a similar mechanism of action.

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## Endothelial Projections as Revealed by Scanning Electron Microscopy

Abstract. *Scanning electron micrographs of the endothelium of the pulmonary artery reveal that the entire surface is covered by a meshwork of irregular projections which vastly increase the surface area. The size and density of the projections suggest that they may function to direct an eddying flow of plasma along the endothelial surface.*

The endothelial lining of blood vessels is remarkable not only for its extensiveness and thinness but also for its position in direct communication with blood. Previous studies of the structure and function of endothelium have concentrated on the selective transport of fluid and solutes in capillaries, the structural studies having focused on cell junctions, pinocytotic vesicles, and endothelial flaps (*1*). Recent evidence indicates that enzymes on or near the surface of endothelial cells are actively engaged in the metabolism of circulating substances, such as the adenine nucleotides and polypeptide hormones, which are degraded rapidly but which are not retained by the cell itself (*2*).

Observation of the endothelial surface by scanning electron microscopy emphasizes a very different aspect which has been overlooked in transmission electron micrographs. The surface is covered by a profuse array of minute, irregular, fingerlike projections. The projections are distinguished from the regular palisades of microvilli which are characteristic of many vertebrate and invertebrate epithelia and from flaps or marginal folds.

Figures 1 and 2A show the inner surface of the pulmonary artery of a

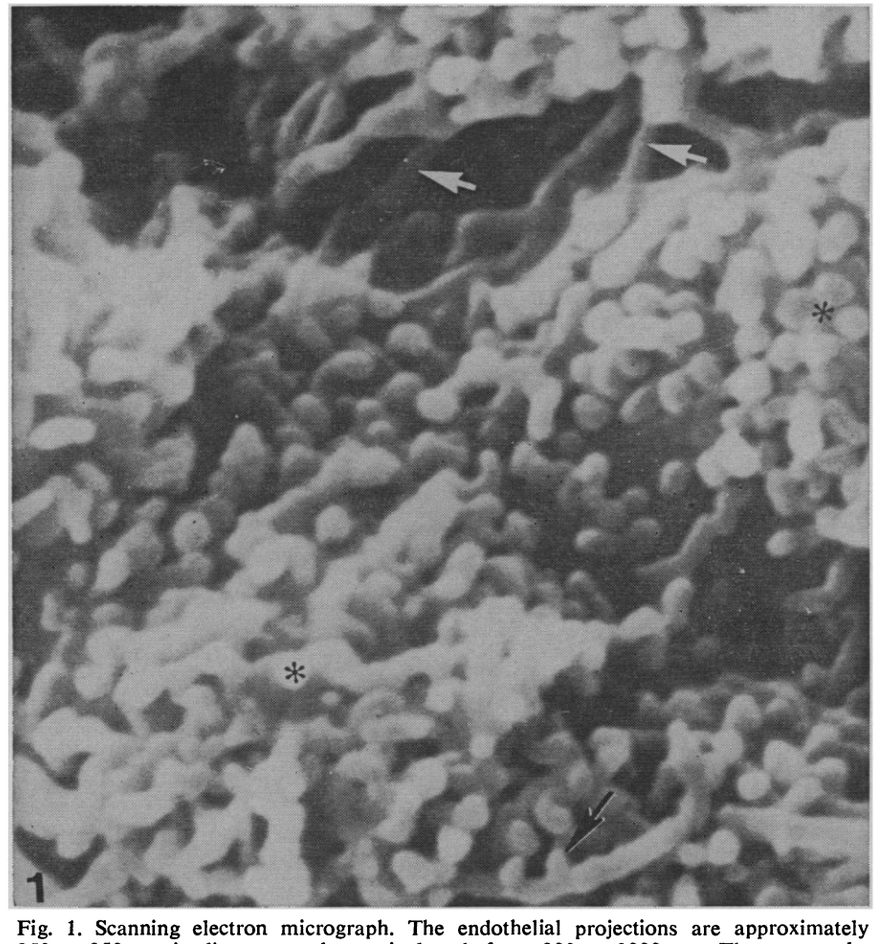


Fig. 1. Scanning electron micrograph. The endothelial projections are approximately 250 to 350 nm in diameter and vary in length from 300 to 3000 nm. They may take the form of knobs or longer arms, some of which branch or bud (black arrow). They are densest over the main body of the cell (*) but extend laterally to overlap adjacent cells (white arrows) (× 14,000).