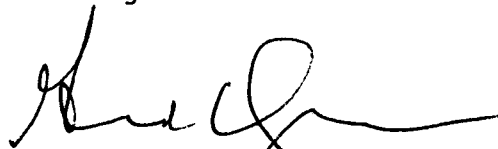


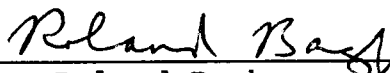
To the Graduate Council:

I am submitting herewith a dissertation written by Robert Kenneth Beam entitled "The Mechanism of Light and MSH-Mediated Color Change in the Lizard, Anolis Carolinensis." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Life Sciences.

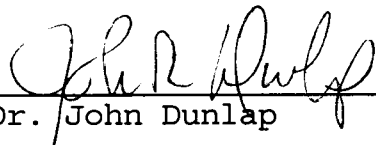


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Associate Vice Chancellor and
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The Mechanism of Light and MSH-Mediated Color Change
in the Lizard, Anolis Carolinensis

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Robert K. Beam

August, 1997

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ABSTRACT

Skin of the lizard, Anolis carolinensis, changes color from green to brown when stimulated with visible light or melanocyte-stimulating hormone (MSH). MSH and light-mediated responses involve melanosome dispersion within dermal melanophores. Color change by these agents was studied reflectometrically in intact skin, and melanophore responses were visually assessed in cultured cells.

The MSH response, but not the dermal photic response (DPR), utilizes adenosine 3',5' cyclic AMP (cAMP). cAMP levels increase in skins darkened with MSH, but not light or calcium ionophore a23187. cAMP-elevating agents also caused dispersion in cultured melanophores. There is no intracellular calcium requirement since the MSH response of skin samples or cultured melanophores remains intact in the presence of lanthanum, and cultured melanophores loaded with the intracellular calcium chelator, BAPTA-AM, respond normally.

The action spectrum of DPR reveals the presence of a blue-light sensitive photopigment ($\lambda_{\max}=425$ nm). Western blotting and immunolocalization suggests the presence of a cone-like opsin protein present on the melanophore plasmalemma and possibly on the melanosome. Calcium is apparently released from intracellular stores during DPR. X-ray microanalysis suggests that this store may be the melanosome. Studies with 8-Bromo-cGMP, which inhibits DPR

but not MSH response, suggests that cGMP may inhibit calcium release. The mechanism of DPR may have similarities to the transduction mechanism of the retinal rod cell which shares a similar embryological origin.

The MSH and DPR responses converge on a common microfilament-based dispersion system. A 57 kd protein (P57) is phosphorylated by light or MSH. Phosphorylation is inhibited by 8-Bromo-cGMP in the assay buffer. Since the protein phosphatase inhibitor, okadaic acid, triggers pigment dispersion in cultured cells, P57 is hypothesized to be the molecular switch which controls pigment movement. Phosphorylation of P57 may be the point at which the two pathways converge.

Finally, we report MSH-sensitivity of the iridophore. Cultured iridophores shift transmittance from red to blue when treated with MSH, cAMP-elevating agents, or colchicine, but not light. Using spectral measurements from xanthophores and iridophores, theoretical skin reflectance spectra were calculated to examine the contribution of iridophore activity to skin color and color change.

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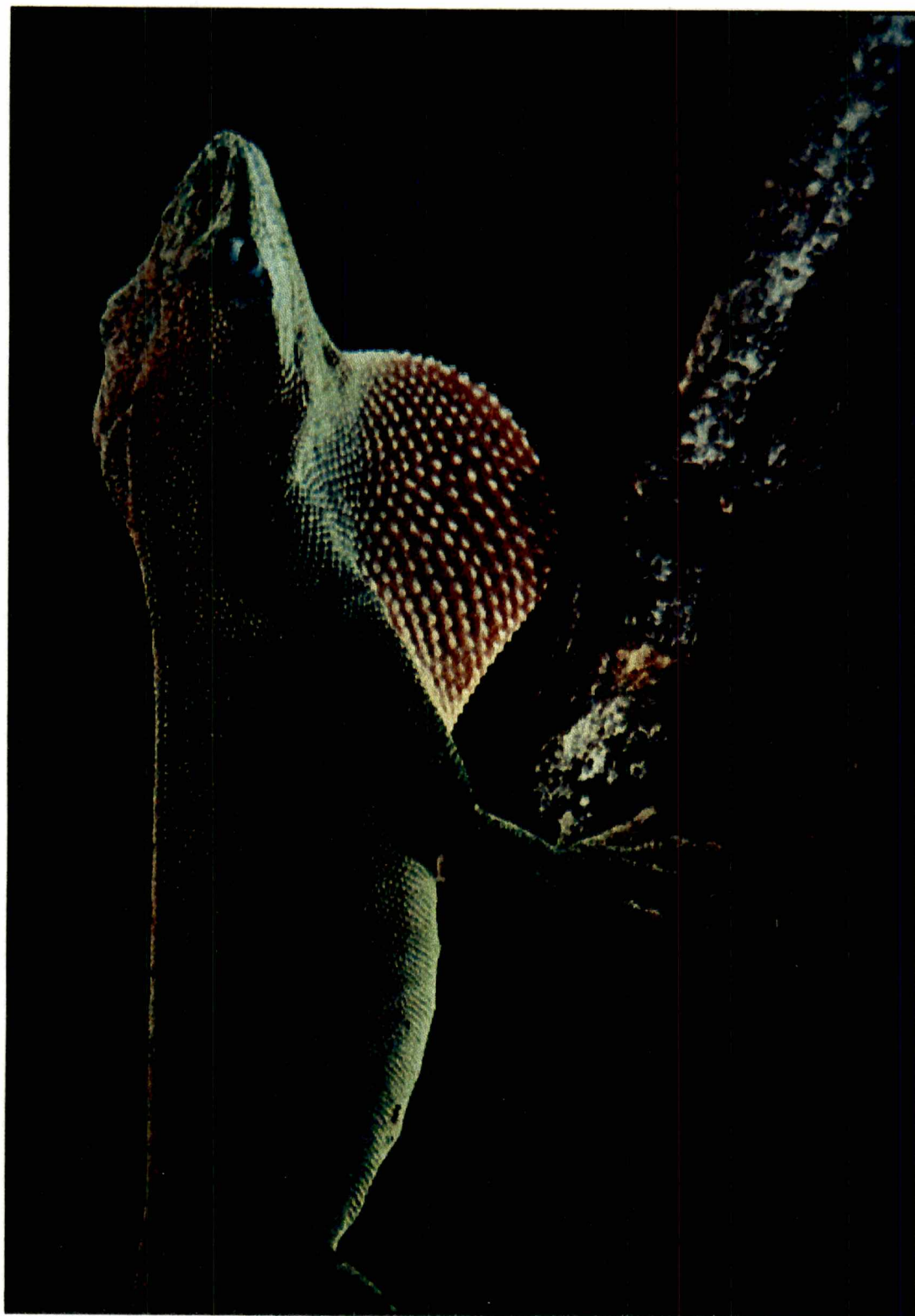
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LIST OF ABBREVIATIONS

A23187	calcium ionophore a23187
ATP	adenosine triphosphate
BAPTA-AM	1,2-bis-(2)aminophenoxythane-N,N,N',N'-tetra acetic acid (acetoxymethylester)
BHQ	di- τ -butylhydroquinone
4BPB	4-bromophenacyl bromide
BSA	bovine serum albumin
CPA	cyclopiazonic Acid
DB-cAMP	N ² ,O ^{2'} -dibutyryl cAMP
DB-cGMP	N ² ,O ^{2'} -dibutyryl cGMP
DMSO	dimethyl sulfoxide
DPR	dermal photic response
DTT	ditheothreitol
cAMP	adenosine 3',5'-cyclic monophosphate
cGMP	guanosine 3',5'-cyclic monophosphate
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol tetraacetic acid
EM	electron microscopy
8-Br-cGMP	8-bromo-cGMP
half-BAPTA	N-(2-methoxyphenyl)iminodiacetic acid
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid
h ν	electromagnetic radiation
G-Kinase	protein kinase G
IBMX	isobutylmethylxanthine
IP ₃	inositol triphosphate
IP ₃ -R	inositol triphosphate receptor
Kd	kilodalton
MOPS	4-morpholinepropanesulfonic acid
MSH	melanocyte stimulating ormone
NB-cGMP	N ² -monobutyryl cGMP
OS-cGMP	O ^{2'} -monosuccinyl cGMP
P57	phosphorylated 57 kd protein
PBS	phosphate buffered saline
PDE	phosphodiesterase
PMSF	phenylmethylsulfonylfluoride
SDS	sodium dodecyl sulfate
SERCA	smooth endoplasmic reticulum Ca ²⁺ -ATPase
Sp-cAMP	adenosine-3',5'-cyclic monophosphorothiate
SSAC	sterile saline/antibiotic cocktail
TEM	transmission electron microscopy
TG	thapsigargin
TPA	12-O-tetradecanoylphorbol 13-acetate
TRIS	tris(hydroxymethy)aminomethane



PART I

INTRODUCTION

Light is a ubiquitous physiological stimulus. Virtually all animals respond to electromagnetic radiation via extra-ocular receptors, a phenomenon generally referred to as the dermal light sense (Steven, 1963). Although these receptors vary in spectral sensitivity, many are maximally sensitive around 500 nm, the solar absorption maximum of the earth (Wolken, 1975). These receptors receive and transduce light energy into other forms, interpreted as behavior (Wolken and Mogus, 1979). For example, phototactic algae respond directly to light, using the stimulus for both energy and orientation (Foster and Smith, 1980). Marine invertebrates demonstrate distinct extra-ocular photodetection and behavior, including photokinesis, phototaxis, and photodiesis (Wolken, 1988).

A common light response is color change. Slow seasonal changes result from the action of the pineal or optic chiasm via the circadian cycles regulated by melatonin (Tamarkin et al., 1985). Mammalian hair and skin color are affected by light (Quevedo, 1969) as is the plumage of birds (Ralph, 1969). This morphological color change is the result of an increased amount of pigment, an increased number of pigmented cells, or both (Bagnara and Hadley, 1969; Lee and Lee, 1971).

In addition to morphological color change, many crustacea and poikilothermic vertebrates are also able to rapidly change color in response to light via a pigment redistribution process, a phenomenon referred to as physiological color change (Taylor and Bagnara, 1972). Physiological color change is possibly effected in some animals by neural or hormonal activity regulated by ocular or pineal light detection, an evolutionarily recent development referred to as the secondary light response (Wolken, 1975).

However, in many cases the chromatophores are themselves photosensitive. This primary light response is thought to be evolutionarily ancient (Wolken and Mogus, 1979). Pigment redistribution occurs in response to the appropriate light stimulus in the absence of any humoral or neural message as can be demonstrated by in vitro physiological recordings from skin tissue or cultured cells (Weber, 1983).

Physiological color changes are thought to be important for various reasons including photoprotection (Burtt, 1981), thermoregulation (Vaughan, 1987a), and crypsis (Hamilton and Peterman, 1971). Thus, it is not surprising that poikilothermic vertebrates also undergo physiological color change in response to light-independent hormone release, primarily the melanotropins (Taylor, 1969), corticosteroids (Edwards

et al., 1981), and catacholamines (Graham, 1961; Hadley and Goldman, 1969). Furthermore, skin color in some fish (e.g. Fundulus heteroclitus) and lizards (e.g. Chameleo pumilus and Phrynosoma) is effected by nervous stimulation (Wyman, 1924; Sand, 1934; Parker, 1938), a mechanism that yields faster responses with greater control of individual chromatophores (Parker, 1948; Fujii and Oshima, 1986).

Because physiological color change can be effected by hormones, nervous stimuli, illumination (Negishi, 1985; Vaughan, 1987), or temperature changes (Smith, 1929; Rahn, 1956; Ross and Odell, 1963), it is often difficult in vivo to elucidate the proximate cause of the common event. In vitro, a variety of methods which monitor reflectance changes (Schizume et al., 1954), absorbance changes (Bitensky et al., 1965), or visual inspection (Hogdem and Slome, 1932; Burgers et al., 1953) of a variety of vertebrate and invertebrate chromatophores have been used to investigate responses. Inherent in most work is the assumption that all signals leading to a common event utilize a common transducer. Thus if a chromatophore can disperse pigment in response to hormone, light, or other mediator it is assumed that the biochemical event leading to this pigment dispersion is identical regardless of stimulus.

Although this unitransducer theory of chromatophore activity appears to have widespread approval, it has yet to be carefully examined. So far, the majority of research on chromatophore activity has utilized a variety of pharmacological agents known to stimulate various pathways or proteins. In keeping with the unitransducer theory, pharmaceuticals that mimic the activity of the stimulus in question have been assumed to activate a vital component of the transduction pathway. However, considering the wide range of stimuli to which chromatophores can respond, a polytransducer model where multiple transduction mechanisms can ultimately effect pigment distribution through a common event (perhaps phosphorylation of a motor protein) is quite plausible. If such a polytransduction model were shown to be correct, some previous studies could prove to be flawed due to the inherent confounding of pathways.

Therefore, the purpose of this project is first, to investigate the dermal photic response and to elucidate the signal transduction pathway in the lizard, Anolis carolinensis, second, to further understand the signal transduction mechanism of hormone-induced color change, and third, to understand how the interaction between cell types produces skin color and color change in this lizard.

This animal model was chosen for several reasons. A. carolinensis lizards are inexpensive, easy to maintain, and produce a pronounced, quantifiable response to visible light as well as to hormones such as melanocyte-stimulating hormone (plate 1.1). Also important is the literature base of hormone-induced color change studies on this animal that will serve as a point of comparison and reference. A key assumption of this research is that the MSH-response and light-response are not necessarily identical, and therefore methods must be carefully employed to examine the light response without perturbing the MSH system.

In designing these experiments, it will be assumed that the light-sensitive dermal chromatophores of lower vertebrates can be thought of as typical neuronal sensory transducers. Although melanophores are not nerve cells, they are neuroectodermal in origin (Stevens, 1954; Bagnara and Hadley, 1972; Bagnara, 1983; Bagnara et al., 1979; Akira and Ide, 1987). Therefore, it should be expected that they convey sensory information via the standard mechanisms, that is, through membrane polarity changes and intracellular second messenger systems. These second messengers may also be responsible for activation or modulation of the chromomotor mechanism, and thus converge with the



Plate 1.1: Lizards Change Color from Green to Brown. A variety of stimuli can effect color change. In this case the brown lizard was injected with 0.1 ml MSH (10^{-7} M). Bar=1 cm.

hormonal pathway, probably via common phosphorylation/dephosphorylation events.

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PART II

LITERATURE REVIEW

DERMAL CHROMATOPHORE UNITS AND COLOR CHANGE MECHANISMS

Skin color results from the interaction of chromatophores within the epidermis (Hadley and Quevedo, 1966) and dermis (Taylor and Bagnara, 1972). Whereas epidermal chromatophores are important for seasonal (morphological) color change, chromomotor mechanisms within dermal chromatophores are responsible for physiological color change in poikilothermic vertebrates (Bagnara et al., 1968; Bagnara and Hadley, 1969; Taylor and Bagnara, 1972).

Many chromatophores contain pigments, contained within specialized subcellular organelles. Pigments include the red pteridines, yellow carotenoids, flavins and, pteridines, white carotenoids, and the red, brown, and black melanins (Bagnara, 1966; Taylor and Bagnara, 1972). Another class of chromatophore, the iridophore, does not contain pigment. Rather, colorless highly reflective crystalline organelles produce iridescent structural colors as a result of Tyndall scattering or thin-layer interference (Huxley, 1968; Rohrich, 1974).

Skin color is primarily the result of the particular layering of chromatophores within the dermis, referred to as the dermal chromatophore unit (Bagnara et al., 1968). Light reflected from the iridophore is filtered by pigmented organelles of overlying chromatophore processes. Color change is effected by the redistribution of pigmented

organelles, an alteration in iridophore reflectance, or both.

The most studied dermal chromatophore units include those of the frogs Hyla cinera, Hyla arborea (Bagnara et al., 1968; Taylor and Bagnara, 1972), Rana pipiens (Hadley, 1966), and the lizard, Anolis carolinensis (von Geldern, 1921; Alexander and Fahrenbach, 1969; Taylor and Hadley, 1970). In these animals the dermal chromatophore unit consists of melanophores, xanthophores, and iridophores.

In A. carolinensis, dendritic deep melanophores surround a layer of superficial xanthophores which cover several layers of iridophores (figure 2.1). White light passes through yellow xanthophores, and blue light is reflected back from the iridophores. As blue light passes through the yellow xanthophore filter, the animal appears green. Stimulation with visible light (Beam and Vaughan, 1995) or the application of hormones such as melanocyte-stimulating hormone (Taylor and Hadley, 1970) causes brown pigment granules (melanosomes) to migrate superficially into vacant dendritic arms, obscuring underlying xanthophores and iridophores and causing the animal to turn brown (plate 2.1), the shade varying with the amount of pigment dispersed. Xanthophores are thought to be passive yellow filters containing either pteridine or carotenoid pigments. Iridophores are dendritic cells with highly ordered stacks

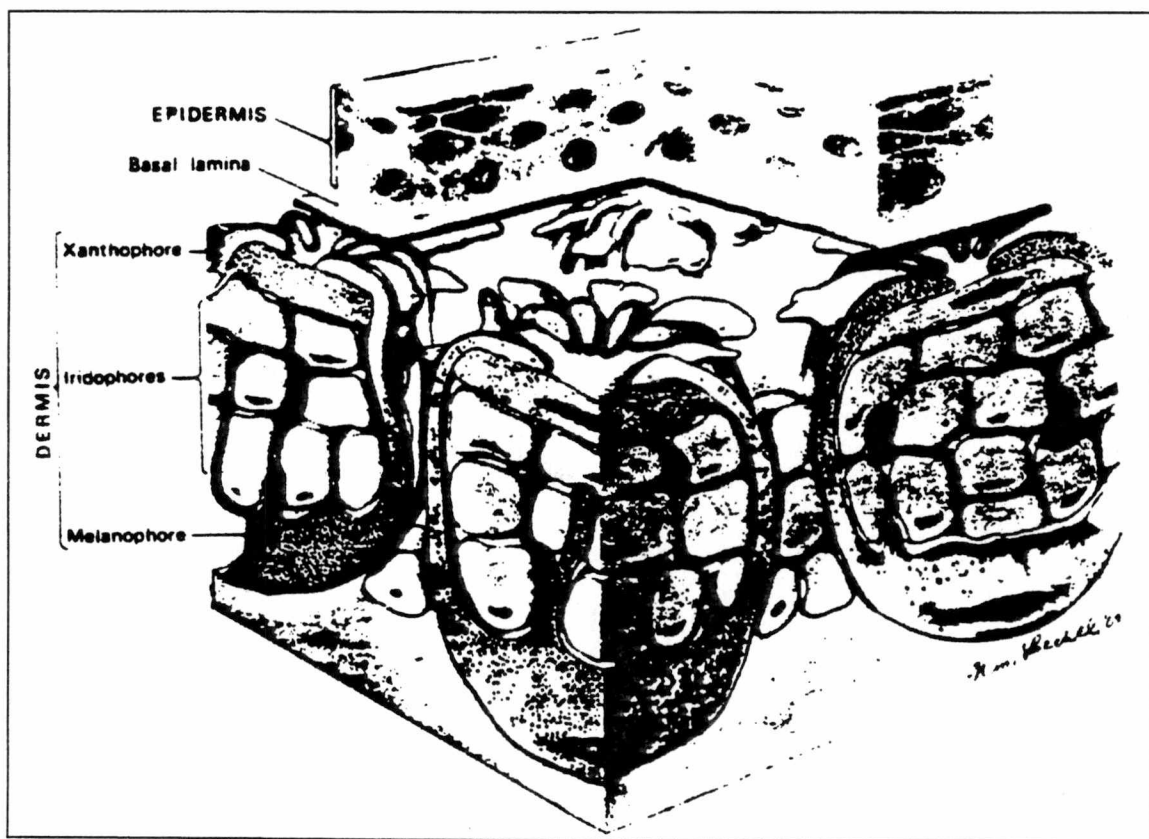
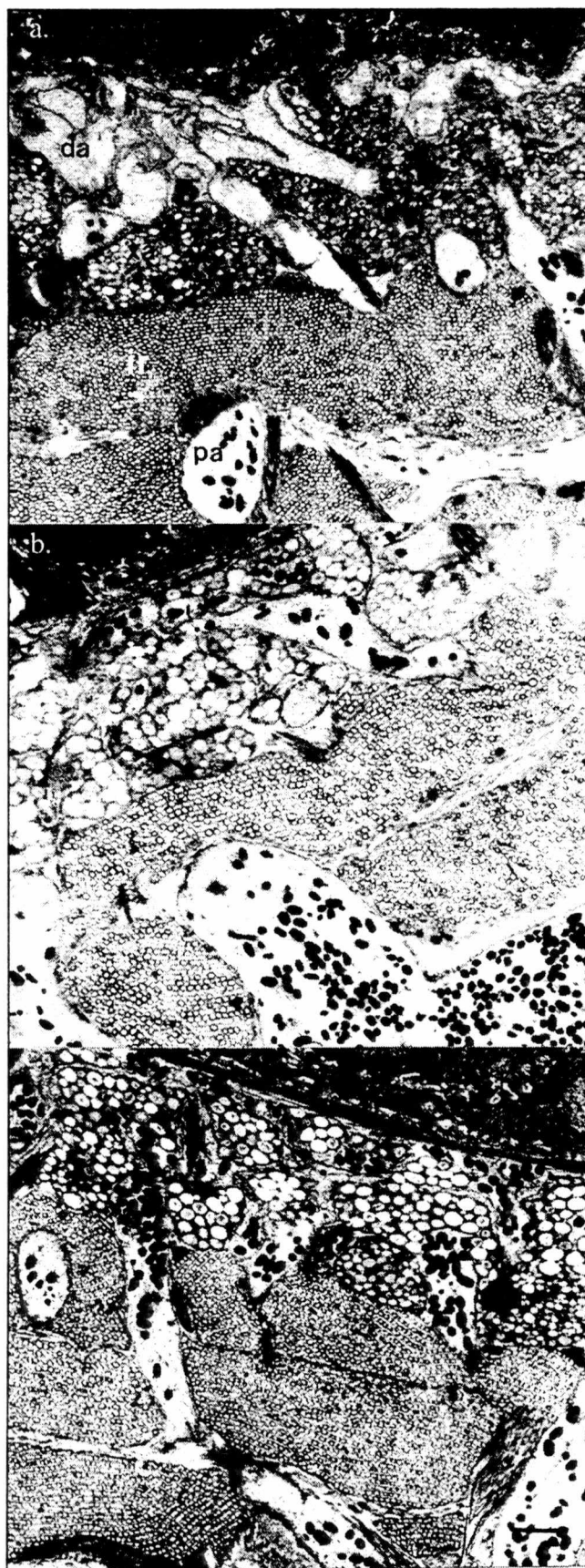


Figure 2.1: Model of Skin of *Anolis Carolinesis*. Dendritic dermal melanophore arms surround and terminate above superficial iridophores and xanthophores. Melanosome migration into these arms causes skin color to change from green to brown (from Taylor and Hadley, 1970, p. 292).

Plate 2.1: Effect of MSH and Light on the Dermal Melanophore of Anolis carolinensis. Electron micrographs (from Beam and Vaughan, 1995) show dermal melanophore distal arms (da) devoid of melanosomes in the control condition (a). When stimulated with light (b) or MSH (c), melanosomes migrate from the body and proximal arms (pa) into distal melanophore arms which terminate above and obscure dermal iridophore and xanthophore layers, causing the animal to change color from green to brown (Bar = 3.3 μ).



of guanine-containing crystalline platelets and are thought to be passive (Rohrlich and Porter, 1972; Rohrlich, 1974).

In frogs, both melanophores and iridophores are actively involved in color change. Reflecting platelets disperse and aggregate much like pigmented organelles (Taylor, 1967) and effect color change by light scattering (Dr. Randall Morrison, personal communication). In the absence of stimulation, guanine platelets are dispersed and melanosomes are aggregated. If the skin is exposed to MSH or other darkening agents, iridophore platelets aggregate whereas melanosomes disperse superficially causing the animal to appear dark (Hadley, 1966; Bagnara et al., 1968; Hadley and Bagnara, 1969; Taylor and Bagnara, 1972).

THE HUMORAL RESPONSE

In amphibians and reptiles a hormone released from the pars intermedia of the hypothalamus (Allen, 1916; Smith, 1916; Atwell, 1919) is responsible for darkening skin. This hormone was identified as melanotropin or melanocyte-stimulating hormone (Lerner and Lee, 1955; for a review, see Lerner, 1993). Skin lightening occurs in the absence of this hormone, although several other hormones including melatonin (Lerner, 1959; Novales, 1963; Novales and Novales, 1965; Messenger and Warner, 1977; Rollag and Adelman, 1992), acetylcholine (Moller and Lerner, 1966; Moellman et al., 1974), and cortisol (Edwards et al., 1981) can also mediate the effect.

Catacholamines sometimes cause darkening e.g. Xenopus laevis (Burgers et al., 1953; Graham, 1961, van de Veerdonk and Konijn, 1970), Scaphiopus couchi (Goldman and Hadley, 1969b) and sometimes lightening e.g. Rana pipiens (Wright, 1947; Abe et al., 1969a,b) depending upon the primary type of adrenergic receptor present. β -adrenergic receptors are linked to a pigment dispersion mechanism, whereas α -adrenergic receptors are linked to a pigment aggregation mechanism. If both receptor types are present as in Anolis carolinensis, Rana temporaria, and Rana tigrina, the classical epinephrine reversal can be observed (Kleinholtz,

1938b; Lee, 1952; Goldman and Hadley, 1969a; Gupta and Bide, 1967).

In contrast to amphibians and reptiles, the dermal chromatophores of fish and crustacea appear to be under bihumoral control. In fish, a stimulating hormone (MSH) and a concentrating hormone (MCH) released from the hypothalamus regulate the dynamics of coloration (Enami, 1955; Fujii and Oshima, 1986; Castrucci et al., 1987). Similarly, the chromatophores of crustacea such as Palaemonetes and Uca pugilator are controlled by concentrating and releasing hormones which are liberated from the eyestalk (Brown, 1939; Fingerman, 1969; Josefsson, 1983; Rao and Riehm, 1993). However, crustacean (Palaemonetes, Callinectes, Uca) chromatophore dispersing hormones have no effect on the chromatophores of Rana pipiens or Rana clamitans, suggesting that the hormones are physiologically dissimilar (Wright and Kohn, 1952).

THE MSH SIGNAL-TRANSDUCTION MECHANISM

In amphibians and reptiles pharmacological evidence implicates a dependence on microtubules for pigment aggregation (Moellman et al., 1973, Hadley and Castrucci, 1987; Vaughan, 1987) and microfilaments for dispersion (Malawista, 1971; McGuire and Moellmann, 1972; Moellman et al., 1973, Obika et al., 1978; Hadley and Castrucci, 1987; Vaughan, 1987). The converse has been documented for a crustacean (Lambert and Fingerman, 1979). Evidence indicates that both dispersion and aggregation of pigmented organelles in the retinal pigment epithelium (Burnside et al., 1983) and dermal chromatophores of various teleosts (Negishi et al., 1985; Rozdzial and Haimo, 1986) utilize microtubules only.

Organelle movement in these teleosts is determined by microtubule polarity (McNiven and Porter, 1986) and may be regulated by phosphorylation of specific axoplasmic motor proteins that are inherently directional (Vale et al., 1985b) such as kinesin (Vale et al., 1985a). MSH-dependent organelle movement in melanophores of the cichlid T. mossambica (Rozdzial and Haimo, 1986) and xanthophores of the goldfish (Lynch et al., 1986a) appear to result from the phosphorylation of a single 57 kd protein. MSH-dependent phosphorylation of the p57 protein in goldfish xanthophores is dependent upon the action of protein kinase A (Lynch et al., 1986b, Yang et al., 1989), the adenosine 3'5' cyclic

monophosphate (cAMP) dependent kinase (Sutherland et al., 1968).

In amphibians, lizards, and fish, elevated cAMP levels alone are sufficient to cause melanosome dispersion as has been shown by the application of exogenous cAMP (Bitensky and Burstein, 1965; Novales and Fujii, 1970; Schliwa and Bereiter-Hahn, 1974), the lipophilic derivative, dibutyryl-cAMP (Hadley and Goldman, 1969; Novales, 1971; Fujii and Miyashita, 1976; Geschwind et al., 1977; Quackenbush, 1981; Burnside and Basinger, 1983; Bruenner and Burnside, 1986), or cAMP-elevating agents such as fluoride, tumor-promoting forskolins (Heath and Hindman, 1988), and the cAMP phosphodiesterase-inhibiting methylxanthine derivatives such as theophylline, caffeine, and isobutylmethylxanthine (Wright and Lerner, 1960; Dikstein and Sulman, 1964; Novales and Davis, 1967; Abramowitz and Chavin, 1974; Ozato, 1977; Negishi and Obika, 1980).

In support of these findings, Abe et al. (1969a) have shown that cAMP levels in the skin of Rana pipiens increase in response to both norepinephrine and MSH in a dose-dependency that correlates with skin darkening. Similarly, Beam and Vaughan (1994) have demonstrated a cAMP increase in MSH-darkened skin of Anolis carolinensis. Finally, skin lightening agents, such as melatonin in Rana pipiens (Abe et al., 1969b) and Xenopus laevis (White et al., 1987) as well as melanin-concentrating hormone in the teleost Labrus

ossifagus decrease skin cAMP content (Svensson et al., 1991).

That the MSH pathway is more complicated than a simple cAMP increase and kinase activation can be seen by examining the ionic requirements for MSH action (Vesely, 1972). Although lipophilic derivatives of cAMP will darken skins without any additional requirements, MSH activity in Xenopus laevis (van de Veerdonk and Brouwer, 1973; van de Veerdonk et al., 1979), Anolis carolinensis (Vesely and Hadley, 1971; Lucas et al., 1987a), amblystoma (Novales and Novales, 1964), Hyla arborea (Dikstein et al., 1963), and Rana pipiens (Vesely, 1972) requires calcium to be effective. Similarly, MCH activity in Poecilia reticulata also requires calcium (Visconti et al., 1989).

It has been shown that MSH acts extracellularly on chromatophores (Horowitz et al., 1980) and requires extracellular calcium for activity (Vesely and Hadley, 1976; Lambert, 1983; Sawyer et al., 1987; Eberle et al., 1987), a similar requirement (Bar and Hechter, 1969) being found for the structurally homologous (Cone et al., 1993) adrenocorticotrophic hormone-receptor interaction. Moreover, in Anolis carolinensis, there appears to be a functional connection between the MSH receptor and the adrenergic receptors, since α -adrenergic agonism (Carter and Shuster, 1981;1982) or β -adrenergic blockade (Vaughan and Greenberg, 1987) inhibits the MSH response, and both the MSH response

(Horowitz, 1956) and the α -adrenergic response (Goldman and Hadley, 1972) require free sulfhydryls.

The location of the calcium requirement is apparently proximal to adenylate cyclase (Vesely and Hadley, 1976; Lucas et al., 1987a). Such an interpretation (figure 2.2) is consistent with the hormone-mimetic action of elevated extracellular calcium (Dikstein et al., 1963) and the demonstration that a superagonistic MSH derivative, [4-norleucine, 7-D-phenylalanine]- α -melanotropin, (Hadley et al., 1981; Lucas et al., 1987a) or a photoaffinity-labeled MSH derivative (de Graan, et al., 1981, 1982) is capable of binding to the MSH receptor in the absence of calcium, yet skin darkening does not occur until the skin is transferred to a calcium-containing saline solution.

Such an interpretation is also attractive because it can explain the absolute calcium requirement (Vesely and Hadley, 1971) without an elevation of intracellular calcium. Several pieces of evidence suggest that intracellular calcium decreases during the MSH response. In Anolis carolinensis, the effect of catecholamines and cAMP agonists is enhanced in the absence of extracellular calcium (Vesely and Hadley, 1976), and Carter and Shuster (1978) demonstrated a potentiating effect of the calmodulin-antagonizing phenothiazines on the MSH response. Similarly, Abrao et al. (1991) demonstrated that the calcium-dependent C-kinase mediates MCH aggregation activity in the teleost

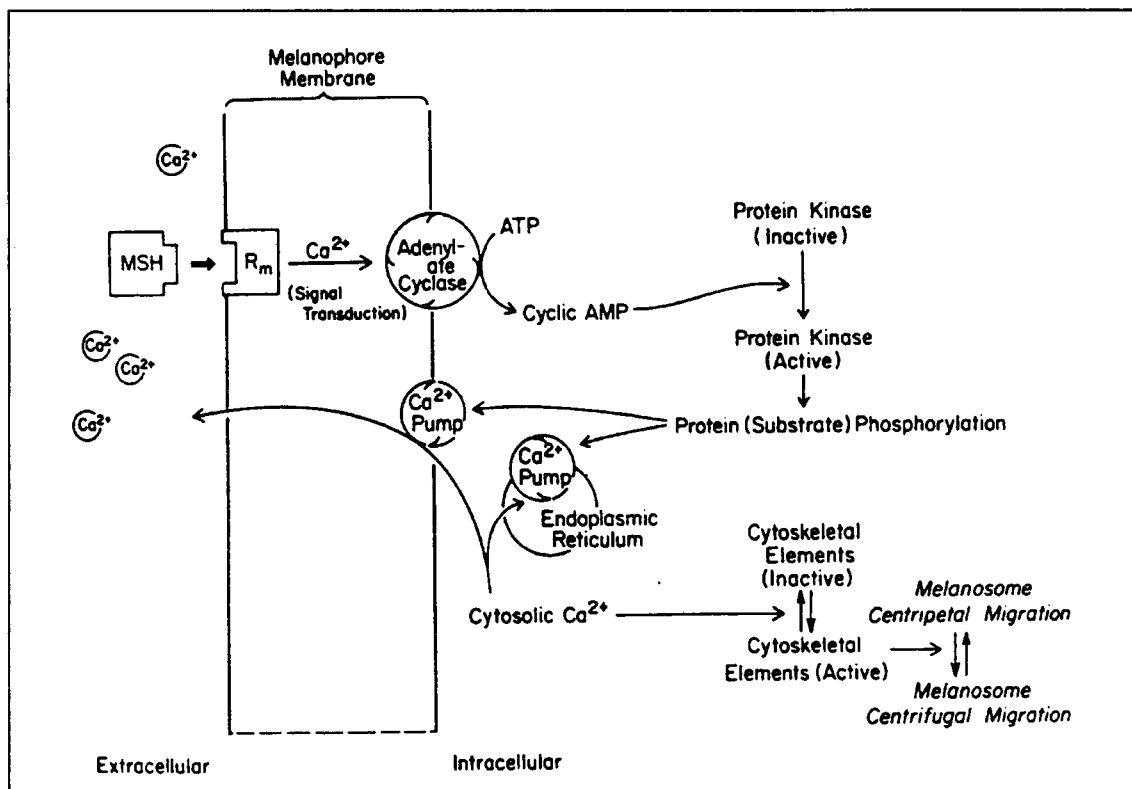


Figure 2.2: Model of Mechanism of Action of MSH on Color Change in *Anolis Carolinensis*. Calcium is required proximal to the action of adenylate cyclase. Stimulation of adenylate cyclase causes an increase in intracellular cAMP, which may decrease intracellular calcium and cause melanosome dispersion (from Sawyer et al., 1983, p. 537).

Synbranchus marmoratus, and Thaler and Haimo (1990) demonstrate a role for calmodulin phosphatase (calcineurin) in pigment aggregation in Tilapia mossambica melanophores.

Considerable evidence, however, argues for an increase in intracellular calcium during MSH-mediated pigment dispersion. In Anolis carolinensis, the calcium ionophore A23187 is hormone-mimetic (Hadley et al., 1981; Lucas et al., 1983). Phorbol esters which activate the calcium-dependent C-kinase (Castagna et al., 1982) potentiate the MSH response (Lucas et al., 1987a), and cromolyn, a known inhibitor of the C-kinase, inhibits the MSH-induced response (Lucas and Shuster, 1987). Furthermore, van de Veerdonk et al. (1979) demonstrated that the calcium channel blockers verapamil and D600 inhibit the MSH response in Xenopus laevis. Using radiolabeled $^{45}\text{Ca}^{2+}$, they were able to demonstrate a decreased calcium efflux in the presence of MSH which may also be interpreted as an increase in intracellular calcium. Elevated cytosolic calcium might be mediated by prostaglandins in Xenopus laevis (van de Veerdonk and Brouwer, 1973), but not in Anolis carolinensis (Lucas et al., 1985). The function of the calcium signal is unknown, but may be related to cytoskeletal events triggering pigment translocation (Sawyer et al., 1983).

D. THE LIGHT RESPONSE

Despite its ubiquity (table 2.1), surprisingly little is known about the physiological mechanism of the dermal photic response. Because of inherent difficulties in examining and recording light responses, the majority of investigators have favored examining hormone-induced color changes. Those investigators who have chosen to examine the light response, schooled in a wealth of literature about the hormonal response, frequently have been slow to shed the dogma of the hormonal system in order to more properly examine what appears be a similar response that might utilize an entirely different mechanism. Indeed, the preponderance of reports to date on light-induced color changes assume the mechanism to be identical to the MSH-induced color change and as a result have utilized experimental design which may confound the activity of the two systems and produce, at best, ambiguous results.

Most studies rely upon observing the effects on DPR of pharmacologically altered cAMP levels. For example, Wakamatsu (1978) and colleagues (1980) demonstrated that the melanophores of the fish, Xiphophorus maculatus, undergo pigment aggregation (rather than dispersion) in response to light. Addition of theophylline or exogenous cAMP caused rapid pigment dispersion in fully aggregated melanophores in the presence of light. Moreover, the rate of dispersion increased when light stimulation was discontinued. Negishi

Table 2.1
Photosensitive Chromatophores

Species	Cell Type	Direction of Response	Light	Reference
<u>Leeches</u>				
<i>Glossiphonia complanata</i>	M	D	V	Wells, 1932
<i>Piscicola geometra</i>	M	D	V	Janzen, 1932
<u>Echinoderms</u>				
<i>Diadema Setosum</i>	M	D	V	Yoshida, 1956 Yoshida, 1957
<i>Centrostephanus longispinus</i>	M?	D	V	Kleinholtz, 1938a Dambach & Jochum, 1968, Weber & Dambach, 1974; 1976
<u>Crustacea</u>				
<i>Palaemon serratus</i>	L	D	V	Knowles, 1940
	L/E	D	V	Stephenson, 1934
<i>Sesarma reticulatum</i>	M	D	V	Fingerman et al., 1960
<i>Uca Pugilator</i>	M	D		Coochill & Fingerman, 1976
<i>Macropipus vernalis</i>	E	D	V	Burgers, 1959
<u>Fishes</u>				
<i>Fundulus heteroclitus</i>	M	C	UV	Spaeth, 1913 Smith, 1939
<i>Anoptichtys jordani</i>	M	D	V	Burgers et al., 1963
<i>Oncorhynchus nerka</i>	RP	D	V	Ali, 1959
<i>Oryzias latipes</i>	M	D	V	Negishi, 1985
<i>Xiphophorus maculatus</i>	M	C	V	Wakamatsu, 1978 Wakamatsu et al., 1980
<i>Zacco temminckii</i>	M	D	V	Naora et al., 1988
<i>Paracheirodon innesi</i>	I		V	Iga & Takabatake, 1986 Lythgoe et al., 1984
<u>Amphibia</u>				
<i>Rana temporaria</i>	M	D	V	Hooker, 1912 Zettner, 1956
<i>Xenopus Laevis</i> -embryonic	M	D	V	Kuleman, 1960 Obika & Bagnara, 1963
-larv. tail fins	M	C	V	Bagnara, 1957 van der Lek et al., 1958
-adult eyes	RP	D	V	Weale, 1956
<i>Rana esculenta</i>	M	D	V	Zettner, 1956
<i>Rana pipiens</i>	M	D	V	Bagnara & Obika, 1967
<i>Amblystoma tigrinum</i> (larvae)	M	D	UV	Laurens, 1915
		C	V	Laurens, 1917
<u>Reptilia</u>				
<i>Chamelio Sp</i>	M	D	V	Brucke, 1852 Zoond & Eyre, 1934
<i>Phrynosoma blainvillii</i>	M	D	V	Redfield, 1918 Parker, 1938
<i>Anolis carolinensis</i>	M	D	V	Hadley, 1931 Vaughan, 1987
<i>Anolis equestris</i>	M	D	V	Smith, 1928 Hadley, 1931
<i>Anolis iodurus</i>	M	D	V	Kleinholtz, 1938a Hadley, 1931
<i>Anolis watsoni</i>	M	D	V	Hadley, 1931

KEY: E erythrophore, I iridophore, L leukophore, M melanophore, RP retinal pigment, D dispersion C contraction, V visible light, UV ultraviolet light (Adapted in part from van der Lek, 1967 and Weber, 1983)

(1985) reported that melanophores of the teleost Oryzias latipes respond to light by pigment dispersion which is reversible in the dark. Theophylline or exogenous cAMP treatment of fully dispersed, light-stimulated melanophores inhibited pigment aggregation upon return to darkness. In both cases, the effect of cAMP or theophylline was to cause or maintain pigment dispersion. Since MSH stimulation, mediated by cAMP, also causes pigment dispersion, and since these investigators did not measure cAMP concentrations in response to light, it is probable that they were activating pigment dispersion through the MSH pathway. The conclusion linking cAMP to the DPR may, thus, be in error. In only one case (embryonic Xenopus laevis melanophores in culture) has the appropriate assay for cAMP been performed (Daniolos et al., 1990).

Although very little is known about the signal transduction mechanism in extra-ocular photoreceptors, it is possible that the photosignal transduction mechanism may share functional homology with photoreceptors of the retina. In fact, the image-forming visual system of the retina may be an advancement of a more ancient dermal photoreceptive system. Although photoreceptive pigments could theoretically include the pyrroles and porphyrins, chlorophylls, hemes, cytochromes, carotenoids, phycobiliproteins, ferredoxins, flavins, melanins, rhodopsins, and porphyropsins (Wolken, 1975), it has been determined immunologically and

spectroscopically that many extra-ocular photoreceptors utilize an opsin or porphyropsin-based pigment (table 2.2). These photoreceptive pigments may be evolutionarily related since all visual opsins share a similar primary sequence and are thought to be offspring of a single ancestral gene (Goldsmith, 1991).

Vertebrate rhodopsin shares membrane motif and sequence homology with a family of G-protein-linked seven-pass biogenic amine receptors including the β -adrenergic receptor (Oprian, 1992) and the MSH receptor (Cone et al., 1993). All of these receptors share common features, including two glycosylation sites near the amino terminus on the extracellular side, several phosphorylation sites near the carboxyl terminus on the cytosolic side, cysteine residues in the extracellular loops which link helices 2-3 and 4-5, as well as conserved residues of the third cytoplasmic loop (linking helices 5-6) which are thought to form the G-protein binding domain (Bowmaker, 1991). The extracellular loops and seven membrane spanning domains are thought to form a ligand-binding domain. In the case of rhodopsin, retinal is bound via a Schiff-base linkage and is activated via isomerization by light.

The retinal chromophore is usually 11-cis, although 13-cis appears in halophilic bacteria, 3-hydroxy-cis occurs in Drosophila, and 11-dehydro-cis occurs in the porphyropsin pigments of euryhaline fishes and, suprisingly,

Table 2.2
Hypothesized Extra-ocular Photopigments

Species	Location	Maximum ABS	Pigment	Method	Reference
<u>Alga</u> <i>Chlamydomonas</i>			opsin	immunological	Foster et al., 1984
<u>Amphibian</u> <i>Xenopus Laevis</i>	Dermal Melanophore	461	porphyropsin	spectrum	Lythgoe and Thompson, 1984
<i>Xenopus Laevis</i>	Dermal Melanophore	460	opsin	spectrum	Daniolos et al., 1990
<u>Birds</u> chicken quail	pineal pineal		opsin opsin	immunological immunological PCR	Deguchi, 1981 Foster et al., 1985; Kramm et al., 1993; Max et al., 1995
<u>Fish</u> <i>Oryzias Latipes</i>	Dermal Melanophore	415	opsin	spectrum	Negishi, 1985
<i>Paracheirodon innesi</i>	Dermal Iridocyte		opsin	immunological	Lythgoe et al., 1984
<i>Xiphophorus maculatus</i>	Dermal Melanophore	400-420	opsin	spectrum	Wakamatsu et al., 1980
<i>Zacco temminckii</i>	Dermal Melanophore	525	porphyropsin	spectrum	Naora et al., 1988
<u>Reptile</u> <i>Anolis Carolinensis</i>	Basal Brain		opsin	immunological	Foster et al., 1993
<i>Anolis Carolinensis</i>	Dermal Melanophore		opsin	immunological	Beam and Vaughan, 1994

the basal brain, pineal, and retina of Anolis carolinensis (Provencio et al., 1992). In all other receptors of this family, a ligand (neurotransmitter or hormone) binds to the ligand-binding domain and activates the receptor (for a review see Oprian, 1992).

The multiple membrane spanning domains of these receptors are reminiscent of the tertiary structure of an ion channel. An interesting hypothesis, then, is that these receptors are descendants of an ancient channel, and a clue to the history of rhodopsin might be the shared utility of retinal as both the chromophore in photosensory tissue and a regulator of membrane permeability in erythrocytes, lysosomes (Dingle and Lucy, 1965) and the egg membranes of fish (Plack et al., 1959). An interesting example that links both osmoregulation with light detection occurs in Halobacterium habolium. Although halorhodopsin is utilized primarily as a proton pump, there is evidence that halorhodopsin is also utilized for sensory transduction (Spudich and Bogomolni, 1984) through cGMP and calcium as intermediates (Schmiz and Hildebrand, 1987).

