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MECHANISM OF MELANOPHORE DISPERSION

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Abstract—The mechanism of the melanophore reaction of the skin of Hyla arborea (tree frog) has been studied. Apart from the specific hormones MSH and ACTH which elicit melanophore dispersion, the following substances produced darkening: theophylline and caffeine regularly, theobromine erratically, but not, however, dyphylline. Sodium iodoacetate and mercuric chloride elicited melanophore dispersion, which fact may be connected with their ability to block active SH groups. Melanophore dispersion is not dependent upon temperature (13–20°); at the unusual temperature of 37° frog melanophore dispersion is, however, inhibited.

On the other hand, melanophore contraction is speeded up with increases in the ambient temperature; this points to the working theory that darkening is a passive reaction whereas lightening is an active reaction requiring energy. The assay of different inhibitors for their interaction with the energy supply has shown that only sodium benzoate inhibits melanophore contraction. This points to the possibility that the energy source should be sought either in the lipid metabolism or in the flavoproteins.

Melanophore contraction (lightening reaction) was elicited by dyphylline and noradrenaline. The effect of adrenaline, melatonin and serotonin was insignificant. This points to a highly specific reaction of the Hyla melanophores. Based upon our experience of the effect of calcium on the melanophore reaction the theory is advanced that the passive stage of melanophore dispersion (darkening) is induced by entrance of Ca^{2+} into the cell and exit of K^+. The active stage of melanophore contraction (lightening), however, needs an energy pump for either removal of Ca^{2+} from the cell or for forcing in of K^+.

The melanophore reaction is a convenient 'slow-motion picture model' for many coupling phenomena occurring in muscles, glands, nerves, etc.

IN EARLIER papers we have shown that the tree frog (Hyla arborea) is most suitable for the observation of melanophore reactions.[1–6] This frog abounds in southern countries where it can be easily caught by the hundreds. The colour of its back skin is homogeneous green when adapted for 2 weeks to a white background and quite stable to external changes. It reacts specifically to the standard hormones used for eliciting the melanophore reaction and changes from a standard bright green in two directions: (a) lemon green, due to extreme melanin contraction; (b) olive green → brown → black, due to melanin dispersion. It has recently been shown that these colour reactions are mediated by the presence of calcium[7] and/or sodium.[8–11] Thus, melanophore reaction places itself in one line with the stimulus-reaction coupling phenomena encountered in muscle contraction, nervous firing and glandular secretion. The latter reactions take place in a split second, thus complicating study of the underlying mechanism, whereas the melanophore reaction takes up to 30 min *in vivo* and *in vitro*.

In the following paper we wish to report on our results with different agents which influence the melanophore reaction *in vitro* and may thus make it possible to draw conclusions with regard to their mode of action and to the general mechanism of the melanophore reaction. This study seems worth while, since its aspects may also be applied to other stimulus-reaction phenomena.

MATERIALS AND METHODS

Tree frogs (Hyla arborea) were kept separately in small glass jars containing up to a height of 2–3 cm tap water—no feeding being required for 3 months. After standing for 2 weeks on a white base, the frogs usually became green adapted and did not react any longer to external colour changes. Those which did not adapt themselves—this may occur during the hot summer months—were discarded. For the experiments the tree frogs were decapitated and pithed. Their back skin was then removed, flattened on filter paper (double the size of the skin) with the skin-colour side facing upward, halved longitudinally and immersed in Petri dishes with 10 ml frog-isotonic solutions, one-half serving for the experiment, the other half for control. The experiments were carried out on a white table at room temperature, except where otherwise indicated. The solutions were always buffered with 2 mM $NaHCO_3$, and the pH was kept between 7·2–7·6.

Observation of the colour changes was effected at intervals of 15, 30, 45 and 60 min. In rare cases observation had to be extended up to 2–3 hr. To obtain a quick and reversible colour change, the following two agents were used and their mode of action studied throughout:

for darkening (melanophore dispersal): 10 mM theophylline in frog Ringer solution (NaCl 110 mM, $CaCl_2$ 2 mM, $NaHCO_3$ 2 mM, KCl 2 mM);

for lightening (melanophore contraction): 10 mM dyphylline in potassium–frog Ringer solution. The latter was prepared by replacing all sodium chloride with an equivalent amount of potassium chloride.

Alternating the theophylline and the dyphylline medium brought about a colour change every time within 30 min. Reversal of these reactions is possible at least 12 times and even more, at room temperature.

The colour changes were recorded with a reflectometer (Photovolt Corp. N.Y., Model 610), which was adapted to the size of the frog skin by an internal and external iris of 0·7 cm dia.[10, 11] White light was used throughout without any filter, and the international standard furnished by the manufacturer was adapted to the needs of our calibration. To increase sensitivity, the reflectometer was calibrated to the threefold scale extension of the international standard; thus 1 RV (reflectance value) used in this paper equals 1/3 of the international reflectance standard (IRS). For measuring, the wet filter paper was put on the external iris of the measuring head of the reflectometer with the frog skin covering the whole area of the iris. Reading began 3 min after starting the reflectometer. It takes a few seconds until the scale reading becomes stable.

