

THE ACTION OF MELATONIN ON SINGLE AMPHIBIAN PIGMENT CELLS IN TISSUE CULTURE

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- 1 Neural crest material from neurula stage *Xenopus* embryos was tissue cultured as small aggregates of cells or a single cell suspension. Isolated pigment cells differentiated after 2 days in culture.
- 2 Melatonin (10^{-15} to 10^{-13} M) induced pigment granule condensation; it was 10,000 times more effective than any other compound tested.
- 3 Tests with appropriate agents showed the pigment cells to have β -adrenoceptors and 5-hydroxytryptamine receptors; these sites could be blocked without affecting the response to melatonin. Phentolamine blocked the effect of melatonin.
- 4 Removal of Na^+ or Ca^{2+} from the bathing medium inhibited melatonin-induced pigment granule condensation; 10 mM K^+ induced pigment granule condensation. D600, Mn^{2+} and La^{3+} , which inhibit Ca^{2+} entry, blocked the effect of melatonin.
- 5 Cyclic GMP induced pigment condensation and cyclic AMP pigment dispersion (10^{-2} to 10^{-4} M).
- 6 It is suggested that the action of melatonin is accompanied by depolarization and the entry of calcium ions.
- 7 Pigment cells in tissue culture could provide a useful bioassay method for melatonin.

Introduction

There is much interest in melatonin, the major product of the pineal gland, because it has recently been proposed that it plays an important neuroendocrine role (Cardinali, 1974; Minneman & Wurtman, 1975). The effect of melatonin on the condensation of granules in pigment cells in amphibian skin has long been known and forms the basis of the tadpole bioassay for melatonin (Ralph & Lunch, 1970), yet little is known about the mode of action of the hormone.

Isolated pigment cells can conveniently be produced by tissue culture of neural crest material from neurula stage *Xenopus laevis* embryos (Jackson, Messenger & Warner, 1975) and are highly sensitive to treatment with melatonin. The present paper describes the effect of melatonin on the condensation of pigment granules in such cultured pigment cells and includes experiments designed to elucidate the mode of action of melatonin.

Methods

Xenopus laevis embryos were produced by adult frogs induced to lay by injection with Pregnyl (Organon Ltd) at a dose of 500 iu/female and 300 iu/male. The embryos were reared in tap water until they reached the required developmental stage; staging follows the normal Table of *Xenopus* (Nieuwkoop & Faber, 1956).

All manipulations were carried out in a laminar flow hood (Hepaire filtration Ltd). The required number of embryos were selected (usually 20) and the superficial jelly coat removed with forceps. They were then soaked in two changes of sterile Ringer solution (for composition see below) for 10 min and finally soaked for 20 min in sterile Ringer solution containing 20 iu/ml penicillin with streptomycin (Gibco-Biocult Ltd). All subsequent manipulations were carried out under sterile conditions.

The embryos were transferred to a petri dish containing Ringer solution for removal of the remaining jelly and vitelline membrane by means of fine watchmaker's forceps. Presumptive neuroectoderm and underlying mesoderm were

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dissected out from late gastrula to early neurula stage embryos (stages 10½–12). The outer pigment layer of the ectoderm was removed manually with or without brief soaking (<5 min) in Ca^{2+} - Mg^{2+} -free Ringer solution with 0.1 mM ethylene glycol-bis tetramino acid (EGTA). The remaining ectoderm and mesoderm tissue was teased into aggregates of 50–100 cells with fine glass needles, left for 5 min to round up and then 6–8 aggregates dispensed into each 35mm Falcon petri dish containing culture medium (see below). *In vitro* culture therefore began before induction of the ectoderm to form neural tissue by mesoderm was complete. Each aggregate became attached to the petri dish, flattened and then differentiated to give a small area of differentiated cells. Single, isolated pigment cells were usually found close to the edge of each group. An example is shown in Figure 1a. Pigment cells within the sheet were not used. This method was used on most occasions since it yielded a higher proportion of pigment cells than that described below.