It should not be surprising that an ancient photoreceptor might be involved in osmotic regulation and monitoring of day length since both of these activities may be equally important in the regulation of reproductive activity, particularly for the euryhaline fishes (Cronly-

Dillon, 1992). Although bacteriorhodopsin shares little sequence homology with visual rhodopsin, the utilization of retinal, and the conservation of the seven-pass membrane motif (Henderson and Unwin, 1975) suggests that this may be an example of selective pressure at the secondary and tertiary level. The lack of sequence homology may be attributed to the divergence of bacteriorhodopsin and visual rhodopsin so long ago (Bowmaker, 1991).

It should also be noted that other conserved features of photosignal transduction include the G-protein, transducin, and the utilization of cGMP as an intracellular messenger. Both of these agents have been shown to be utilized in a wide variety of extra-ocular photoreceptors (table 2.3).

Further clues to the link between dermal photoreceptors and ocular photoreceptors come from an embryological examination. The retina develops from patches of photosensitive surface ectoderm that invaginate to form the neural tube (Balfour, 1881). Chromatophores, as previously stated, are neural crest in origin. Therefore, interesting comparisons have been made between chromatophores and neurosensory tissue. Lacour et al. (1992) argue that studies of modulation of melanophore dendricity in vitro are useful

Table 2.3
Utilization of Transducin and cGMP in Extra-ocular
Photoreception

Organism	Tissue	cGMP?	Transducin?	Reference
<u>Amphibians</u>				
<i>Rana Esculenta</i>	pineal	unknown	yes	Veen et al., 1986
<i>Triturus Vulgaris</i>	pineal	unknown	yes	Veen et al., 1986
<u>Birds</u>				
chicken	pineal	yes	yes	Dryer and Henderson, 1991; 1993; Veen et al., 1986
<i>Coturnix coturnix</i>	pineal	unknown	yes	Veen et al., 1986
<u>Ciliates</u>				
<i>Blepharisma japonicum</i>		yes	unknown	Fabczak et al., 1993a
<i>Stentor coeruleus</i>		yes	unknown	Fabczak et al., 1993b
<u>Halobacterium</u>				
<i>Halobacterium halobium</i>		yes	unknown	Schmiz and Hildabrand, 1987
<u>Mammals</u>				
Cow	pineal	unknown	no	Veen et al., 1986
Wistar rat	pineal	unknown	no	Veen et al., 1986
<u>Mollusc</u>				
<i>Onchidium verruculatum</i>	abdominal ganglion	yes	unknown	Nishi and Gotow, 1989; Gotow and Nishi, 1991
<u>Reptile</u>				
<i>Anolis carolinensis</i>	skin	yes	yes	Vaughan and Tallent, 1988; Beam and Vaughan, 1994; Beam and Vaughan, 1996

models of neurite outgrowth. Lerner and colleagues (1988) demonstrated that melanophores of Xenopus laevis contain an odorant-sensitive pigment movement that is apparently homologous to the odorant-sensitive adenylate cyclase system of the olfactory bulb (Pace et al., 1985). Comparisons have also been made between visual and tactile encoding mechanisms in the vertebrate central nervous system (Cronly-Dillon, 1991).

Finally, in many animals there continues to be an intimate association between chromatophores and the retina. Photosensitive retinal cells of reptiles and birds contain oil droplets that act as spectral cut-off filters. These oil droplets are bright red, orange, yellow, very pale, or translucent, and some are made of the same carotenoid pigments that occur in dermal chromatophores (Bowmaker, 1991). Equally intriguing, a melanin-pigmented epithelial layer (the retinal pigment epithelium or RPE) is closely associated with the retinae of virtually all vertebrates and invertebrates as well as the eye spot of unicellular organisms (Cronly-Dillon, 1992). In fishes and amphibians, the melanosomes of the RPE are known to disperse in the presence of light and aggregate in darkness (Bruenner and Burnside, 1986; Burnside and Basinger, 1983; Wagner and Douglas, 1983). Furthermore, an invertebrate-like opsin has been found in bovine retinal pigment epithelium (Jiang et al., 1993). Similarly, guanine crystals within iridophores

of the ocular tapetum of some fishes are known to migrate in response to light (Heath and Hindman, 1988). Finally, melanophores are directly involved in the rod cell light response. RPE cells contain a retinol-binding protein. Retinol is transferred from the blood to the pigmented cell, isomerized to cys-retinol via retinol isomerase, and shuttled to the rod cell (Bridges and Alvarez, 1987). Considering the evolutionary/embryological heritage, and the continued functional role of chromatophores in the retina, it is, thus, not unreasonable to expect similarities between mechanisms of ocular and dermal photoreception (Vaughan and Tallent, 1988; Clotheir and Lythgoe, 1987).

THE RETINAL SIGNAL TRANSDUCTION MECHANISM

The signal transduction mechanism of the vertebrate retinal rod cell has already been worked out and suggests a testable paradigm. The outer segment of the retinal rod, derived from a ciliary structure (Cronly-Dillon, 1992), is depolarized in the dark-adapted state due to high levels of cGMP. cGMP opens cation-selective gates in the rod outer segment (Yau and Nakatani, 1984; Cobbs and Pugh, 1985). During photoexcitation, the photoreceptor, rhodopsin, is activated (for a review see Lewis and Kilger, 1992). In the presence of light, 11-cis retinal isomerizes to all-trans retinal and dissociates (for a review see Lewis and Kilger, 1992). This isomerization causes a conformational change in the opsin protein from which a previously attached G-protein, transducin, is liberated. The α -subunit of transducin stimulates cGMP phosphodiesterase (Sitaramayya et al., 1986). The decrease in cGMP closes cation-sensitive channels and hyperpolarizes the cell, which is the message of photoexcitation.

This decreased cation conductance, along with the activity of a $\text{Na}^+/\text{Ca}^{++}, \text{K}^+$ exchanger (Cervetto et al., 1989), decreases intracellular calcium levels (Yau and Nakatani, 1985; McNaughton et al., 1986). Decreased intracellular calcium activates guanylate cyclase (Koch and Stryer, 1988), causing the cell to repolarize (figure 2.3).

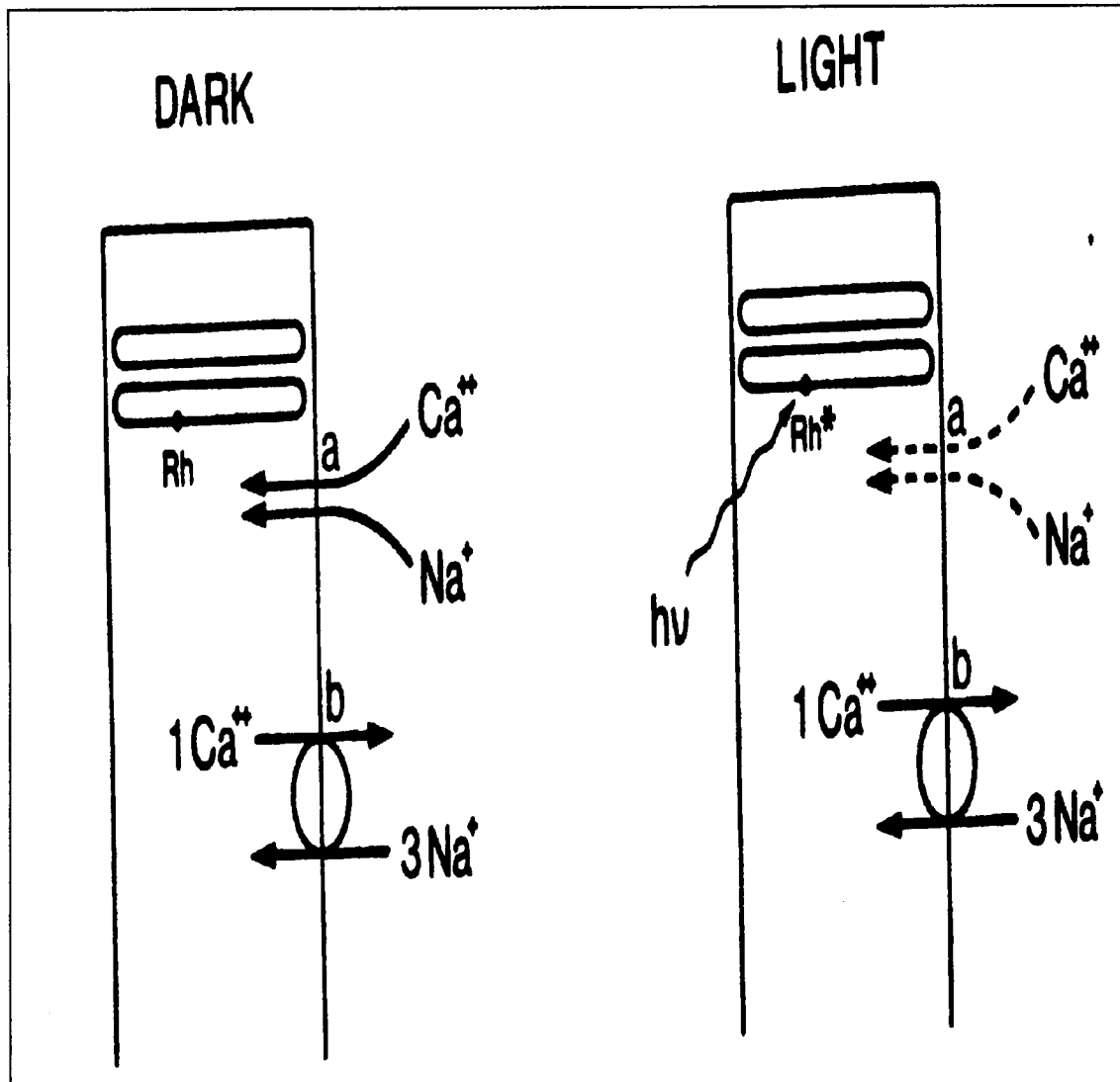


Figure 2.3: Model of Photosignal Transduction in the Vertebrate Rod Cell. In the dark, high levels of cGMP cause a cGMP-gated $\text{Na}^{+}/\text{Ca}^{2+}$ channels to be open. The rod cell is depolarized. As a result of photostimulation, transducin activated cGMP phosphodiesterase reduces intracellular cGMP which closes cGMP-gated $\text{Na}^{+}/\text{Ca}^{2+}$ channels, hyperpolarizing the cell (from Yau and Baylor, 1989, p. 293).

It has often been assumed that invertebrate photosignal transduction is very different from the vertebrate model. This is because invertebrate photosensory structures are very different. They are derived from microvilli rather than cilia, and their response to light is depolarizing rather than hyperpolarizing. However, recent biochemical evidence suggests that the two pathways may not be as evolutionarily isolated as originally thought.

The tertiary structure of invertebrate rhodopsin appears similar to vertebrate rhodopsin, although sharing only about 15-30% sequence homology. A key difference in the photoreaction is that trans-retinal never dissociates (for a review see Rayer et al., 1990). Invertebrate transduction involves a G-protein (Calhoon et al., 1980; Bolsover and Brown, 1982; Fein, 1986). It has been shown that invertebrate transducin can bind to vertebrate opsin in vitro, stimulate cGMP hydrolysis, and is immunologically cross-reactive (Vandenberg and Montel, 1984a; Stieve and Lumme, 1989).

Furthermore, as in other photosensitive tissues (table 2.3) both cGMP and calcium have been implicated as second messengers (for a review see Rayer et al., 1990). In the visual photoreceptors of the squid, Loligo (Vandenberg and Montel, 1984b), and the horseshoe crab, Limulus (Payne et al., 1986), both cGMP and calcium increase in response to light. Intracellular calcium is released via the

phosphatidyl inositol pathway (Brown et al., 1984; Fein et al., 1984) which is linked in Limulus to a Na⁺-dependent depolarization (Bolsover and Brown, 1985) modulated by cGMP-gated channels (Johnson et al., 1986; Bacigalupo et al., 1991). A cGMP-gated channel has also been demonstrated in the ocular photoreceptors of Sepia officinalis (Huppertz, 1995). In contrast, in the extra-ocular photoresponsive neuron of the gastropod mollusc, Onchidium, light triggers a decrease in cGMP (Nishi and Gotow, 1989). This decrease in cGMP closes a K⁺ channel and, thus, depolarizes the cell, the depolarization being modulated by an apparently parallel phosphatidyl inositol pathway (Gotow and Nishi, 1991).

The relationship between the phosphatidyl inositol pathway and cGMP pathway in invertebrate photoreception has not yet been determined. A similar interaction, however, appears to be present in vertebrate smooth muscle cells which, interestingly, undergo visible and UV light-induced relaxation (Ehrreich and Furchgott, 1968) due to a nitric oxide induced cGMP increase (Karlsson et al., 1984). In smooth muscle, cGMP is known to lower cytosolic calcium (Felbel et al., 1988) by inhibiting calcium release from the sarcoplasmic reticulum (SR) via cGMP-dependent phosphorylation of an SR channel protein (Twort and Breeman, 1988; Cornwell et al., 1991), by inhibiting the production of inositol triphosphate (Ruth et al., 1993), by inhibiting calcium influx (Yu et al., 1990), by blocking L-type calcium

channels which may be utilized to refill SR calcium stores (Low, 1993), and by stimulating a $\text{Na}^+/\text{Ca}^{2+}$ exchange mechanism (Furukawa et al., 1991).

SUMMARY OF KNOWN INFORMATION

The putative mechanism of action of MSH on Anolis carolinensis skins has been previously reviewed (pages 14-27). Although the light response had been recognized by Hadley (1931) and Kleinholtz (1938c), no detailed examination of the response appeared until the late 1980s. Vaughan (1987) showed that the proximal mechanism of pigment dispersion is the same for both the light and MSH responses. Cytochalasin B, an inhibitor of microfilaments, prevents pigment dispersion, whereas colchicine, an inhibitor of microtubules prevents pigment reaggregation. Furthermore, the two responses appear to be compartmentalized since skin, challenged with a submaximal dose of MSH and light simultaneously, darkens to a greater extent than with MSH alone. Upon cessation of light stimulation, the skin will partially recover such that the final darkening is no greater than that induced by MSH alone (figure 2.4). Later, Vaughan and Tallent (1988) further demonstrated the compartmentalization of the skin responses by showing that the light-induced response is inhibited by 8-Bromo-cGMP, whereas the MSH response remains unaffected. A summary of published effects of all agents tested on A. carolinensis skins and their presumed mode of action is presented in table 2.4.

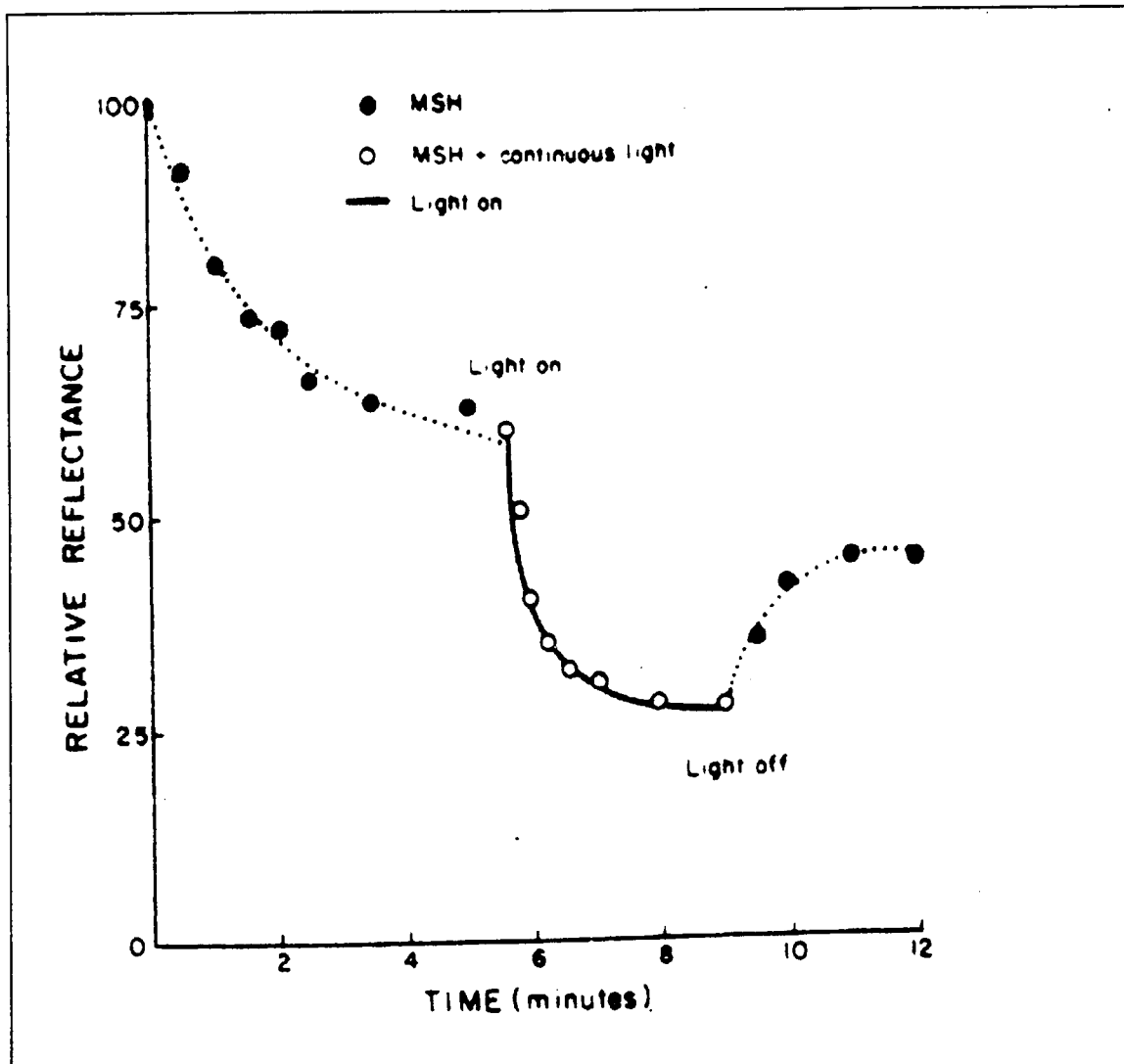


Figure 2.4: Demonstration of the Compartmentalization of the Light and MSH Responses in Anolis carolinensis. Skins darkened submaximally with MSH will darken further when stimulated with visible light. Upon cessation of light stimulus, skins partially recover such that the final darkening is no greater than that induced by MSH alone (From Vaughan, 1987, p. 112).

Table 2.4
Pharmacological Indicators of MSH and DPR Pathways in
Anolis Carolinensis

Drug	Mode of Action	Effect on MSH Response	Effect on DPR	Reference
<u>CAMP Drugs</u> CAMP dibutyryl cAMP IBMX	2nd Messenger mimmicks cAMP inhibits PDE	lightens Darkens Skin * Darkens Skin*	? 	Hadley and Goldman, 1969a Goldman and Hadley, 1972 Hadley and Goldman, 1969b
<u>cGMP Drugs</u> 8-Br-cGMP	mimmicks cGMP	no effect	inhibits	Vaughan and Tallent, 1988
<u>PKC Drugs</u> cromolyn phorbol ester	inhibits PKC stimulates PKC	inhibits potentiates	? ?	Lucas and Shuster, 1987 Lucas et al., 1987b
<u>Prostaglandins</u> PGF ₂ PGI ₂ PGE ₂ indomethacin	2nd Messenger 2nd Messenger 2nd Messenger Inhibits PG synthesis	potentiates no effect darkens (additive) no effect	? ? ? ?	Lucas et al., 1985 Lucas et al., 1985 Lucas et al., 1985 Lucas et al., 1985
<u>Ionic</u> Lanthanum A23187 phenothiazines	Blocks Ca ⁺⁺ channels Ca Channel inhibit calmodulin	inhibits (high conc.) Darkens Skin * Darkens Skin *	? ? ?	Vesely and Hadley, 1971 Hadley et al., 1981; Lucas et al., 1983 Carter and Shuster, 1978
<u>Receptor Effects</u> clonidine propranolol	decrease cAMP β -blocker	inhibits inhibits	? ?	Carter and Shuster, 1982 Vaughan and Greenberg, 1987
<u>Misc Drugs</u> sulfhydryl inhibitors	reduce free sulfhydryls	inhibits	?	Horowitz, 1956; 1957; Goldman and Hadley, 1972

KEY ?=no information known; *=authors assumed to be activating the MSH response, but since skin darkens in response to applied agent, it is impossible to know if the MSH or DPR response has been activated.

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Part III.

Cyclic-GMP and Calcium, but not Cyclic-AMP, are implicated
in the Dermal Photic Response of the Lizard,
Anolis carolinensis

ABSTRACT

The dermal melanophore of the lizard, Anolis carolinensis, responds directly to light and melanocyte-stimulating hormone (MSH) by dispersion of melanin from a perinuclear position into superficial dendritic processes. This results in a change in skin color from bright green to brown. The dermal photic response (DPR) produces a reduction in reflectance of approximately 35% which is complete within 3 minutes and is completely reversible within 2 minutes.

Although both the DPR and MSH responses have been reported in other animals to be mediated by cyclic adenosine monophosphate (cAMP), changes in cAMP levels characteristic of the MSH response do not accompany DPR. However, a poorly metabolizeable cyclic guanosine monophosphate (cGMP) analog, 8-bromo-cGMP, inhibits DPR by approximately 80% in isolated skin but does not appear to alter pigment dispersion elicited by MSH.

Furthermore, when skins are darkened with the calcium ionophore a23187, they are no longer light-responsive, whereas MSH-sensitivity remains intact. This observation, combined with the dose-dependent effect of Ca^{2+} on DPR magnitude and the inability to assay cAMP increases in skins darkened with a23187, suggests that a23187 is DPR-mimetic, rather than MSH-mimetic as previously suggested.

The action spectrum of DPR is indicative of a blue cone-type opsin with maximal sensitivity at 425 nm. The

presence of a 40 kd cone-type photopigment was confirmed via western blot analysis and immunolocalized to the melanophore plasma membrane as well as to a cytosolic locale, possibly the melanosome. It appears that DPR signal transduction in Anolis melanophores may involve changes in cGMP levels and calcium, perhaps in a manner analogous to the signal transduction mechanism in retinal rod cells.

INTRODUCTION

Extra-ocular photosensitivity has been found in essentially every group of animal organism (Wolkun and Mogus, 1979; Weber, 1983). Extra-retinal systems responding to timing, duration, and intensity of illumination are often important regulators of biological rhythms of activity and reproduction (Tamarkin et al., 1985). The ubiquitous dermal light sense, or dermal photic response (DPR) may be involved in crypsis in fishes and larval amphibians, in photoprotection (Burttt, 1981) and as a mechanism to gain thermal energy for animals such as the small iguanid lizard, Anolis carolinensis (Vaughan, 1987b). DPR in this animal involves a color change from brilliant green to brown with a shift in albedo of 30-50% within 3 minutes of exposure to visible light at intensities above 50 watts per meter² (Vaughan, 1987a). This color change is effected by a redistribution of melanin within dermal melanophores (Beam and Vaughan, 1995a) presumably in a manner similar to the MSH response already described (Taylor and Hadley, 1970). Partially because of the thermal benefit afforded by DPR, A. carolinensis, a subtropical reptile at the northern edge of its distribution near Knoxville, Tennessee, remains active on bright winter days, even when air temperature hovers at 0° C. around rock faces where they live (Gatten et al., 1988; Vaughan, 1987b).

The skin of A. carolinensis will also turn brown in response to melanocyte-stimulating hormone (MSH). The actual physical mechanism of pigment movement itself appears to be the same for both the DPR and MSH responses (Vaughan, 1987a). Although adenosine 3',5'-cyclic monophosphate (cAMP) has been implicated in the control of both responses in other animals (Wakamatsu et al., 1980; Sawyer et al., 1983; Rollag, 1993), we describe evidence suggesting that in A. carolinensis the proximal control of DPR, in contrast to that for the MSH response, does not involve cAMP but possibly involves cGMP (Vaughan and Tallent, 1988) and calcium (Beam and Vaughan, 1995b). We suggest that the process of signal transduction in DPR may be analogous to the system in vertebrate retinal rod cells which share a similar embryonic origin.

MATERIALS AND METHODS

Animals

The animals used in this study were male lizards of the species, Anolis carolinensis, recently captured from wild populations in coastal Louisiana and purchased from Snake Farm, Laplace, LA. Individuals were maintained in group colony on a 12 hour light/dark cycle at 21° C.

Physiological Recordings

For each experiment an animal was killed by decapitation. Dorsal torso skin was removed and floated on physiological saline (in mM/L: 111 NaCl, 2.0 KCl, 1.0 CaCl₂, 2.0 NaHCO₃, 5.0 glucose at pH 7.2) in the dark for at least 45 minutes prior to use. Under these conditions skin stabilizes to exhibit a tonic green color. Variation from this color of maximal reflectance in the visible spectrum was measured using the tungsten light source of an Oriel variable monochromatic fiber optic illuminator (model 77503) and an Oriel photodiode-equipped detection system (model 7072). Light was delivered through one branch of a bifurcated fiber optic bundle placed normal to and 2.0 mm above skin floating over a saline-filled depression in a wax dish. Reflected light returned through the other branch of the fiber optic bundle to illuminate the photodiode detection system. Output was recorded on a chart recorder and logged via computer. Although responses were defined as complete when two identical reflectance values were logged

at 10 second intervals, experiments were occasionally carried out for 5 minutes past completion.

Relative reflectance change, defined as the percent change in reflectance from the initiation to the termination of the response, was used to compare treatments in which skin was transferred to a second dish containing test solutions of drug, hormone, and/or nucleotide.

Full spectrum light at an average intensity of 100 watts/meter² from the tungsten light source was used both to induce DPR and to measure albedo in DPR experiments. Using a series of gelatin filters (Eastman Kodak Company), we determined that light at 600 nm is ineffective for inducing DPR. This wavelength was, therefore, chosen to monitor MSH-induced reflectance changes to avoid confounding DPR responses.

Pharmacological Agents

All test reagents were purchased from Sigma Chemical Company (St Louis, MO) unless otherwise noted. Stock dilutions were made in either saline or dimethyl sulfoxide (DMSO). The final concentration of DMSO in saline was 1.0% in all experiments requiring the solvent (a23187). DMSO had no intrinsic effect on skin at this concentration. Unless otherwise noted, skins were equilibrated in test solutions for 20 minutes before testing. During this time, skin reflectance was observed at 600 nm to determine the effects on unilluminated skin.

Student's T-test ($p=.05$), or where appropriate, paired T-test, was used to detect treatment effects. Results are expressed as mean $\pm$ standard error of the mean, where appropriate.

Cyclic Nucleotide Assays

Cyclic-AMP levels were measured using a competitive binding assay purchased in the form of a kit (catalog #TRK.432) from Amersham Corporation (Arlington Heights, Il.). Four skin samples (approximately 1.5 cm X 1.5 cm, 50 mg) from each animal were assayed for cAMP, at the point of maximal DPR, maximal MSH response, maximal response to calcium ionophore a23187 (100 μ M, 1 hour), or untreated and after dark adaptation (control). Upon attainment of maximal physiological response, skin samples were immediately frozen in liquid nitrogen. Frozen samples were ground with the aid of glass beads in a ceramic mortar precooled with liquid nitrogen. Powdered skin was resuspended in 0.5 ml aliquots of ice-cold TRIS/EDTA (10 mM/5mM at pH 7.4), and centrifuged (4000G, 5 minutes at 4 C). The supernatant was boiled (5 minutes) and assayed for cAMP in duplicate. Protein content was measured by the method of Bradford (1976) against a standard curve prepared from bovine serum albumin (BSA). Results were expressed as picomoles cAMP per microgram protein.

Attempts to assay cGMP changes in skin homogenates treated as above were unsuccessful. Possible explanations are presented in the discussion.

Determination of Action Spectrum

Utilizing a Leitz microscope equipped with a photometer and a Baush and Lomb monochromator attached to an epi-illuminating mercury lamp, we utilized the photometer calibration curve supplied by the manufacturer and the adjustable monochromator entrance slit width so as to attain near equivalent, but unknown, light output from 400 to 575 nm in 25 nm increments. This light source was then used to both induce and record DPR as previously described (Vaughan, 1987a). Normalized magnitude of DPR as a function of wavelength was measured and averaged for skin tissue from 5 animals.

Cell Culture

The cell culture technique will be described in detail in part VI. Cells for these experiments were grown on acid washed sterilized glass coverslips.

Immunofluoresence

Coverslips containing cell cultures were briefly rinsed in saline, and attached cells were simultaneously fixed and permeabilized by submersion in acetone (-80 °C, 1 minute). Nonspecific binding sites were blocked by equilibration for 30 minutes in blocking solution (PBS/.05% Tween-20, 2% normal goat serum, 0.5% BSA) at room temperature. Following

a brief rinse in PBS/.05% Tween-20, coverslips were probed 1 hour to overnight with primary antisera at various dilutions in blocking solution. Coverslips were then rinsed 3 times in PBS/.05% Tween-20 for 5 minutes each, and probed 1 hour with a secondary fluorescein-labeled antibody at manufacturer's recommended dilution in blocking solution. In control experiments the primary antibody was omitted, replaced with normal serum, or replaced with primary antibody prebound to antigen.

Following extensive rinsing in three changes of blocking solution followed by double-distilled water, coverslips were mounted in fluorescence mounting medium (90% glycerol, 10% PBS, 0.1% p-phenylamine diamine, pH 8.7) and viewed on a Nikon microscope with appropriate epifluorescent equipment. Fluorescence was photographed with 3200 ASA Technical Pan film (Eastman Kodak Company) and processed per manufacturer's recommendation.

Western Blotting

Whole skin from five lizards was pooled, frozen in liquid nitrogen, and ground with the aid of glass beads in a mortar pre-cooled with liquid nitrogen. Powdered skin was resuspended in a plastic conical centrifuge tube containing 10 ml of homogenization buffer (10 mM TRIS, 1 mM DTT, 1mM PMSF, 1 mM benzamide, 5 mM EDTA, 5 µg/ml leupeptin, pH 6.5). Following centrifugation (20,000 x G, 30 minutes, 4 °C), lipophilic proteins were extracted from the pellet with 2 ml

of homogenization buffer containing 1% lauryl maltoside (Anatrace, Maumee, OH) by gentle inversion for 24 hours at 4 °C. Following centrifugation (20,000 x G, 30 minutes, 4°C), the soluble extract was concentrated with an Amicon (Beverly, MA) microprep device.

Concentrated proteins were assayed via the BCA method (Pierce, Boston, MA) using a BSA standard (Sigma). Twenty micrograms of protein in a volume of 10 µl was mixed 1:1 with SDS-sample buffer (40 mM TRIS-base, 10% glycerol, 4% sodium dodecyl sulfate, 400 mM DTT, 0.4% bromophenol blue), boiled 5 minutes, and loaded onto a high-resolution 10-20% gradient acrylamide mini-gel (Zaxis, Hudson, Ohio). Following electrophoresis (150 V, 2 hours), proteins were electroblotted (4 °C, .1A, 12 hours) onto nitrocellulose paper.

Nitrocellulose blots were blocked overnight in blocking solution (PBS/.05% Tween-20 containing 10% dry milk) at room temperature. Following three washes at room temperature (10 minutes each) in PBS/.05% Tween-20, blots were probed overnight at room temperature with CERN-906 or CERN-886 antisera diluted 1:1000 in antibody solution (PBS/.05% Tween-20 containing 0.5% normal goat serum), washed overnight in 6 changes of blocking solution, and then probed 1 hour at room temperature with 10^6 CPM I^{125} -labeled secondary antisera in antibody solution. Blots were then washed overnight at room temperature in 6 changes of

blocking solution, followed by three 10 minute washes in doubled-distilled water at room temperature, dried overnight, and used to expose Medcor XR-4 X-ray film (-80 °C, 6 weeks, with intensifying screens).

Antisera

Polyclonal antisera directed against bovine rod opsin and bovine alpha-transducin were gifts of Dr. Barry Knox (Syracuse University, Syracuse, NY). Monoclonal antibody CD4 directed against the N-terminus of bovine rod opsin was a gift of Dr. Robert Molday (University of British Columbia, Vancouver, British Columbia, Canada). Polyclonal antisera directed against chicken rods, CERN-886, and cones, CERN-874 and CERN-906, were gifts of Dr. Willem DeGrip (University of Nijmegen, The Netherlands). All other supplies were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

RESULTS

The magnitude of the DPR we are reporting here (approximately 35%) is less than what we reported (50%) previously (Vaughan, 1987a). In earlier work, we used a microscope equipped with a photometer to measure reflectance from the central portion of single scales. Change in color is effected when melanin in melanophores lying beneath xanthophores and reflective iridophores is redistributed into dendritic cell processes that terminate above and obscure those cells. Most iridophores and xanthophores are located in the central portion of the scale and, thus, reflectance changes are greatest there. Reflectance measurements reported herein involved dozens of scales and include interscale areas where no pigment cells other than melanophores are located and, therefore, produce lower DPR values. With that single exception, the response is similar. On exposure to light, skin rapidly darkens to reach a minimum reflectance within 3 minutes (figure 3.1a). Returned to darkness, skin recovers to its original tonic green color within 2 minutes.

Effect of 8-Br-cGMP on DPR and MSH Responses

A cell permeable, poorly hydrolyzable analog of cGMP, 8-Br-cGMP (1.0 mM), significantly inhibited DPR (figure 3.1b). A normal DPR (in the absence of 8-Br-cGMP) involved a change in reflectance of $33.2 \pm 1.4\%$ (n=9 trials). After

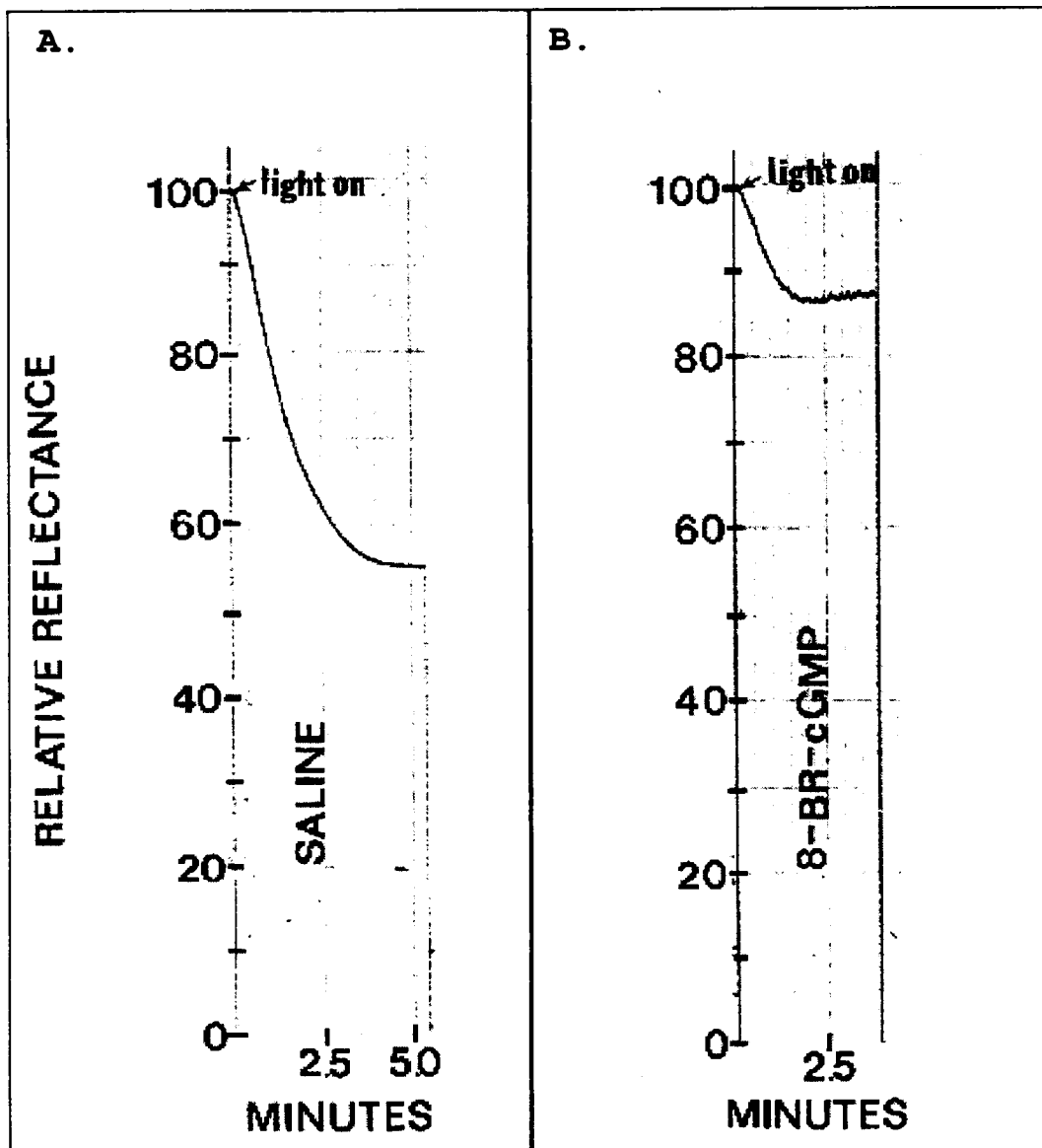


Figure 3.1: 8-Bromo-cGMP Inhibits DPR. Relative reflectance as a percent of the initial dark-adapted value is shown as a function of the duration of the illumination in minutes. Panel (A) is from skin in saline, and panel (B) is from the same skin sample after a 3 minute preincubation in 8-Br-cGMP (1 mM). These traces are typical of responses recorded from the skin of nine individuals.

incubation with 1.0 mM 8-Br-cGMP, subsequent DPR was reduced to $6.7 \pm 1.3\%$ (n=9 trials), a mean inhibition of $80.6 \pm 3.6\%$ (n=9 trials). Maximum effect was observed at concentrations around 1 mM. Inhibition was dose-dependent (figure 3.2), maintained as long as 8-Br-cGMP was left on the skin, and effective within 3 minutes of incubation. As a control, 8-bromo-guanosine was substituted for 8-bromo-cGMP. At 1 mM, 8-bromo-guanosine inhibited DPR by only $12.6 \pm 3.5\%$ (n=8 trials). Finally, skins were challenged with 8-Br-cGMP (1 mM) at the point of maximal DPR in the presence of continued light stimulation. If added at the point of maximal DPR, 8-Br-cGMP causes skin to recover $69.0 \pm 7.8\%$ (n=3 trials).

At a concentration of 10^{-7} M, MSH produced a $70.6 \pm 1.6\%$ (n=5 trials) change in reflectance (figure 3.3A). Skins pretreated for 20 minutes with 1 mM 8-Br-cGMP and then challenged with 10^{-7} M MSH in the presence of 1 mM 8-Br-cGMP resulted in a $71.8 \pm 1.8\%$ (n=5 trials) change in reflectance (figure 3.3B). We conclude that 8-Br-cGMP inhibits DPR ($p < .05$) but has negligible effect on chromatic response to MSH.

Effect of Other cGMP Analogs on DPR

Trials were conducted with various cGMP analogs at 1 mM (table 3.1). We report that 8-Br-cGMP was the most potent

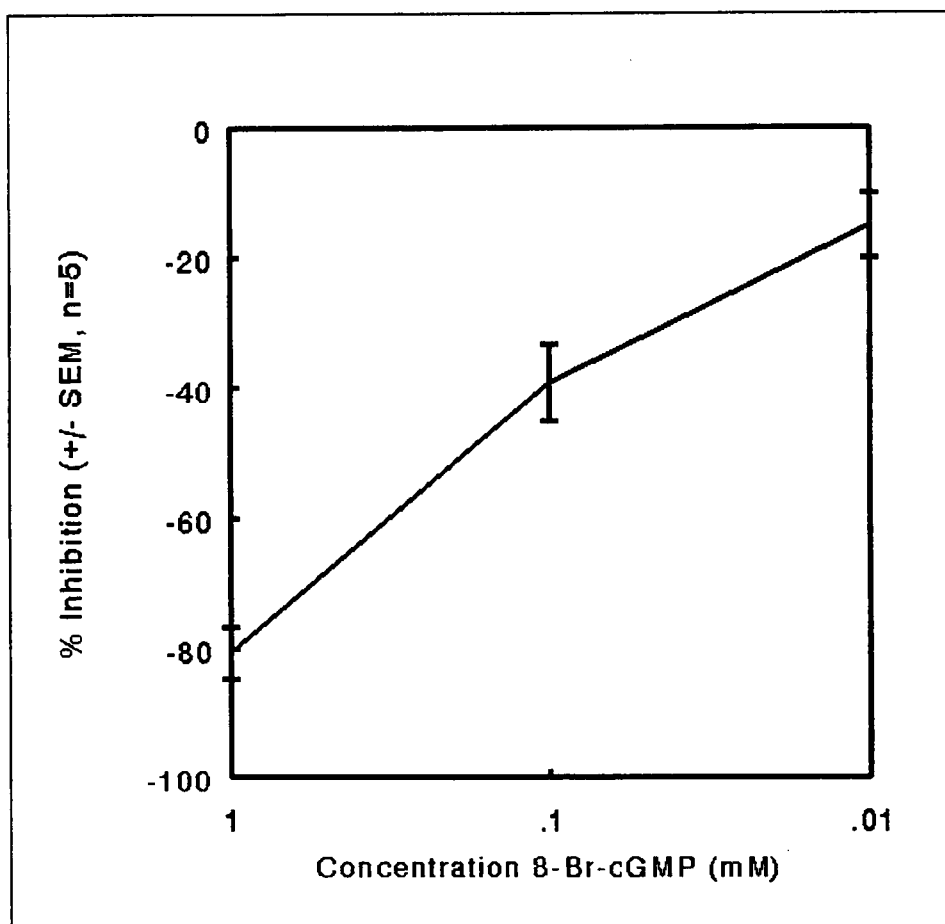


Figure 3.2:Dose Response Effect of 8-Br-cGMP on DPR

Table 3.1
Effect of cGMP Analogs on DPR

Analog (1.0 mM)	% Inhibition of DPR ($\pm$ SEM)	Sample Size
cGMP	-2.5 $\pm$ 4.4%	5
N ² ,O ^{2'} -dibutyryl cGMP	-5.3 $\pm$ 5.6%	5
N ² -monobutyryl cGMP	-54.0 $\pm$ 4.0%	5
O ^{2'} -monosuccinyl cGMP	-25.9 $\pm$ 3.1%	5
8-Br-cGMP	-80.6 $\pm$ 3.6%	9

*p<.05, paired T-test

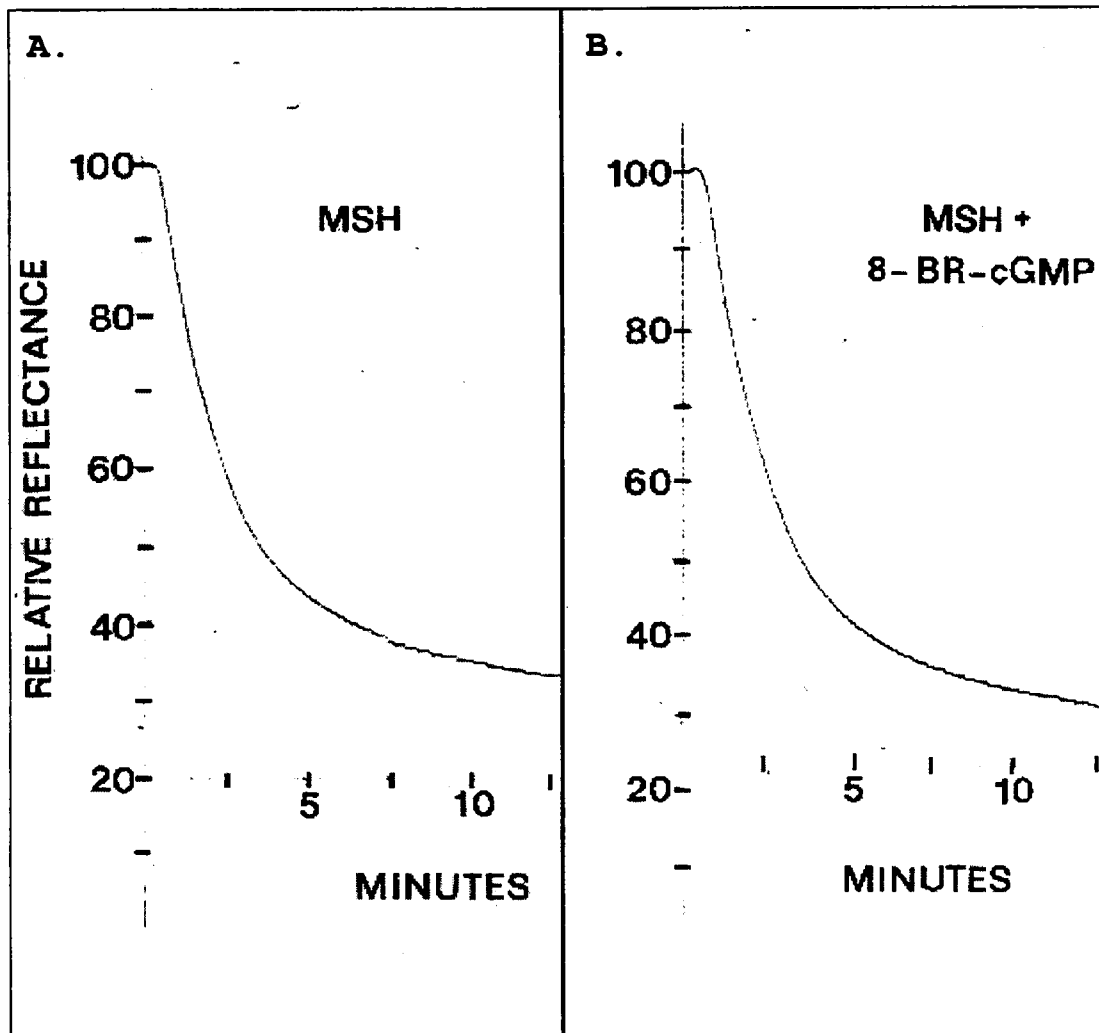


Figure 3.3: 8-Bromo-cGMP has No Effect on the MSH Response. Relative reflectance, expressed as a percent of the initial dark-adapted value, is shown as function of the duration of exposure to MSH (10^{-7} M). Reflectance was measured using 600 nm light to avoid confounding DPR. Panel (A) shows the response to MSH alone, and panel (B) shows the response to MSH of skin pretreated 20 minutes and in the presence of 8-Br-cGMP (10^{-3} M). These traces are typical of recorded responses from the skin of five individuals.

inhibitor of DPR whereas dibutyryl-cGMP (DB-cGMP) and unmodified cGMP were without effect. Other analogs tested (N-monobutyryl cGMP and O-succinyl cGMP) were intermediate in effect.