The agents and enzyme inhibitors studied are listed below. With few exceptions their reactions were not relevant to the mechanism of the reaction studied by us, and they are, therefore, not mentioned any further.

Substances promoting melanophore dispersion. ACTH (Armour), MSH (Armour), theophylline, caffeine, theobromine, sodium iodoacetate ($CH_2I.COONa$), mercuric chloride ($Hg\ Cl_2$).

Substances inducing melanophore contraction. Dyphylline, noradrenaline, potassium-ions.

Substances which did not significantly influence melanophore movement in vitro. Melatonin, adrenaline, serotonin, glycerol, D,L-glyceraldehyde ($HO.CH_2$—$CH.OH$—CHO Sigma), potassium cyanide (KCN), sodium arsenite ($Na\ As\ O_2$, BDH), carbonyl-cyanide-*m*-chloro-phenylhydrazone (CCP—Dr. P. G. Heytler, E.I. Dupont de Nemour & Co.), dihydrostreptomycin, cystine, cysteine, tryptophane, glutathione reduced (Hoffmann–La Roche), glutathione oxidized (Hoffmann–La Roche), bradykinin (Sandoz), relaxin (Releasin, Warner–Chilcott), angiotensin (Hypertensin, Ciba), lactose, glucose, urea, uric acid, chlorothiazide, ATP (Calbiochem), ADP, AMP, sodium barbital, oleic acid, alpha-glycerophosphate (BDH), alpha-amino-nicotinamide (Frank W. Horner), thiamine HCl, procaine HCl, histamine, 3-acetyl pyridine (N.B. Co.), reserpine, chlorpromazine, chlordiazepoxide, delyside, methysergide, hydergine, ergotamine, ergotoxine, iproniazid, isoniazid, isocarboxazid, nialamide, tranylcypramine, pheniprazine, phenelzine, etryptamine, methyldopa (Aldomet, Merck), oxytocin (Syntocinon, Sandoz), vasopressin (Octapressin, Sandoz), heparin, cortisone, hydrocortisone.

Substances which significantly inhibited melanophore contraction. Sodium benzoate C_6H_5COONa.

RESULTS

A. Mechanism of melanophore contraction

1. *Substances promoting melanophore contraction.* These substances induce a lemon-coloured skin in the tree frog. They include: adrenaline, noradrenaline, melatonin, dyphylline. The results are summarized in Table 1, which shows that noradrenaline was the most active melanophore-contracting substance. We preferred dyphylline as

TABLE 1. MELANOPHORE CONTRACTION OF ISOLATED FROG SKIN BY DIFFERENT AGENTS, AFTER DARKENING OF THE SKIN WITH 10mM THEOPHYLLINE

Substance	Concentration mM	Degree of melanophore contraction in RV*				
		0	15	30 (min)	45	60
Noradrenaline	0·1	10	18	38	46	51
	1	15	22	35	40	45
	5	13	17	26	36	38
Dyphylline	2	10	30	35	47	53
	5	9	30	37	57	58
Frog Ringer	—	11	13	16	25	32
K^+-frog Ringer	—	12	20	27	34	41

Melatonin, adrenaline and serotonin did not differ from the controls at concentrations ranging between 0·01–10 mM.

* Reflectance values (see Methods).

standard substance for inducing melanophore contraction because of its high stability, easy solubility and the quick reversibility of its effect. It should be mentioned already here that the dyphylline reaction in itself is contrary to that obtained by its congeners theophylline, caffeine and theobromine which are melanophore dispersants. Melatonin, adrenaline and serotonin did not produce any significant melanophore contraction as compared with the control. This unexpected result will be discussed later.

2. *Effect of temperature on melanophore contraction (and dispersal).* Table 2 shows that the rate of darkening of the frog skin is not affected by ambient temperatures such as 13 or 20°; only at 37°—which should be considered a high temperature for a cold-blooded animal—darkening is definitely inhibited.

TABLE 2. EFFECT OF TEMPERATURE ON CONTRACTION AND DISPERSAL OF MELANOPHORES *in vitro* BY THEOPHYLLINE AND DYPHYLLINE

(Standard procedure at 10 mM concentration)

Substance	Temperature (°C)	Degree of melanophore contraction in RV*				
		0	15	30 (min)	45	60
Theophylline (for darkening)	13	34	20	17	12	9
	20	32	13	11	9	7
	37	32	27	25	23	21
Dyphylline (for lightening)	13	5	7	10	13	14
	20	6	15	24	30	33
	37	5	20	46	55	57

* Reflectance values (see Methods).