Effect of Nitric Oxide Elevating Agents on DPR

Trials were conducted in the presence of 1 mM nitroprusside or 1 mM nitroglycerin purchased from a local pharmacy. We report that nitroprusside had no effect on DPR, whereas nitroglycerin enhanced DPR (table 3.2). If a nitric oxide stimulated guanylate cyclase were involved in DPR, we would predict that both agents would be inhibitory. Thus, nitric oxide is probably not involved in DPR.

Role of Calcium in DPR and MSH Responses

Previous researchers (Hadley et al., 1981; Lucas et al., 1983) have demonstrated that the calcium ionophore a23187 will darken lizard skins and, thus, assumed the ionophore to be hormone-mimetic. In order to test this hypothesis, we performed control DPRs on lizard skins (n=5), darkened these skins maximally with a23187 (100 μ M, 1 hour), and then attempted to elicit a DPR and MSH response from these same skins (figure 3.4). DPR before a23187 treatment was $21.1 \pm 1.6\%$. These same skins darkened $28.1 \pm 5.9\%$ when challenged with 100 μ M a23187 (not statistically different), but would not darken further when challenged with light

Table 3.2
Effect of Nitric Oxide Elevating Agents on DPR

Treatment	% of Control DPR ($\pm$ SEM, n=5)
Nitroglycerin	136.6 $\pm$ 11.6*
Nitroprusside	100.9 $\pm$ 3.1

*p<.05, 2-tailed t-test

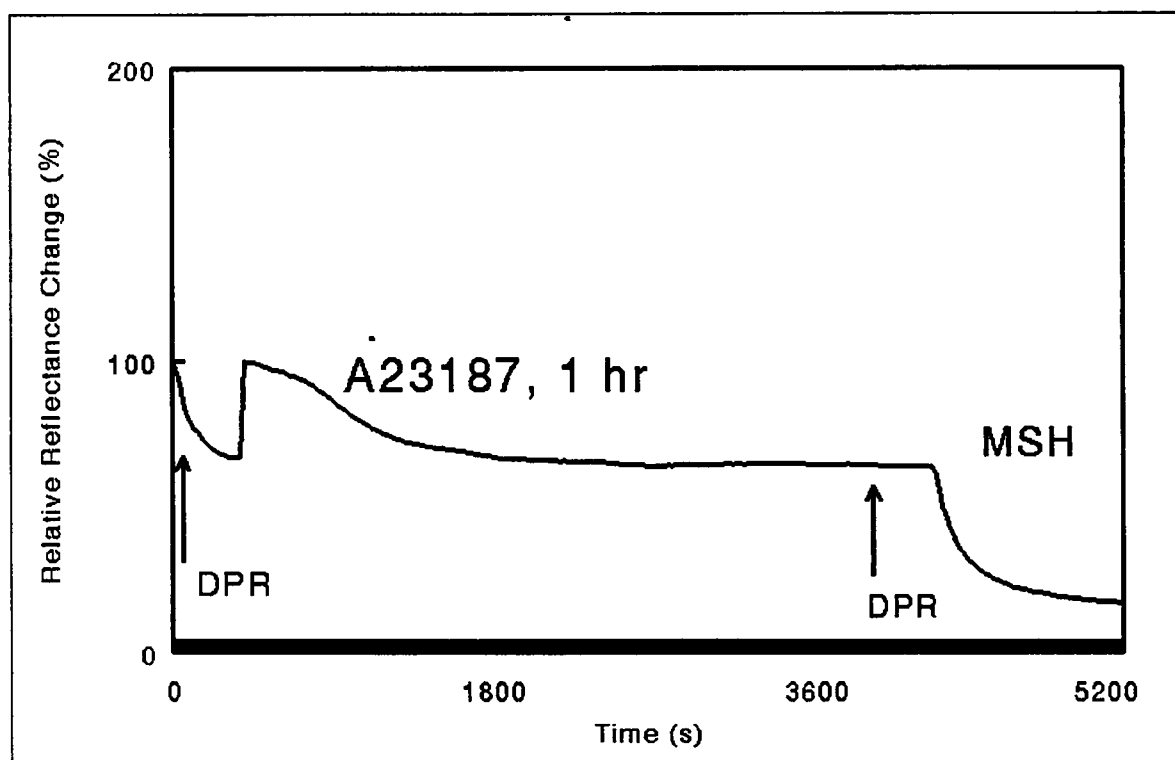


Figure 3.4: Effect of Calcium Ionophore A23187 on Light and MSH Responses. Skins (n=5) were tested for light-sensitivity, allowed to recover, then treated with calcium ionophore a23187 (100 μ M, 1 hour). Ionophore-dependent darkening was monitored with 600 nm light so as not to induce DPR. Following ionophore darkening, skins were challenged to respond to light and then MSH (10^{-7} M). The light response was completely abolished, whereas the MSH response remained intact.

($-0.1 \pm 3.0\%$). These same skins, however, darkened $46.8 \pm 14.8\%$ when challenged with MSH (10^{-7} M), which was not statistically different from the $59.0 \pm 5.6\%$ darkening of a control group of skins. Our data suggest that the effect of a23187 may be DPR-mimetic, rather than MSH-mimetic.

In order to further investigate the calcium-requirement for DPR, skins (n=5) were tested for DPR in saline containing no calcium (1 mM EGTA), 5 mM Ca^{2+} , 10 mM Ca^{2+} , and 10 mM Ca^{2+} with 8-Br-cGMP (1 mM). We report (table 3.3) that calcium-free saline inhibits DPR, whereas increasing calcium 10-fold enhances DPR. Furthermore, in the presence of 8-Br-cGMP, DPR is abolished, even in the presence of increased extracellular calcium.

Assay of cAMP Levels in Skin

cAMP levels were measured in control skins, skins darkened by calcium ionophore a23187, light, and MSH. From these results (table 3.4), we conclude that, although the MSH response involves a cAMP intermediate ($p < .05$), DPR does not. Furthermore, the ability of calcium ionophore a23187 to darken skins without increasing cAMP, in conjunction with our demonstration that a23187 darkened skins will not respond to light but respond normally to MSH, strongly suggests that the ionophore exerts its effect via perturbations of DPR and not the MSH pathway.

Table 3.3
Effect of Calcium or Calcium + 8-Br-cGMP on DPR

Treatment	% of Control DPR ($\pm$ SEM, n=5)
EGTA	67.0 $\pm$ 6.2%*
5 mM Ca	98.3 $\pm$ 7.5%
10 mM Ca	144.6 $\pm$ 5.1%*
10 mM Ca + 1 mM 8-Br-cGMP	13.2 $\pm$ 4.3%*

p<.05, paired T-test

Table 3.4
cAMP Levels in Lizard Skin

Treatment	cAMP Concentration (pmole/ μ G protein) ($\pm$ SEM, n=5)
Control	.034 $\pm$.005
MSH	.346 $\pm$.106*
Light	.034 $\pm$.005
A23187	.024 $\pm$.005

p<.05, one-tailed paired T-test

Action Spectroscopy and Photopigment Identification

The action spectrum of DPR suggests a blue photopigment with maximal sensitivity of 425 nm (figure 3.5). Previously (Beam and Vaughan, 1994), we described experiments demonstrating the presence of an opsin-type pigment in lizard skin. For that work, we utilized a polyclonal antibody directed against bovine opsin. Labeling required very high antibody concentrations (1:50), the absence of detergent from all solutions, and heroic efforts.

With the demonstration of an action spectrum indicative of a blue cone, and the recent demonstrations of primarily cone-like photopigments in the basal brain (Foster et al., 1993) and retina (McDevitt et al., 1993) of this animal, it seemed profitable to reinvestigate the nature of the skin photopigment with cone and rod-discriminating antisera. As expected, we found that western blots of lizard skin and retina react more strongly with anti-cone antisera, labeling a 40 kd monomer as well as characteristic oligomers, whereas the reaction with anti-rod antisera, under exactly the same conditions, is quite weak. Curiously, lizard skin opsin does not comigrate with lizard retinal opsin, suggesting that skin opsin is a distinct gene or protein product (plate 3.1).

Immunofluorescent results (plate 3.2) confirm the presence of a cone-like photopigment apparently associated

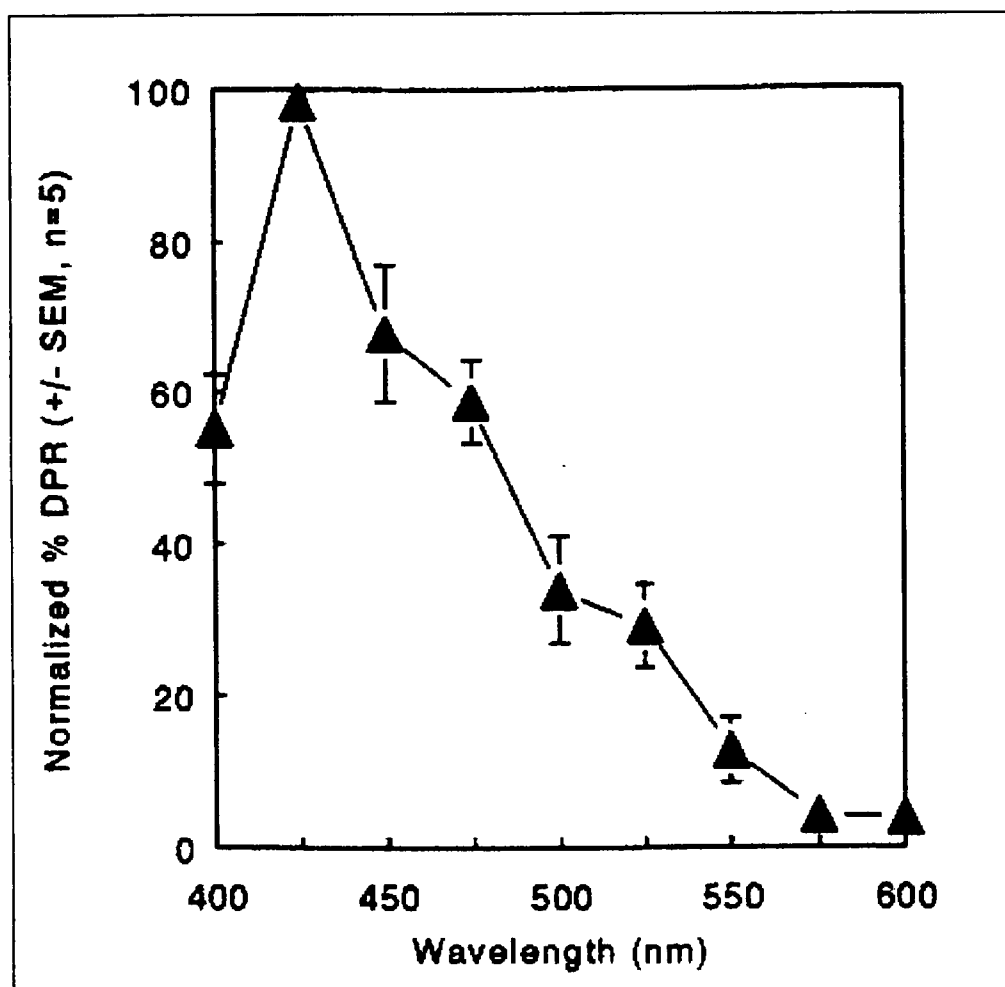


Figure 3.5: Action Spectrum of DPR. The shape of the action spectrum of DPR suggests an opsin-type pigment with maximal sensitivity in the blue region.

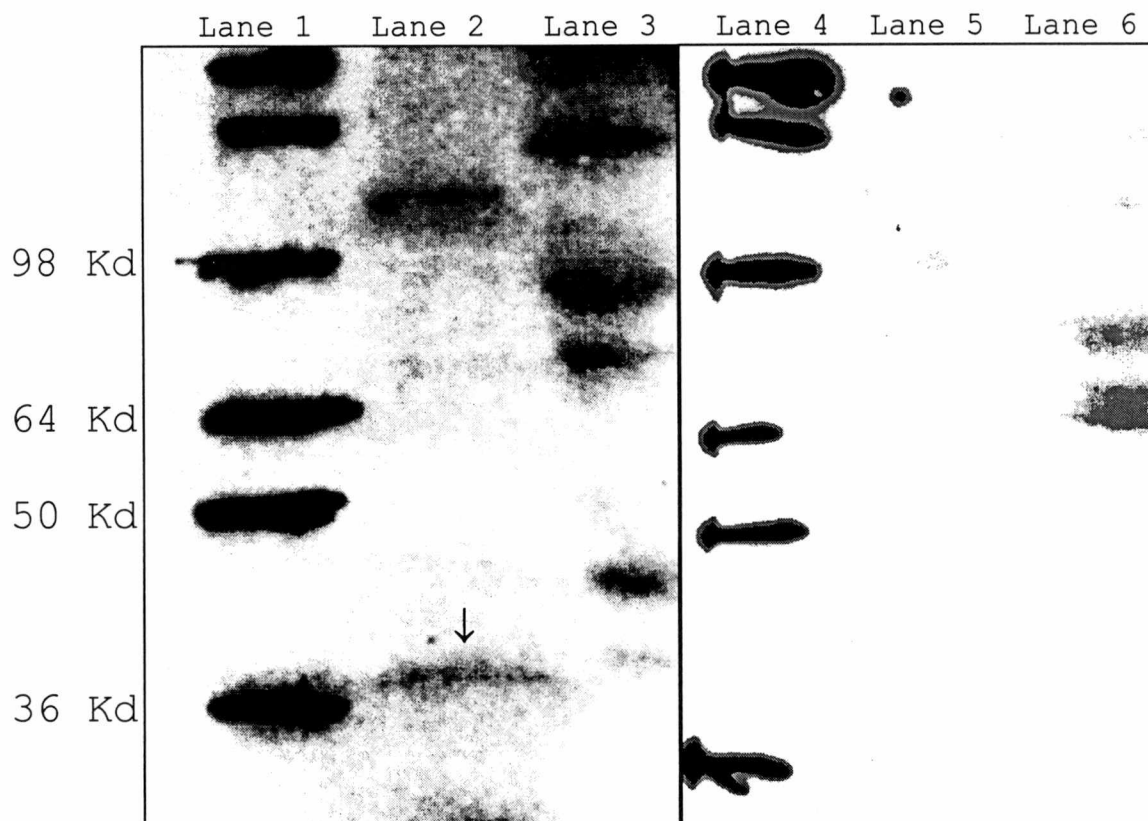
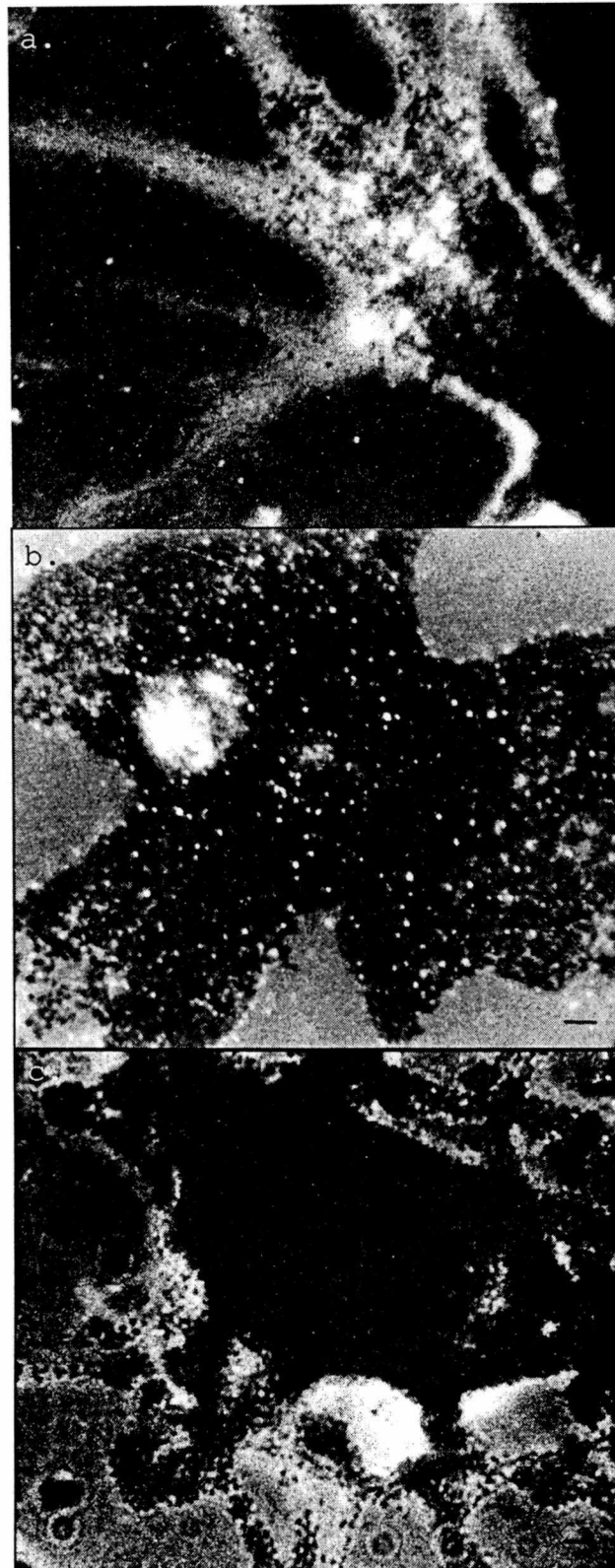


Plate 3.1: Western Blot of Lizard Skin. (A) Using anti-cone opsin immune serum (CERN-906), a 40 kd band (arrow), as well as higher molecular weight oligomers, are labeled in lizard skin extract (lane 2) and retina extract (lane 3). (B) Using anti-rod opsin immune serum (CERN-886) no labeling is apparent in lizard skin extract (lane 5), and only light labeling is apparent in retina extract (lane 6). Lanes 1 and 4 are molecular weight markers.

Plate 3.2: Immunofluorescent Localization of Opsin. Immunofluorescent localization using anti-cone opsin serum (CERN-906) suggests that the photopigment is localized on (a) the plasma membrane and (b) dispersed throughout the cytoplasm, possibly on melanosome membranes. In control experiments (c), no labeling was present (Bar = 2 μ).



with the melanophore plasma membrane as well as dispersed throughout the cytoplasm, apparently attached to melanosomes, as previously suggested (Beam and Vaughan, 1995a). Labeling was much more intense with cone antisera than rod antisera, and is effective at a greater dilution (table 3.5).

Using the western blot technique, we previously identified a 40 kd band that cross-reacts with an antibody directed against bovine alpha-transducin (Beam and Vaughan, 1996). However, antibody concentrations had to be very high (1:10), and the band was too faint to photograph. The presence of a cone-like photopigment may explain our previous difficulty probing for alpha transducin, since cone and rod alpha-transducins have been shown to be immunologically distinct (Lerea et al., 1986).

Table 3.5
Immunofluorescent Results with Opsin Antibodies

Antibody	Dilution	Labeling Intensity
monoclonal CD4	1:10	-
anti-bovine rod opsin	1:50	+
CERN-886	1:50	+
CERN-874	1:250	+++
CERN-906	1:250	+++

DISCUSSION

Sensitivity to light is a relatively universal phenomenon which occurs in many diverse forms: phototactic withdrawal in marine vertebrates, photomorphogenesis in plants, the dermal photic response in lower vertebrates (Weber, 1983). It may reach its highest level of sophistication and complexity in the vertebrate retinal system. Dermal photoreception appears to have evolved prior to the retinal system since it is found in lower invertebrates where it functions primarily as a primitive light sense associated with phototaxis (Wolkun and Mogus, 1979). The dermal response also appears earlier in ontogeny than the retinal system in fish, amphibian, and reptile species which have been studied (Weber, 1983). Since photoreceptive cells in both the dermis and eye develop from neuroectoderm, it is not surprising that they may exhibit similar mechanisms for photoreceptive mechanisms (Clotheir and Lythgoe, 1987; Vaughan and Tallent, 1988).

Considering the ubiquity of the phenomenon, little is known about the signal transduction mechanism involved in extra-retinal photoreception. Recent progress has, however, been made in elucidating the signal transduction cascade in the retina, revealing a central role for cGMP and calcium. Cyclic GMP levels are regulated through the action of guanylate cyclase and cGMP phosphodiesterase (PDE). Guanylate cyclase acts to convert GTP to cGMP while PDE

hydrolyzes cGMP to 5'-GMP. These two enzymes, acting antagonistically, regulate cGMP levels by controlling synthesis and degradation. Cyclic GMP acts in the retina to maintain open cation channels in the plasma membrane of the rod outer segment. The hydrolysis of cGMP results in closure of these channels, hyperpolarization of the rod cell, and a message of photoexcitation to surrounding cells (for a review see Stryer, 1991).

If rod cells and melanophores share aspects of a common transduction mechanism, one would expect opsin, cGMP and calcium to be involved in the control of DPR. Although non-functional opsins have been demonstrated in the pineal of the mouse (Kramm et al., 1993), the demonstrated blue opsin-like action spectrum of A. carolinensis skin, in conjunction with the recent cloning and sequencing of an A. carolinensis opsin with a maximal sensitivity of 420 nm (Kawamura and Yokoyama, 1996) argues strongly for an opsin type photoreceptor in the skin of this lizard.

Furthermore, the photoreceptor is apparently linked to intracellular changes in cGMP. By mimicking the effect of increasing cGMP levels with cGMP analogs, we have shown inhibition of DPR in Anolis carolinensis without a significant effect on the hormonal response. 8-Br-cGMP is a highly potent agonist for two major cGMP-binding proteins, the cGMP-gated channel of the vertebrate rod cell (Zimmerman et al., 1985; Matesic and Liebman, 1987) and the cGMP-

dependent protein kinase (Lincoln and Corbin, 1983), whereas it is an antagonist of cGMP phosphodiesterase (Zimmerman et al., 1985). The DB-cGMP derivative is ineffective as an agonist of the cGMP-gated channel (Mastic and Liebman, 1987) and the G-kinase (Dr. Tom Lincoln, personal communication). Our demonstration of the effectiveness of 8-Br-cGMP and the ineffectiveness of DB-cGMP on DPR inhibition is consistent with a functional role of cGMP in DPR.

There are a number of reasons why we may have been unable to assay cGMP changes in lizard skin. First, only a small amount (less than 10%) of cells in whole-skin samples assayed are light-sensitive melanophores. Since intracellular cGMP levels are typically 1-2 logs lower than cAMP levels, it is difficult to detect cGMP changes against a high background. Also, cGMP changes may be pulsatile. cGMP increases in UV-stimulated smooth muscle within a few seconds and falls to baseline within a minute. Our measurements at maximal physiological response (5 minutes) may thus have occurred too late to detect any differences (Dr. Tom Lincoln, personal communication). Finally, Lincoln and Keely (1981) have demonstrated an increase in cGMP in cardiac tissue when it is stimulated with either acetylcholine or nitroprusside. However, only in acetylcholine-stimulated tissue could an increase in protein kinase G activity be observed. They postulate the presence of cGMP pools within tissues, only some of which are coupled

to the G kinase. A corollary to this idea is that there may be functional cGMP elevations too small to measure (Dr. Tom Lincoln, personal communication).

Changes in cGMP levels are typically linked either to changes in levels of nitric oxide or intracellular free calcium. Although we have been unable to demonstrate a nitric oxide intermediate in DPR, we have demonstrated that the calcium ionophore a23187 darkening effect on skin is DPR-mimetic, rather than MSH-mimetic. Although we have not determined the exact relationship between cGMP and calcium in DPR, it seems likely that the utilization of cGMP/calcium but not cAMP can explain, at least in part, the compartmentalization of DPR previously observed (Vaughan, 1987).

The MSH response is thought to be mediated by cAMP (Sawyer et al., 1983), and our results support this claim. Abe et al. (1969) reported a cAMP increase in response to MSH in the skin of the frog, Rana pipiens. Several investigators have shown that the application of exogenous cAMP or its lipophilic derivatives causes skin darkening in the frog and lizard (Bitensky and Burstein, 1965; Hadley and Goldman, 1969).

In direct contrast to our findings, several investigators have reported that DPR in other species may be cAMP-dependent. In only one case (embryonic Xenopus laevis melanophores in culture) has the appropriate assay been

performed (Daniolos et al., 1990). All other studies (Wakamatsu et al., 1980; Negishi, 1985; Rollag, 1993) rely upon observing the effects of pharmacologically altered cAMP levels on DPR. We believe that any conclusions drawn from these studies are somewhat tenuous since it is impossible to know whether the investigator was actually perturbing the DPR or the MSH response.

That two separate signal transduction pathways in the melanophore may cause a similar physiological effect is not without precedent. Abrao et al. (1991) report that protein kinase C mediates melanocyte-concentrating hormone (MCH) melanosome aggregation in the teleost Synbranchus marmoratus whereas melanosome aggregation induced by norepinephrine is most likely a result of a reduction in intracellular cAMP levels (Negishi et al., 1982).

Cyclic-GMP has long been suspected to be involved in melanophore regulation. cGMP has been detected in the skin of the bullfrog (Ishikawa, 1969). Ochoa (1928) observed that guanidine compounds have a paling effect on the melanophores of R. temporaria. Abramowitz and Chavin (1974) observed that cGMP produces reversible melanosome dispersion in the teleost, Carassius auratus. DB-cGMP has a paling action on frog skin, but is unable to reverse or inhibit the MSH darkening response (Novales, 1971). It is, however, able to reverse the darkening effect of DB-cAMP and theophylline (Novales and Novales, 1979). Dibutyryl-cGMP is more

effective than dibutyryl-cAMP in producing pigment dispersion in the melanophores of the teleost, Oryzias latipes. Furthermore, dispersed melanosomes, when challenged to aggregate with epinephrine, are unable to do so in the presence of dibutyryl-cGMP but not dibutyryl-cAMP (Negishi, 1985). Finally, Negishi and Obika (1980) report that O. latipes melanophores undergo a partial pigment dispersion when strongly illuminated in the presence of guanine nucleotides. Other extra-ocular light-sensitive cells apparently utilize the cGMP pathway as well. Light induces a decrease in cGMP in molluscan extra-ocular photoreceptors (Nishi and Gotow, 1989) and in dissociated chick pinealocytes (Dryer and Henderson, 1991).

In summary, although increased levels of cAMP accompany pigment dispersion in response to MSH, cyclic-GMP and calcium, instead, are implicated in mediation of the DPR after their manner of involvement in retinal phototransduction. Considering this and the similar origin of both retinal rod cells and melanophores, it is reasonable to propose that some degree of homology exists in the mechanism of phototransduction in the two systems.

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Part IV

The Role of Calcium in the Dermal Photic Response
of the Lizard, Anolis Carolinensis

ABSTRACT

We have previously demonstrated that calcium is a necessary mediator of the dermal photic response (DPR) of the lizard, Anolis carolinensis (part III). In this work, we further examine the role of calcium with experiments utilizing calcium-substituted saline and the application of various calcium channel blockers. We suggest that a primary light-sensitive intracellular calcium pool exists in the melanophore. Secondary extracellular pools apparently replenish this primary source.

As identified by calcium precipitation and x-ray microanalysis, the primary calcium pool is apparently located within the melanosome. The melanosome is derived from smooth endoplasmic reticulum, and is a putative source of calcium in other systems. Previous studies (part III) suggest that it may be a location of the photoreceptive opsin pigment as well.

Application of smooth endoplasmic reticulum calcium ATPase (SERCA) blocking agents cause light-independent skin darkening and inhibits the dark recovery of skins challenged with light. Furthermore, the light-independent skin darkening effect of 2 out of 3 of these agents is inhibited by 8-bromo-cGMP.

The mediator of calcium release remains unknown. Pharmacological and immunological evidence presented argues against the utilization of inositol triphosphate. The

inhibitory effect of 8-Br-cGMP on DPR (part III) and on the light-independent skin darkening induced by SERCA inhibiting agents makes cyclic 3'-5' guanosine monophosphate (cGMP) a likely candidate. Two models of the mechanism by which cGMP could modulate cytosolic free calcium concentration are presented.

Finally, the MSH response does not appear to require elevated levels of intracellular calcium. In the presence of extracellular lanthanum, a potent inhibitor of calcium flux, multiple DPRs undergo successively greater inhibition until skins are completely insensitive to light. These same skins darken normally when tested with MSH. The previously described role of calcium in transducing the MSH-signal is apparently related to membrane stabilization, or some other extracellular requirement.

INTRODUCTION

Intact skin of the lizard, Anolis carolinensis changes color from green to brown in the presence of a variety of stimuli including prostaglandins (Lucas et al., 1985), beta-acting catacholamines (Hadley and Goldman, 1969; Vesely and Hadley, 1976), melanocyte-stimulating hormone (MSH) and visible light (Vaughan, 1987). Both the MSH and light-induced color change are effected by a redistribution of pigment within dermal melanophores (Taylor and Hadley, 1970; Beam and Vaughan, 1995a). The light activated color change, or dermal photic response (DPR), appears to involve the same mechanism of pigment movement as the MSH response but is smaller in magnitude and appears to be functionally compartmentalized (Vaughan, 1987), perhaps due to a different signal transduction mechanism (part III). Previously, we have reported that the cAMP changes accompanying the MSH response do not accompany DPR. Rather cGMP and/or calcium appear to be the mediator of DPR (Beam and Vaughan, 1995a; part III).

Ionic requirements of the hormone-mediated responses have previously been determined through ion substitution, concentration variation, and the application of various channel blockers (Novales, 1962; Vesely and Hadley, 1976; Vesely and Hadley, 1979) and an ionophore (Hadley et al., 1981; Lucas et al., 1983). Through these techniques, it has been determined that the only extracellular ion absolutely

necessary for MSH-induced darkening is calcium (Vesely and Hadley, 1971; Vesely and Hadley, 1976; Vesely and Hadley, 1979).

The role of calcium in the MSH response is not well understood. That extracellular calcium is not implicitly required for melanosome movement can be inferred from the ability of beta-acting catecholamines or exogenously applied cAMP derivatives to disperse melanosomes in its absence (Hadley and Goldman, 1969). Extracellular calcium may be necessary for hormone/receptor interaction (Lucas et al., 1987) and as a mediator for some transduction event proximal to adenylate cyclase activation (Vesely and Hadley, 1976; Sawyer et al., 1983). One study implies that an increase in cytosolic calcium may transduce the MSH response (Lucas et al., 1983).

However, our recent work (Beam and Vaughan, 1996; part III) has called this latter point into question for A. carolinensis. We have verified previous reports (Hadley et al., 1981; Lucas et al., 1983) that A. carolinensis skins darken in response to the calcium ionophore a23187. However, we have further demonstrated that ionophore-darkened skins are no longer light-responsive, whereas the MSH-sensitivity remains intact. We have also shown that, although cAMP increases in skins darkened with MSH, there is no apparent change in cAMP levels when skins are darkened with either

light or the calcium ionophore. In this work, we further discuss the role of calcium in the dermal photic response.

MATERIALS AND METHODS

Animals

The animals used in this study were male lizards of the species, Anolis carolinensis, recently captured from wild populations in coastal Louisiana and purchased from Snake Farm (LaPlace, LA). Individuals were maintained in group colony on a 12 hour light/dark cycle at 21° C. For each experiment an animal was decapitated, and the dorsal torso skin was removed and floated on physiological saline (in mM/L: 111 NaCl, 2.0 KCl, 1.0 CaCl₂, 2.0 NaHCO₃, 5.0 glucose at pH 7.2) in the dark for at least 45 minutes prior to use. Under these conditions, skin stabilizes to exhibit a tonic green color. Variation from this condition of maximal reflectance in the visible spectrum was measured reflectometrically as previously described (part III).

Pharmacological Agents

Alpha-melanocyte stimulating hormone (MSH), 8-bromoguanosine 3'-5'-cyclic monophosphate (8-Br-cGMP), 4-bromophenacyl bromide (4BPB), indomethacin, ionophore a23187, neomycin sulfate, nicardipine, nifedipine, thapsigargin (TG), and verapamil were purchased from Sigma Chemical Company (St. Louis, MO). ω -Conotoxin was purchased from Research Biochemicals Incorporated (Natick, MA). 2,5-di- τ -butylhydroquinone (BHQ) and cyclopiazonic acid (CPA) were purchased from Calbiochem (La Jolla, CA). All other reagents were of standard laboratory grade.

Stock reagents were either dissolved in saline or DMSO (a23187, BHQ, 4BPB, CPA, indomethacin, nicardipine, nifedipine, TG, verapamil) and stored frozen. DMSO concentrations were maintained at 1.0% in all experiments requiring the solvent. DMSO at this concentration has no effect on light or MSH responses. Student's T-test, or where appropriate, paired t-test, was used to determine significance ($p < 0.05$).

For attempts to immunolocalize the inositol triphosphate receptor (IP₃R), a polyclonal antibody directed against Xenopus IP₃R (Parys et al., 1992) was provided by Dr. Kevin P. Campbell (Howard Hughes Medical Institute, Iowa City, IA). For attempts to localize a retinal rod cGMP-gated channel analog, monoclonal antibody 1D1 directed against bovine retinal rod cGMP-gated channel (Molday et al., 1990) was provided by Dr. Robert Molday (University of British Columbia, Vancouver, Canada).

Calcium Localization

Putative intracellular calcium stores were observed by transmission electron microscopy (TEM) of calcium precipitated skin sections. Control skins and skins depleted of intracellular calcium by equilibration in multiple changes of calcium-free saline containing 1 mM EGTA for at least 24 hours (until no light response could be elicited)

were subjected to calcium precipitation based upon the method of Griffiths et al. (1985).

Briefly, control and Ca^{2+} -depleted tissues from lizards (n=5 experiments) were fixed overnight at 4° C in 3% EM-grade glutaraldehyde (Electron Microscopy Services) in the presence of 90 mM potassium oxalate, pH 7.4 (adjusted with KOH). Skins were then briefly rinsed in cold 7.5% sucrose in 90 mM potassium oxalate (pH 7.4), postfixed 2 hours at 4 °C in 1% osmium tetroxide, 2% potassium pyroantimonate (pH 7.4), dehydrated through a graded ethanol series, and embedded in Spurr's resin (Electron Microscopy Services). Using a diamond knife, thin (100 nm) sections were cut on a Riechert model OMU-3 ultramicrotome, collected on copper grids, and viewed without further staining at 100 Kv on an Hitachi H-800 electron microscope. For x-ray microanalysis, the x-ray spectra of precipitants were collected using a Si(Li) detector (Link) interfaced with the microscope. Antimony and calcium peaks were separated by "peak stripping" a potassium pyroantimonate standard.

Immunolocalization

Several attempts were made to immunolocalize the IP_3R and cGMP-gated channel in lizard skin. Using conventional protocols, we were unable to identify IP_3R or the cGMP-gated channel via immunofluoresence, immunogold electron

microscopy, or western blotting with an enzyme-linked secondary antibody.

RESULTS

Variation of Extracellular Calcium Concentration

Extracellular calcium levels affect albedo. In the absence of extracellular calcium, skins become lighter, whereas when extracellular calcium is increased from 1 mM to 10 mM, skins darken significantly (table 4.1). Furthermore, increasing extracellular calcium 10-fold enhances DPR $44.6 \pm 5.1\%$ ($n=9$), whereas a 0 mM calcium (1 mM EGTA or EDTA) saline is inhibitory (table 4.2). Finally, successive photostimulations in the absence of extracellular calcium produce smaller and smaller DPRs (figure 4.1) as would be expected if intracellular calcium sources were being utilized, but not being replenished.

Divalent Ion Substitution for Calcium

By substituting the chloride salt of various divalent ions for calcium we determined that Ba^{2+} , Mg^{2+} and Sr^{2+} can all substitute for extracellular Ca^{2+} with no inhibition to initial DPR (table 4.3). However, successive DPRs diminish in size exactly as in the presence of EDTA or EGTA. Furthermore, Co^{2+} and Ni^{2+} were inhibitory to the initial DPR with increasing inhibition to successive DPRs. Other divalent ions tested (Mn^{2+} , Cd^{2+} , Hg^{2+}) were apparently melanotoxic as previously reported (Vesely and Hadley, 1979).

Table 4.1.
Effect of Extracellular Calcium on Skin Albedo

Treatment (10 Minutes)	% Change in Albedo ($\pm$ SEM, n=5)
0 mM Ca^{2+} (1 mM EDTA)	$18.2 \pm 6.9^*$
0 mM Ca^{2+} (1 mM EGTA)	$18.5 \pm 10.5^*$
5 mM Ca^{2+}	0.0 ± 3.9
10 mM Ca^{2+}	$-20.2 \pm 2.1^*$

* $p < .05$, 1-tailed test

Table 4.2.
Effect of Extracellular Calcium Concentration on DPR

Treatment	Percent of Control DPR ($\pm$ SEM, n=9)
0 mM Ca^{2+} (1 mM EDTA)	$-57.7 \pm 4.3^*$
0 mM Ca^{2+} (1 mM EGTA)	$-33.0 \pm 6.2^*$
5 mM Ca^{2+}	-1.7 ± 7.5
10 mM Ca^{2+}	$44.6 \pm 5.1^*$

* $p < .05$, 1-tailed test

Table 4.3
Effect of Extracellular Calcium Ion Substitution on DPR

Treatment (1 mM)	% of Control DPR ($\pm$ SEM, n=5)
Ba^{2+}	89.4 ± 9.2
Co^{2+}	$61.2 \pm 7.9^*$
La^{3+}	$45.0 \pm 10.2^*$
Mg^{2+}	96.0 ± 2.8
Ni^{2+}	$80.8 \pm 5.8^*$
Sr^{2+}	90.7 ± 9.1

* $p < .05$, 1-tailed test

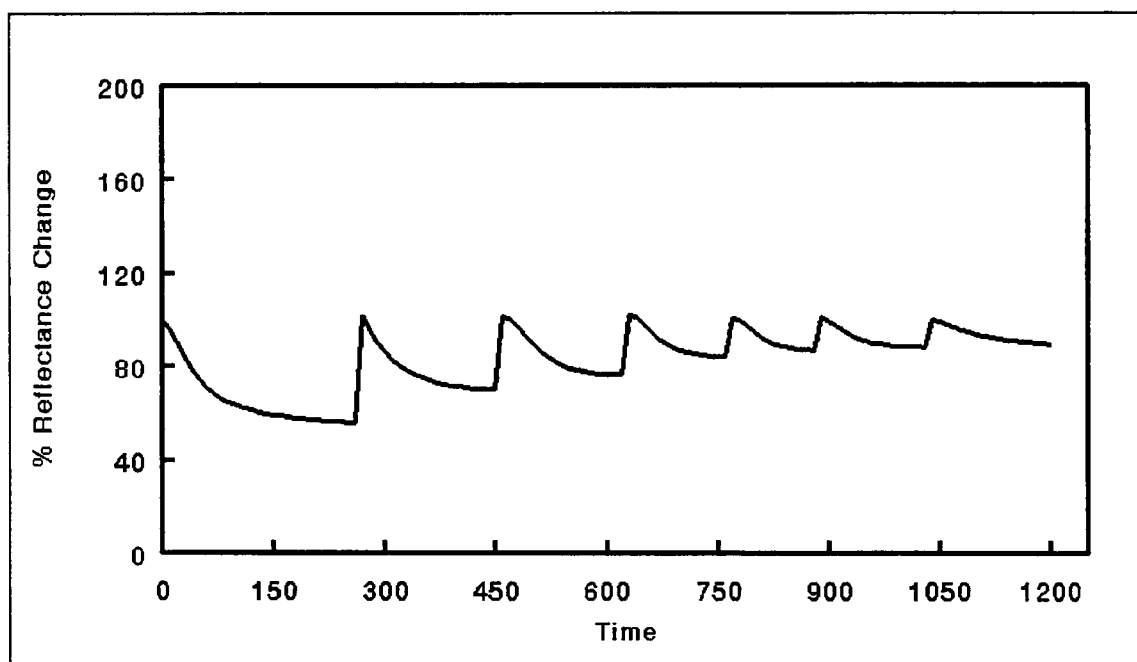


Figure 4.1: Representative Tracing of the Effect of 0 mM Ca^{2+} Saline on DPR. Skins were initially challenged to respond to light in normal saline (control). The saline was replaced with 1 mM EGTA saline without calcium, and the skin challenged repeatedly to respond to light.

Calcium Channel Blockade

In the presence of 1 mM Ca^{2+} , the calcium channel blocker diltiazem (100 μM) inhibits DPR by $31.4 \pm 5.7\%$ ($n=5$) without inhibition of recovery. However, unlike ion substitution, successive DPRs are not inhibited to any greater extent than the initial DPR. Other L-type Ca^{2+} channel blockers (verapamil, nifedipine, nifedipine) were without effect (all at 100 μM) on DPR (table 4.3), whereas higher doses of verapamil (1 mM) were apparently melanotoxic. The L-type and N-type calcium channel blocker, ω -Conotoxin (McCleskey et al., 1987), also was without effect on DPR (table 4.4).

Lanthanum chloride in a buffer-free calcium-containing saline was used as a general inhibitor of calcium flux (Weiss, 1974). The effect of the lanthanum ion was to inhibit successive DPRs in a manner similar to the calcium-free experiments previously discussed. Also, the La^{3+} ion apparently does not interfere with (or perhaps substitutes for) the calcium necessary for the MSH response. Therefore, we were able to test responsiveness of lanthanum-treated skins to MSH. Skins apparently depleted of intracellular calcium via multiple DPRs in $\text{La}^{3+}/\text{Ca}^{2+}$ saline still respond normally to MSH (figure 4.2, table 4.5), even in the presence of high (10 mM) extracellular La^{3+} concentrations.

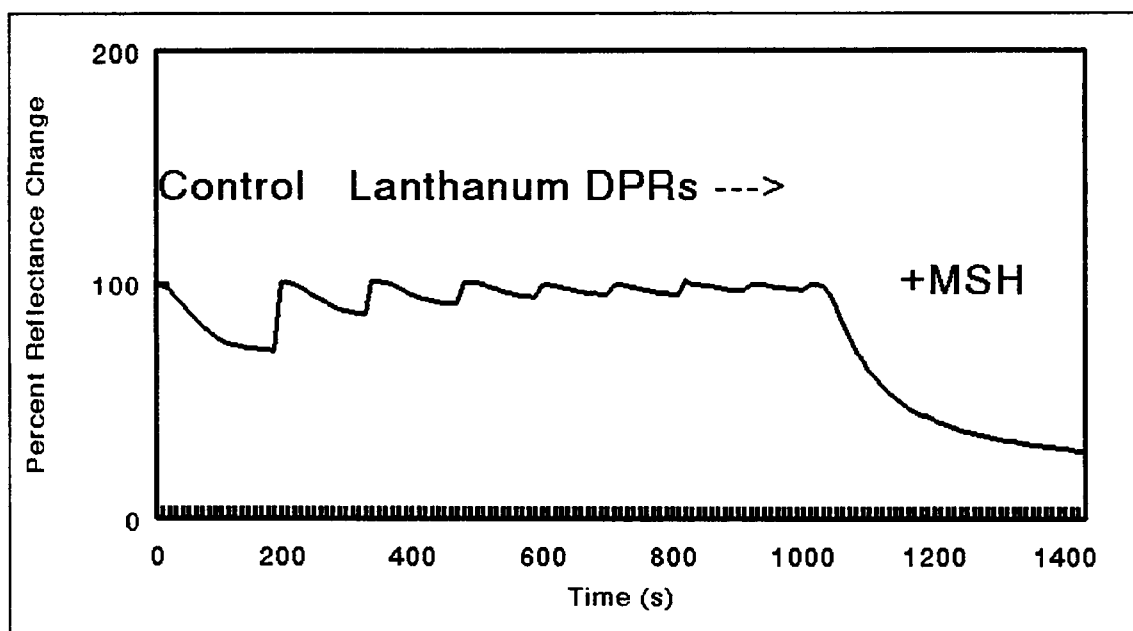


Figure 4.2: Representative Tracing of the Effect of Lanthanum Chloride on DPR and MSH Responses. In the presence of 10 mM lanthanum chloride, successive DPRs diminish in size until skins are completely unresponsive to light. These same skins, when challenged with MSH (10^{-7} M), respond normally.

Table 4.4
Effect of Calcium Channel Blockers on DPR

Treatment	Dose	% of Control DPR ($\pm$ SEM, n=5)
ω -Conotoxin	10 μ M	109.5 $\pm$ 8.5
Diltiazem	100 μ M	68.6 $\pm$ 5.7*
Nicardipine	100 μ M	125.7 $\pm$ 26.7
Nifedipine	100 μ M	122.2 $\pm$ 12.3
Verapamil	100 μ M	90.1 $\pm$ 5.1

*p<.05, 1-tailed test

Table 4.5
Inhibitory Effect of Lanthanum Chloride + Multiple DPRs on
Light and MSH Responses

Treatment	%Inhibition of Response ($\pm$ SEM, n=5)
Light (5th Light Response)	98.5 $\pm$ 1.9*
MSH	1.8 $\pm$ 3.6

*p<.05, 1-tailed test

Depletion and Refilling of Intracellular Calcium Stores

Skins could be depleted of intracellular calcium stores via multiple photic responses in EGTA-saline as previously described, or by equilibrating skins in multiple changes of EGTA-saline for greater than 24 hours. By equilibrating Ca^{2+} -depleted skins in multiple changes of Ca^{2+} -containing saline, we were then able to demonstrate a partial refilling of calcium stores. Skins ($n=3$) totally unresponsive to light begin regaining responsiveness after 20 minutes in Ca^{2+} -saline. Furthermore, these skins regain over half-maximal responsiveness within 2 hours of Ca^{2+} treatment (figure 4.3).

Depletion of Intracellular Calcium Depletes Melanosomal Calcium Stores

Control tissues treated for EM-calcium localization had dense calcium/antimonate precipitants in the cytoplasm and within melanosomes (plate 4.1A). These precipitants were completely absent in the light-insensitive EGTA-pretreated tissues (plate 4.1B). The content of control precipitants was primarily antimony and calcium as revealed by x-ray microanalysis. The calcium peak was easily visible after "peak stripping" a potassium antimonate standard (figure 4.4). As further proof of their calcium content, these

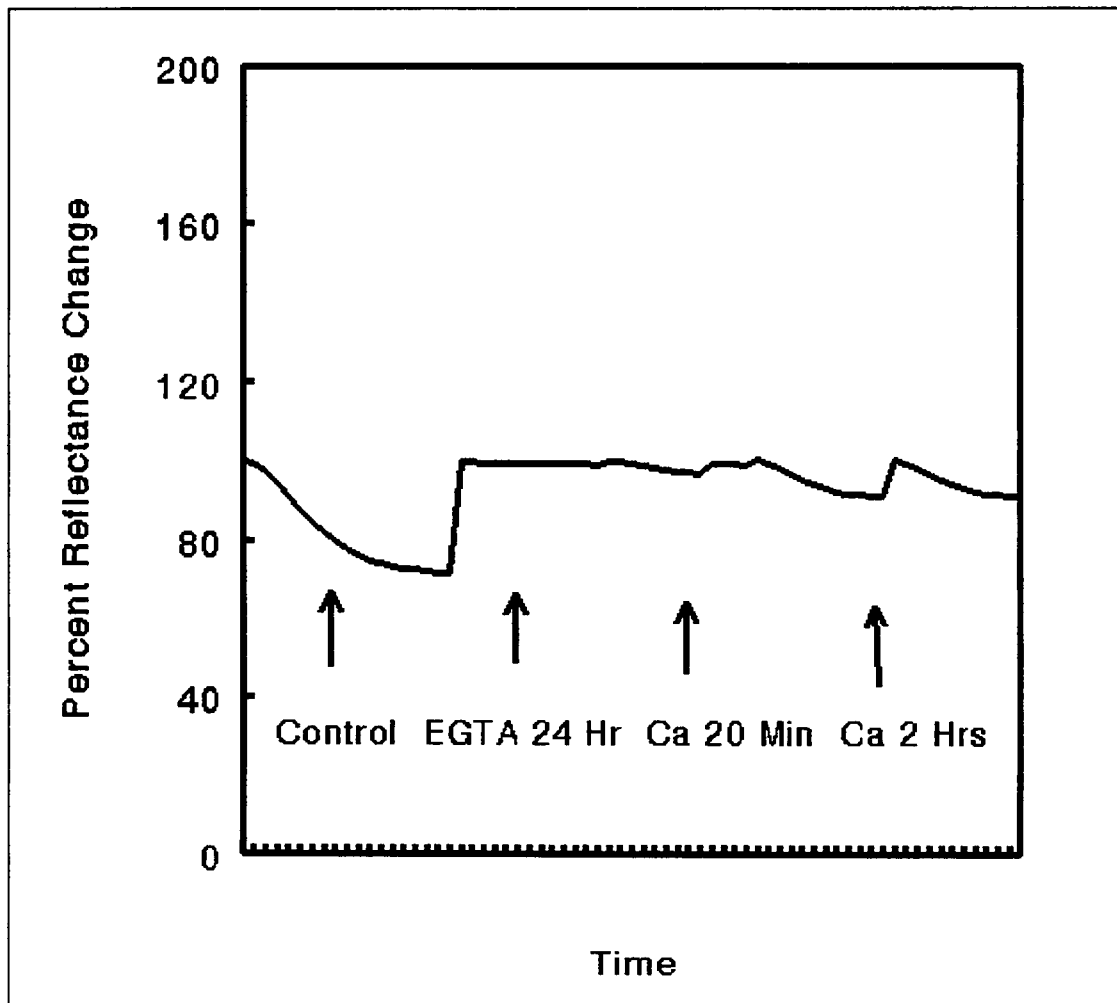


Figure 4.3: Effect of EGTA Depletion and Refilling of Calcium Stores on DPR. Skins were tested for DPR (control), then equilibrated in multiple changes of EGTA-saline for 24 hours. These skins no longer respond to light, but can regain their light sensitivity after equilibration in Ca^{2+} -containing saline.

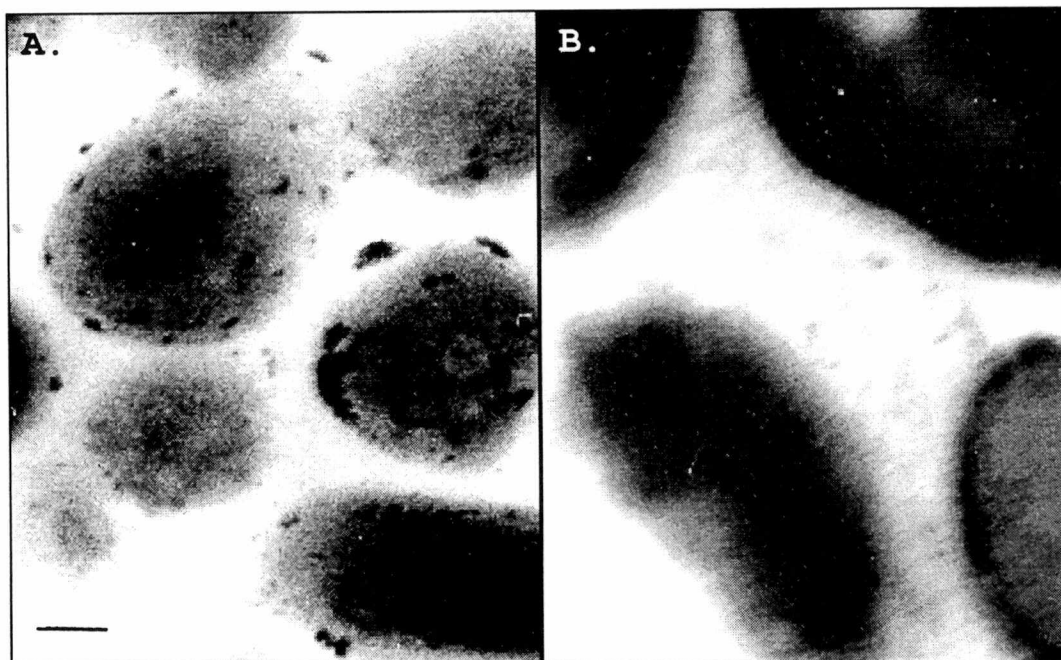


Plate 4.1: Localization of Calcium Antimonate Precipitants. Calcium antimonate precipitants are localized (A) in melanosomes of control tissues. EGTA-depleted light-insensitive tissues (B) show no calcium precipitant within melanosomes (Bar = .05 μ).

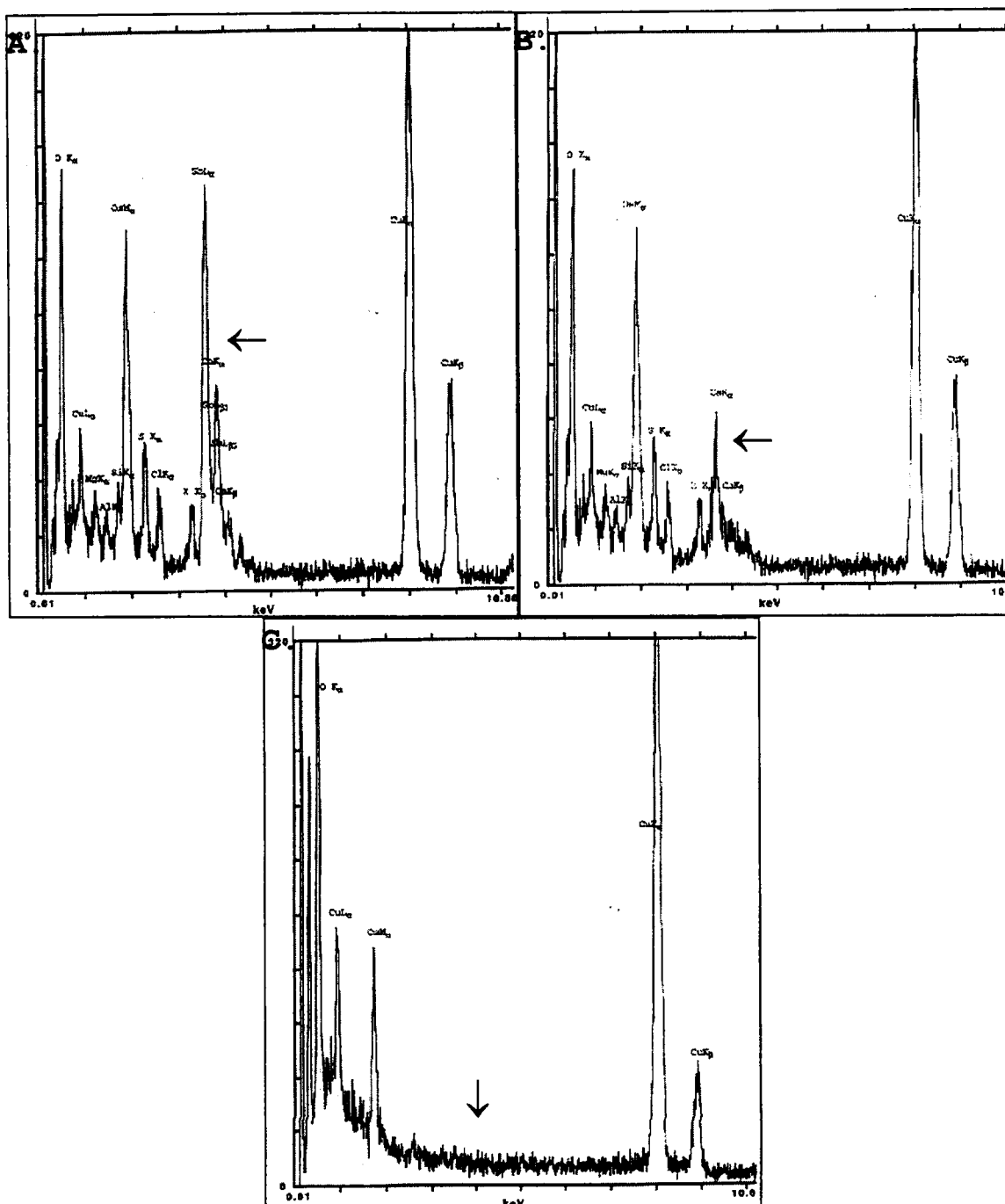


Figure 4.4: X-ray Microanalysis of Calcium Antimonate Precipitants. (A) arrow at calcium/antimonate peak (B) arrow at calcium peak after subtracting an antimonate standard (C) arrow at background area showing no calcium or antimonate peaks.

precipitants were completely soluble in EGTA (10 mM, 60° C, 1 hour), but not distilled water.

Effect of Phospholipase C Inhibitors and Indomethacin

A prominent agonist of intracellular calcium release is inositol triphosphate (IP₃), a cleavage product produced by the action of phospholipase C upon the phosphotidyl inositol membrane phospholipid (Berridge and Irvine, 1984). Several pharmacological agents were used to determine if the IP₃ pathway is involved in intracellular calcium release during DPR. Neomycin sulfate (10 mM) and 4-bromophenacyl bromide (100 µM) were used by other researchers to antagonize IP₃ formation in fish melanophores, due to their inhibitory effect on phospholipase C (Abrao et al., 1991). Lithium chloride (10 mM) was used as an antagonist of IP₃ turnover. Following a 30 minute incubation, DPR was only modestly inhibited by IP₃ antagonists, whereas the MSH response was completely unaffected. Furthermore, lithium chloride did not appear to affect either response (table 4.6).

Prostaglandins are also potential mediators of calcium release. Several prostaglandins have been shown to darken A. carolinensis skins (Lucas et al., 1985). Since it was shown in these studies that indomethacin, an inhibitor of prostaglandin synthesis, does not inhibit the MSH response, it seemed profitable to investigate its effect on DPR. We

report here (table 4.6) that indomethacin has no effect on DPR. Thus, the skin-darkening effects of prostaglandins appear unrelated to DPR or MSH pathways.

Effect of SERCA Ca-ATPase Inhibitors

Recently, several agents have been discovered (Thastrup et al., 1990; Wictome et al., 1992) that increase cytoplasmic calcium by inhibiting the smooth endoplasmic reticulum Ca^{2+} -sequestering ATPase (SERCA). These agents have proven quite useful for perturbing cytosolic calcium regulation. In order to examine the effect of inhibiting calcium sequestration, skins were treated with SERCA inhibitors cyclopiazonic acid (CPA), thapsigargin (TG), τ -butylhydroquinone (BHQ) or calcium ionophore a23187 in the presence and absence of 8-Br-cGMP (1 mM). Reflectance at 600 nm was monitored during the 60 minute incubation.

Following the incubation, skins were challenged to respond to light stimulation. All agents darkened skins independently of light. Moreover, the light-independent darkening effect of 2 of the 3 (BHQ and TG) SERCA inhibitors could be inhibited by 8-Br-cGMP, whereas the darkening effect of a23187 was unaffected (table 4.7). Finally, skins darkened with any SERCA inhibitor would darken further when stimulated with light, but successive DPRs diminished in magnitude as recovery was apparently inhibited (figure 4.5).

Table 4.6
Effect of IP₃ Agents and Indomethacin on DPR and MSH
Responses

Agent	Dose	%of Control DPR	%of Control MSH (10 ⁻⁷ M) (±SEM, n=5)
4BPB	.1 mM	70.7 ± 6.2*	105.0 ± 2.9
Neomycin Sulfate	10 mM	73.2 ± 7.2*	95.3 ± 4.4
Lithium Chloride	10 mM	101.5 ± 7.2	101.1 ± 3.2
Indomethacin	.1 mM	85.2 ± 4.9	no effect (Lucas et al., 1985)

*p<.05, 1-tailed test

Table 4.7
Effect of SERCA Inhibitors or A23187 on Albedo

Drug	Dose	Effect on Albedo** (±SEM, n=5)
2,5-di-t-Butylhydroquinone	10 µM	-38.6 ± 10.0
+8-Br-cGMP	1 mM	-9.2 ± 4.8*
Cyclopiazonic Acid	10 µM	-30.0 ± 8.1
+8-Br-cGMP	1 mM	-19.0 ± 1.7
Thapsigargin	10 µM	-45.6 ± 5.7
+8-Br-cGMP	1 mM	-9.6 ± 8.2*
Ionophore a23187	100 µM	-32.0 ± 7.1
+8-Br-cGMP	1 mM	-34.8 ± 10.4

*p<.05, 1-tailed test

**negative numbers denote decreased albedo

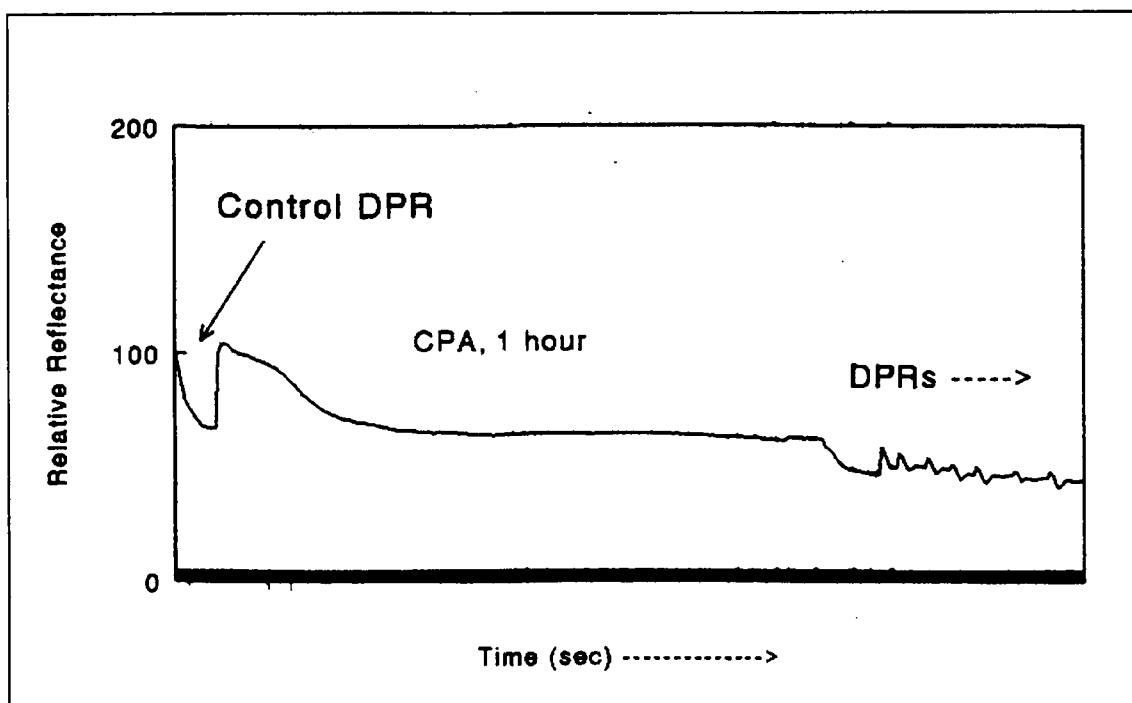


Figure 4.5: Effect of CPA on DPR. DPR diminishes in size as skin fails to completely recover. Similar effects are seen with thapsigargin and τ -butylhydroquinone (n=5 skins per treatment).

DISCUSSION

The results presented strongly suggest that intracellular calcium stores are utilized for DPR. That a single normal uninhibited DPR can occur after the transfer of skins to calcium-free saline containing Ba^{2+} , Mg^{2+} , or Sr^{2+} suggests that either extracellular calcium is not required for DPR, or that the necessary extracellular calcium is present in interstitial pools. Ba^{2+} and Sr^{2+} are generally regarded as permeable to Ca^{2+} channels, whereas Mg^{2+} is impermeable but does not function in Ca^{2+} -channel blockade because the ion has little affinity for the calcium channel (for a review, see Hess, 1990). The successively greater inhibition of multiple DPRs without an inhibition in skin recovery suggests that intracellular calcium stores are being depleted, perhaps due to the transport or leak of cytosolic calcium across the plasma membrane during skin recovery. Extracellular calcium would then presumably be used to replenish intracellular calcium stores. Competing permeable divalent ions and/or diminished extracellular calcium might prevent these stores from refilling.

The initial inhibition caused by calcium-free saline containing EDTA, EGTA, Co^{2+} , or Ni^{2+} is possibly due to the complete depletion of interstitial calcium pools by EDTA or EGTA, or the blockade of calcium channels by Co^{2+} or Ni^{2+} . It may also be possible that a depolarizing calcium current

triggers the release of calcium from intracellular stores. In this case the Ba^{2+} or Sr^{2+} current might elicit this same response. Mg^{2+} might not interfere with a current mediated by interstitial calcium, whereas the blocking effect of Co^{2+} or Ni^{2+} , or the presence of divalent chelators, would inhibit the response.

The type of plasma membrane calcium channel utilized is not distinguishable with our data. Pharmacological blockers normally associated with the L-type and N-type channels are without effect, even at the very high concentrations tested (100 μM). The only exception is diltiazem which inhibits DPR at very high concentrations (100 μM), but does not cause successively greater inhibition to subsequent DPRs as would be expected. Since other L-type channel-blocking agents are without effect, the effect of diltiazem may be unrelated to calcium blockade. Pharmacological inhibitors of the T-type channel were not tested, leaving this channel type a possibility by default.

Because of its size and trivalent charge, the lanthanum ion is a potent inhibitor of calcium flux (Weiss, 1974). Since skins depleted of intracellular calcium via multiple DPRs in the presence of lanthanum still respond normally to MSH, we conclude that the MSH response does not utilize a calcium current, or perhaps does not utilize a calcium

current which is lanthanum-sensitive. Furthermore, although we are unable to rule out the utilization of intracellular calcium stores for the MSH response, it is clear that any stores used are independent of the light-sensitive stores.

Previous reports have suggested that the lanthanum ion is melanotoxic at doses greater than 40 μ M (Lucas et al., 1987). On rare occasions, we have noticed skin darkening in response to lanthanum-saline. These skins were omitted from further consideration in our studies. However, in general, we have noticed no toxic effects at doses as high as 10 mM.

Other investigators have utilized the lanthanum ion at a concentration of 1 mM without any toxic effect (Vesely and Hadley, 1979). These investigators state that lanthanum pretreatment was inhibitory to the MSH response of A. carolinensis skins, but no data or methodology were documented for comparison. At present we are unable to explain this contradiction.

Previous investigators have reported that the calcium ion plays a bifunctional role in the MSH-mediated response, possibly necessary for the hormone/receptor interaction and necessary as a transducer of the MSH activity itself (Lucas et al., 1987). The calcium-requiring transduction event is believed to occur prior to activation of adenylate cyclase

(Hadley et al., 1981), a plasma membrane bound protein (Ross and Gilman, 1980).

Since Ba^{2+} , Sr^{2+} , and to some extent Be^{2+} can substitute for Ca^{2+} to permit MSH-mediated melanosome dispersion (Vesley and Hadley, 1979), and since these ions are not generally substitutable for calcium-mediated signal transduction mechanisms (Hille, 1992), we believe the reported calcium-requiring transduction event may be related to the membrane stabilizing properties of calcium.

Such an interpretation is in complete agreement with our demonstration of an intact MSH response in the presence of extracellular lanthanum/calcium, and agrees with studies demonstrating that the superagonistic MSH analogue, Nle^4 -D-Phe⁷- α -MSH, does not require calcium for receptor binding, but still requires calcium for pigment translocation (Hadley et al., 1981b; Lucas et al., 1987). We believe that previous reports (Hadley et al., 1981; Lucas et al., 1983) which assume that the calcium-dependent skin-darkening effect of ionophore a23187 is MSH-mimetic should now be reinterpreted as DPR-mimetic in light of this and previous (Beam and Vaughan, 1996; part III) studies.

The similar effects of the Ca-ATPase inhibitors, cyclopiazonic acid, τ -butylhydroquinone, and thapsigargin, on tonic skin color and DPR suggest that the calcium

released from intracellular stores during DPR is apparently sequestered within smooth endoplasmic reticulum or a derivative during recovery. That skins darken in the presence of these drugs without photostimulation, and that light-stimulated skins do not fully recover in the presence of these drugs suggests a central role for calcium release and sequestration during DPR and recovery.

It is tempting to speculate that the calcium released during DPR is stored within melanosomes. Similarly, the light-induced reorganization of pigment granules within the giant neurons of Aplysia is thought to be related to calcium release from the granules (Brown et al., 1974). Also, Muller and Bereiter-Hahn (1991) have identified calcium pools within dermal melanosomes of the frog (Xenopus laevis) and the fish (Poecilia reticulata) which could be involved in pigment translocation processes.

A Ca^{2+} -binding phosphoprotein similar to the endoplasmic reticulum protein, calnexin, has been found on melanosomes from normal and malignant human melanocytes (Dakour et al., 1993). This protein has multiple phosphorylation sites and is hypothesized to play a role in melanogenesis, maintenance of calcium levels within the melanosome, and possibly organelle translocation (Vinayagamoorthy et al., 1993).

Our studies have confirmed that an EGTA-depletable calcium pool exists within the dermal melanosomes of A. carolinensis. The limiting membrane of melanosomes is thought to be derived from the endoplasmic reticulum (Turner et al., 1975). Also, we have previously reported (Beam and Vaughan, 1994; part III) the presence of an opsin-like protein which is apparently localized on the plasmalemma, and possibly on the melanosome (Beam and Vaughan, 1995). This close geometric organization of the photoreceptor with the calcium pool may provide the previously identified (Vaughan, 1987) compartmentalization of the DPR.

Alternatively, melanin is known to bind to cations with high affinity (Potts and Au, 1976). Melanin of the eye (Panessa and Zadunaisky, 1981), ear (Gottesberge, 1988), and skin (Muller and Bereiter-Hahn, 1991) has been hypothesized to serve as a cation sink, chelating extra calcium as well as other toxic elements. In support of this, we have also detected aluminum, magnesium, potassium and sulfur within lizard melanosomes.

It should be noted, however, that the antimonate precipitable calcium pool appears distinct from the organic melanosomal calcium pool. Calcium and other metals present within the melanosome result in electron opacity in unstained sections, even when tissues were previously depleted by EGTA (plate 4.1B). We believe this opacity is

due to biologically inactive melanometal chelates which are not antimonate precipitable. Conversely, control tissues appear to have, in addition to the melanometal pool, a distinct, EGTA-depletable, antimonate-precipitable, calcium pool which may be biologically accessible.

The agent responsible for releasing calcium from intracellular stores is presently unknown. Logical candidates include prostaglandins, inositol triphosphate or cGMP. That indomethacin, an inhibitor of prostaglandin synthesis, does not inhibit DPR (table 4.6) argues against a prostaglandin intermediate.

Since phospholipase C inhibitors were able to partially inhibit the light response, we are unable to rule out this pathway. However, the amount of inhibition we achieved is minimal in comparison to that of Abrao and colleagues (1991) who demonstrated greater than 70% inhibition of the effect of melanocyte-concentrating hormone in the teleost, Synbranchus marmoratus, at doses of neomycin sulfate which only inhibited DPR 26% in A. carolinensis. Furthermore, our inability to identify an IP₃ receptor via immunolocalization techniques argues against the use of an IP₃ intermediate. It should be noted, however, that the extremely sensitive radiolabeled secondary antibody techniques employed in the identification of a cone photoreceptor (part III) were not utilized in these studies.

An interesting hypothesis is that cGMP is the intermediate controlling calcium release. Since 8-Br-cGMP inhibits both the light-independent skin-darkening effect of Ca-ATPase inhibitors and DPR, we suggest that high levels of 8-Br-cGMP may somehow antagonize calcium release, stimulate calcium sequestration, or both.

The interaction between cGMP and calcium may be similar to that which occurs in the retinal rod cell, which may be evolutionarily related to DPR (Vaughan and Tallent, 1988; part III). Thus, in the absence of photic stimulation, high levels of cGMP may act to somehow block calcium flux. Photic stimulation may activate a transducin like G-protein which stimulates the enzyme, cGMP phosphodiesterase. Decreasing cGMP levels during photic stimulation may indirectly increase intracellular calcium (figure 4.6).

This model loses some attractiveness because the function of cGMP is exactly backwards of its action in the retinal rod photoreceptor, and, although cGMP is known to sometimes increase (Payne et al., 1986; Huppertz, 1995) and sometimes decrease (Nishi and Gotow, 1989; Gotow and Nishi, 1991) in invertebrate photosignal transduction, no known system utilizes a calcium channel inhibited by cGMP. In the ventral photoreceptors of the horseshoe crab, Limulus, intracellular calcium is released via the phosphatidyl inositol pathway (Payne et al., 1986) during a Na^+ -dependent

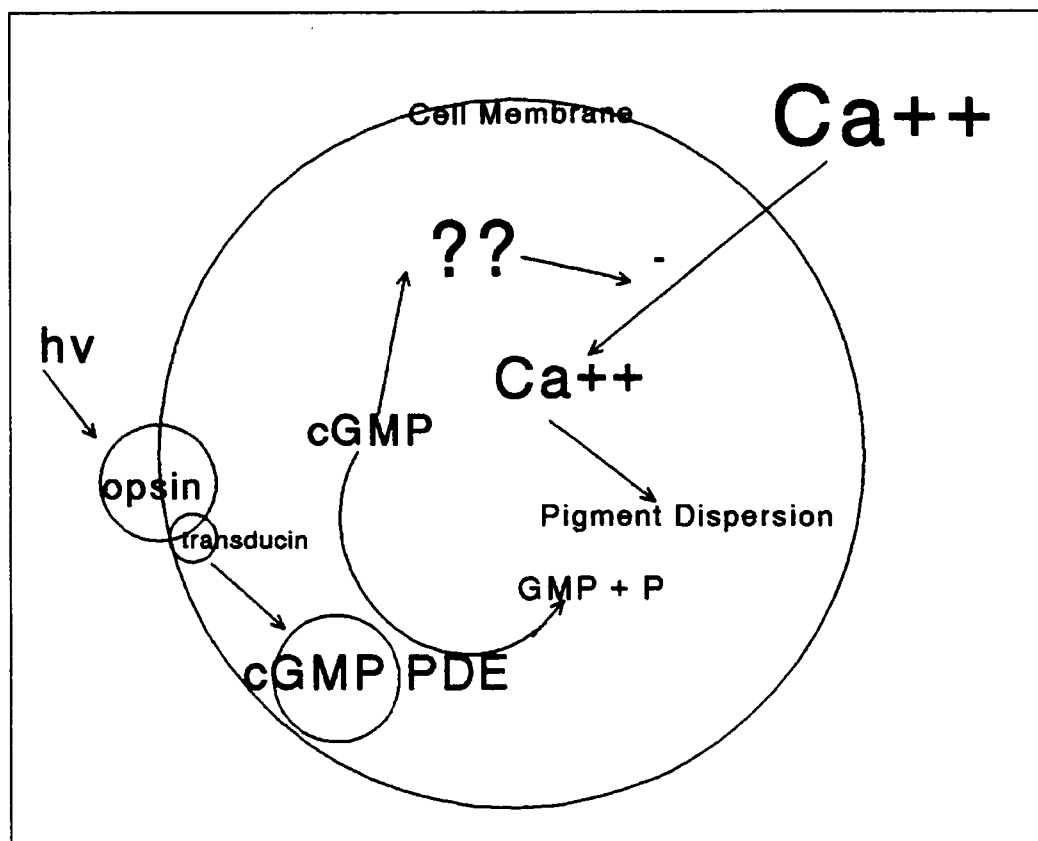


Figure 4.6: Hypothetical Model of cGMP Inhibition of DPR Based Upon the Vertebrate Photoreceptor Model. Light activates cGMP phosphodiesterase. Decreasing cGMP increases cytoplasmic calcium through a variety of mechanisms including release from intracellular and extracellular stores (see text).

depolarization (Bolsover and Brown, 1985) modulated by cGMP-gated channels (Johnson et al., 1986; Bacigalupo et al., 1991). Also arguing against this hypothesis is our inability to immunologically demonstrate the presence of a cGMP-gated channel. However, the monoclonal bovine retinal antibody utilized may be too specific to detect the skin variant.

Alternatively, the interaction between cGMP and calcium may be similar to the pathway utilized by the smooth muscle cell whose mechanism of contraction has been used as a model for melanosomal movement (Dikstein et al., 1963; Hadley et al., 1981). Although cGMP in DPR is apparently not linked to nitric oxide (part III) as in smooth muscle, the calcium released during DPR may stimulate a calcium-sensitive guanylate cyclase. Increased cGMP levels may activate the cGMP-dependent protein kinase which has been shown to decrease cytoplasmic free calcium by increasing calcium sequestration (Twort and Breemen, 1988) and efflux (Furukawa et al., 1991) within smooth muscle cells (figure 4.7). If this model is correct, the inability of 8-Br-cGMP to inhibit skin darkening induced by the calcium ionophore a23187 could be due to the tremendous increases in cytosolic calcium induced by this agent, and/or the short-circuiting of the plasma membrane as a barrier during cGMP-stimulated efflux.

The ultimate effect of increased calcium is presently unknown. Whether calcium directly stimulates a kinase or

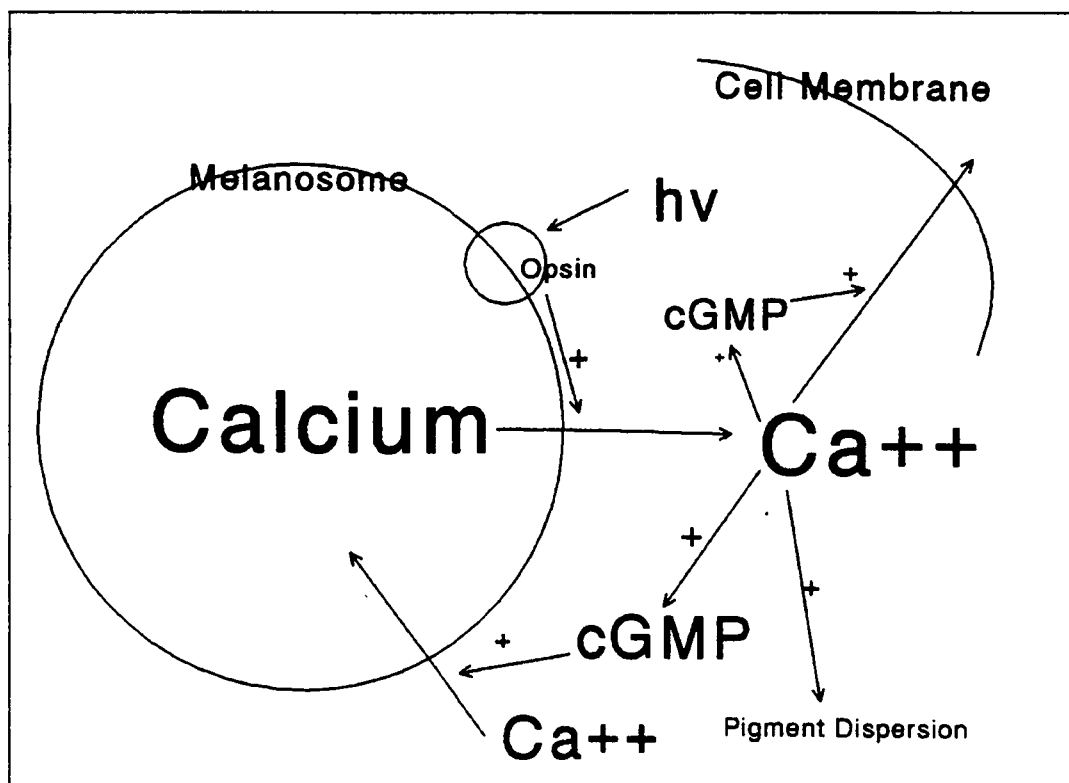


Figure 4.7: Hypothetical Model of cGMP Inhibition of DPR Based Upon the Smooth Muscle Cell Model. Cytoplasmic calcium increases cGMP via activation of guanylate cyclase. cGMP triggers calcium sequestration by melanosomal stores as well as extrusion via a plasma membrane pump.

phosphatase (Thaler and Haimo, 1990) which results in pigment movement, or whether the calcium activates an as yet unknown third messenger remains to be investigated.

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Part V.

Other Ionic Requirements
of the Dermal Photic Response
in the Lizard, Anolis Carolinensis

ABSTRACT

Previously, we have demonstrated a calcium ion requirement for the dermal photic response (DPR) in the lizard, Anolis carolinensis, although the requirement is not absolute, since a single uninhibited DPR will occur in its absence (part IV). The purpose of this work is to determine if any other extracellular ions are essential for DPR. By ion substitution, we have been unable to identify a requirement for the chloride or bicarbonate ion. We have demonstrated that sodium free saline is inhibitory to DPR, but interpret the requirement to be secondary, since DPR still occurs in its absence, albeit inhibited. The magnitude of DPR correlates with the presence and concentration of potassium, suggesting that a depolarization event may modulate DPR. Finally, DPR remains reasonably intact over a wide range of pH and saline osmolarity. We conclude that there is no extracellular ion absolutely required for DPR.

INTRODUCTION

Previously, ionic requirements of the hormone-mediated response have been determined via ion substitution, concentration variation, and the application of various channel blockers (Novales, 1962; Vesely and Hadley, 1979; Vesely, 1972) and an ionophore (Hadley et al., 1981; Lucas et al., 1983). Through these techniques, it has been determined that the only extracellular ion absolutely necessary for the MSH-induced darkening of A. carolinensis skin is calcium (Vesely and Hadley, 1979).

It has been suggested that calcium has a bifunctional role, necessary for MSH binding to its receptor (Lucas et al., 1987, and necessary for transducing the MSH signal (Hadley et al., 1981). The calcium requirement for transducing the MSH-response is proximal to cAMP production, and may be related to membrane stability (part IV).

Calcium is not implicitly required for melanosome movement as can be inferred from the ability of β -acting catecholamines or exogenously applied cAMP derivatives to disperse melanosomes in the absence of calcium (Hadley and Goldman, 1969). In fact, the observation that β -catecholamines or cAMP derivatives are more effective dispersing agents in Ca^{2+} -free saline has led to the suggestion that cytoplasmic calcium may decrease during pigment dispersion (Hadley et al., 1981).

In contrast to the hormone-mediated response, ionic requirements of the dermal photic response have not been previously elucidated. From our previous work (Beam and Vaughan, 1995, 1996; part III; part IV), it is apparent that DPR utilizes calcium. Skins darkened by equilibration in saline containing 100 μ M calcium ionophore a23187 for one hour no longer respond to light, whereas they respond normally to MSH. Furthermore, successive photostimulations in the absence of extracellular calcium (1 mM EGTA) result in increasingly diminished photic responses with complete recovery of each DPR. In the present paper, we further examine the ionic requirements of the dermal photic response and compare the deduced requirements with the published requirements for the MSH response.

MATERIALS AND METHODS

Animals

The animals used in this study were male lizards of the species, Anolis carolinensis, recently captured from wild populations in coastal Louisiana and purchased from Snake Farm (Laplace, LA). Males were maintained in group colony on a 12 hour light/dark cycle at 21° C. For each experiment an animal was decapitated and the dorsal torso skin was removed and floated on physiological saline (in mM/L: 111 NaCl, 2.0 KCl, 1.0 CaCl₂, 2.0 NaHCO₃, 5.0 glucose at pH 7.2) in the dark at least 45 minutes prior to use. Under these conditions skin stabilizes to exhibit a tonic green color. Variation from this condition of maximal reflectance in the visible spectrum was measured reflectometrically as previously described (part III).

Pharmacological Agents

All reagents were of standard laboratory grade. To determine the requirement for sodium, we substituted the chloride salts of cesium, choline, lithium, or rubidium for sodium chloride in otherwise normal saline. To examine the importance of potassium we examined the effect of varying potassium levels (0 mM, 5 mM, 10 mM) on the photic response.

To determine the importance of chloride, we examined the effect of replacement of sodium chloride with the

acetate, bromide, fluoride, iodide, nitrate, nitrite, or sulfate salt of sodium. To examine the importance of the bicarbonate ion, we substituted 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), 4-morpholinepropanesulfonic acid (MOPS), phosphate, and tris(hydroxymethyl)aminomethane (TRIS) buffers, all at pH 7.2. Preliminary observations of the effect of pH were made with a bicarbonate buffer adjusted from pH 4.0-10.0.

We examined the importance of osmotic strength by adding sucrose or water to saline so as to vary the osmotic strength of the saline from 168 to 312 milliosmolar (mOsm), with 240 mOsm being the control value. All skins were equilibrated in test solutions 10 minutes prior to testing. Significance was usually determined using Student's paired T-test ($p < .05$), in which the magnitude of the control photic response was paired with the magnitude of DPR after treatment. All tabulated data are expressed as percent of control $\pm$ standard error of the mean.

RESULTS

Effect of Sodium Replacement

Replacement of sodium with the chloride salt of choline, cesium, lithium, or rubidium inhibits DPR by approximately 40% (table 5.1). This inhibition is effective with the first DPR after treatment and remains effective until skins are washed in normal saline.

Effect of Potassium Concentration Variation

We found that increasing K^+ 5-fold (10 mM) enhances DPR, whereas a 0 mM K^+ saline is inhibitory (table 5.2).

Effect of Chloride Replacement

Replacement of the chloride ion with acetate, bromide, iodide, or nitrate anions inhibits DPR, whereas nitrate or sulfate anions can substitute for chloride, suggesting that the Cl^- ion is not absolutely required for DPR (table 5.3).

Effect of Buffer Replacement and pH Variation

Since MOPS and TRIS buffers but not HEPES or phosphate, all at pH 7.2, could substitute for bicarbonate (table 5.4), we conclude that there is no immediate requirement for this ion during DPR. Furthermore, our brief examination of the effect of pH on DPR reveals that DPR remains reasonably intact over the wide range of H^+ concentration tested (pH 4-10). The most obvious effect is that acidic pH tends to increasingly inhibit successive DPRs without inhibiting recovery (figure 5.1).

Table 5.1
Effect of Sodium Substitution on DPR

Substitution	Percent of Control DPR ($\pm$ SEM, n=5)
choline	41.7 $\pm$ 8.9*
cesium	63.3 $\pm$ 9.0*
lithium	64.3 $\pm$ 5.1*
rubidium	56.2 $\pm$ 6.8*
sucrose	33.4 $\pm$ 11.4*

*p<.05 (2-tailed paired t-test)

Table 5.2
Effect of Variation of Potassium Concentration on DPR

K ⁺ Concentration	Percent of Control DPR ($\pm$ SEM, n=7)
0 mM	88.8 $\pm$ 3.9*
5 mM	99.4 $\pm$ 15.2
10 mM	118.8 $\pm$ 5.4*

*p<.05 (1-tailed, paired t-test)

Table 5.3
Effect of Chloride Substitution on DPR

Substitution	Percent of Control DPR ($\pm$ SEM, n=5)
acetate	82.3 $\pm$ 4.5*
bromide	85.6 $\pm$ 6.3*
iodide	66.6 $\pm$ 5.3*
nitrate	100.5 $\pm$ 6.7
nitrite	68.8 $\pm$ 10.8*
sulfate	94.8 $\pm$ 11.1

*p<.05 (2-tailed, paired t-test)

Table 5.4
Effect of Buffer Substitution on DPR

Substitution	Percent of Control DPR ($\pm$ SEM, n=5)
HEPES	80.6 $\pm$ 7.5*
MOPS	87.6 $\pm$ 19.4
TRIS	91.7 $\pm$ 13.7
Phosphate	74.0 $\pm$ 5.7*

*p<.05 (2-tailed, paired t-test)

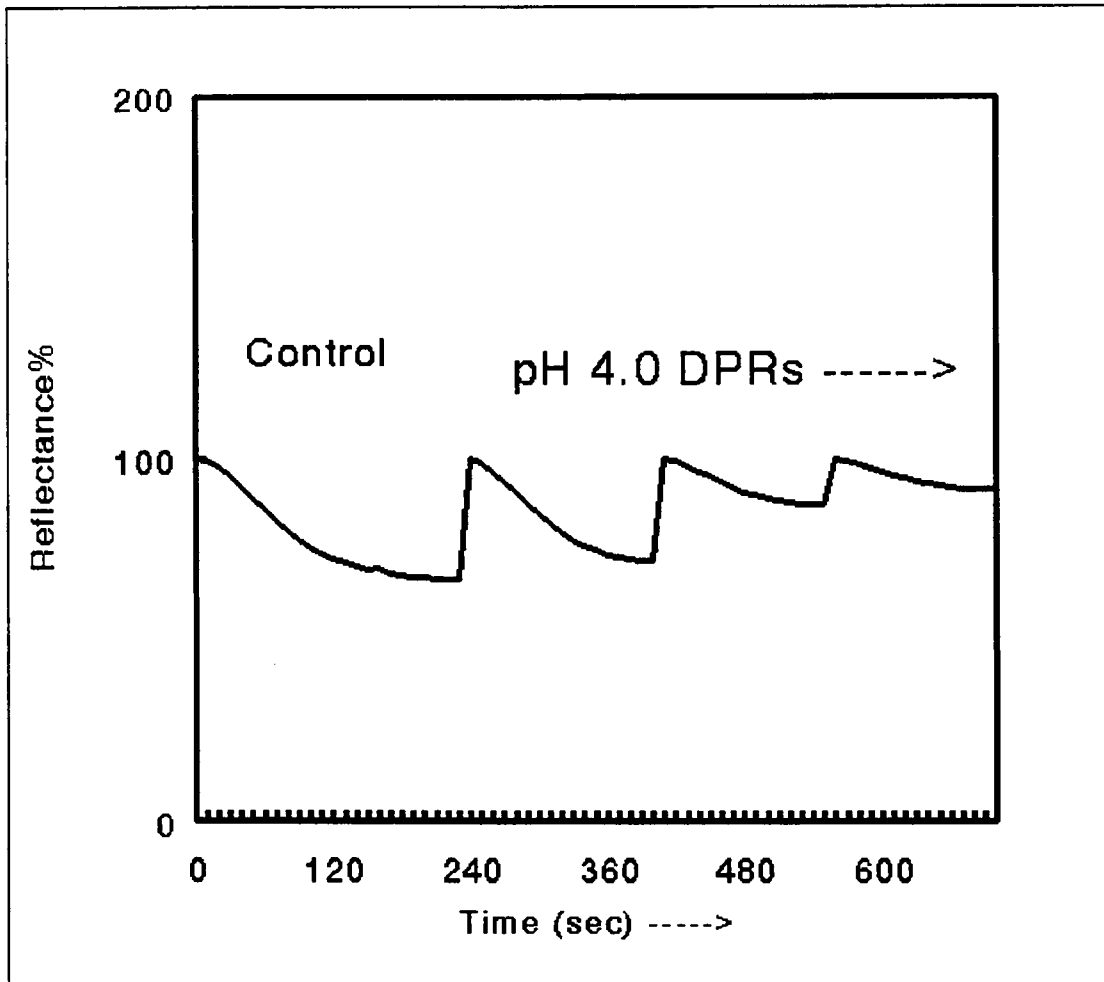


Figure 5.1: Effect of Acidic pH on DPR. After performing control DPRs, lizard skins (n=3) were transferred to normal saline adjusted to pH 4.0 with HCl. These skins were then challenged to respond to visible light. Successive DPRs diminished in magnitude in a manner reminiscent of previous experiments with calcium-free saline (part IV).