With regard to lightening, Table 2 shows that with increase of ambient temperatures the rate of melanophore contraction is considerably speeded up. Therefore, it is logical to assume that darkening is a passive reaction which does not require thermosensitive enzymatic processes. On the other hand, lightening is an active reaction requiring a metabolism-borne energy source, dependent upon temperature.

The energy of activation required can be calculated from the rates of reaction at different temperatures, as expressed by the Arrhenius' formula: $\log k = \text{constant} \; A/RT$,

where A is the energy of activation,

R is 1·987 cal per degree,

T the absolute temperature.

Plotting $\log k$ against $1/RT$ allows the calculation of A from the slope which results in the case of dyphylline lightening in approx. 10 kcal/mole. This value falls well within the range of an enzymatic reaction.

3. *Agents inhibiting the lightening effect.* Table 3 shows the effect of various substances studied for inhibition of the melanophore-contracting activity. The standard inhibitors of glycolysis (sod. iodoacetate and D,L-glyceraldehyde), a respiratory poison (potassium cyanide), the uncouplers of oxidative phosphorylation (sod. arsenite and CCP) and a blocker of the Krebs' cycle (sodium fluoroacetate) exerted little or no

activity in preventing melanophore contraction. The most active substance at its commonly used level of activity was sodium benzoate which, according to Avigan, Quastel and Scholefield,[12] combines with CoA and inhibits its catalytic effect at concentrations of 10^{-2} M. This fact points to the probability that the upkeep of melanophore contraction is based upon the lipid metabolism. This assumption is

TABLE 3. EFFECT OF SUBSTANCES STUDIED FOR INHIBITION OF THE MELANOPHORE-CONTRACTING ACTIVITY

The frog skin was darkened by the standard procedure with theophylline and subsequently lightened at 37 ° with dyphylline with and without presence of the following substances

Substance	Concentration (M)	Inhibition of lightening rate (%)
Pot. cyanide	10^{-3}	0
	10^{-4}	0
D,L-glyceraldehyde	5×10^{-3}	0
	10^{-3}	0
Sod. iodoacetate	10^{-3}	65
	10^{-4}	30
Sod. arsenite	10^{-3}	75
	10^{-4}	20
	10^{-5}	0
CCP (Carbonyl cyanide *m*-chlorophenylhydrazone)	10^{-5}	60
	10^{-6}	0
Sod. benzoate	10^{-2}	100
	5×10^{-3}	50
	10^{-3}	20
Sod. fluoroacetate	10^{-3}	0

further strengthened by the relative insensitivity of this reaction to the inhibitors of other energy-providing systems. However, sodium benzoate inhibits flavoproteins as well. We have found that the sodium benzoate inhibition is not brought about by low-chain fatty acids, a fact which does not favour the flavoprotein inhibition concept.[13]

B. Mechanism of melanophore dispersion

4. *Substances promoting melanophore dispersion.* It is well known that substances which block active SH groups cause melanophore dispersion (Horowitz[14]). Moreover, it is also known that xanthine derivatives are active dispersers. We, therefore, studied compounds of these two groups. The results are compiled in Table 4. The first two compounds (mercuric chloride and sodium iodoacetate) block active SH groups.

The mechanism of action of the xanthine derivatives which promote melanophore dispersal has still to be defined. In our comparative tests theobromine always yielded erratic results, but caffeine and theophylline gave constant melanophore dispersion, the latter being the most active xanthine derivative. On the other hand, dyphylline (cf. Table 1) proved to be a strong melanophore contractor.

5. *The effect of calcium ions on melanophore dispersion.* From the studies of Bianchi[15] on striated muscle it is known that caffeine-produced contraction is independent of

TABLE 4. MELANOPHORE DISPERSION OF ISOLATED FROG SKIN BY DIFFERENT AGENTS

Substance	Concentration (mM)	Degree of melanophore dispersion in RV*				
		0	15	30 (min)	45	60
Mercuric chloride	1	37	35	25	22	21
		31	31	27	26	26
Sod. iodoacetate	1	40	39	27	22	22
Theobromine	5	32	42	41	38	35
	10	36	48	43	35	38
Caffeine	2	25	22	23	20	17
	5	29	26	24	18	11
	10	35	25	23	21	15
Theophylline	1	30	16	13	11	9
	2	38	18	12	11	10
	5	45	20	12	11	10

* Reflectance values (see Methods).

extracellular calcium. Novales[8] has shown that the melanophore dispersal of caffeine is independent of extracellular sodium. We, therefore, compared the melanophore dispersion provoked by theophylline and caffeine in the presence and absence of 10 mM calcium in frog isotonic sucrose solution. Table 5 shows that caffeine dispersion is indeed not enhanced by calcium ions, whereas that of theophylline is augmented.