Effect of Tonicity

We examined the effect of saline tonicity on skin tone and DPR (table 5.5). We report that hypotonic saline inhibited DPR in direct proportion to the level of hypotonicity, while having no detectable effect on basal skin tone. DPR was unaffected by hypertonic saline within the range tested. Hypertonic saline also had no detectable effect on skin albedo, except for 130% saline which caused significant skin darkening.

Table 5.5
Effect of Osmolarity on Skin Tone and DPR

Ionic Strength (mOsm)	% of Control Albedo ($\pm$ SEM, n=5)	% of Control DPR ($\pm$ SEM, n=5)
168	117.6 $\pm$ 7.7	25.7 $\pm$ 6.4*
192	117.8 $\pm$ 8.7	40.7 $\pm$ 5.7*
216	114.6 $\pm$ 6.9	44.4 $\pm$ 1.9*
240	control	control
264	98.0 $\pm$ 2.7	106.0 $\pm$ 2.1
288	105.0 $\pm$ 6.0	117.4 $\pm$ 14.7
312	86.4 $\pm$ 3.4**	124.1 $\pm$ 44.6

*p<.05 (2-tailed, paired t-test)

**p<.05 (2-tailed, univariate t-test)

DISCUSSION

The partial inhibition of DPR in the absence of sodium agrees with data previously presented (Vesely, 1972; Vesely and Hadley, 1979) for the MSH response. The partial inhibition of the MSH response was interpreted as the absence of an implicit sodium requirement, since skins could partially respond in the absence of sodium.

Whether sodium is permissive to the action of calcium or is, perhaps, directly involved in a membrane depolarization event is not known. However, it is known that the Anolis melanophore membrane is permeable to potassium (Vesely and Hadley, 1979), and, according to the Nernst equation, increasing potassium 5-fold should depolarize the cell membrane 29 mV, whereas decreasing potassium should cause hyperpolarization. Our finding that increased potassium enhances DPR, whereas decreased potassium inhibits DPR argues in favor of a depolarization type event as a potential component of DPR. Indeed, a light-sensitive 10-20 mv depolarization of similar timecourse to DPR has been recorded from skins of this animal (Dr. Gerald Vaughan, personal communication).

If DPR is modulated by a Na^+ -induced depolarization event, it would be interesting to speculate how depolarization affects DPR. Kinosita (1963) proposed an electrophoretic theory of pigment movement in which

depolarization of the cell membrane would electrostatically attract negatively charged melanosomes. Conversely, Novales (1959), observing that hypotonicity decreases skin reflectance and hypertonicity inhibits the MSH response in frogs, suggested that melanosome dispersion is triggered by the osmotic uptake of water that would accompany a sodium current. In order to test this hypothesis the effect of osmotic variation on basal skin tone and DPR was investigated.

Hypotonic saline, which should favor the uptake of water, did not darken lizard skins or enhance DPR. Hypotonic saline actually inhibited DPR in proportion to the degree of hypotonicity (table 5.5). A similar finding has been documented for the MSH-response of A. carolinensis (Vesely and Hadley, 1979).

Furthermore, the hypertonicity induced inhibition of the MSH response in the frog (Novales, 1959) was not observed by us for A. carolinensis DPR. In fact, contrary to predictions of the osmotic model, unstimulated skins actually darken in very hypertonic (130%) saline, and DPR remains intact. Thus, it seems reasonable to conclude from our data and others (Vesley and Hadley, 1972) that the osmotic model of Novales (1959) is not correct.

An alternative hypothesis is that Na^+ ions permeate $\text{Na}^+/\text{Ca}^{2+}$ -channels during DPR, in a manner analogous to visual

photoreceptors, which may be evolutionarily related (Vaughan and Tallent, 1984; part II). The combined depolarizing effect of Na^+ and Ca^{2+} (part IV) might somehow be coupled to an increased calcium influx, the release of calcium from intracellular stores, or both (part IV). In the ventral photoreceptor cells of the horseshoe crab, Limulus, intracellular calcium is released via the phosphatidyl inositol pathway (Payne et al., 1986) during a Na^+ -dependent depolarization (Bolsover and Brown, 1985) modulated by cGMP-gated channels (Johnson et al., 1986; Bacigalupo et al., 1991).

Although our failure to implicate the chloride ion as necessary for DPR is similar to the lack of a requirement of this ion for the MSH response, our data as to which ions can substitute for chloride conflict with the findings of Vesely (1972) who found that either bicarbonate or phosphate, but not sulfate could substitute for chloride and allow MSH-induced skin darkening. However, it should be emphasized that no statistical tests were reported for Vesely's work. Even so, it is interesting that Vesely (1972) found that sulfate Ringer completely inhibited A. carolinensis skin responses to MSH whereas we were unable to detect a treatment effect on DPR.

By buffer substitution, we were unable to demonstrate any immediate requirement for the bicarbonate ion (table

5.4). Furthermore, our preliminary studies on the effect of pH suggest that acidic pH causes successively greater inhibition to multiple DPRs, an effect somewhat analogous to EGTA-saline (part IV) and perhaps working through a similar mechanism, since acidic pH has been shown to be an inhibitor of calcium flux (Hille, 1992).

In summary, although extracellular calcium is the only ion implicitly required for MSH-mediated dispersion (with sodium apparently playing a secondary role), there is no extracellular ion implicitly necessary for DPR. Clearly, in the absence of extracellular calcium, sufficient calcium exists within intracellular stores to elicit DPR (Beam and Vaughan, 1995; part IV). Extracellular calcium may partially contribute to DPR and is responsible for refilling intracellular stores. Furthermore, sodium is a contributing factor to DPR, and it may exert its effect through a membrane depolarization event. That event may be the stimulus necessary to trigger calcium influx or to trigger calcium release from intracellular stores.

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Part VI.

Physiologically Active Reptilian Melanophores in Culture

ABSTRACT

Melanophores from adult lizards of the species, Anolis carolinensis, were cultured and tested for physiological activity when stimulated with light, hormones, or other agents. We report that cultured melanophores behave similarly to melanophores in intact skin. Melanophores disperse pigmented melanosomes when stimulated with blue light, melanocyte-stimulating hormone, or β -acting catecholamines, whereas α -acting catecholamines trigger pigment aggregation.

In all cases ($n > 50$), any melanophore that would disperse pigment in response to light would also disperse pigment in response to MSH (10^{-10} M). One-half (10 out of 20) cells tested were unable to undergo photodispersion in the presence of 10 μ M 8-Br-cGMP, whereas the MSH response remained intact in all cases.

Cells ($n = 5$) also disperse pigment when challenged with the highly specific cAMP agonist, Sp-cAMP (500 μ M), or with the phosphodiesterase inhibitor, isobutylmethylxanthine (IMBX, 10 μ M), indicative of a role for cAMP in pigment dispersion. Previous work suggests that cAMP is the mediator of the MSH response.

Pigment will also disperse when cells (n=10) are treated with the calcium ionophore, a23187 (1 μ M). The pigment-dispersing effect of a23187 is suggested to be due to perturbations of the light-sensitive pathway. Melanophores loaded with the calcium chelator BAPTA-AM (0.5 μ M) are unable to undergo light-mediated dispersion in 5 out of 12 cases whereas the MSH-mediated dispersion remains intact. Furthermore, when cells (n=5) are treated with lanthanum (1 mM) saline, pigment partially photodisperses then reaggregates, whereas pigment disperses normally when stimulated with MSH.

Regardless of the stimulus, pigment dispersion apparently involves microfilaments, since cytochalasin B (50 μ M) will completely inhibit light, MSH, IBMX, Sp-cAMP, or a23187-mediated pigment dispersion (n=5). Pigment aggregation after a light response apparently requires microtubules since aggregation is inhibited in the presence of 1 mM colchicine (n=5). These results suggest that melanophores in culture behave similarly to melanophores in intact skin.

Furthermore, numerous melanophores were identified that respond well to MSH but not light, or have pigment dispersed in the control state and would undergo aggregation when

treated with α -acting catacholamines, but would undergo dispersion again when the agent was removed. These cells were regarded by us as being damaged by the culturing process and were omitted from further studies. It is possible, however, that they make up distinct subpopulations of melanophores whose differential control adds subtle complexities to the control of skin color.

INTRODUCTION

Physiological color change has fascinated investigators since the time of Aristotle. Although the majority of the ancient literature is concerned with the color change of the African chameleon, most work in this century has focused on species indigenous to major research centers (Zoond and Eyre, 1934) such as the North American lizard, Anolis carolinensis, whose color change from green to brown was first documented by Carlton (1903).

Since then, investigators have identified a variety of stimuli that can effect color change in the intact animal as well as isolated skin. Visible light (Carlton, 1903; Kleinholtz, 1938a; Vaughan, 1987), and hormones such as melanocyte-stimulating hormone (MSH) and epinephrine have been thoroughly documented agonists of color change (Kleinholtz, 1938a,b; Goldman and Hadley, 1969; Hadley and Goldman, 1969a).

Although not completely understood, the mechanism of the MSH-induced skin darkening has been the subject of most investigations. Change in skin color is effected when melanin in dermal melanophores lying beneath xanthophores and reflective iridophores is redistributed into dendritic cell processes that terminate above and obscure those cells (Alexander and Fahrenbach, 1969; Taylor and Hadley, 1970).

The same mechanism is apparently involved in the light response (Beam and Vaughan, 1995b), making the dermal melanophore both hormone and light-sensitive.

Researchers have quantitated color change of entire animals or intact skin samples visually by using the Hogben-Slome index (1931), and by measuring the transmittance (Wright, 1948) or, more commonly, the reflectance of skin samples (Schizume et al., 1954). Using these methods and appropriate pharmacology, investigators have demonstrated the necessity of microfilaments for MSH or light-stimulated melanosome dispersion, and microtubules for aggregation (Vaughan, 1987).

Using similar techniques investigators have suggested a role for cAMP (Hadley and Goldman, 1969b; Beam and Vaughan, 1994; part III) and calcium (Vesely and Hadley, 1971; Lucas et al., 1987) in MSH-mediated skin darkening; whereas, calcium and cGMP, but not cAMP, appear to be involved in the light-mediated response (Vaughan and Tallent, 1988; Beam and Vaughan, 1994; part III). Since, however, the dermal melanophore represents only 5-10% of the cell total in lizard skin, it is difficult to localize pharmacological responses to the dermal melanophore. In order to evaluate controlling mechanisms of pigment movement more carefully,

it would be useful to examine individual melanophores in culture.

Although photosensitive melanophores have been previously cultured from the amphibian Xenopus laevis (Seldenrijk et al., 1979; Daniolos et al., 1990) and the teleosts Xiphophorus maculatus (Wakamatsu et al., 1980) and Oryzias Latipes (Negishi, 1985), previous attempts utilizing the skin of adult Anolis carolinensis have produced cells of many types which remain viable for several days, but no chromomotor activity of the melanophore was documented (Rohrlich and Rubin, 1975).

Recently, we have successfully cultured A. carolinensis melanophores which remain viable for several weeks, rather than days, and have demonstrated both light and hormonal responses of these cells (Beam et al., 1995). In this study, we demonstrate the utility of the cultured melanophore by verifying many experiments previously performed on intact lizard skin and introduce further evidence for compartmentalization of the light and MSH responses.

MATERIALS AND METHODS

Animals

Animals used in this study were adult male lizards of the species, Anolis carolinensis, recently captured from wild populations in coastal Louisiana and purchased from Snake Farm (Laplace, LA). Animals were maintained in group colony on a 12 hour light/dark cycle at 21 C.

Cell Culture Reagents

Antibiotic cocktail, collagenase, hyaluronidase, isobutylmethylxanthine (IBMX), Leibovitz L-15 medium, 12-O-tetradecanoylphorbol 13-acetate (TPA), trypsin, and alpha-melanocyte stimulating hormone (MSH) were purchased from Sigma Chemical Company (St. Louis, MO). Fetal bovine serum (FBS), Nu-Serum IV, and iron supplemented calf serum were purchased from Gibco (Grand Rapid, NY). Synthetic human insulin (Eli Lilly) was obtained from a local pharmacy. Stock solutions of all agents were dissolved directly in saline solution or DMSO where appropriate (IBMX). When DMSO was used as a solvent, the final concentration was 1.0%, a dose that had no intrinsic effect on chromatophore activity.

Cell Culture Technique

Lizards were briefly rinsed with 70% ethanol followed by decapitation. The dorsal skin was aseptically removed and transferred to sterile saline (in mM/L: 111 NaCl, 2.0 KCl,

1.0 CaCl₂, 2.0 NaHCO₃, 5.0 glucose at pH 7.2) containing antibiotic cocktail (200 IU/ml penicillin, 200 µg/ml streptomycin and 5 µg/ml amphotericin B). Skins were rinsed with agitation for five minutes in three changes of sterile saline/antibiotic cocktail (SSAC) and then incubated one hour in SSAC containing 0.1% trypsin at 37° C. With sterile forceps, the epidermis was then carefully separated from the dermis and discarded. The dermis was minced with a sterile razor blade and digested (30° C overnight) in SSAC containing 0.01% collagenase/0.01% hyaluronidase. After suspension by gentle trituration, dermal cells were pelleted and washed twice in SSAC by centrifugation (500G, 5 minutes), and resuspended in medium containing 3 parts water, 1 part fetal bovine serum (or 1/2 part Nu-Serum IV, 1/2 part iron-supplemented calf serum), and 6 parts Leibovitz L-15 medium (pH 7.4) supplemented with antibiotic cocktail. In an effort to induce proliferation, culture media were occasionally supplemented with 10⁻⁷ M melanocyte-stimulating hormone (MSH), 100 µM IBMX, 10 µM TPA, or insulin (10 U/ml). Cell suspensions were placed in sterile 96-well microplates (Corning) and held at 30° C in humidified air.

Pharmacological Agents

Colchicine, cytochalasin B, 8-bromo-3'-5'-cyclic guanosine monophosphate (8-Br-cGMP), calcium ionophore a23187, clonidine, epinephrine, isobutylmethylxanthine (IBMX), isoproterenol, lanthanum chloride, melatonin, norepinephrine, and octopamine were purchased from Sigma Chemical Company (St. Louis, MO). The Sp-isomer of adenosine-3',5'-cyclic monophosphorothiate (Sp-cAMP) was purchased from Research Biochemicals Incorporated (Natick, MA). 1,2-bis-(2)aminophenoxythane-N,N,N',N'-tetraacetic acid (BAPTA-AM), N-(2-methoxyphenyl)iminodiacetic acid (half-BAPTA) and the surfactant, Pluronic F-127, were purchased from Molecular Probes, Inc. (Eugene, OR). Stock solutions were prepared by dissolving agents directly into saline or DMSO where appropriate (a23187, BAPTA-AM, IBMX, Pluronic F-127). When DMSO was used as a solvent, the final concentration was 1.0%.

Pharmacological Experiments

After 4-6 days, melanophores were evaluated for sensitivity to light and other agents using a Nikon inverted microscope with camera attachment. After identifying a melanophore of interest, a heated steel dissection probe was used to make a hole in the lid covering the well. With this access, the incubating medium was removed and replaced with

normal saline at room temperature (20° C). The cell was then photographed in the control state, light-stimulated state, or after the addition of appropriate pharmacological agents.

Using a series of gelatin cut-off filters (Eastman Kodak Company), we determined that light > 540 nm was insufficient to induce DPR in a single cell (plate 6.1), as has been previously demonstrated in whole skin (part III). A red gelatin filter (Kodak #2A) was, therefore, placed over the microscope viewing light source when the effect of MSH or any other light-independent agent was tested.

At the completion of each experiment, the solution in the experimental well was replaced with 0.2% sodium azide, and the hole sealed with molten paraffin. In this way, the microplate could then be returned to the incubator with the sterility of unused wells maintained for later use. A minimum of five cells from five separate wells from a minimum of three lizards was examined for each experiment.

BAPTA Loading

For the purpose of chelating intracellular free calcium, cells were loaded with the cell-permeable calcium chelator, BAPTA-AM. Light responsive cells were identified and photographed in the light-dispersed and dark-aggregated state. Cells were then incubated with loading solution (normal saline containing 0.5 μ M BAPTA-AM/0.1% Pluronic F-

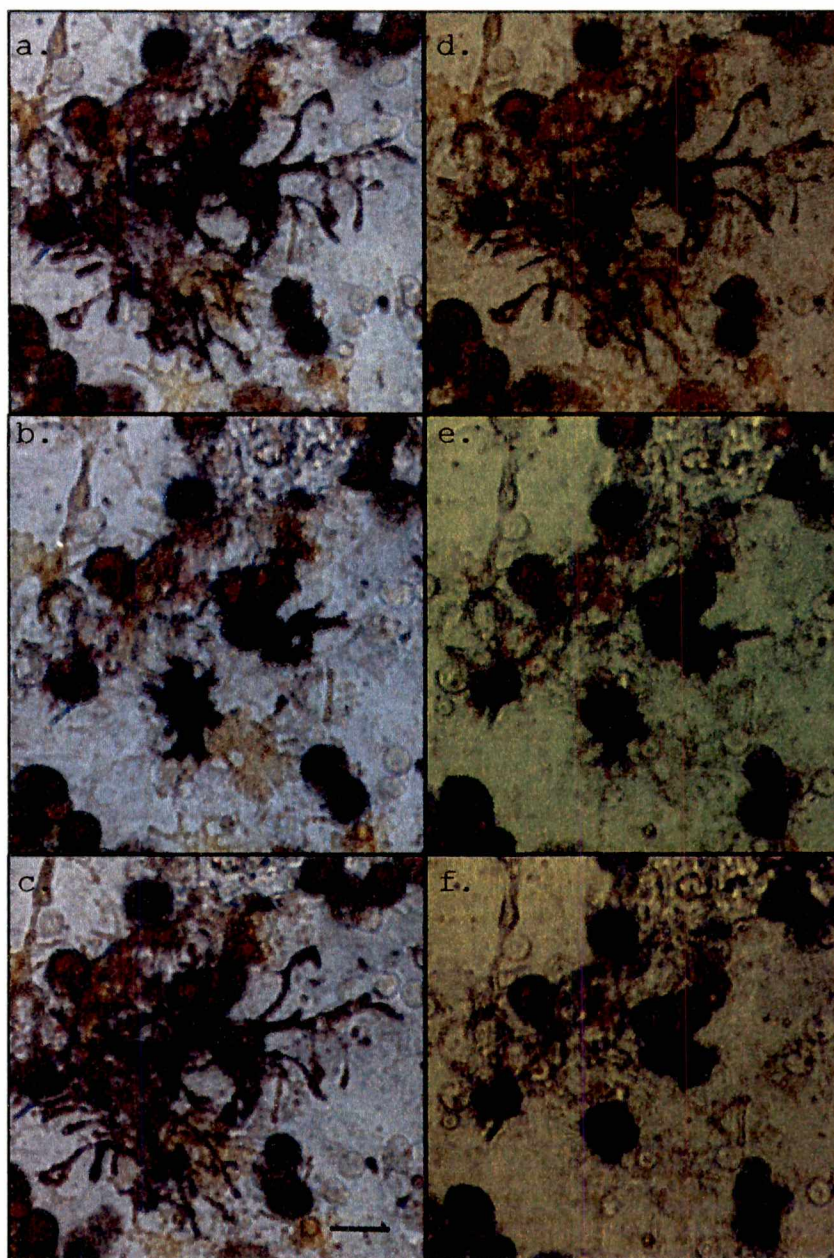


Plate 6.1: Wavelength Dependence of Light Response. Using a set of spectral cut-off filters, cells were examined after 10 minutes in the presence of (a) white light, (b) darkness, (c) light >400 nm, (d) light >460 nm, (e) light > 560 nm, (f) light > 600 nm. Bar = $10\ \mu$.

127) for 1 hour at room temperature. During the loading process, melanosomes tend to disperse initially. In 5 out of 12 cells examined, melanosomes are super-aggregated within 1 hour. Cells continuing to display dispersed melanosomes were regarded by us as being damaged by the loading process and were not examined further. After loading, cells were washed with normal saline for ten minutes prior to testing. Control cells were loaded with loading solution without BAPTA-AM, or containing half-BAPTA, a BAPTA-like analogue with no chelating ability. In all cases (n=5), the controls were without effect on melanosome dispersion, DPR, or MSH-induced melanosome dispersion.

RESULTS

Melanophores from freshly digested tissue are initially round and non-dendritic. They attach slowly to plastic or glass and thus seem to require a matrix before bonding. Upon attachment, approximately 20% of the melanophores regain their dendritic shape within 4-6 days. Virtually all of those that regain their dendricity have pigment dispersed into the dendritic arms.

Most of these cells can be induced to aggregate their pigment completely by exposure to the tungsten light source of the microscope for 10 minutes, which facilitates further dispersion, followed by aggregation in the dark for 5 minutes. In dark-aggregated melanophores, intact distal arms, devoid of pigment, could be seen with phase-contrast microscopy. All melanophores were tested for photosensitivity before utilization for any other experiments. Any melanophore that could undergo photodispersion and dark aggregation was assumed to be physiologically normal.

Other agents tested for pigment aggregating ability include epinephrine, melatonin, norepinephrine, and octopamine. Of these, norepinephrine (1 μ M) could cause complete pigment aggregation. All other agents tested were without effect (n=5) at the highest dosage tested (100 μ M).

Effect of Visible Light on Melanophores

In response to a tungsten light source, pigment disperses into the dendritic arms, a process that is essentially complete within 5 minutes. Without stimulation, pigment returns to the cell center within 2.5 minutes, a process that can be repeated many times (plate 6.2).

At least two photosensitive, morphologically distinct, kinds of melanophores can be observed within culture (plate 6.3). The most common type, comprising roughly 75% of all photosensitive melanophores tested, has a distinct perikaryon with numerous, elongated, branched or unbranched dendritic arms. Unstimulated cells either assume a round shape with pigment fully retracted into the perikaryon, or assume a stellate shape with pigment aggregated into the perikaryon and proximal arms. The second type of melanophore has a less distinct perikaryon and dendrites. During photodispersion, pigment spreads out in all directions, with little definition of individual dendritic arms, reminiscent of a neural growth cone and appearing similar to Xenopus melanophores cultured by Rollag and Adelman (1993). The time course of light response is the same for both cell types.

Effect of MSH and Catacholamines on Melanophores

All melanophores ($n > 50$) that would respond to visible light would also respond to MSH (plate 6.4) within a similar

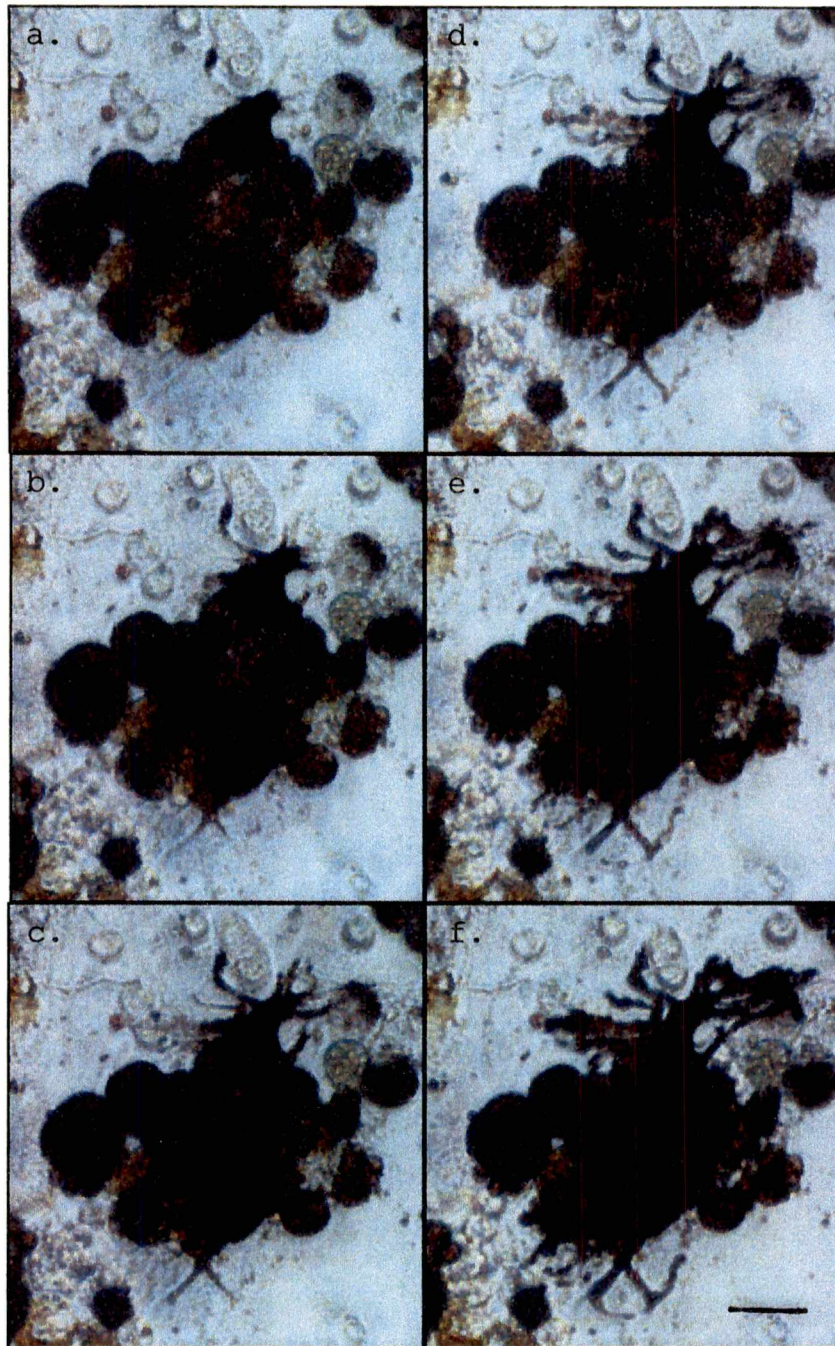


Plate 6.2: Photosensitivity of Melanophores. Melanophores were stimulated with white light and photographed at time (a) 0, (b) .5, (c) 1, (d) 2.5, (e) 5, and (f) 10 minutes. Upon cessation of stimulation, pigment returns to the dark-adapted condition. Bar = 10 μ .

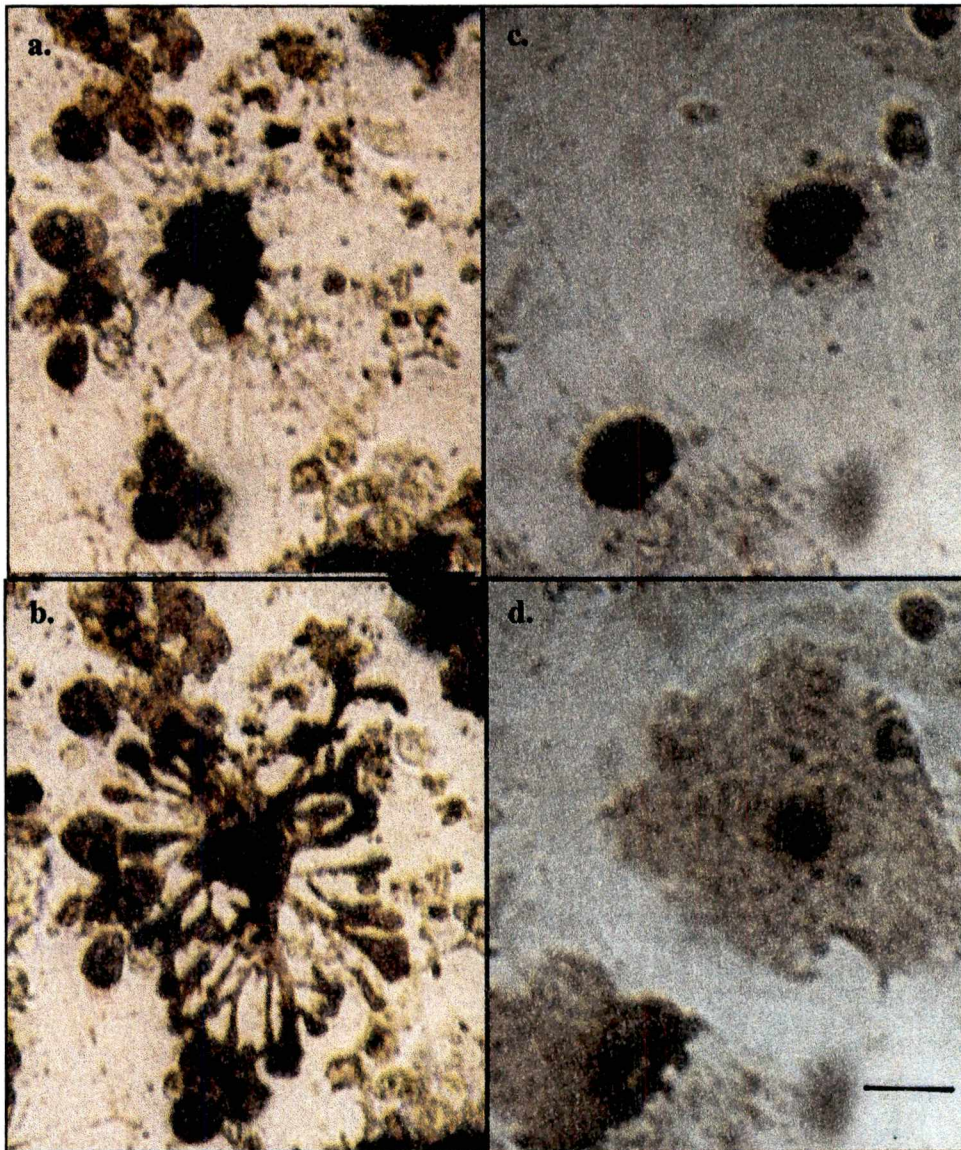


Plate 6.3: Identification of Melanophore Sub-types. Two morphologically distinct classes of light-sensitive melanophores exist in culture. The predominant class is either round or stellate in darkness with pigment aggregated into proximal arms (a). Stimulated cells disperse pigment into distinct arms (b). In the other class, dark-adapted melanophores are punctate (c), whereas stimulated melanophores (d) disperse melanosomes in all directions. Bar = 10 μ .



Plate 6.4: MSH-sensitivity of Melanophores. The same melanophores shown in plate 2 were stimulated with MSH (10^{-10} M) in the absence of white light and photographed at time (a) 0, (b) .5, (c) 1, (d) 2.5, (e) 5, and (f) 10 minutes. Bar = 10 μ .

time period. However, MSH stimulation produced a much greater magnitude of pigment dispersion than light stimulation, as could be predicted from earlier work with intact skin (part III). β -acting catecholamines also produce dispersion. We found that epinephrine (1 μ M) was a weakagonist of pigment dispersion (n=5), whereas isoproterenol (1 μ M) was stronger (n=5). Norepinephrine (1 μ M) and the potent alpha-agonist, clonidine (1 μ M), both stimulated pigment aggregation (n=5, plate 6.5). These results agree with earlier animal and intact skin studies (Goldman and Hadley, 1969; Carter and Shuster, 1982).

Role of cAMP and cGMP in Melanophores

Adenosine 3'-5' cyclic monophosphate (cAMP) levels can be elevated by the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). We found that IBMX (10 μ M) was a potent melanosome dispersing agent (n=5). As further evidence of the effect of cAMP, the highly specific cAMP agonist, Sp-cAMP (Rothermel et al., 1983), completely disperses melanosomes (plate 6.6) of challenged cells (n=5).

It has been shown that 8-Br-cGMP inhibits light-induced but not MSH-induced skin darkening (Vaughan and Tallent, 1988; Beam and Vaughan, 1994; part III). We tested 8-Br-cGMP (10 μ M) on cultured melanophores and found that the

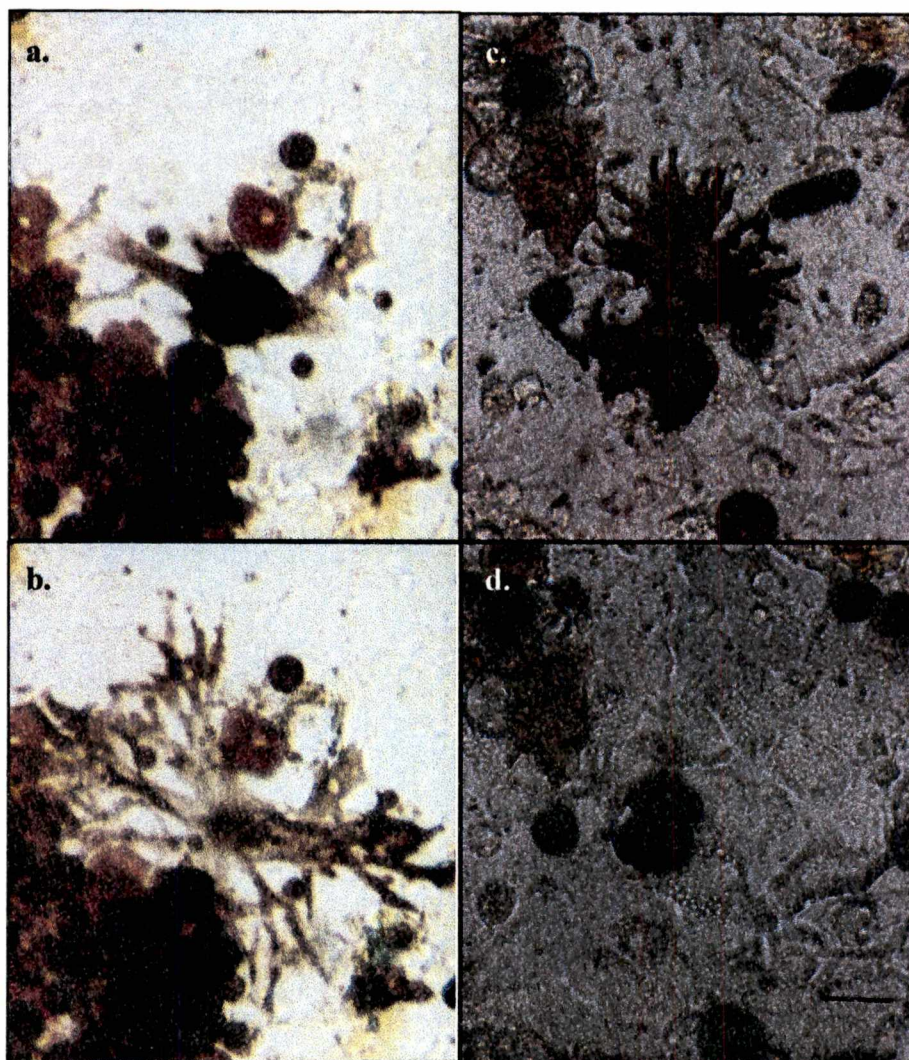


Plate 6.5: Effect of Catecholamines on Melanophores. Dark-adapted melanophores with pigment aggregated (a) undergo pigment dispersion (b) when treated with β -acting catecholamines such as isoproterenol ($1\ \mu\text{M}$, 10 minutes). Conversely, dark-adapted melanophores in the dispersed state (c) undergo aggregation (d) when treated with α -acting catecholamines such as norepinephrine ($1\ \mu\text{M}$, 10 minutes). Bar = $10\ \mu$.

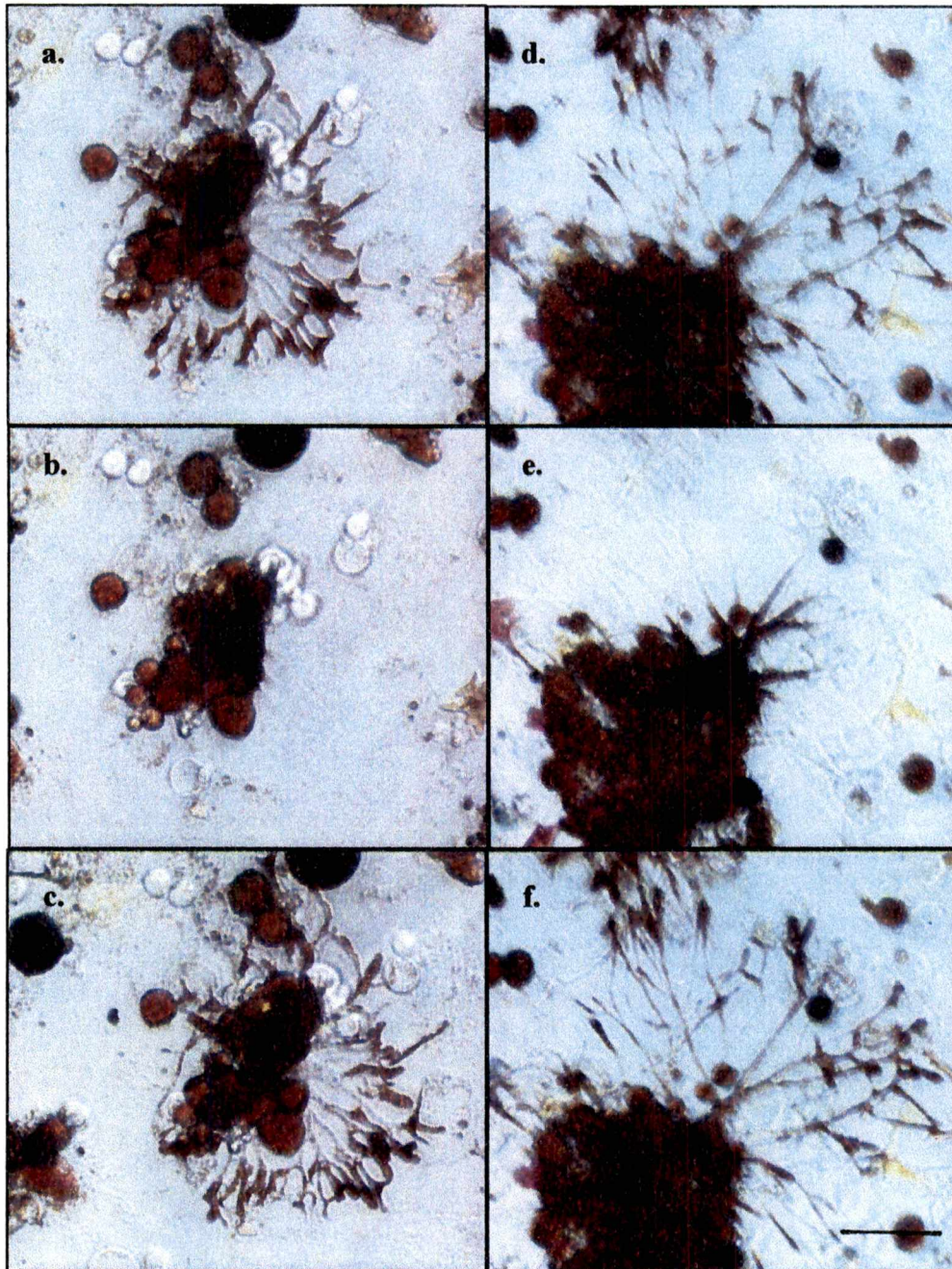


Plate 6.6: Effect of IBMX or Sp-cAMP on Melanophores. Melanophores which undergo normal photodispersion (a,d) and dark-aggregation (b,e) undergo dispersion when treated with 10 μ M IBMX (c) or 400 μ M Sp-cAMP (f) for 10 minutes in darkness. Bar = 10 μ .

light-mediated but not the MSH-mediated dispersion is completely inhibited in one-half (10 out of 20) of the melanophores tested (plate 6.7). Higher doses (1 mM) of 8-Br-cGMP were ineffective at blocking the light response of cells resistant to lower doses. The effect of 8-Br-cGMP on the response of melanophores to other agents (e.g. IBMX, a23187) was not investigated.

Effect of Calcium on Melanophores

It has been demonstrated that intact skin samples darken in the presence of the calcium ionophore, a23187 (Hadley et al., 1981; Lucas et al., 1987; Beam and Vaughan, 1996; part III). In this study, we found that all melanophores tested (n=10) would undergo dispersion when treated with this agent (plate 6.8).

In order to further examine the role of intracellular calcium on MSH and light responses, cells of interest were loaded with the cell-permeable calcium chelator BAPTA-AM. Cells were then challenged to respond to light or MSH (10^{-10} M). Cells treated with BAPTA-AM which maintain pigment aggregation (5 out of 12) are unable to respond to light, whereas they respond normally to MSH (plate 6.9).

It had been suggested that extracellular calcium is used secondarily to calcium from intracellular stores during DPR. The inhibitory effect of lanthanum or EGTA on DPR in

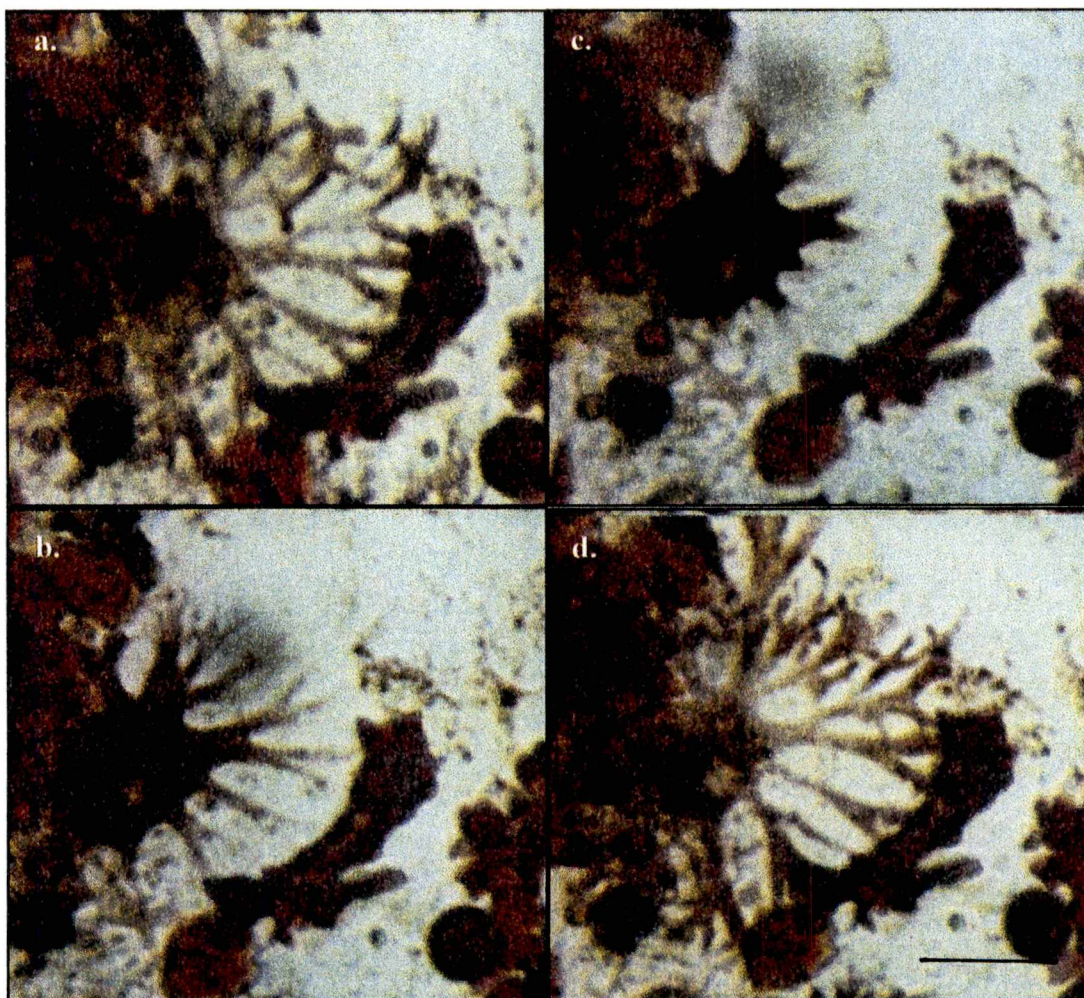


Plate 6.7: Effect of 8-Br-cGMP on Light Response. Cultured melanophores undergo dispersion when stimulated 10 minutes with visible light (a) and aggregation in darkness (b). After treatment with 10 μ M 8-Br-cGMP for 10 minutes in darkness, cells (10 out of 20) are unable to respond to light (c), but respond normally to MSH (10^{-10} M). Bar = 10 μ .