TABLE 5. MELANOPHORE DISPERSION OF ISOLATED FROG SKIN BY 10 mM CAFFEINE AND THEOPHYLLINE SOLUTIONS IN PRESENCE OF 10 mM Ca^{2+} PRECEDED BY SOAKING FOR 3hr WITH SUCROSE SOLUTION

Substance	Concentration	Degree of melanophore dispersion in RV*				
		0	15	20 (min)	45	60
Caffeine	5	50	44	40	39	35
Caffeine + 10 mM CaCl	5	50	41	40	37	35
Theophylline	5	40	35	28	22	20
Theophylline + 10 mM CaCl	5	40	35	23	19	14

* Reflectance values (see Methods).

6. *Substances inhibiting melanophore dispersion.* Five standard inhibitors of enzyme reactions (potassium cyanide, sodium arsenite, CCP, sodium benzoate and sodium fluoroacetate) were examined for their capacity to inhibit melanophore dispersion evoked by theophylline. The results were negative with regard to KCN, C_6H_5COONa and FCH_2COONa. Positive results were only obtained by excessively high concentrations of SH-group blockers, as e.g. $Na.As.O_2$ and CCP. This effect is, however, non-specific as it appeared at the same concentration also with melanophore-contracting substances (cf. Table 3).

DISCUSSION

The frog skin melanophore reaction is an excellent model for the study of the stimulation reaction mechanism because of its slow reaction time. All phenomena studied on muscles, nerves, glands, etc. are so fast that an analysis of their individual phases is almost impossible. The frog skin provides an easily accessible slow motion picture of the different stages of action.

TABLE 6. EFFECT OF SUBSTANCES STUDIED FOR INHIBITION OF THE MELANOPHORE-DISPERSING ACTIVITY INDUCED BY 10 mM THEOPHYLLINE

Substance	Concentration (M)	Inhibition of darkening rate (%)
Potassium cyanide	10^{-3}	0
Sod. arsenite	10^{-4}	25
	10^{-3}	70
CCP	10^{-6}	20
	10^{-5}	40
Sodium benzoate	10^{-2}	0
Sod. fluoroacetate	10^{-3}	0

The fact that the stimulus of dispersion, provided by all known agents, depends on the presence of calcium and that caffeine dispersion is independent of it, contributes to the suitability of this model reaction.

The experimental findings fit into the following picture of melanophore dispersion: Dispersion is provoked by increase of intracellular calcium ions and simultaneous decrease of intracellular potassium ions. This movement corresponds to the direction of the respective cation gradients in the organism and does not, therefore, require any enzymatic energy (Fig. 1). Contraction, however, requires cation movements in the

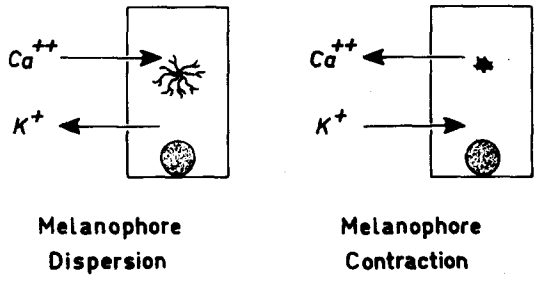

FIG. 1. Hypothesis for the mechanisms of melanophore movements. For dispersion Ca^{2+} enters and K^+ leaves the cell. For contraction Ca^{2+} leaves and K^+ re-enters the cell. The latter stage requires energy.

opposite direction and, therefore, requires energy. The most likely explanation for this phenomenon is the existence of a calcium and/or potassium pump system which is based on the lipid metabolism. If we accept this hypothesis, it is obvious that those agents which provoke dispersion interfere with the selective membrane permeability by 'loosening' it, and those which promote contraction 'tighten' it.

Hyla frog skin differs from Rana and Bufo frog skin in that it does not react to melatonin *in vitro*, but does so *in vivo* in rather high concentrations. This reaction is not dependent on the pituitary, hypothalamus or epiphysis since it appears in the decapitated Hyla frog just as well as in the living frog. Thus it seems that the typical melatonin reaction is dependent upon the presence of an intact blood or lymphatic system of the frog.

Adrenaline and serotonin are not active in the melanophore movement, whereas noradrenaline induces contraction. We, therefore, studied 8 MAO inhibitors (see Materials) but none of them induced melanophore movement either *in vivo* or *in vitro*. Methyldopa was not active either.

Delyside which is active in fish[16] and reserpine which is active in living Rana[17] were not active in Hyla, neither *in vivo* nor *in vitro*. This characterizes the melanophore system of Hyla as highly specific and selective in its responses. Cortisone and hydrocortisone did not act upon melanophore contraction or dispersion *in vivo* or *in vitro*. This is in contradistinction to the results of Lerner and his group[10, 11] but in confirmation of results published more recently.[18]

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