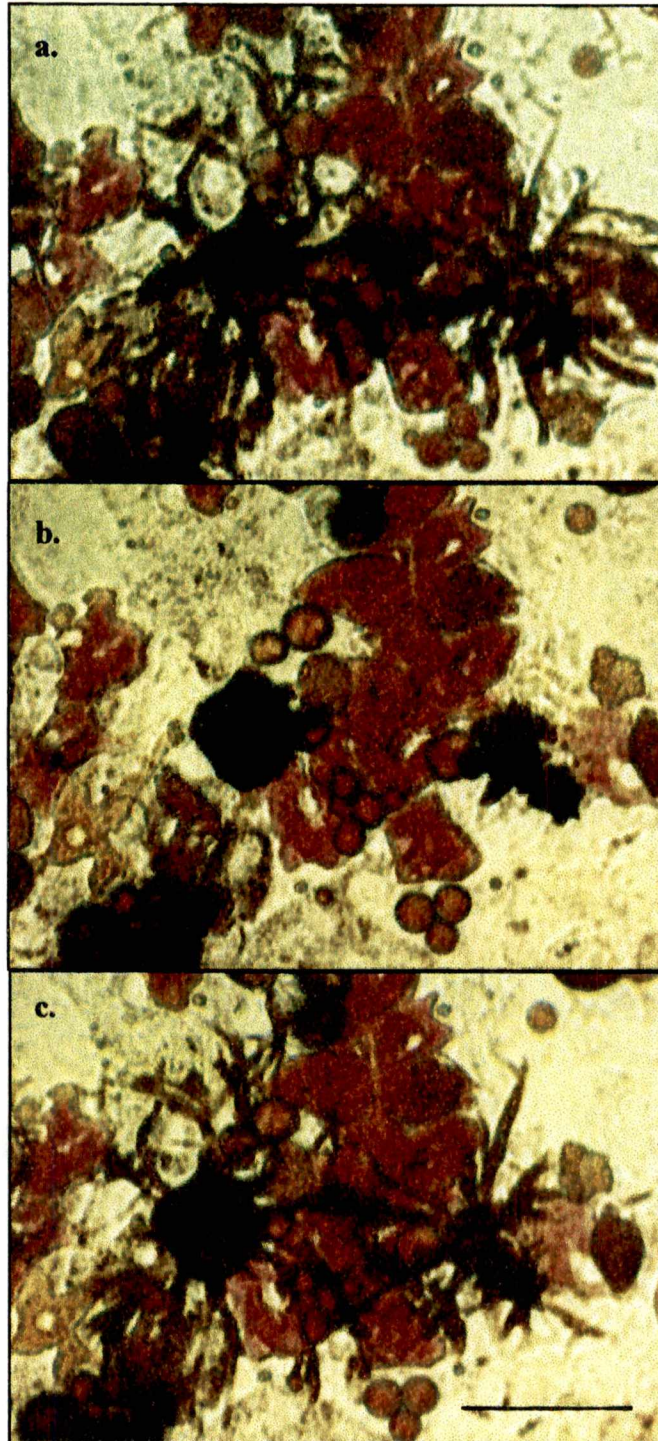


Plate 6.8: Effect of Calcium Ionophore a23187 on Melanophores. Melanophores that undergo dispersion when stimulated with light (a) undergo aggregation in darkness (b). Upon the addition of calcium ionophore a23187 ($1\text{ }\mu\text{M}$, 10 minutes) melanophores undergo pigment dispersion in the absence of light (c). Bar = $10\text{ }\mu$.

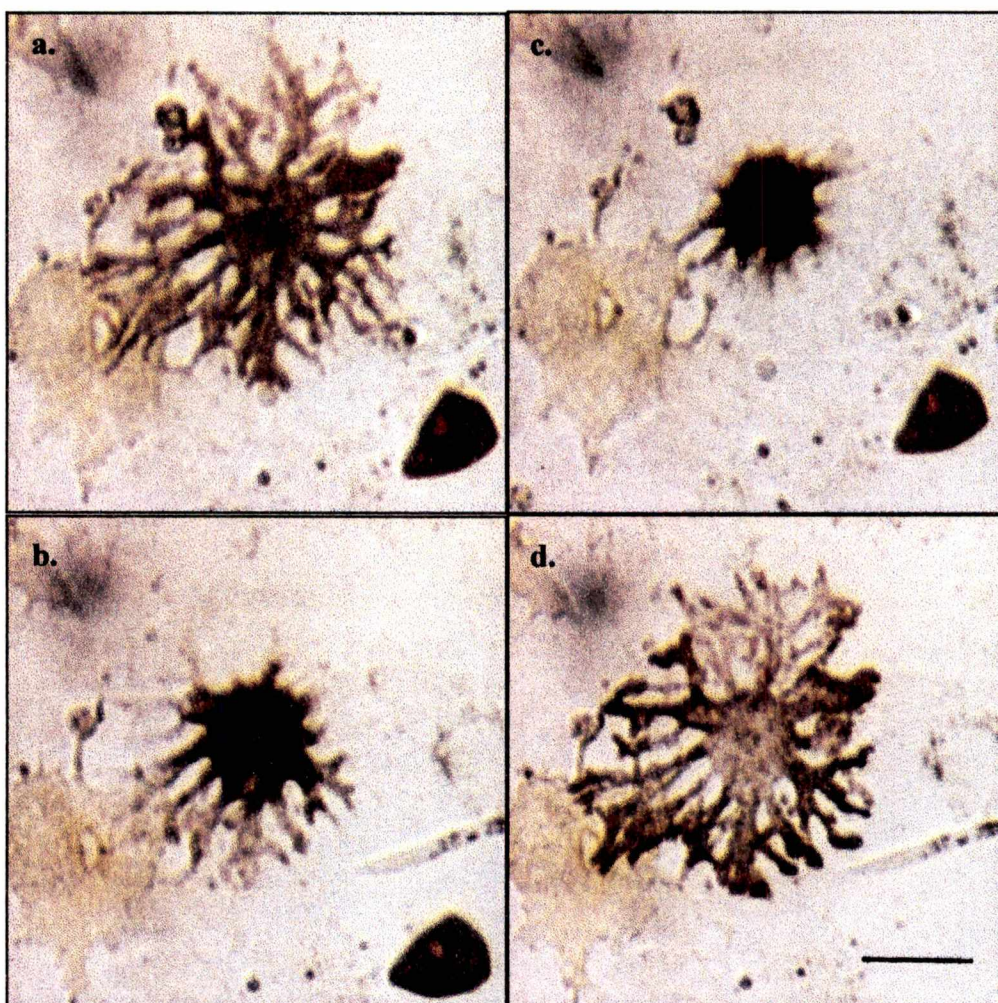


Plate 6.9: Effect of Intracellular Calcium Chelation on Melanophore Responses. A Melanophore that responds normally to light (a) and darkness (b) is unable to respond to light when loaded with the intracellular calcium chelator, BAPTA-AM (c). This same cell responds normally to MSH (10^{-10} M). Bar = 10 μ .

intact skin is thought to be due to depletion of intracellular stores (Beam and Vaughan, 1995a; part IV). Consistent with this hypothesis, when cultured melanophores are light-stimulated immediately after the addition of either of these agents, pigment disperses nearly completely but immediately reaggregates in the presence of continued light stimulation (n=5). No further light-dispersion is possible. However, lanthanum-treated melanophores will disperse pigment normally in response to MSH (plate 6.10).

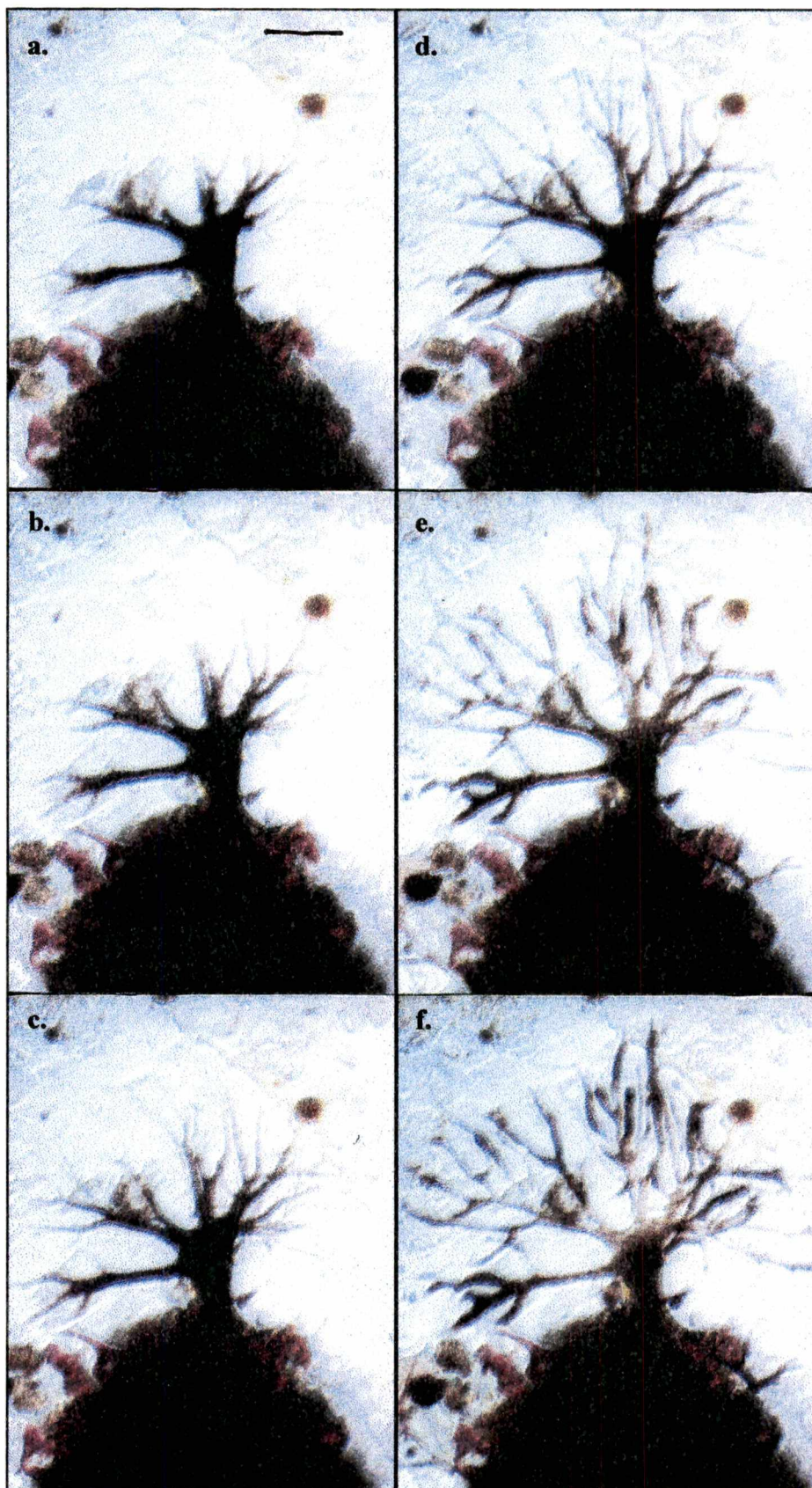
Role of Microfilaments and Microtubules in Pigment Movement

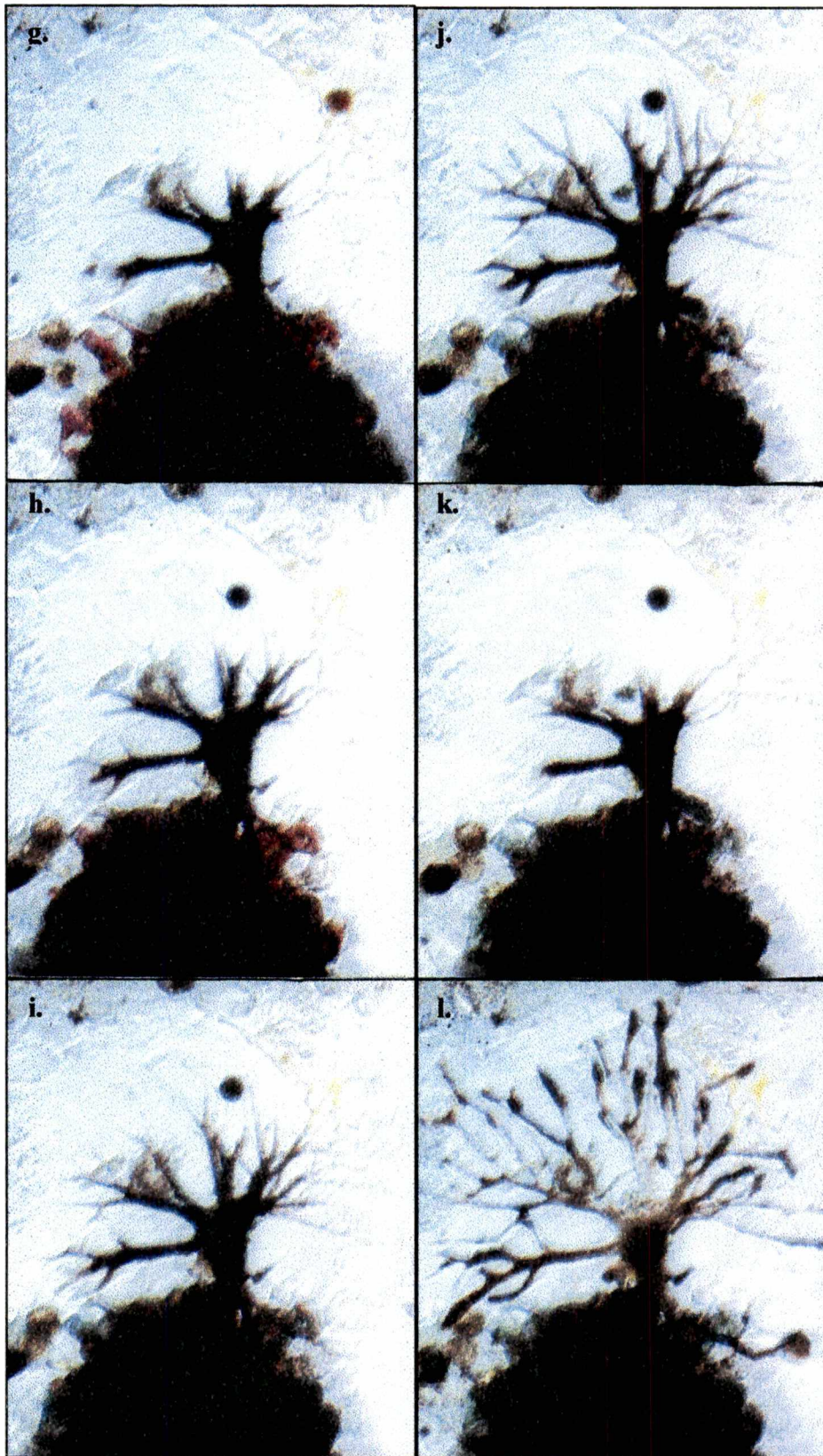
Cytochalasin B has previously been shown to inhibit MSH or light-induced melanosome dispersion in intact skin and colchicine to inhibit retrograde pigment movement upon cessation of light stimulation (Vaughan, 1987). We report similar effects of both agents on cultured melanophores. Cytochalasin B causes complete pigment aggregation and inhibits the dispersing effect of all agents tested (table 6.1). Similarly, cells treated with colchicine (1 mM, 20 minutes, n=5) will disperse pigment in response to light, but do not reaggregate pigment in dark conditions (plate 6.11). The effect of colchicine on reaggregation after MSH stimulation was not examined.

Table 6.1.
Effect of Cytochalasin B on Pigment Dispersion

Dispersing Agent	Dose	Inhibited by CCB?	n
a23187	1.0 μM	yes	5
IBMX	10 μM	yes	5
Isoproterenol	100 μM	yes	5
Light	>100 W/m^2	yes	10
MSH	0.1 μM	yes	10
Sp-cAMP	500 μM	yes	5

Plate 6.10: Effect of La^{3+} on Light and MSH Responses. A melanophore was stimulated with light and photographed at time 0 (a), 0.5 (b), 1.5 (c), 2.5 (d), 5 (e), and 10 (f) minutes. After 10 minutes in darkness, the saline was replaced with buffer-free saline containing 1mM La^{3+} / 1 mM Ca^{2+} and photographed at time 0 (g), 0.5 (h), 1.5 (i), 5 (j), and 10 (k) minutes. The cell was subsequently stimulated 10 minutes with MSH (10^{-10} M) in $\text{La}^{3+}/\text{Ca}^{2+}$ saline and found to respond normally (l). Bar = 10 μ .





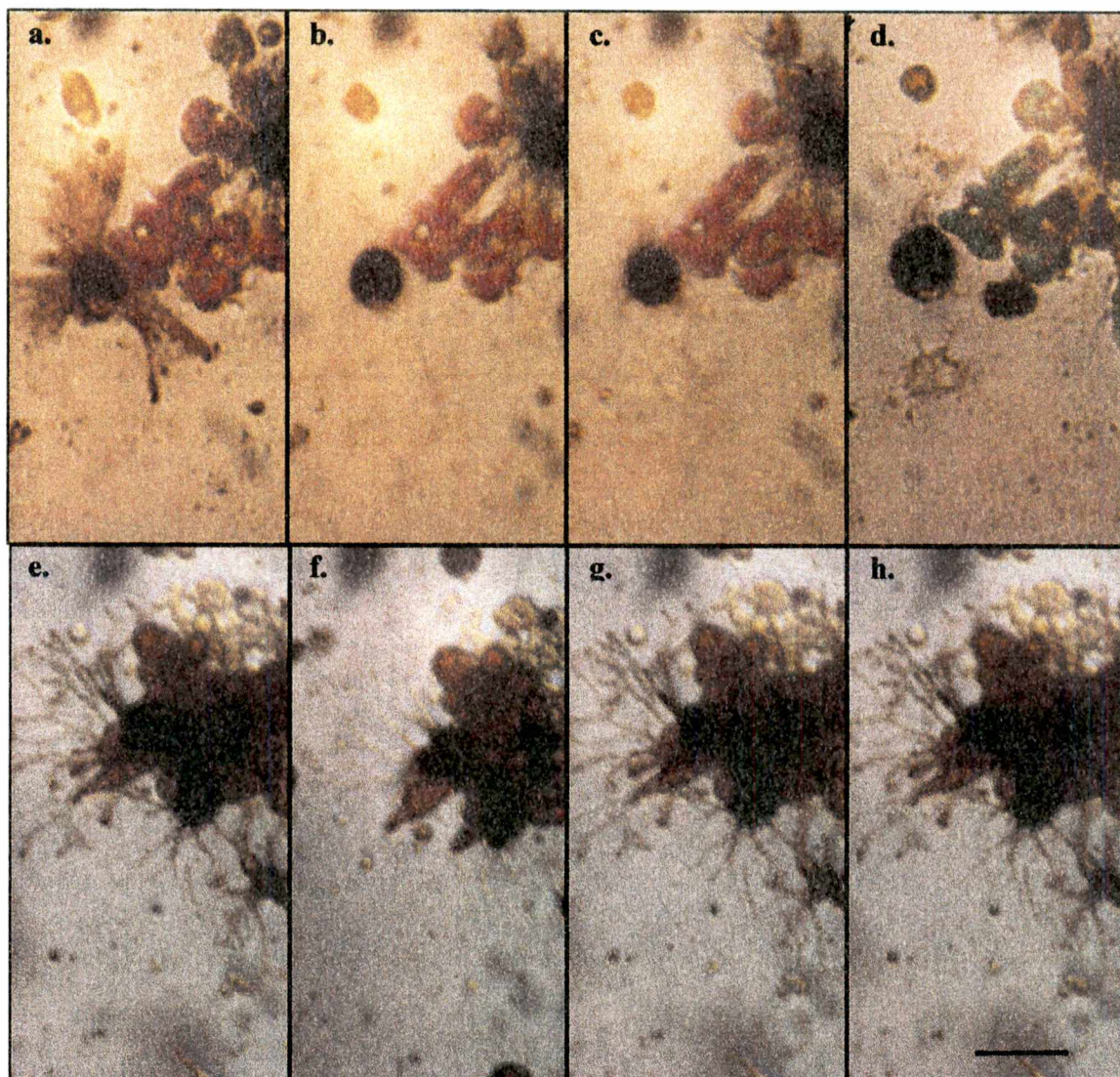


Plate 6.11: Effect of Cytochalasin B and Colchicine on Melanophore Responses. Melanophores stimulated 10 minutes with light (a,e) were then dark adapted and incubated 10 minutes with 50 μ M cytochalasin B (b) or 1 mM colchicine (f). Melanophores were then stimulated 10 minutes with light. Cytochalasin B completely inhibited the light response (c) and the MSH response (d). Colchicine had no effect on the light response (g), but inhibited dark recovery (h). Bar = 10 μ .

DISCUSSION

All pharmacological agents tested typically function at a concentration 2-3 logs lower than in intact skin. The higher concentrations required for intact skin experiments probably reflects the difficulty of drug penetration of the subcutaneous adipose layer which is necessary for the drug to reach the melanophores.

This study demonstrates that cultured melanophores behave similarly to melanophores of intact skin samples. Individual melanophores are both light and hormone sensitive. In response to blue light, melanocyte-stimulating hormone (MSH) or beta-acting catacholamines, melanosomes disperse into distal melanophore arms.

In this study we have identified two morphologically distinct classes of melanophores, both of which are hormone and light-sensitive. The studies presented herein included melanophores of both populations, although the vast majority of melanophores examined were of the distinct-arm type, since they were the most prevalent. It is not known if these two classes represent real melanophore populations in vivo, or if they are an artifact of culture. It should be noted, however, that based upon melanosome size, Taylor and Bagnara (1970) were able to identify two types of MSH-responsive melanophores in the skin of this lizard.

Pigment dispersion, regardless of stimulus, is sensitive to cytochalasin B and, thus, presumably, requires microfilaments (Malawista, 1971), whereas pigment aggregation after light stimulation apparently requires microtubules (Vaughan, 1987), which have been shown to be present in the melanophore arms of this lizard (Alexander and Fahrenbach, 1969). Similar microfilament and microtubule requirements have been reported for amphibian melanophores (Moellman et al., 1973, Hadley and Castrucci, 1987).

As in intact skin, cAMP and calcium both trigger melanosome dispersion. Numerous previous reports have identified the cAMP pathway as a component of the hormone-mediated response in both A. carolinensis (Vaughan and Beam, 1994; part III) and other animals (Sawyer et al., 1983). However, in the teleost T. Mossambica, calcium antagonizes cAMP-mediated pigment dispersion (Oshima et al., 1988; Thaler and Haimo, 1990), whereas decreased calcium during aggregation of P. scalare melanophores is not causal (Sammak et al., 1992). Hadley et al. (1981) suggest decreasing cytosolic calcium may accompany the MSH response in A. carolinensis. Since the MSH response remains intact in the presence of lanthanum or BAPTA-AM, our results do not dispute this claim. Indeed, an alternative explanation for the BAPTA-AM induced pigment dispersion in 7 of 12

melanophores tested is that the agent is MSH-mimetic. However, the inhibitory role of both BAPTA-AM and lanthanum on light-mediated pigment dispersion provides clear evidence for the necessity of elevated intracellular calcium during the light response.

It should be pointed out that a limitation of our study is that because we were unable to induce proliferation, we were, by necessity, working with a dishomogeneous primary culture, hence our sample sizes were necessarily small, and our observations were qualitative only. Thus, subtle effects of agents on rate or magnitude of dispersion may have gone unnoticed.

Despite numerous attempts to induce proliferation, photosensitive melanophores have only been maintained for up to 3 weeks in culture with our technique. Rohrllich and Rubin (1975) reported that Anolis chromatophores only live several days. They attributed the short lifetime in part to cell damage during their enzymatic and mechanical digestion. Our method is less harsh, using one fifteenth the enzyme diluent, and we have noticed that the most highly responsive melanophores are usually associated with nearly undigested tissue, probably indicating that mild digestion conditions best preserve cell integrity.

A second limitation to our study is that only melanophores that would respond to light were tested for responsiveness to other agents. We have casually observed many melanophores in culture that would respond to MSH while only responding modestly, if at all, to light. Furthermore, we have observed melanophores with dispersed pigment that will aggregate in response to norepinephrine, but will disperse pigment again when norepinephrine is removed from the bathing saline.

These melanophores that did not produce good light responses or would not remain dark-aggregated in the absence of norepinephrine were regarded by us as being damaged by the culturing process. However, it is possible that they make up distinct populations of melanophores whose controlling mechanisms may add subtle complexities to the production of skin color.

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Part VII

Energy Utilization and Phosphorylation
in the Dermal Photic Response of the Lizard, Anolis
carolinensis

ABSTRACT

Skin of the lizard, Anolis carolinensis, changes color from green to brown when stimulated with visible light, melanocyte-stimulating hormone, or β -acting catecholamines. Hormone-induced darkening appears to utilize a cAMP intermediate, whereas the light-induced response apparently utilizes calcium and cGMP. However, the pathways apparently converge at a common microfilament-mediated dispersion mechanism.

Utilizing a protein-phosphorylation assay, we determined that a 57 kd protein is phosphorylated in vitro when lizard skins are treated with light or melanocyte stimulating hormone. Phosphorylation of this protein is inhibited when 8-Br-cGMP, an inhibitor of DPR, is included in the phosphorylation buffer. Previous researchers have demonstrated the phosphorylation of a 57 kd protein in fish chromatophores in response to MSH and have hypothesized this protein to be the molecular switch whose phosphorylation state determines the direction of pigment movement. We suggest a similar mechanism in A. carolinensis melanophores and further suggest that phosphorylation of this protein may be the point of convergence for light and hormone-mediated dispersion.

In the presence of the phosphatase inhibitors okadaic acid or sodium β -glycerophosphate (10 μ M, n=5), melanophores undergo light-independent dispersion, suggesting that a phosphatase is active in maintaining the aggregated state. Inhibition of a phosphatase and/or stimulation of a kinase might bring about phosphorylation of the molecular switch.

Finally, although phosphorylation triggers pigment dispersion, utilizing the metabolic inhibitors, 2,4 dinitrophenol, potassium cyanide or sodium azide, we demonstrate that since pigment disperses in the presence of any of these agents (10 μ M, n=5), aggregation is apparently the energy-requiring step, a conclusion in complete agreement with studies on fish chromatophores.

INTRODUCTION

The skin of the lizard, Anolis carolinensis, can change color from green to brown when stimulated with visible light or hormones such as melanocyte-stimulating hormone (MSH) or β -acting catecholamines (Goldman and Hadley, 1969; Vaughan, 1987; part III). The tonic skin color (green) results from the selective reflection of blue light from dermal iridophores and its subsequent filtering through superficial yellow xanthophores. Skin darkens when pigmented melanosomes within dermal melanophores migrate distally into dendritic arms superficial to the xanthophore and iridophore layers (Taylor and Hadley, 1970). Using visible light or appropriate pharmacology, dispersion and reaggregation of melanosomes can be induced many times in vivo, in isolated skin samples (Vaughan, 1987), and in individual melanophores in culture (part VI).

The light response appears to be functionally compartmentalized from the MSH-response, possibly due to different signal transduction mechanisms. Hormone-induced darkening appears to utilize a cAMP intermediate, whereas the light-induced response apparently utilizes calcium and cGMP (part III, part IV; Beam and Vaughan, 1994; Beam and Vaughan, 1995; Beam and Vaughan, 1996). However, the

pathways apparently converge at a common microfilament-mediated dispersion mechanism (Vaughan, 1987, part VI).

An important issue yet to be examined is the mechanism by which the two pathways intersect. Somehow, either calcium or cAMP will activate pigment dispersion, possibly via a bifunctional motor protein. The purpose of this paper is to attempt to elucidate this agent.

MATERIALS AND METHODS

Animals

The animals used in this study were male lizards of the species, Anolis carolinensis, recently captured from wild populations in coastal Louisiana and purchased from Snake Farm (LaPlace, LA). Individuals were maintained in group colony on a 12 hour light/dark cycle at 21°C. For each experiment an animal was decapitated, and the dorsal torso skin was removed and floated on physiological saline (in mM/L: 111 NaCl, 2.0 KCl, 1.0 CaCl₂, 2.0 NaHCO₃, 5.0 glucose at pH 7.2) in the dark for at least 45 minutes prior to use. Under these conditions, skin stabilizes to exhibit a tonic green color. Variation from this condition of maximal reflectance in the visible spectrum was measured reflectometrically as previously described (part III).

Cell Culture

The cell culture reagents and technique were previously described (part VI).

Pharmacological Agents

In order to examine the effects of phosphatase inhibition on melanophore dispersion, 100 μ M sodium β -glycerophosphate (Sigma Chemical Company, St Louis, MO) or 10 μ M okadaic acid (Calbiochem, La Jolle, CA) were tested for effects on cultured melanophores. In order to examine

the energy requirements of pigment movement, the effects of metabolic poisoning with 10 μ M 2,4 dinitrophenol, 10 μ M sodium azide, or 10 μ M potassium cyanide (Sigma) were examined.

Protein Phosphorylation Assay

Three skin samples (approximately 1.5 cm X 1.5 cm, 50 mg) from each animal (n=3) were assayed for protein phosphorylation, at the point of maximal DPR, maximal MSH response, or untreated and after dark adaptation (control).

Upon attainment of maximal physiological response, skin samples were immediately frozen in liquid nitrogen. Frozen samples were ground with the aid of glass beads in a ceramic mortar precooled with liquid nitrogen. Powdered skin was resuspended in 0.80 ml aliquots of ice-cold kinase buffer (10 mM sodium acetate, 10 mM magnesium chloride, 0.1 mM calcium acetate, 1 mM PMSF, 1 mM DTT, adjusted to pH 6.5) and centrifuged (4000G, 5 min. at 4°C). Protein concentration was determined using the method of Bradford (1976) and a bovine serum albumin (BSA) standard curve. Twenty micrograms of protein homogenate was diluted to a final volume of 20 μ l in kinase buffer or kinase buffer containing 1 mM 8-Br-cGMP (Sigma) in plastic 1.5 ml microcentrifuge tubes.

Phosphorylation reactions were initiated by the addition of 5 μ l (specific activity 1000 Ci/mmol) γ P³²-ATP (New England Nuclear, Boston, MA). After 1 hour in a 37°C water bath, reactions were terminated by the addition of 25 μ l SDS-sample buffer (40 mM TRIS-base, 10% glycerol, 4% sodium dodecyl sulfate, 400 mM DTT, 0.4% bromophenol blue), submerged in a boiling water bath (5 minutes), and centrifuged (14K x G, 5 minutes). A 25 μ l volume of the supernatant (total = 10 μ g per lane) was loaded into precast 10-20% high resolution gradient minigels (Zaxis, Hudson, Ohio), and subjected to SDS-polyacrylamide electrophoresis (155 V, 2 hours).

Upon completion of electrophoresis, gels were stained with colloidal coomassie blue (Zaxis, Hudson, Ohio) per manufacturers recommendation. Following destaining, gels were dried overnight and subsequently used to expose Medcor XR-4 X-ray film (12 hours, -80°C, with intensifying screens).

RESULTS

Treatment of intact, cultured, melanophores with okadaic acid (10 μ M) or sodium β -glycerophosphate (10 μ M) elicits rapid and complete light-independent pigment dispersion (plate 7.1). This result suggests that protein phosphorylation is necessary for pigment dispersion and that a phosphatase is active in maintaining pigment aggregation under control conditions.

Phosphorylation of a single 57 kd protein (P57) was detected when skins were darkened with MSH or light. This phosphorylation was completely inhibited in the presence of 8-Br-cGMP. Rather, phosphorylation of a >250 kd protein was detected in the presence of this agent (figure 7.1). Whether phosphorylation of either of these proteins is due to an increase in kinase activity, or a decrease in phosphatase activity (or both) has not been determined.

Finally, experiments utilizing metabolic inhibitors suggest that energy is required to maintain the aggregated state, but not the dispersed state. Using 2,4 dinitrophenol, sodium azide, or potassium cyanide, we were able to demonstrate that equilibration with any of these agents (10 μ M, 30 minutes) produces pigment dispersion (plate 7.2) in cultured melanophores.

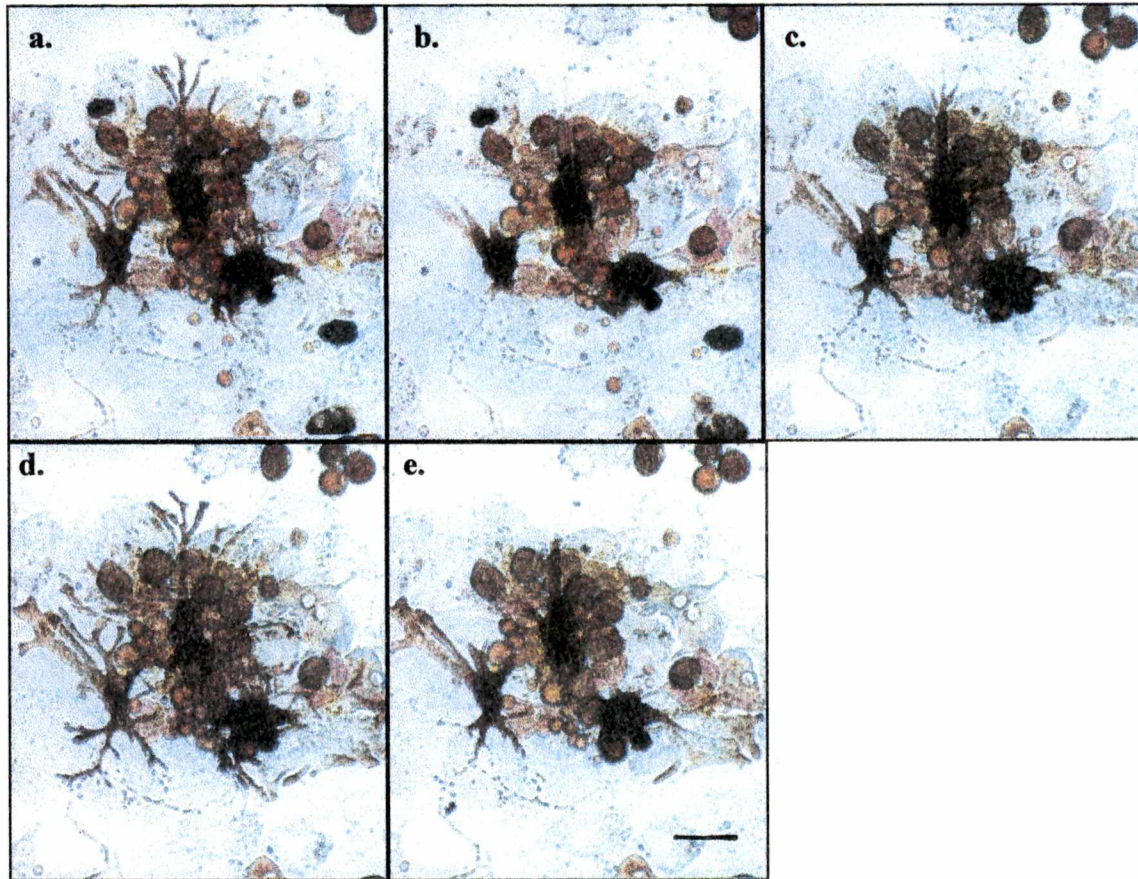


Plate 7.1: Typical Effect of the Phosphatase Inhibitor, Okadaic Acid, on Cultured Melanophores. Melanophores (n=5) undergo photodispersion(a), dark-aggregation (b), disperse pigment in the dark after 10 minutes incubation with 10 μM okadaic acid (c), undergo further photodispersion (d), with very little dark aggregation (e). Similar effects are seen with sodium β -glycerophosphate. Bar = 10 μ .

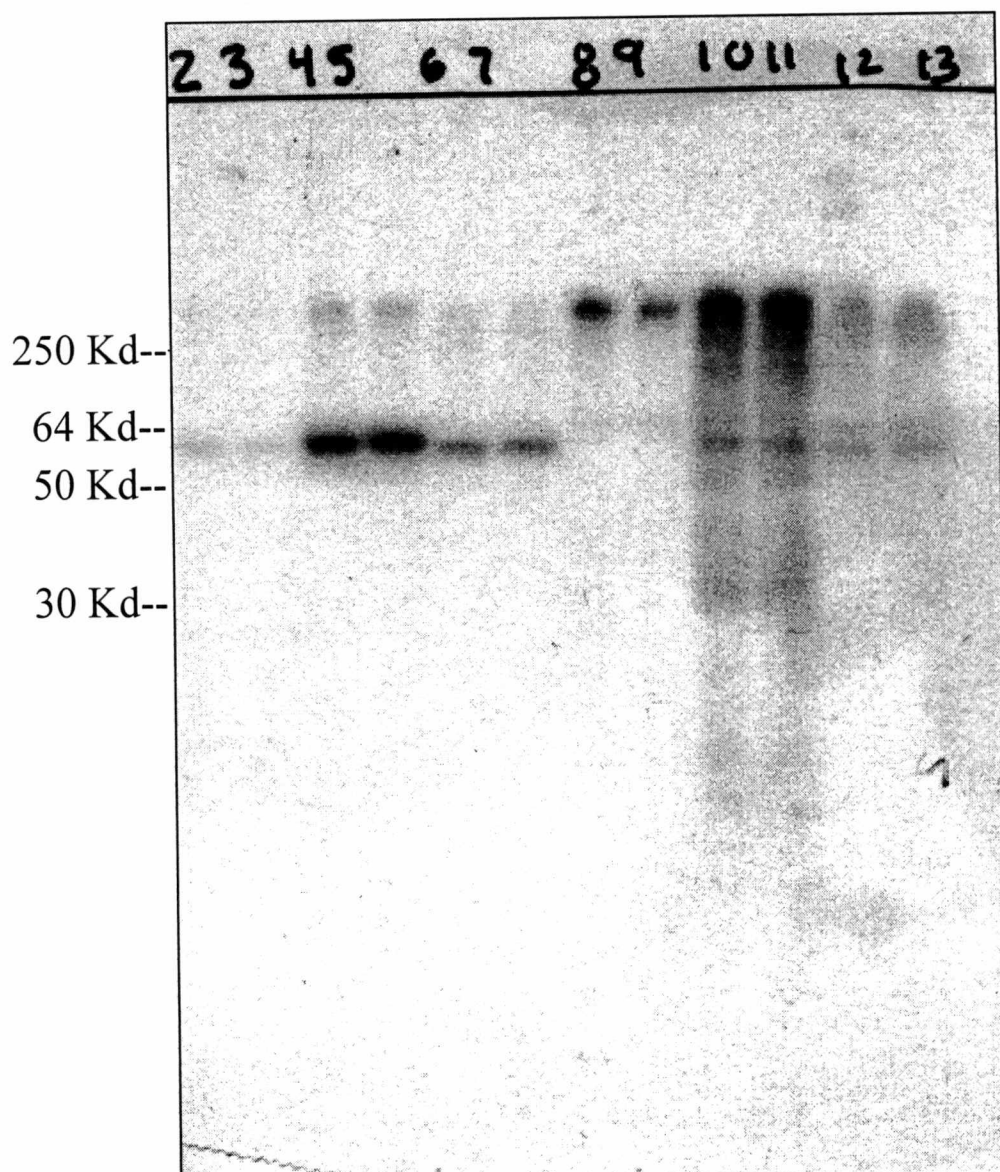


Figure 7.1: Autoradiogram Showing Protein Phosphorylation in Lizard Skin. Protein phosphorylation is shown in the control (lanes 2-3), light-stimulated (lanes 4-5), MSH-stimulated (lanes 6-7), control + 8-Br-cGMP (lanes 8-9), Light + 8-Br-cGMP (lanes 10-11), and MSH + 8-Br-cGMP (lanes 12-13) conditions.

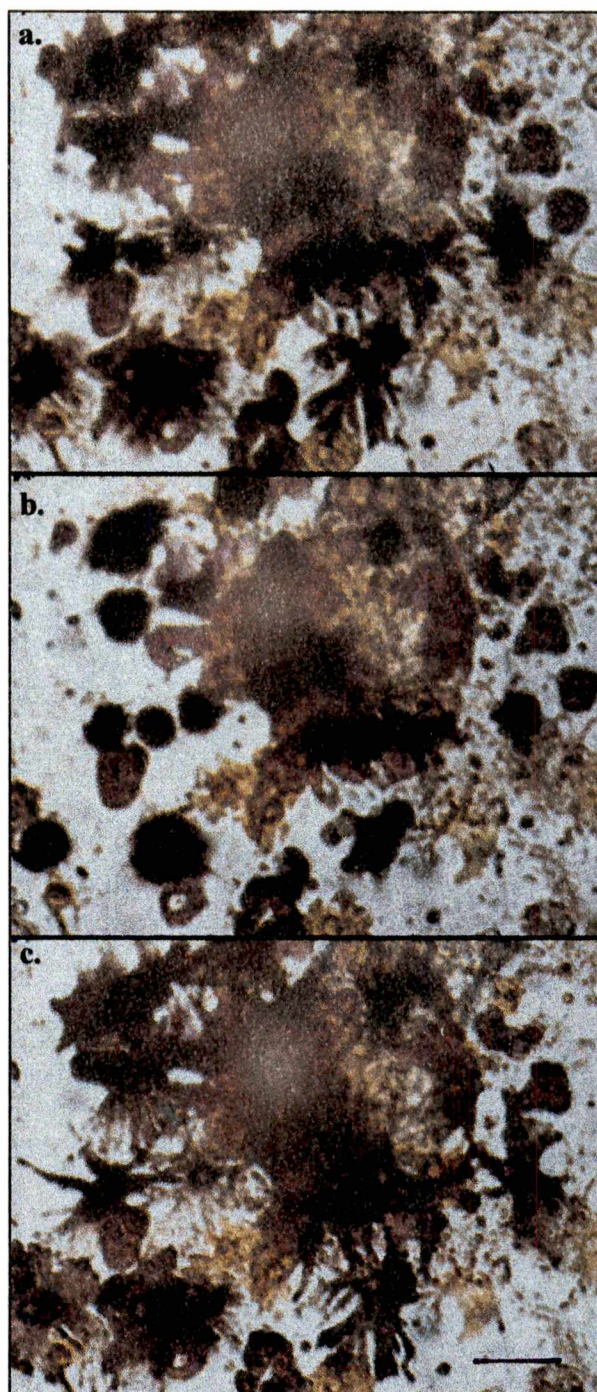


Plate 7.2: Typical Effect of 2,4 Dinitrophenol on Melanophores. Melanophores undergo photodispersion (a), dark-aggregation (b), and dark dispersion (c) in the presence of 2,4 dinitrophenol ($10\ \mu\text{M}$, 1 hour). Similar effects are seen with sodium azide or potassium cyanide ($n>5$). Bar = $10\ \mu$.

DISCUSSION

It has been previously reported that okadaic acid mimics MSH-induced dispersion in melanophores of Xenopus laevis (Cozzi and Rollag, 1992) and that changes in phosphorylation of a 58 kd protein correlate with MSH-induced melanosome dispersion in this animal (de Graan and Eberle, 1987). Similarly, phosphorylation of a 57 kd protein initiates pigment dispersion in goldfish xanthophores (Lynch et al., 1986a). It was further demonstrated that this protein is directly phosphorylated by the cAMP-dependent protein kinase (Lynch et al., 1986b) with little or no phosphorylation occurring from alternate kinases (Yang et al., 1989). Finally, phosphorylation of a 57 kd protein has been shown to be causal to pigment dispersion in the melanophores of the cichlid, Tilapia mossambica, and is thought to be the molecular switch which determines the direction of pigment movement (Rozdzial and Haimo, 1986).

In A. carolinensis, either visible light or MSH will initiate pigment dispersion (Vaughan, 1987; part III). Although the signal-transduction pathways are apparently compartmentalized, previous evidence suggests that the proximal mechanism of pigment movement is the same for each (Vaughan, 1987; part VI). In this study, we demonstrate the phosphorylation of a 57 kd protein in response to light or MSH, and infer from the effects of phosphatase inhibitors on

pigment dispersion that phosphorylation of this protein is causal to melanosome dispersion. Furthermore, if this protein is in fact the biochemical switch, phosphorylation of this protein may be the point of intersection of the light and MSH pathways.

The MSH pathway has been shown to involve a cAMP intermediate (Beam and Vaughan, 1994; parts III,IV), and it seems logical to suggest that P57 is phosphorylated by the cAMP-dependent protein kinase. The light-dependent pathway appears to utilize calcium as well as cGMP (Beam and Vaughan, 1994, 1995, 1996; parts III, IV, VI). Whether the calcium/calmodulin kinase can also phosphorylate P57, or whether calcium acts in a different mechanism (perhaps by inhibiting a phosphatase?) to bring about phosphorylation is not known. Furthermore, it is possible that light utilizes another as yet unknown intermediate since calcium alone in the control assay buffer does not induce phosphorylation of P57.

In any event, the mechanism of the inhibitory effect of 8-Br-cGMP on DPR can now be hypothesized, since this agent inhibits phosphorylation of P57. Interestingly, this agent is as effective at inhibiting light-dependent phosphorylation as it is at inhibiting MSH-dependent phosphorylation, although we have been unable to demonstrate an inhibitory effect of 8-Br-cGMP on the MSH response

(Vaughan and Tallent, 1988; Beam and Vaughan, 1994; part III; part VI). Furthermore, in the presence of 8-Br-cGMP, a >250 kd protein is phosphorylated instead of the presumed molecular switch. The function, action, and implications of phosphorylation of this protein are as yet unknown.

The ability of 8-Br-cGMP to inhibit MSH-dependent phosphorylation of P57 in vitro may be further evidence for the compartmentalization of the light response in vivo (part III). In the presence of cGMP or a bioactive analog, compartmentalization of a cGMP effector molecule would result in a net decrease in the light-dependent phosphorylation only. In the phosphorylation assay, cell disruption may destroy this compartmentalization, and the cGMP effector molecule is present homogenously throughout solution where it can inhibit net MSH and light-dependent phosphorylation.

Although phosphorylation is necessary to trigger pigment dispersion, studies with metabolic inhibitors suggest that pigment aggregation, and not dispersion, is the energy-dependent process. Previously, Horowitz (1958) demonstrated the reversible darkening effect of 2,4 dinitrophenol or pyocyanin on A. carolinensis skins, and, combined with the observation that dead skin is always brown, suggested that pigment aggregation is the energy consuming phase of the aggregation/dispersion cycle.

Our demonstration of the melanosome dispersing effect of sodium azide, 2,4 dinitrophenol, or potassium cyanide in cultured cells agrees perfectly with the observations of Horowitz. However, we have noticed that dead skins are always brown only if calcium is present. Skins equilibrated in EGTA saline for one week at room temperature remain green even after decomposition. That dead skins can be either brown or green suggests that the existence of melanosomes in the aggregated or dispersed state is not energy requiring per se. Rather, the dispersing effect of metabolic inhibitors may be due to an increase in cytosolic calcium, perhaps via cell depolarization (parts III, IV) and inhibition of calcium sequestering ATPases (part IV). Similarly calcium released during tissue death may activate the DPR mechanism. Therefore, the energy requirement for maintaining aggregation in vivo may be related to inhibiting the light-dependent calcium-release mechanism of pigment dispersion.

The utilization of energy for MSH-dependent dispersion (and subsequent aggregation) is not known. However, all chromatophores examined appear to share a common energy requiring dynein-like motor protein for aggregation (for a review, see Schliwa, 1987). For example, pigment aggregation in Fundulus melanophores (Clark and Rosenbaum, 1982) and Holocentrus erythrophores (Beckerle and Porter, 1982) has

been shown to be sensitive to dynein inhibitors. Dynein ATPase inhibitors also inhibit both orthograde and retrograde melanosome movements in melanophores of the teleost Oryzias latipes (Negishi and Obika, 1978; Negishi et al., 1985).

Although elevated intracellular calcium does not trigger aggregation in angelfish melanophores (Sammak et al., 1992), aggregation movements in Holocentrus erythrophores are triggered by an increase in cytosolic calcium (Luby-Phelps and Porter, 1982; Stearns and Ochs, 1982; McNiven et al., 1986). Furthermore, studies utilizing lysed, ATP-depleted melanophores of T. mossambica indicate that aggregation apparently requires elevated cytosolic calcium, dephosphorylation of the molecular switch by calcineurin, and ATP energy input. Dispersion, however, appears to only require a phosphorylation event. Studies utilizing the ATP analog, ATP γ S, which is a substrate for the cAMP-dependent protein kinase but not the known force-generating ATPases, suggests that phosphorylation of the 57 kd protein alone may be sufficient to induce dispersion (Rozdzial and Haimo, 1986; Thaler and Haimo, 1990). The similarities in the use of a 57-58 kd molecular switch (A. carolinensis, T. mossambica, and X. laevis) probably

represent a high degree of conservation of the chromomotor mechanism.

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Part VIII.

Demonstration of Physiologically Active Iridophores
in the Skin of the Lizard, Anolis carolinensis

ABSTRACT

Physiological activity of the iridophore in the skin of Anolis carolinensis has previously escaped detection. Previous studies have suggested that the iridophore serves as a passive, selective, reflector of blue light. Utilizing our technique to culture A. carolinensis chromatophores, we demonstrate that the iridophore, normally transmitting red light, transmits blue light when stimulated with melanocyte-stimulating hormone (10^{-10} M). Visible light has no effect.

The mechanism of color change appears to involve microtubules and elevated levels of adenosine 3',5'-cyclic monophosphate (cAMP) but not calcium or guanosine 3',5'-cyclic monophosphate (cGMP) since isobutylmethylxanthine (IBMX), Sp-cAMP, or colchicine are all hormone-mimetic, whereas the calcium ionophore a23187 and 8-Bromo-cGMP are without effect.

By measuring the transmittance spectra of control and MSH-stimulated iridophores, and assuming the thin layer interference model to be operative, we construct theoretical reflectance spectra and demonstrate a maximal reflectance shift from blue-green to red, with the total amount of reflected light remaining unchanged.

Although there are many inherent difficulties which make measuring the spacing of iridophore crystals unfeasible, assuming thin layer interference and a non-ideal

reflector, we calculate that sheets of iridosomes are separated by 41 nm of cytoplasm in the control state. The spacing increases to 97 nm in stimulated iridophores. The demonstration that hypotonic saline, which presumably increases crystal spacing, shifts reflectance to the red, whereas hypertonic saline shifts reflectance toward the blue further supports the thin-layer interference model.

Although the actual role of the physiologically active iridophore in color change is not clearly understood, it seems probable that many of the color states not previously explainable by the current model involving only a physiologically active melanophore may be attributed to iridophore activity.

INTRODUCTION

Highly reflective chromatophores called iridophores or gaunophores are nearly as reflective as polished metal (Huxley, 1972) and are integral components of the dermis of many vertebrates and invertebrates (Bagnara, 1966; Cloney and Brocco, 1983). Color is produced structurally as can be verified by the absence of colored pigment (Rayleigh, 1919).

All iridophores contain highly refractive organelles, called iridosomes or iridophore platelets. Most of these contain crystallized purines, primarily guanine, enveloped in a membrane originating from the endoplasmic reticulum (Cloney and Brocco, 1983; Morrison and Frost-Mason, 1991). In some animals, particularly several frog species (Hadley, 1966; Bagnara et al., 1968; Taylor and Bagnara, 1972) and at least one species of fish (Matsuno and Iga, 1989), the iridosomes aggregate and disperse into dendritic arms, and thus affect intensity of light reflection.

In other animals, such as many fish (Fujii, 1993), cephalopods (Brocco and Cloney, 1980; Cooper et al., 1990), Sceloporus lizards (Morrison, 1995), and the iguanid lizard, Anolis carolinensis, iridosomes are arranged in multiple layers or stacks in a well organized lattice (Rohrlich and Porter, 1972) maintained by an extensive cytoskeleton (Rohrlich, 1974) within non-dendritic cells. In these cases,

the lattice suggests that color production is due to thin-layer interference (Rohrlich and Porter, 1972).

According to the thin-layer interference model, light reflecting off boundaries between substances of low and high refractive indices will interfere constructively so as to have very high reflectivity for the ideal wavelength, with very high transmittance for non-ideal wavelengths (Land, 1966, 1972). The ideal wavelength is a function of the optical thickness (actual thickness X refractive index) of each crystal sheet. Since light reflected from the bottom surface must undergo a half-wavelength phase change in order to interfere constructively with light reflected from the top surface, the optical thickness of the crystal must, therefore, be approximately 1/4 the ideal wavelength (figure 8.1).

Maximal reflectivity is obtained when the reflector is ideal. In an ideal reflector, the optical thickness of the crystal and cytoplasmic layers are equivalent. Assuming normal incidence, the wavelength of maximal reflectance for such a system is given by the equation, $\lambda_{\max} = 4 \cdot NA \cdot DA = 4 \cdot NB \cdot DB$, where NA and NB are the refractive indices of the cytoplasmic layer and crystal layer, and DA and DB are the actual thickness (Huxley, 1968; Land, 1972). The intensity of reflected light can be greatly increased by utilizing

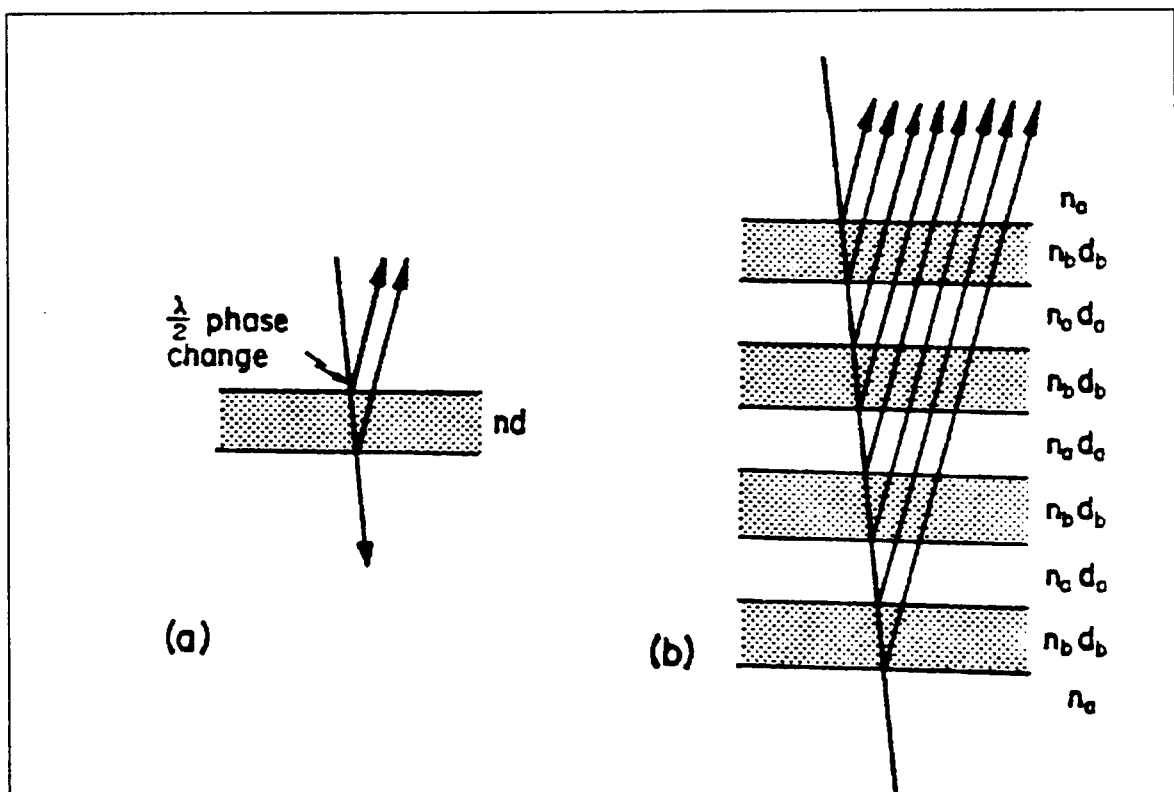


Figure 8.1: Schematic Representation of Thin Layer Interference. (a) Light reflected from the bottom surface must undergo a half-wavelength phase change in order to interfere constructively with light reflected from the top surface. (b) The intensity of reflected light can be greatly increased by utilizing multiple layers of high and low refractive index, with ten or more layers acting as a near perfect reflector for the ideal wavelength. Figure adapted from Land (1966).

multiple layers of high and low refractive index, with ten or more layers acting as a near perfect reflector for the ideal wavelength.

As iridophores deviate from ideality, reflectivity decreases, and the wavelength of maximal reflectance shifts. The peak wavelength is then given by the equation, $\lambda_{\max}=2(N_A \cdot DA + N_B \cdot DB)$, assuming normal incidence. For nonideal reflectors, bandwidth of reflectance is a function of the number of interface layers and the refractive index difference ($N_B - N_A$), which translates to roughly one third the visible spectrum for most biological systems (Huxley, 1968; Land, 1972).

Although most physiologically passive iridophores can be assumed to be ideal reflectors (Fujii, 1993; Morrison, 1995), physiologically active iridophores, which are capable of changing the color of reflected light, must, of necessity, be of the non-ideal type. Color change is effected when the optical thickness of either the crystal or cytoplasmic layers change. Changes in the optical thickness of the crystal layer can be effected by varying the tilt of the crystal (Rohrlich, 1974), whereas changes in the cytoplasmic layer can be effected osmotically (Bone and Denton, 1971; Clothier and Lythgoe, 1987) or by mechanical changes of the cytoskeleton (Rohrlich, 1974; Fujii, 1993).

An obvious conclusion of the non-ideal equation is that increasing the optical thickness of either layer will shift reflection toward longer wavelengths. A spectral shift toward longer wavelengths has been designated an "LR-response," whereas a spectral shift toward shorter wavelengths has been designated an "SR-response" (Fujii, 1993).

The skin of A. carolinensis is ordinarily green, but can change to brown when stimulated with hormones such as melanocyte-stimulating hormone (MSH) or visible light (Vaughan, 1987). The tonic green color is produced when iridophores, which had been thought to be physiologically passive, reflect blue light which is filtered by overlying yellow xanthophores to produce a green color. Red light transmitted through iridophores is absorbed by underlying melanophores (von Geldern, 1921; Alexander and Fahrenbach, 1969; Taylor and Hadley, 1970; Rohrlich and Porter, 1972).

It is generally agreed that the color change from green to brown is effected when underlying dermal melanophores disperse pigmented melanosomes into superficial dendritic arms to obscure underlying iridophores (Taylor and Hadley, 1970). This model, however, explains green and brown color only, whereas the lizard is capable of changing shades of yellow, gray, brown, and green (von Geldern, 1921). Clearly, another physiologically active chromatophore, in addition to

the dermal melanophore, must be present in the skin of A. carolinensis to account for these color changes.

A likely candidate is the iridophore. Although, previous researchers have been unable to demonstrate physiological activity of A. carolinensis iridophores (Rohrlich and Porter, 1972; Taylor and Hadley, 1972), the presence of an elaborate cytoskeletal framework attached to the iridosomes (Rohrlich and Porter, 1972) may provide the machinery necessary to tilt crystals or alter the spacing within the crystal lattice.

Previous failures to identify physiological activity may be due to the limitations of studies which examined only fixed specimens. Since differences between treatments may only involve a minute change in the horizontal spacing of platelets, without heroic effort, artifacts induced by fixatives and dehydration may obscure treatment differences (Bone and Denton, 1971; Morrison, 1995). Thus, with the recent advances in the technique to culture A. carolinensis chromatophores (Beam et al., 1996; part VI), we have been able to demonstrate physiological activity of the iridophore.

MATERIALS AND METHODS

Animals

Animals used in this study were adult males of the species, Anolis carolinensis, recently captured from wild populations in coastal Louisiana and purchased from Snake Farm (Laplace, LA). Animals were maintained in group colony on a 12 hour light/dark cycle at 21° C.

Pharmacological Agents

Colchicine, cytochalasin B, 8-bromo-3',5'-cyclic guanosine monophosphate (8-Br-cGMP), calcium ionophore a23187, isobutylmethylxanthine (IBMX), alpha-melanocyte stimulating hormone (MSH), and melatonin were purchased from Sigma Chemical Company (St. Louis, MO). The Sp-isomer of adenosine-3',5'-cyclic monophosphorothiate (Sp-cAMP) was purchased from Research Biochemicals Incorporated (Natick, MA). Stock solutions of all agents were dissolved directly in lizard Ringer's solution or DMSO where appropriate (a23187, IBMX). When DMSO was used as a solvent, the final concentration was maintained at 1.0%, a dose that had no intrinsic effect on chromatophore activity.

Cell Culture

The cell culture reagents and technique are as previously described (part VI) with one difference. Although cultured melanophores become physiologically active within

4-6 days, iridophores do not gain physiological activity for 10-14 days. Initially, iridophores appear brown to transmitted light. With time, the cells begin to transmit red light and gain hormone sensitivity.

Pharmacological Experiments

After 10-14 days in culture, iridophores were evaluated for physiological activity microscopically, according to the method described in part VI. For all pharmacological experiments, a minimum of 30 iridophores, from at least 3 wells and representing at least 3 animals, was examined.

Spectroscopy

Cells grown on glass coverslips were briefly rinsed in saline and mounted upside down on deep-welled glass slides filled with saline. The edges of the coverslip were sealed with finger nail polish to prevent evaporation. Cells were examined with a 67X objective (final magnification 670X on a Leica microscope equipped with a photomultiplier (4.0 μM^2 aperture) and a substage quartz-halogen lamp (100 W) attached to a Bausch and Lomb grating monochromator. Light transmission from 400-700 nm in 10 nm increments (bandwidth <5 nm) was compared to blank transmission through clear areas of the slide. Transmittance was expressed as percent of the blank value.

Intact Skin Recordings

Intact skin responses (n>20 animals) to MSH (10^{-7} M) were recorded reflectometrically with 600 nm light as previously described (part III).

RESULTS

Pharmacology

Cultured iridophores are typically orthogonal and clustered in groups of 10 or more. Ordinarily, these cells appear red to transmitted light, but can change to blue 3-5 seconds after the addition of MSH at very low doses (plate 8.1). We were able to visually detect color changes at doses as low as 10^{-14} M. We were unable to detect any sensitivity to stimulation with visible light (table 8.1).

The second messenger utilized by iridophores for color change appears to be adenosine 3',5'-cyclic monophosphate (cAMP), since the highly specific cAMP agonist, Sp-cAMP, or the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX), are MSH-mimetic. Although 8-Br-cGMP and the calcium ionophore a23187 had pronounced effects on cultured melanophores (part VI), these agents were without effect on cultured iridophores (table 8.1).

Spectroscopy

Spectroscopic studies of iridophore transmittance confirm a shift in transmittance from red to blue when stimulated with MSH (figure 8.2). Assuming negligible absorption, a theoretical reflectance spectrum (figure 8.3) is an inverse of the transmittance spectrum (Dr. Randall Morrison, personal communication), and appears to agree

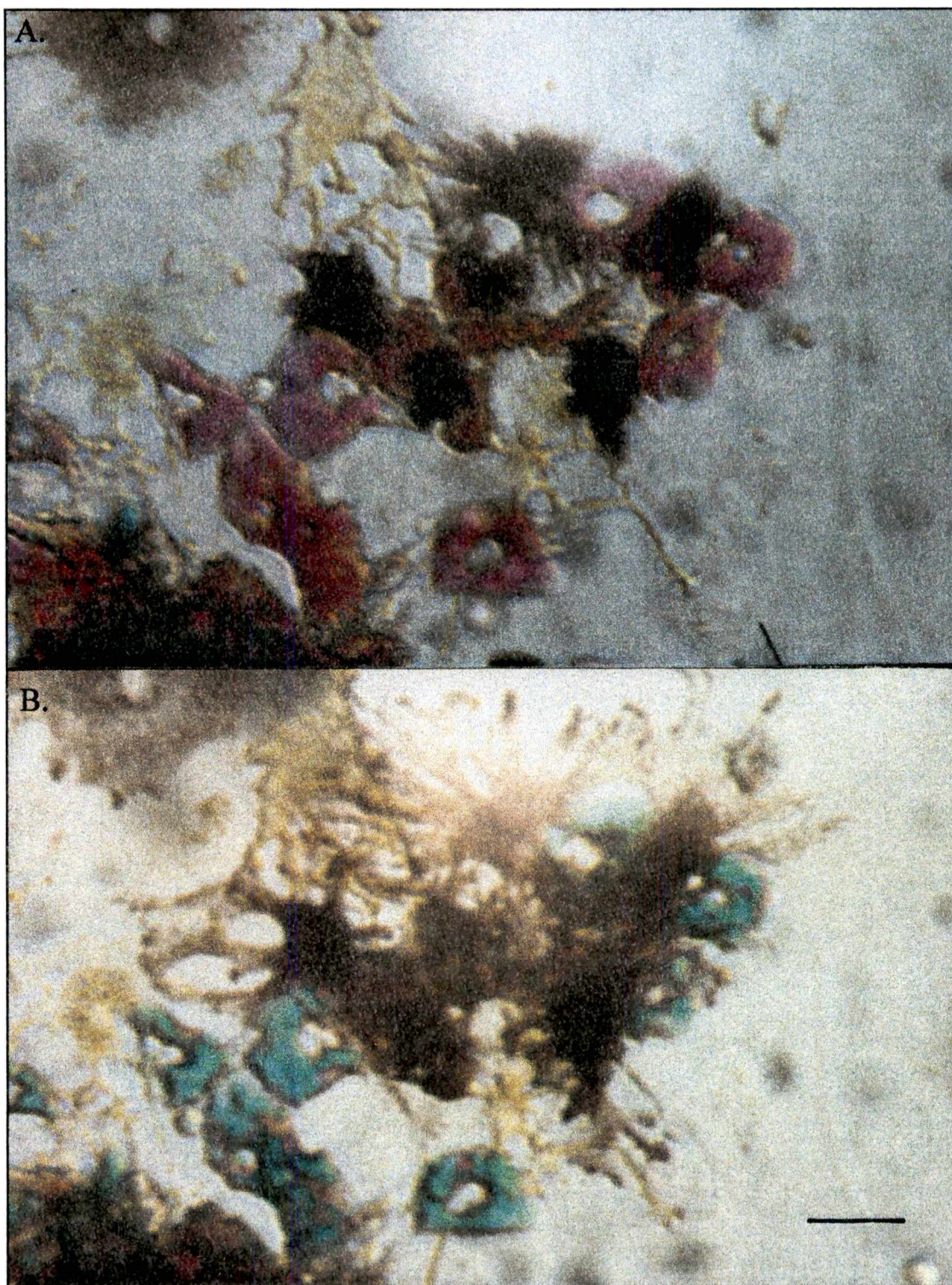


Plate 8.1: Effect of MSH on Cultured Iridophores. Iridophores in the (A) control state undergo transmittance change when (B) stimulated with MSH (10^{-10} M). Bar = 10 μ m.

Table 8.1
Effect of Various Agents on Iridophore Transmittance

Treatment	Transmitted Light	Sample Size
Control	red	>200
Visible Light (100W, 10 min)	red	50
MSH (10^{-10} M, 10 min)	blue	50
MSH + melatonin	blue	50
MSH + 8-Br-cGMP	blue	50
8-Br-cGMP (10 μ M)	red	50
a23187 (10 μ M, 10 min)	red	50
Sp-cAMP (500 μ M, 10 min)	blue	50
IBMX (10 μ M, 10 min)	blue	50

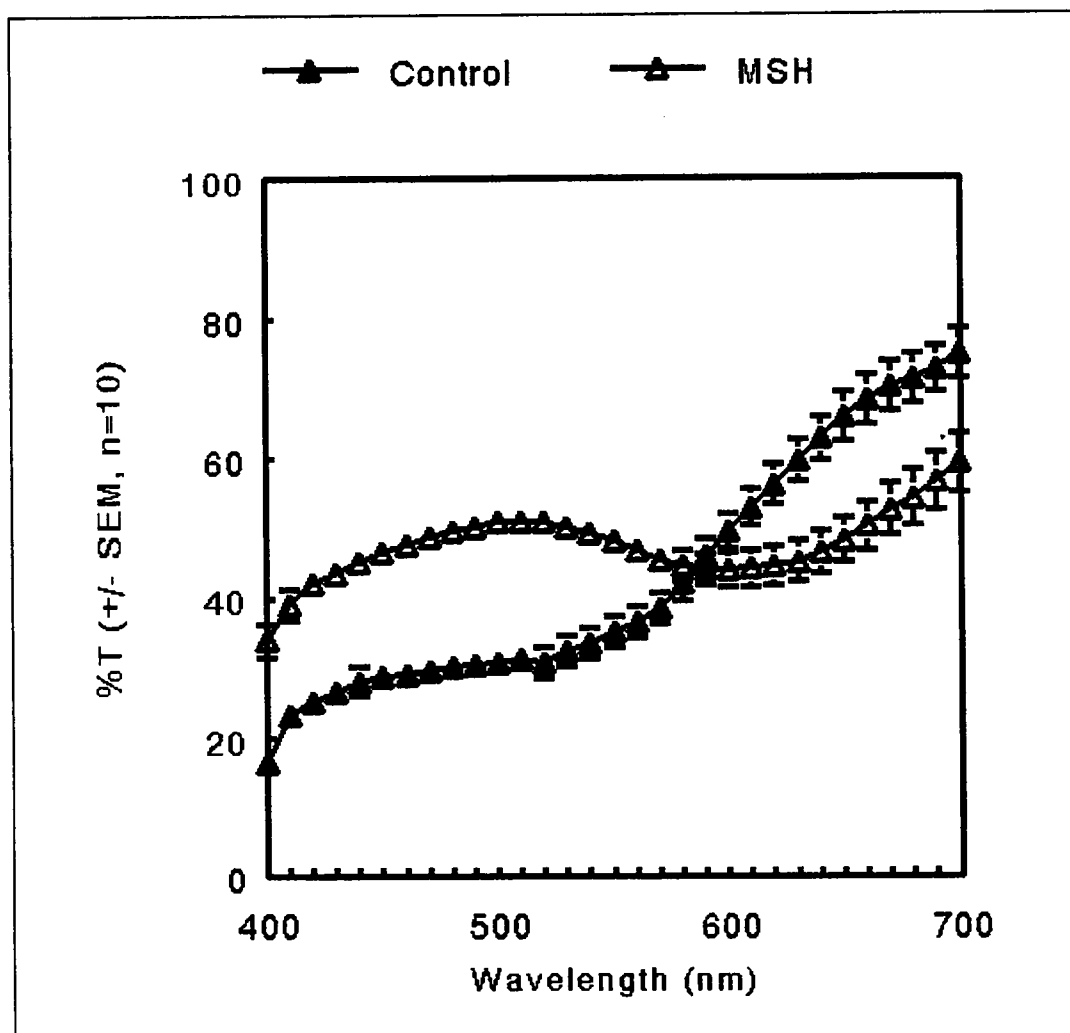


Figure 8.2: Change in Transmittance of Iridophores when Stimulated by MSH. The iridophore transmittance spectrum shifts from red (open triangles) to blue (closed triangles) when iridophores are stimulated with MSH (10^{-10} M).

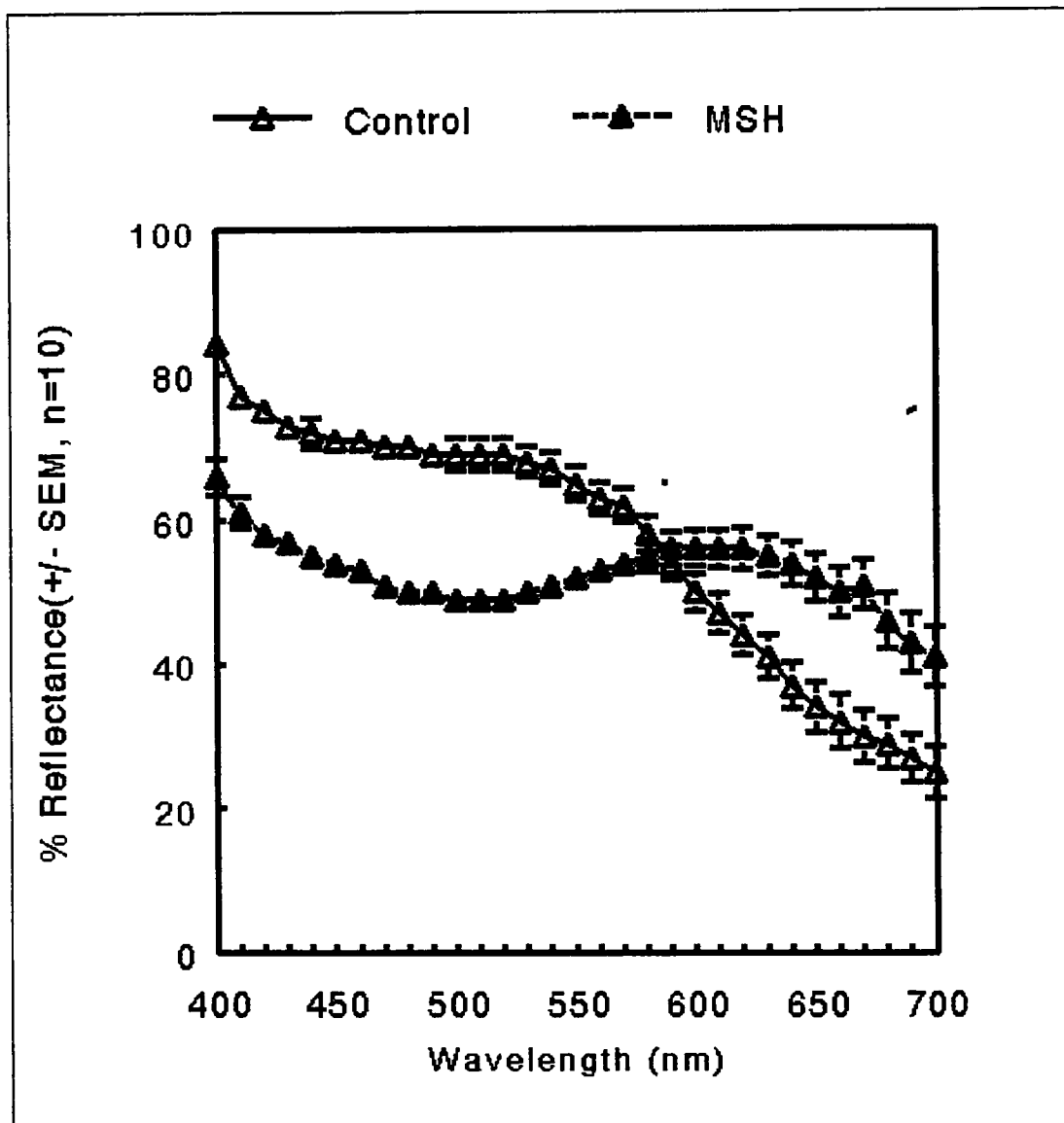


Figure 8.3: Calculated Reflectance Spectrum of Iridophores. Iridophores shift reflectance from blue (open triangles) to red (closed triangles) when stimulated with MSH (10^{-10} M).

fairly well with the observation that unstimulated iridophores reflect blue-green light (Rohrlich and Rubin, 1972). Subtraction of the transmittance spectra yields a difference spectrum (figure 8.4) which shows a shift in maximal reflectance from <500 nm to >650 nm for MSH stimulated iridophores, a "LR-response."

Presumably, reflectance changes are brought about by alterations in the spacing between horizontal sheets of iridosomes. If the thin-layer interference model is correct, variation in saline concentration should affect the transmittance/reflectance by osmotically altering the spacing between horizontal rows of platelets (Bone and Denton, 1971; Clothier and Lythgoe, 1987). Hypotonic saline should increase spacing between platelets and thus increase the wavelength of reflected light and decrease the wavelength of transmitted light. We report here that hypotonic saline (6 parts normal saline + 4 parts distilled water) shifted iridophore transmittance from red to blue ($n=15$), whereas replacement with hypertonic saline (normal saline + 96 mOsm sucrose) shifted transmittance back to red.

The actual mechanism of spacing alteration appears to involve microtubules, since colchicine alone could mimic MSH-induced color change, whereas cytochalasin B, an antagonist of microfilament-mediated mechanisms, was without

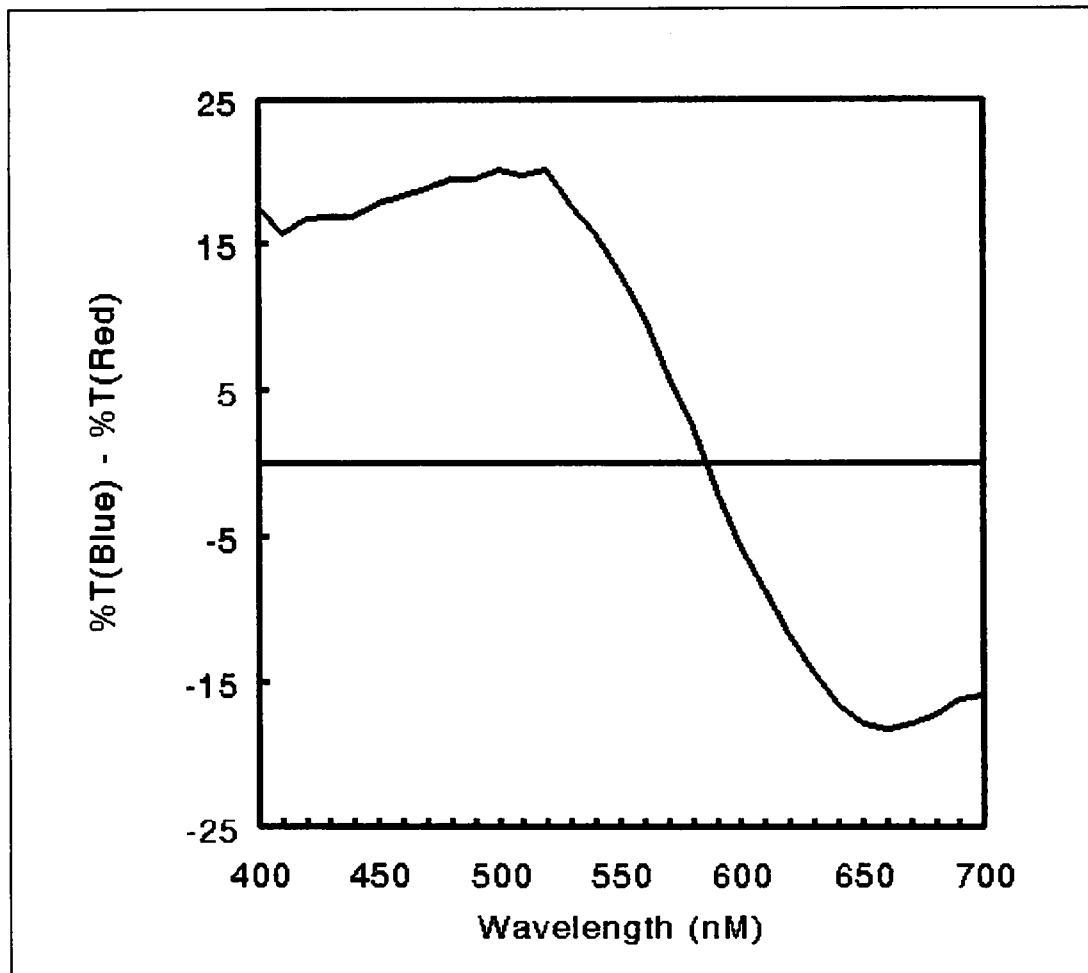


Figure 8.4: Demonstration of Iridophore "LR-response" by MSH. Difference spectrum created by subtracting the control reflectance spectrum from the MSH reflectance spectrum (figure 8.3) shows a shift in maximal reflectance from blue to red.

effect on iridophore color or MSH-mediated color change. Unlike the case of A. carolinensis melanophores (Vaughan, 1987), cytochalasin B, an antagonist of microfilament mediated mechanisms, had no obvious effect on color or MSH-mediated color change in iridophores (table 8.2).

Intact Skin Recordings

When monitoring the effect of MSH (10^{-7} M) on intact skin with 600 nm light, skins typically increase albedo 10-40% (Figure 8.5) for a short time (20-60 seconds) before undergoing the characteristic darkening response (part III). The significance of this result and its probable relationship to the activity of the iridophore are discussed later.

Table 8.2
Effect of Colchicine, Cytochalasin B, and Cytochalasin B + MSH on
Iridophore Transmittance

Treatment (10 min)	Transmitted Light	Sample Size
colchicine (1 μ M)	blue	50
cytochalasin B (100 μ M)	red	50
cytochalasin B + MSH (10^{-10} M)	blue	50

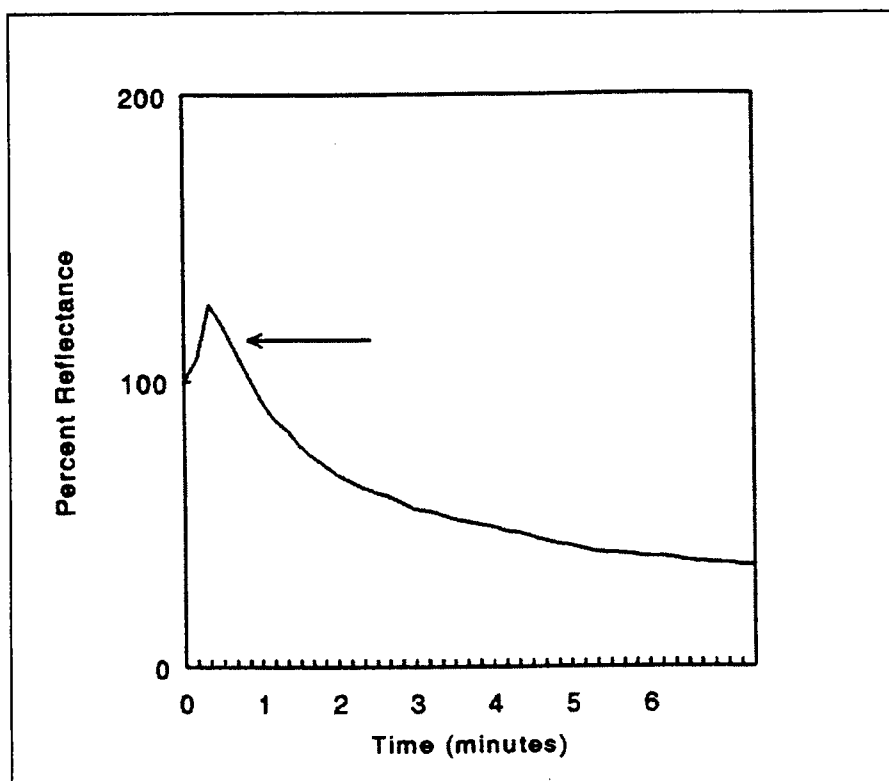


Figure 8.5: Demonstration of Iridophore "LR-response" by MSH in Intact skin. Typical chart recording of the response of intact lizard skin to MSH (10^{-7} M) as measured with 600 nm light. Skins typically increase albedo initially (arrow), before darkening.

DISCUSSION

Physiologically active iridophores have been previously found in a wide variety of animals including cephalopods (Hanlon et al., 1990), frogs (Taylor, 1969), and fish (Bone and Denton, 1971; Lythgoe and Shand, 1982; Matsuno and Iga, 1989; Nagaishi et al., 1989). In many cases, such as A. carolinensis, the presence of multiple layers of crystals arranged in a well-defined lattice suggested that color production may be due to thin-layer interference.

Rohrlich and Porter (1974) report the horizontal thickness of A. carolinensis iridosomes and the cytoplasmic spacing to be 100 nm. The cytoplasmic spacing can be assumed to be in error due to fixation artifact (Bone and Denton, 1971, Morrison, 1995), nevertheless, some elementary assumptions can be made based upon the thickness and composition of the iridosome crystal.

The iridosome crystal is primarily guanine (Rohrlich and Rubin, 1975) with a refractive index of 1.83. A cytoplasmic refractive index is typically estimated to be 1.33, the refractive index of water (Denton and Land, 1971). If one assumes A. carolinensis iridophores are ideal reflectors, $\lambda_{\max} = 4 \cdot NA \cdot DA = 4 \cdot NB \cdot DB = 4 \cdot 1.83 \cdot 100 = 732 \text{ nm}$, which is far from the blue-green (475 nm) reflectance observed.

Thus, as expected, A. carolinensis iridophores are not ideal.

If one uses the non-ideal equation and solves for cytoplasmic spacing (DA) assuming 475 nm reflection, $\lambda_{\max} = 2(N_A \cdot DA + N_B \cdot DB) = 475 \text{ nm} = 2(1.33 \cdot DA + 100 \cdot 1.83) = 41 \text{ nm}$. Calculated in the same way, an "LR-response" with a shift to 625 nm reflectance would thus require 97 nm spacing, both, when accounting for fixation artifact, within reasonable range of the observed 100 nm spacing.

Total reflectance of red and blue reflecting iridophores can be compared by calculating the area under each curve (figure 8.3), and comparing the groups with an unpaired t-test. The area under the blue-reflecting curve is calculated as being $17,676 \pm 673 \text{ nm\%}$, whereas the area under the red-reflecting curve is $16,307 \pm 603 \text{ nm\%}$, which is not statistically different ($p=.1467$, $n=10$).

Although the "LR-response" does not decrease the total reflectance of iridophores, it may still contribute to the greater magnitude (Vaughan, 1987; part III) of MSH-mediated skin darkening since less reflected blue light would decrease skin hue i.e. less blue light reflected would result in a darker green color, and red-reflected light might be quenched when filtered through the xanthophore or epidermal layers.

Furthermore, while recording MSH responses from intact skin reflectometrically with a 600 nm light, we have observed skins to increase reflectance 10-40% before the characteristic darkening response. Since the iridophore reflectance shift occurs in a matter of seconds whereas the melanophore response is complete in several minutes, it is probable that the increased reflectance may be caused by the iridophore "LR-response" which is ultimately masked by melanosome dispersion.

The actual mechanism involved in shifting iridophore reflectance may involve an increase in cytoplasmic spacing between crystal sheets. Clothier and Lythgoe (1987) suggested that such an alteration might take place by an osmotic mechanism. Other researchers have demonstrated that a tubulin-dynein mechanism is essential for spectral shifts in fish (for a review, see Fujii, 1993). Our observation that colchicine activates the spectral shift seems to implicate a microtubule system as well.

Previously, we suggested that the MSH and light responses of A. carolinensis skin are compartmentalized (Vaughan, 1987). Our further research has shown that melanophores respond to light and MSH through different signal transduction mechanisms (part III). With this work, we can now state that further compartmentalization of responses can be attributed to the type of cells involved,

since the light response only utilizes pigment dispersion in the melanophore whereas the MSH response utilizes both melanosome dispersion and spectral changes in iridophore reflectance. Furthermore, since the MSH-induced color change in both the iridophore and melanophore appear to involve elevations of intracellular cAMP, our previous work demonstrating cAMP increases in MSH-darkened lizard skins (part III) should now be interpreted as a combination from both the iridophore and melanophore populations.

In conclusion, it is interesting to speculate that color changes not previously explained in A. carolinensis skin may be attributable, at least in part, to iridophore effects. Excitement-darkening is an event clearly distinguishable from MSH-induced or light-induced skin darkening. When skins are darkened with MSH or light, the skin is uniformly dark brown. Skins darkened with epinephrine are mottled, dark green, with patches of black or brown. It is tempting to speculate that the dark green color may be caused by a shift in iridophore reflectance to longer wavelengths without blockade by superficial melanosome pigments. If iridophores reflect red light, much of this might be quenched by the xanthophore and/or epidermis, which would cause the skin to appear dark green. Verification awaits testing of catecholamine effects on iridophores. Furthermore, similarly to cultured Rana pipiens

iridophores (Bagnara and Fukuzawa, 1990), iridophores transmitting a variety of colors (particularly various shades of red, blue, purple, and white) have been seen in our cultures, leaving open the possibility that the iridophore may contribute to other skin colors.

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Part IX

Spectroscopic Verification of the Mechanism of Color
Production and Color Change in the Skin of the Lizard,
Anolis Carolinensis.

ABSTRACT

According to the current model, the tonic (green) skin color of the lizard, Anolis carolinensis, results from the selective reflection of blue-green light from iridophores which is filtered by overlying yellow xanthophores. When skin is stimulated with melanocyte-stimulating hormone or visible light, dark-pigmented melanosomes within dermal melanophores disperse into dendritic melanophore arms which terminate above and obscure lower iridophore and xanthophore layers, causing the skin to turn brown. Gray, dark green, and yellow lizards have also been observed, but the mechanism of color production in these cases is not well explained by the current model.

With our recent discovery of the physiologically active iridophore (part VIII), it is necessary to reexamine the contributions of the iridophore to skin color production and change. Iridophores undergo an "LR-response", shifting reflectance from blue-green to red, when stimulated with MSH. In order to understand the significance of the shift to color production, a reflectance spectrum of green skin and the transmittance spectra of cultured xanthophores and melanophores were measured. Using the calculated reflectance spectra of control and MSH-stimulated iridophores (part VIII), a theoretical reflectance spectrum of control skin

was constructed, which agreed quite well with the measured spectrum.

The calculated skin spectrum using the MSH-stimulated iridophore reflectance was shifted to the right, with the additional red-reflected light presumably being absorbed by dispersed melanosomes, the epidermis, or some as yet unidentified cell type. By calculating the difference of area under the calculated control and stimulated iridophore reflectance spectra, we determined that 23% less green light is reflected when the iridophores are stimulated. This may cause the darker green color produced during the "excitement-darkening" catecholamine-mediated response. Other colors, in theory, could be produced by the iridophore in response to other, as yet unknown, stimuli.

Finally, with an understanding of the mechanics of the dermal chromatophore unit and with examination of the measured xanthophore transmittance spectrum, the paradoxical method by which the xanthophore simultaneously transmits enough blue light inward to allow stimulation of blue-light sensitive melanophores while simultaneously preventing the transmission of enough blue light outward to overwhelm the production of green color can be explained.

INTRODUCTION

The color that an object presents depends upon the wavelength of reflected light. Chemical substances which transmit specific light wavelengths are known as pigments. Pigments are abundant in nature and are heavily utilized by the dye and ink industries (Simon, 1971). Pigmented organelles, contained within many dermal chromatophores, are responsible for most animal color.

Chromatophores include the red pteridine-containing erythrophores, the yellow pteridine, carotenoid, or flavin-containing xanthophores, the white carotenoid-containing leukophores, and the red, brown, and black melanin-containing melanophores (Bagnara, 1966; Taylor and Bagnara, 1972). Of these, the melanophores are the most prevalent in nature, occurring commonly in both the epidermis (Hadley and Quevedo, 1966; Quevedo, 1972) and dermis (Bagnara et al., 1968). Functional significance of melanization includes protection from ultraviolet light, free radical scavenging, thermoregulation, and concealment (Hadley, 1972; Burtt, 1981; Breathnach, 1988).

Another biological mechanism of color production is structural. These often iridescent colors are not characterized by the presence of a pigmented molecule. Rather, colors are produced by light interference as a

result of microstructure, and are characterized by the observation that the reflected color will vary with the incidence of illumination (Rayleigh, 1886).

The thin film interference model suggests that as light waves strike a crystal lattice, some wavelengths pass through the lattice whereas others are reflected back toward the source. These reflected light waves interfere with one another such that some wavelengths are enhanced while others are eliminated. Physical studies suggest that varying the spacing between lattice crystals and/or the tilt of the crystals can alter the reflected spectrum (Land, 1972).

Chromatophores that utilize structural color include iridescent iridophores and guanophores common in vertebrate eyes and the dermis of poikilothermic vertebrates. Iridophores contain stacks of crystalline adenine, guanine, or hypoxanthine reflecting platelets generally organized in a tightly bound array as the thin film interference model predicts (Setoguti, 1967; Rohrich, 1974; Cloney and Brocco, 1983).

In many animals, skin color is a result of the interaction of both pigmented and structural dermal chromatophores which are ordinarily arranged in a particular pattern referred to as the dermal chromatophore unit (Bagnara et al., 1968). The response of chromatophores to

stimuli is an alteration in the morphology of the dermal chromatophore unit. Some chromatophores are passive, contributing only to basic animal color and morphological color change via seasonal changes in pigment production. Others actively contribute to color change via pigment translocation processes or via structural alterations of the crystal lattice. Translocated pigment granules either obscure or reveal pigments of underlying chromatophores, whereas the alteration of a crystal lattice can shift the entire spectrum of reflectance (Lythgoe and Shand, 1982). The quantity, type, and activity of chromatophores varies with species (Bagnara et al., 1968).

The lizard, Anolis carolinensis, can change color from green to brown when stimulated with visible light or melanocyte-stimulating hormone (Vaughan, 1987). Additionally, the skin takes on a dark green (unpublished observation) and black mottled appearance when stressed or stimulated with β -acting catecholamines (Kleinholtz, 1938). Other colors, including shades of gray, green and yellow are observed occasionally (Carlton, 1903; von Geldern, 1921).

The tonic green skin color is produced when blue-green light, selectively reflected from iridophores, is filtered by overlying yellow xanthophores. Skin changes from green to brown when deep melanophores disperse melanosomes into

superficial dendritic arms that terminate above and obscure underlying xanthophore and iridophore layers (von Geldern, 1921, Alexander and Fahrenbach, 1969; Taylor and Hadley, 1970).

Although this model explains the tonic color and skin-darkening effect of light or MSH, it is unable to explain the darker green color produced by β -catacholamines or the sometimes observed shades of yellow (Carlton, 1903; von Geldern, 1921). Moreover, it assumes that the iridophore is a passive reflector of blue-green light (Taylor and Hadley, 1970; Rohrlich and Porter, 1972; Rohrlich, 1974). With our recent advances in the cell culture technique, we have discovered that iridophores are physiologically active, capable of undergoing a long wavelength reflectance shift from blue-green to red (part VIII). Therefore, it seems necessary to investigate the model of color production spectroscopically, in order to more carefully ascertain the role of the iridophore in color production and color change in this lizard.

MATERIALS AND METHODS

Animals

Animals used in this study were adult males of the species, Anolis carolinensis, recently captured from wild populations in coastal Louisiana and purchased from Snake Farm (Laplace, LA). Animals were maintained in group colony on a 12 hour light/dark cycle at 21° C.

Cell Culture

The cell culture reagents and technique were described in detail in part VI with modifications for iridophore culture in part VIII.

Spectroscopy

Intact Skin

Skin preparation and the photometric recording technique were described in part III. Using the variable monochromatic filter feature, reflectance spectra from control skins and these same skins darkened with MSH (10^{-7} M) were measured (n=5). In order to avoid inducing DPR, skins were illuminated only long enough to obtain reflectance measurements. Reflectance at each wavelength (400-700 nm in 20 nm increments) was normalized to the reflectance of a barium chloride standard.

Cultured Cells

Spectral transmittance measurements of melanophores and xanthophores (n=5) were accomplished essentially as described for iridophores in part VIII. No attempt was made to distinguish between the two classes (Alexander and Fahrenbach, 1969) of xanthophores distinguishable with electron microscopy.

RESULTS

Intact Skin

Figure 9.1 shows the reflectance spectrum for intact lizard skin in the control and MSH-treated states. Green (control) skin reflects predominantly in the yellow-green region (560 nm), with a small peak in the blue (420 nm). The shape of the MSH-darkened skin reflectance spectrum is nearly flat.

Cultured Cells

Figures 9.2 and 9.3 show transmittance spectra for xanthophores and melanophores respectively. As expected, the xanthophore transmits very little blue light, acting as a spectral cut-off filter passing wavelengths greater than 500 nm. Conversely, the melanophore acts as a light sink, transmitting very little light at any wavelength.

Calculation of Skin Reflectance Spectrum

We have previously reported the transmittance spectra for control and MSH-treated iridophores, as well as their calculated reflectance spectra (part VIII). These spectra, along with the xanthophore transmittance spectrum (figure 9.3) can be used to calculate a theoretical skin reflectance spectrum.

The spectrum of light reflected off the iridophore is a function of the xanthophore transmittance spectrum, which is

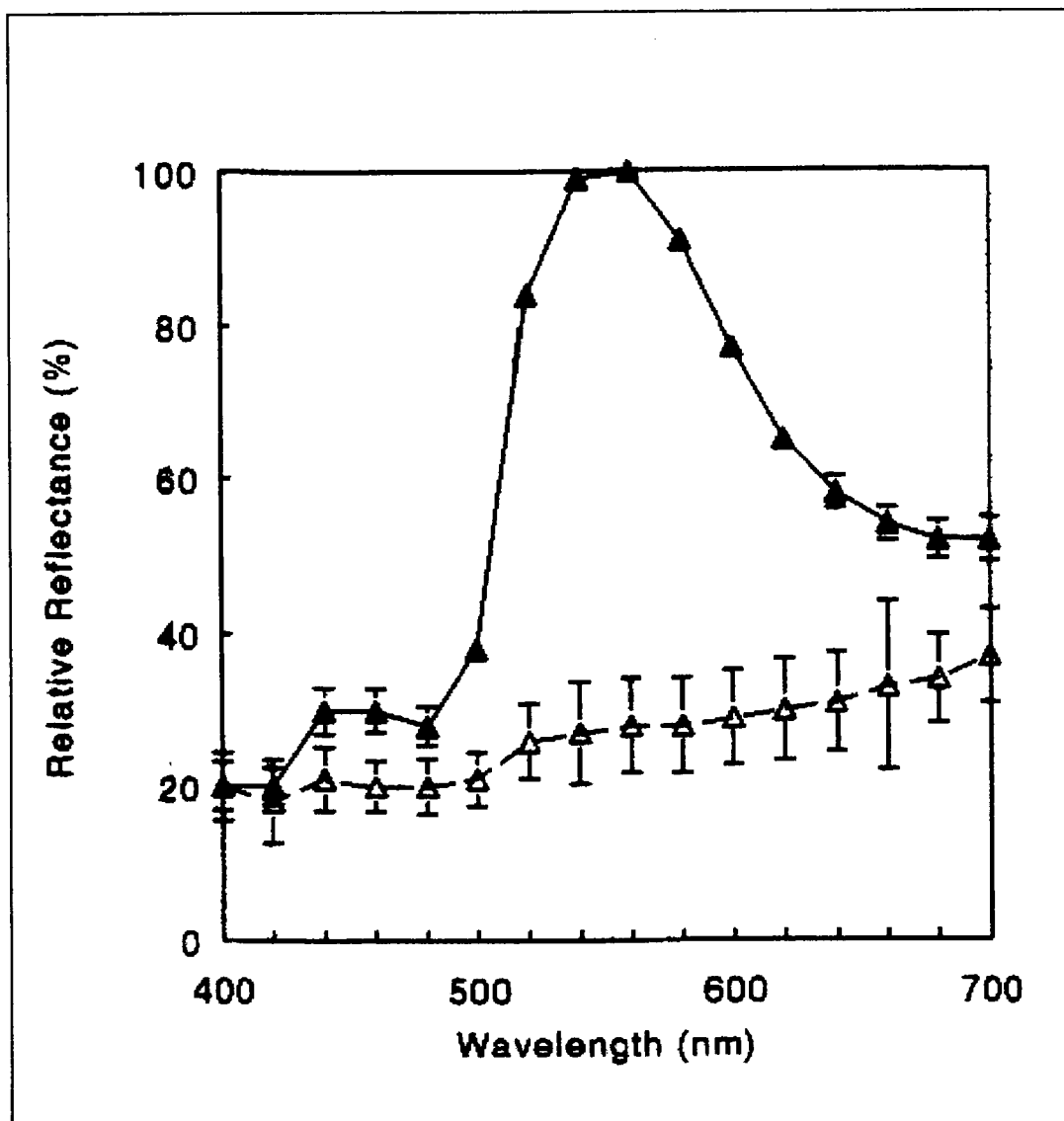


Figure 9.1: Reflectance Spectra of Green and Brown Skin. Reflectance spectra were measured from control skin (solid triangles) and skins darkened (open triangles) with MSH (10^{-7} M).

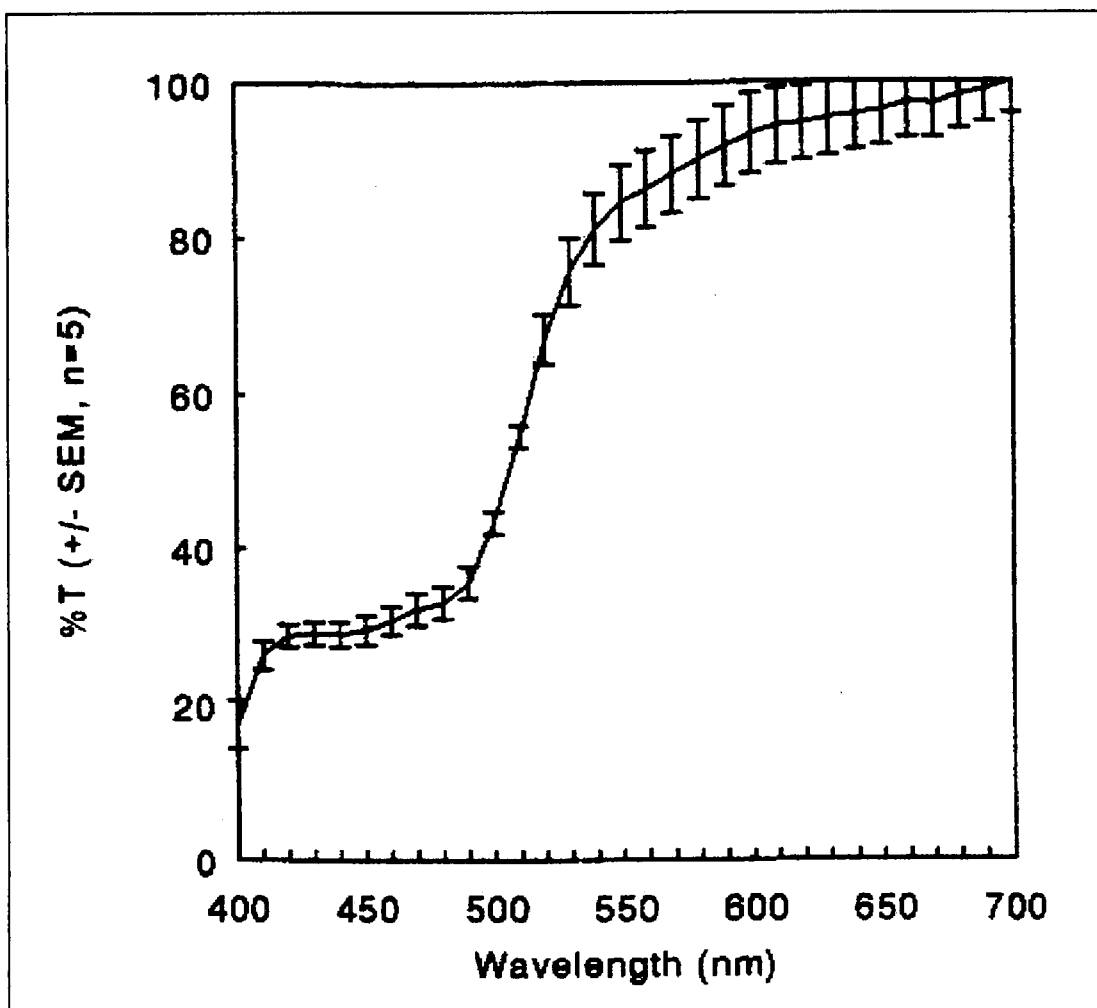


Figure 9.2: Xanthophore Transmittance Spectrum

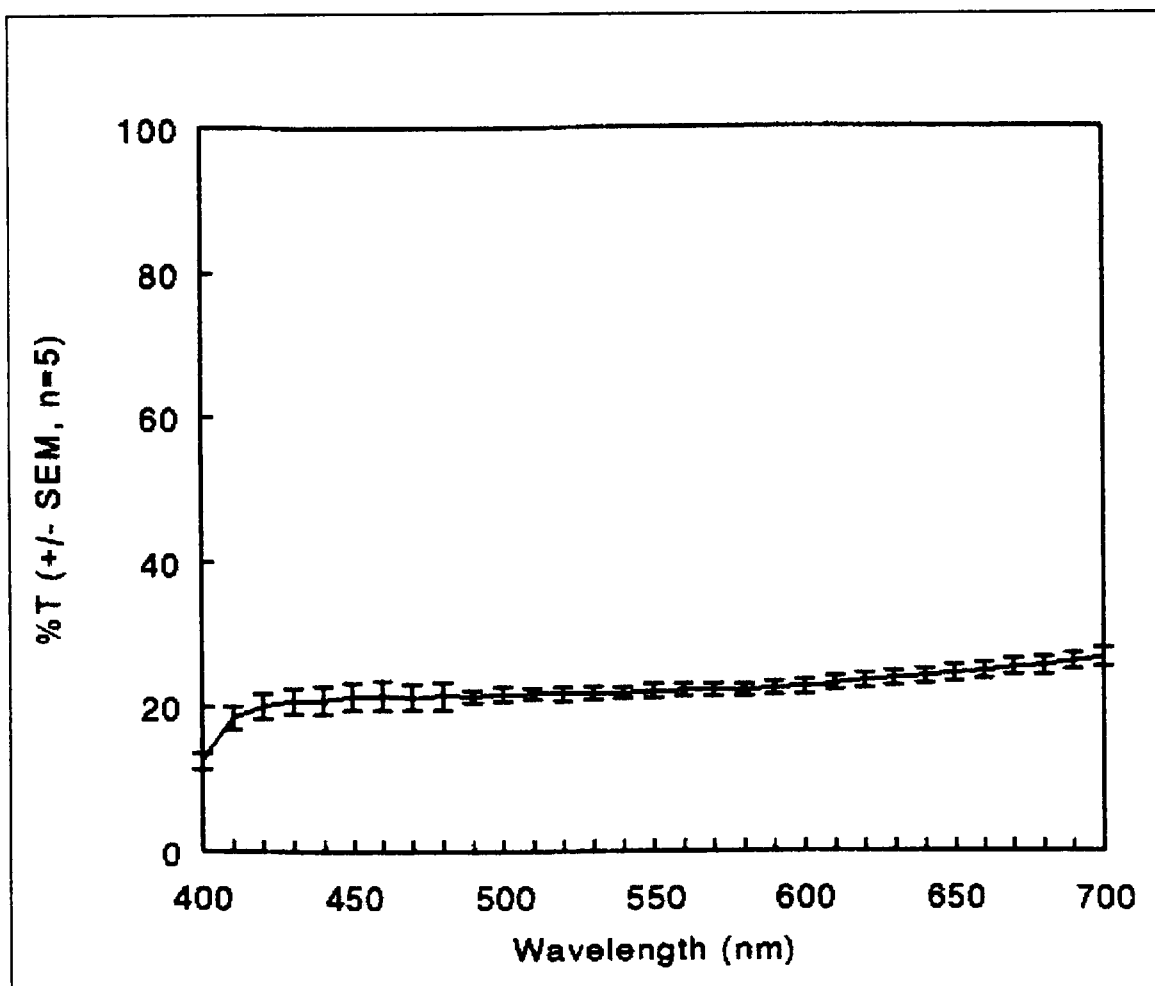


Figure 9.3: Melanophore Transmittance Spectrum

the percent of light reaching the iridophore at each wavelength, and the iridophore reflectance spectrum, which is the percent of light reaching the iridophore that is reflected at each wavelength. The reflected light can be expressed mathematically as the multiplication of 2 single-dimension matrices:

$$\text{Percent Reflected Light} = T_{\text{xanthophore}} \cdot R_{\text{iridophore}} / 100 \quad (\text{Equation 9.1})$$

where T and R are the percent transmittance and reflectance of xanthophores and iridophores respectively. The reflected light is filtered again by the overlying xanthophore, so that light reflected to the observer can be expressed as:

$$\text{Observed Light} = \text{Reflected Light} \cdot T_{\text{xanthophore}} / 100 \quad (\text{Equation 9.2})$$

where "reflected light" is the solution to equation 9.1, and "observed light" is the percent of light reflected to the observer at each wavelength.

Using these equations, the calculated green skin reflectance spectrum can be constructed, and the theoretical effect of iridophore color change on overall skin reflectance can be examined (figure 9.4).

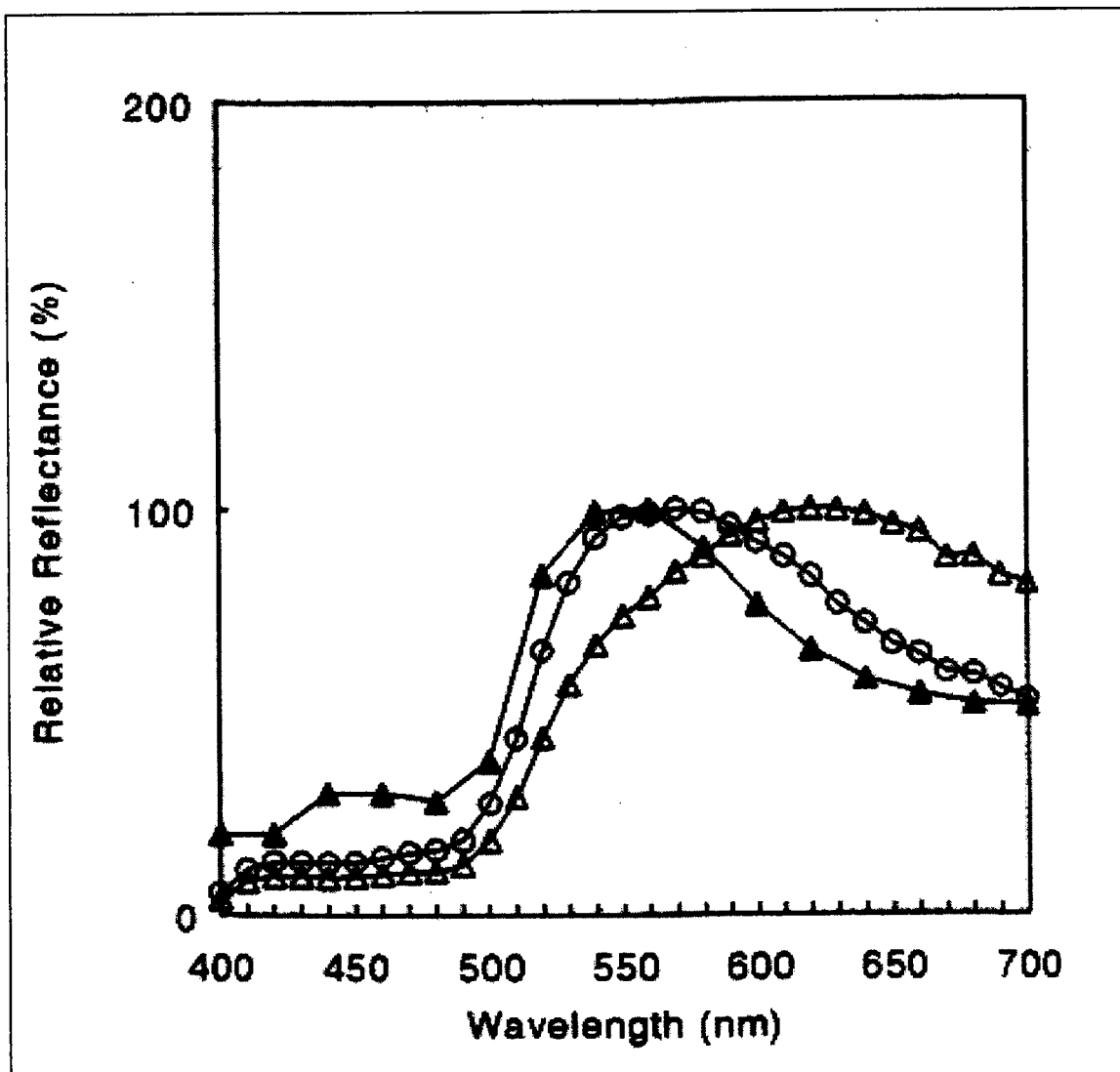


Figure 9.4: Calculated Green Skin Reflectance Spectrum Agrees with Measured Skin Spectrum. Normalized skin reflectance spectrum (solid triangles) agrees well with the calculated spectrum (open circles). Hormone-stimulated iridophores shift the calculated spectrum to the right (open triangles), decreasing reflectance in the green (500-560) region.

DISCUSSION

The calculated green skin reflectance spectrum agrees quite well with the spectral reflectance measured from control skin (figure 9.4). Differences may be attributed to extracellular and epidermal contributions, and the added filtering/reflectivity of intact skin due to multiple cell layers.

When skin is stimulated with MSH, melanosomes are dispersed into superficial dendritic arms (Taylor and Hadley, 1970), and the iridophores undergo a long-wavelength "LR" reflectance shift (part VIII). Red reflected light is absorbed by the dispersed melanosomes, and the animal appears brown.

The LR shift results in a decreased reflectance of green light during the MSH response (figure 9.4). The decrease can be estimated by calculating the area difference under the green region (500-560 nm) of the calculated skin reflectance curves (figure 9.4) for iridophores reflecting blue and red light:

$$\left(\int_{500^{\text{red}}}^{560^{\text{red}}} - \int_{500^{\text{blue}}}^{560^{\text{blue}}} \right) / \int_{500^{\text{blue}}}^{560^{\text{blue}}} = -.23 \quad (\text{Equation 9.3}).$$

Thus, physiological activation of the iridophores could result in a 23% decrease in skin reflectance in the green region with a corresponding reflectance increase in the red region.

Since melanin is an effective light sink at all visible wavelengths (figure 9.3), it is unclear what effect, if any, the LR shift has on MSH-induced color change. However, it should not go unnoted that the typical MSH response results in a greater albedo decrease than DPR, on the magnitude of about 20-40%, or roughly equivalent to the decreased green reflectance resulting from the LR shift.

It is clear, however, that if a mechanism existed to induce the LR shift independent of melanosome dispersion, skin would appear dark green, assuming that the red-reflected light were absorbed by overlying structures. In "excitement-darkening," skins or lizards treated with epinephrine appear mottled (Kleinholtz, 1938) dark green and black, due to the dispersion of select melanophore populations. It is tempting to speculate that sensitivity of the iridophore to catecholamines could produce the dark green color. The β -adrenergic antagonist, propranolol, has been shown to inhibit excitement darkening and to retard the rate of MSH-induced color change in vivo without affecting maximal darkening (Vaughan and Greenberg, 1986). Since the LR shift occurs much faster than melanosome dispersion (part VIII), it is possible that the retarded rate of MSH-induced color change is due to an effect of propranolol on the iridophore.

Since we have been unable to demonstrate any effect of visible light on the iridophore, the reflectance change

elicited during DPR appears to be caused entirely by dispersion of melanosomes superficial to the xanthophore. It is somewhat paradoxical that the xanthophore must sufficiently filter out blue light so as to allow the animal to appear green, yet also allow sufficient blue-light transmission to stimulate blue-light sensitive melanophores.

The paradox is solved in the following way. By transmitting light through the xanthophore twice, once before being reflected from the iridophore, and once afterwards, the effective reflectance of the skin in the blue region is greatly inhibited in two passes while still transmitting a significant amount of blue light in one pass through the xanthophore to the underlying melanophore arms.

By combining equations 9.1 and 9.2, the light reflected off the skin as seen by an observer would be

$$\begin{aligned}\text{Observed Light} &= (T_{\text{Xanthophore}} \cdot R_{\text{Iridophore}}) T_{\text{Xanthophore}} / 100^2 \\ &= T_{\text{Xanthophore}}^2 \cdot R_{\text{Iridophore}} / 100^2 \quad (\text{Equation 9.4}).\end{aligned}$$

Where T and R are percent transmittance and reflectance respectively, and "Observed Light" is the percent of illuminating light reaching the observer at each wavelength.

Thus, the actual filtration accomplished by transmitting light through the xanthophore twice is proportional to the xanthophore transmittance spectrum to

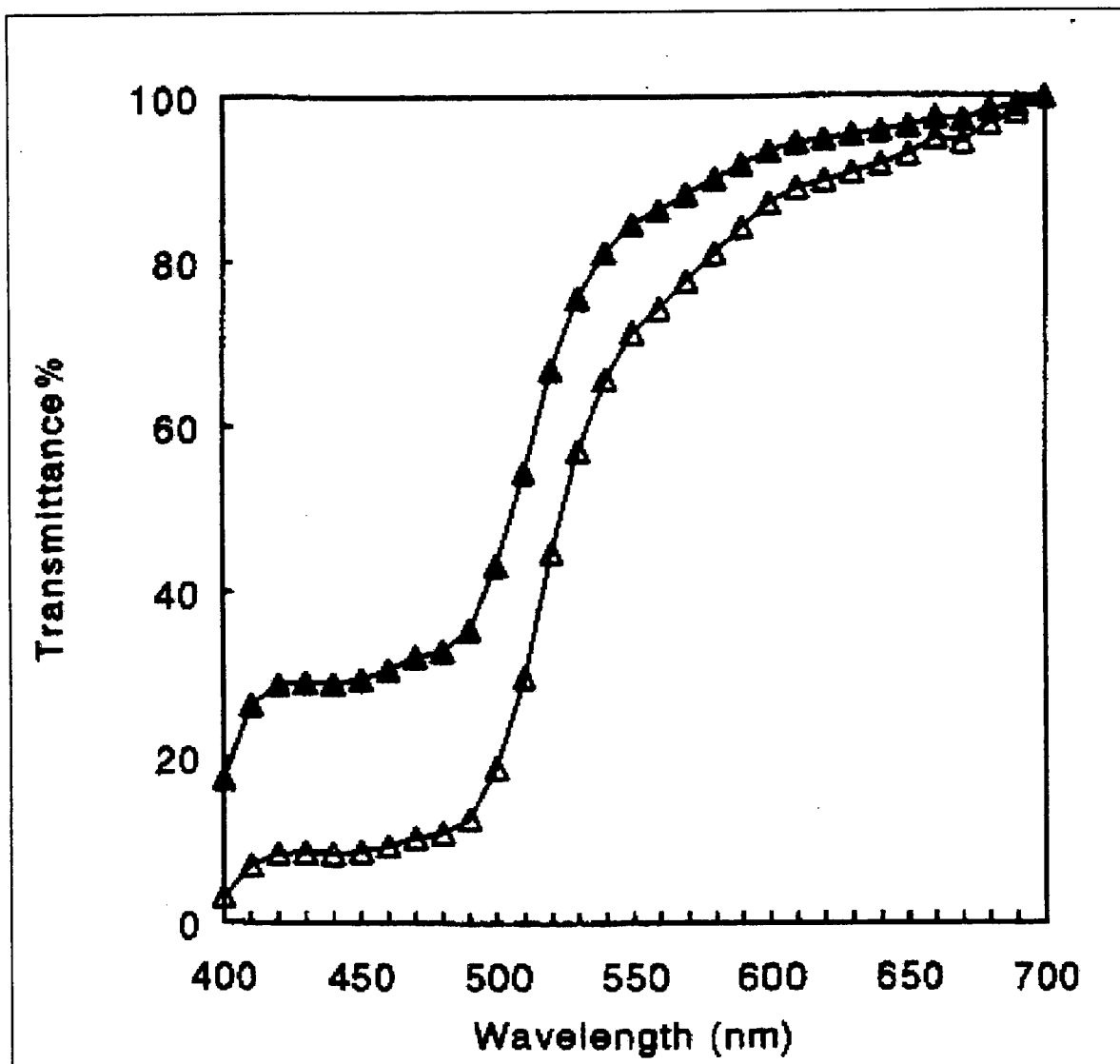


Figure 9.5: Comparison of "One-pass" and "Two-pass" Xanthophore Transmittance. The actual "one-pass" transmittance (solid triangles) and the functional "two-pass" transmittance (open triangles) of the xanthophore differ in the amounts of blue light transmitted to the melanophore and to the observer (see text).

the second power. As a result, only 5-10% blue light (400-450 nm) is reflected to the observer (figure 9.5) with green and red light transmitted effectively, whereas 25-30% blue light is transmitted to lower cell layers. Presumably, this light is sufficient to induce DPR.

The impact of the xanthophore on filtering blue-light suggests possible mechanisms of morphological and physiological regulation of DPR. Seasonal modulation of the concentration of carotenoid pigment found within xanthophores could alter blue light transmittance and, thus, be a mechanism for regulating the magnitude of DPR. Furthermore, xanthophores have been observed in culture with pigment aggregated and dispersed (unpublished observation). Although we have been unable to identify a hormone or second messenger that can induce pigment translocation within xanthophores, a physiologically active xanthophore, as occurs in other species (Lynch et al., 1986), could provide another mechanism for modulating skin color and DPR.

Previously, Vaughan (1987b) has argued that DPR may be a thermoregulatory mechanism, allowing the animal to increase absorbance of solar energy. Intrinsic regulation may involve temperature-dependent melanosome dispersion (Hadley and Goldman, 1969) and light-stimulated dispersion. It is possible that extrinsic regulation, such as a humoral signal stimulating the iridophore LR shift, could further increase the capture of blue-light. Such an effect would

have two consequences: increased absorbance of high energy short-wavelength light, and increased stimulation of photosensitive pigment dispersion which might further increase light absorption.

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Part X

Conclusion

CONCLUSION

In this work, we have demonstrated that skin of the lizard, Anolis carolinensis undergoes both light and hormone-dependent physiological color changes (part III). The light-dependent color change is effected by a dispersion of melanosomes within dermal melanophores (part VII), whereas the MSH-mediated color change utilizes both melanosome dispersion (Taylor and Hadley, 1970; part VII) and a long-wavelength shift in the iridophore reflectance spectrum (part VIII). Other color changes might be mediated by the selective activation of the iridophore by other hormones.

In both the melanophore (parts III, VI) and the iridophore (part VIII), MSH activity appears to utilize an increase in intracellular cAMP. In the melanophore, cAMP may cause phosphorylation of a 57 kd molecular switch (part VII) which may initiate the chromomotor response.

It has been previously suggested (Vesely and Hadley, 1971; Lucas et al., 1987) that there is a specific extracellular calcium requirement for MSH-mediated melanosome dispersion. Calcium may be necessary for hormone/receptor interaction and/or for some transduction event proximal to activation of adenylate cyclase. Furthermore, some (Sawyer et al., 1983) have argued that the effects of MSH on melanosome dispersion involve decreased

intracellular calcium. Others (Lucas et al., 1983) have implied that intracellular calcium may increase. By demonstrating the normal MSH response of cultured melanophores in lanthanum saline (part IV) or in the presence of an intracellular calcium chelator (part VIII), we have conclusively shown that elevated intracellular calcium is not a prerequisite for MSH-mediated melanosome dispersion.

The mechanism of MSH-mediated pigment dispersion, thus, appears to utilize a simple cAMP cascade terminating in the activation of a molecular switch (P57) via phosphorylation, which somehow triggers pigment dispersion (figure 10.1). In the absence of MSH, phosphorylation of P57 is inhibited by a phosphatase (part VII).

The signal transduction mechanism utilized by DPR is very different. DPR, but not the MSH response, utilizes increased intracellular calcium released primarily from intracellular stores (parts III, IV) and possibly from extracellular source as well (parts IV, V). A depolarization event may be involved in calcium release (parts IV, V). This calcium presumably is released from the melanosome (part IV), which may also contain the photopigment (part III). Mimicking the effect of increased cGMP with bioactive cGMP analogs is inhibitory to DPR (part III). However, it is unclear from these studies if a decrease in cGMP triggers

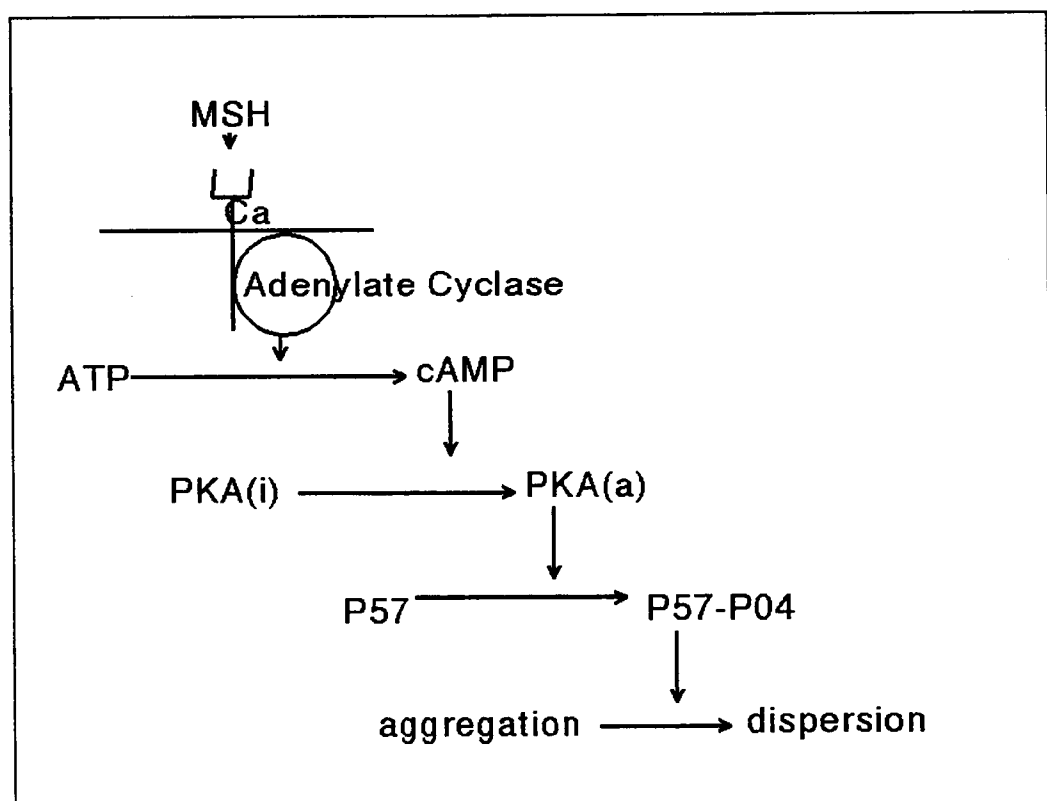


Figure 10.1: Model of the Mechanism of Action of MSH on Dermal Melanophores. MSH activates adenylate cyclase. cAMP activates protein kinase A. Protein kinase A phosphorylates P57, which causes pigment dispersion.

pigment dispersion, or if cGMP increases during DPR and acts as a feedback inhibitor of DPR (part III). Accordingly, two models of light action on the dermal melanophore are offered (figurea 10.2, 10.3).

In the first model (figure 10.2) light triggers a decrease in cGMP. Decreased cGMP triggers an increase in cytosoloic free calcium, perhaps by allowing calcium release from the melanosome (part IV). Increased calcium might effect melanosome dispersion by phosphorylation of the same molecular switch activated by MSH, and thus convergence of the two mechanisms may occur at this level (part VII). Phosphorylation might occur by activation of a kinase or inhibition of a phosphatase. In the dark, high levels of cGMP might somehow act to prevent phosphorylation of P57, either by activating a phosphatase or inhibiting a kinase (part VII). The effect might be direct (part VII), or through inhibition of a calcium signal (parts III, IV), or both. The role of cGMP-dependent phosphorylation of the >250 kd protein in the tranduction event may be related to a direct cGMP-mediated mechanism of P57 dephosphorylation.

In the second model (figure 10.3), light directly triggers an increase in cytosolic calcium, perhaps by a direct coupling of the photoreceptor to the melanosome calcium store (part IV) or via cell depolarization (parts IV, V). Increased cytosolic calcium might trigger

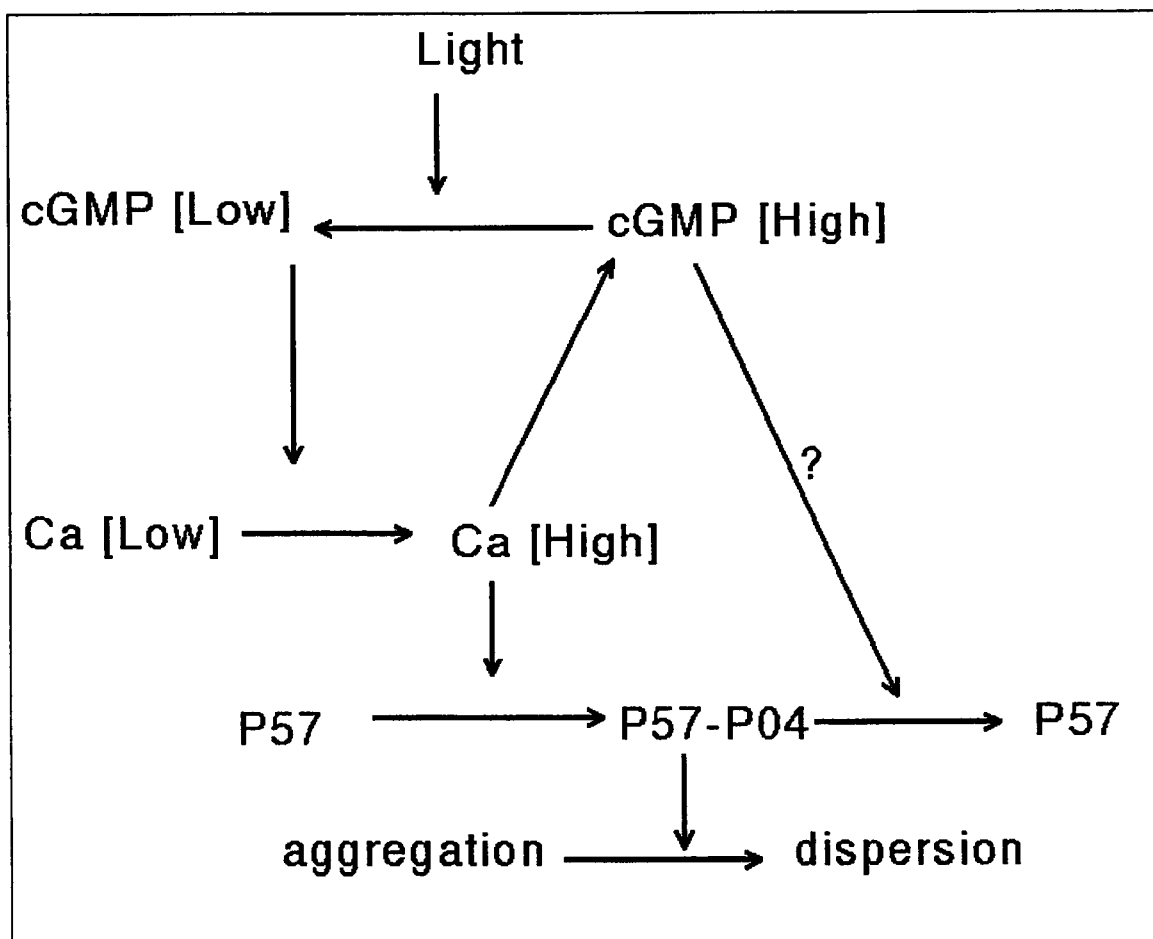


Figure 10.2: Model of the Mechanism of DPR I. Light may trigger a decrease in cGMP. Decreasing cGMP may trigger an increase in cytosolic Ca^{2+} . Increased cytosolic Ca^{2+} may trigger phosphorylation of P57 and pigment dispersion. Increased Ca^{2+} may also stimulate guanylate cyclase, thus increasing levels of cGMP. cGMP may inhibit dispersion by decreasing cytosolic Ca^{2+} , by directly dephosphorylating P57, or both.

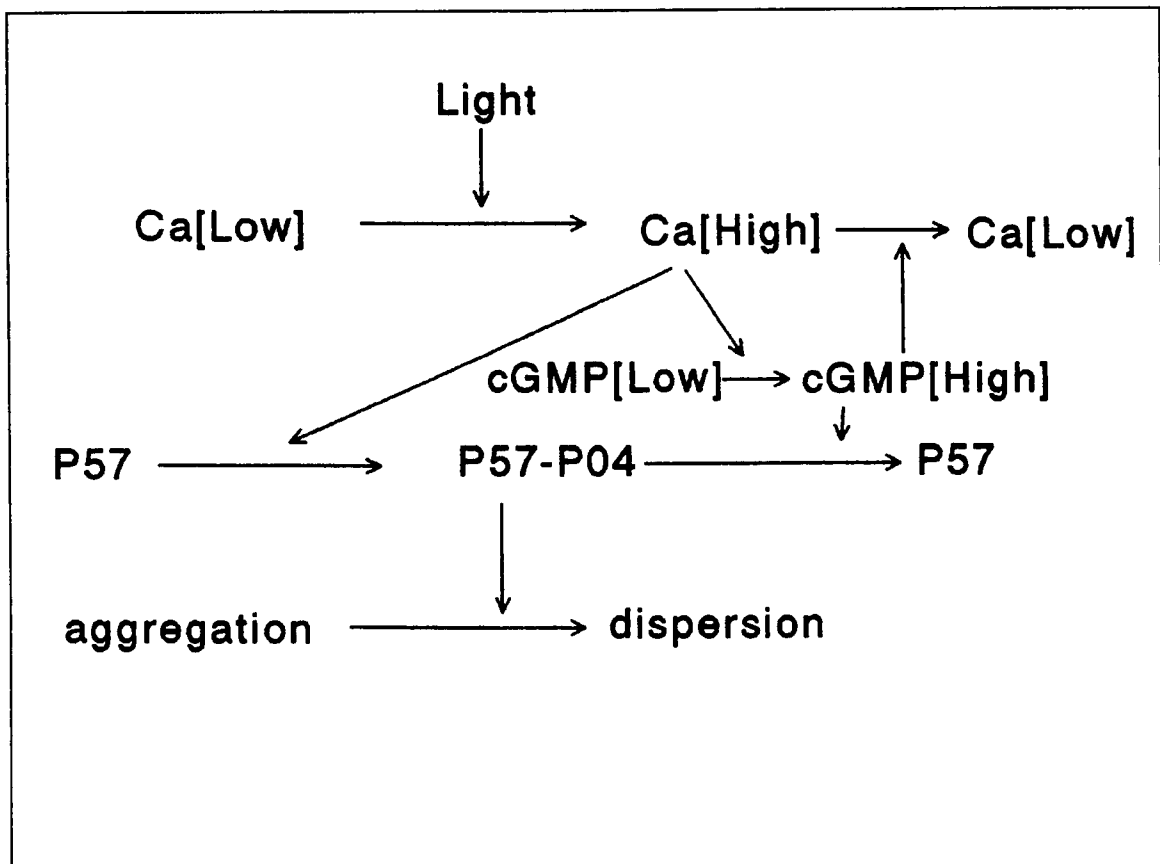


Figure 10.3: Model of the Mechanism of DPR II. Light may directly trigger a cytosolic Ca^{2+} increase. Increased cytosolic Ca^{2+} may trigger phosphorylation of P57 and pigment dispersion. Increased Ca^{2+} may also stimulate guanylate cyclase, thus increasing levels of cGMP. cGMP may inhibit dispersion by decreasing cytosolic Ca^{2+} , by directly dephosphorylating P57, or both.

phosphorylation of P57 and melanosome dispersion as described above. Furthermore, increased cytosolic calcium might activate guanylate cyclase, causing increased levels of cGMP. Increased cGMP might act in a negative feedback mechanism, triggering the sequestration of calcium and favoring dephosphorylation of P57. cGMP might also directly trigger dephosphorylation of P57 as in the previous model.

We have suggested (parts II, III) that the mechanism of DPR may be similar to that of visual photoreception, which shares a common embryological origin. In support of this argument, recent evidence suggests that the mechanism of invertebrate visual photoreception is even more strikingly similar to our model of dermal photoreception than previously thought. For example, in the visual photoreceptors of many invertebrates, illumination causes the release of calcium from intracellular stores. In some cases, such as the bee and locus, the smooth microvillar endoplasmic reticulum, which acts as a calcium source during illumination, is attached to a microfilament network, and undergoes a rearrangement during illumination (Walz and Baumann, 1996). Similarly, pigmented granules in crayfish photoreceptor cells contain calcium, and may act as a calcium source during illumination (Schroder et al., 1980). In other invertebrates such as the fruit fly, Drosophila

(Hofstee and Stavenga, 1996), and the crab, Sesarma cinereum (King-Smith and Cronin, 1996), pigmented organelles within the visual photoreceptors migrate superficially in response to light, acting as a primitive pupil. The migration is dependent on elevated intracellular calcium. Furthermore, in many invertebrates, cGMP has been implicated as a mediator of cytosolic free calcium concentration (Bacigalupo et al., 1991; Hupperz, 1995).

Further research into the mechanism of invertebrate photoreception and into the mechanism of dermal light sensitivity in A. carolinensis may provide further evidence of homology between the two systems. Determination of the level of conservation of the mechanism awaits further examination of the mechanism of dermal photosensitivity in other organisms.

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VITA

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Upon completion of the degree, he enrolled in the University of Tennessee Graduate School where he pursued the Ph.D. degree in Life Sciences which was awarded in August, 1997, with highest honors. Currently, he and his wife, Shannon, live in Memphis, Tennessee, where he is pursuing the M.D. degree as a Clinical Scholar at the University of Tennessee College of Medicine.