Alternatively, neural plate and underlying notochord and somites were dissected from late neurula stage (stages 18–20) embryos and then treated for 2–5 min with Ca^{2+} - Mg^{2+} -free Ringer solution containing 1 mM EGTA, which loosened the adhesion between different layers. Dissected tissue from 3 embryos was transferred into each petri dish containing culture medium with a mouth pipette. Complete dispersal into single cells was achieved by gently sucking the aggregates up and down the mouth pipette. Cells produced in this way differentiated to give a relatively uniform monolayer culture.

In both culture systems differentiation into the cell types normally produced by the dissected areas of the embryo followed the same time course as in the whole embryo. Pigmentation of the melanocytes began after 2 to 3 days in culture.

Solutions

All solutions were made up in glass distilled water and autoclaved at 15 lb/sq inch for 10 minutes. The composition of the solutions was as follows: (i) Ringer solution (mM): NaCl 120, KCl 2.5, CaCl_2 2.0 and Tris 2.0, titrated to pH 7.4 with HCl. (ii) Disaggregating solutions: calcium was omitted from Ringer solution and either 0.1 mM EGTA or 1 mM EGTA added, pH 8.0. (iii) Culture medium (mM): NaCl 100, KCl 2.5, CaCl_2 2.0, MgCl_2 2.0 and NaHCO_3 5.0, titrated to pH 7.4, plus 10% foetal calf serum and 20 iu/ml penicillin with streptomycin. The addition of 10% foetal calf serum brought the final concentrations of Na^+ to 120 mM and K^+ to 3 mM.

Further supplementation of the medium was not necessary because the cells contain yolk platelets, which provide the substrate for differentiation.

The degree of condensation of the pigment granules was assessed quantitatively with a Vickers scanning microdensitometer model No.M85. The spot (5 μm

diameter) was focused on the centre of a single cell to one side of the nucleus, and readings taken after application of different concentrations of melatonin. The results were plotted as a dose-response curve, setting the reading obtained for the maximum effective dose of melatonin as 100. Alternatively a cell (or cells) was (were) observed with a compound microscope during treatment with melatonin and the degree of pigment condensation assessed visually. Estimation of the dose which brought about 50% condensation of pigment was difficult. We therefore determined the dose which produced withdrawal of pigment from the outermost processes and the lowest dose which produced maximal condensation of the pigment granules. These two points correspond to the bottom and top of the dose-response curve and gave, for melatonin, results comparable with those obtained by densitometry. Potencies of drugs causing pigment condensation, relative to melatonin were determined in this way (see Figure 4). The method allowed convenient and reliable assay even though it departs from the established principle in pharmacology that assays should be based on the linear portion of the sigmoid dose-response curve corresponding with responses between 20 and 80% of the maximum. It has the advantage that experiments may be done when an expensive densitometer is not available. Before starting any measurements, the cells were washed with fresh Ringer solution containing 10% foetal calf serum. This invariably produced transient condensation of the pigment and tests were delayed until the condensation had passed away. All solutions contained 10% foetal calf serum which improved cell viability and hence reproducibility.

Source of materials

Melatonin was kindly donated by Dr Josephine Arendt, who also gave us samples of *N*-acetyl-5-methoxykynuramine (NAMK) and *N*-acetyl-5-methoxyformylkynuramine (NAMFK). Methysergide was obtained from Sandoz Products Ltd, D-2 bromolysergic acid diethylamide (BOL) from Hoffman-La-Roche Ltd, who also gave us A23187. Methoxamine came from Ciba Laboratories, thymoxamine from William R. Warner & Co. Ltd, and (\pm)-propranolol from I.C.I. All other chemicals were obtained from Sigma Ltd. Foetal calf serum and penicillin/streptomycin came from Gibco-Biocult Ltd.

Results

Figure 1 shows the effect of melatonin on a group of pigment cells. Initially (Figure 1a) the pigment was fully dispersed in all cells. Melatonin 0.1 femtogram/ml (4.3×10^{-16} M) produced withdrawal of pigment from the outer processes of the most sensitive cells (Figure 1b). As the melatonin concentration was increased all the cells began to respond. Melatonin

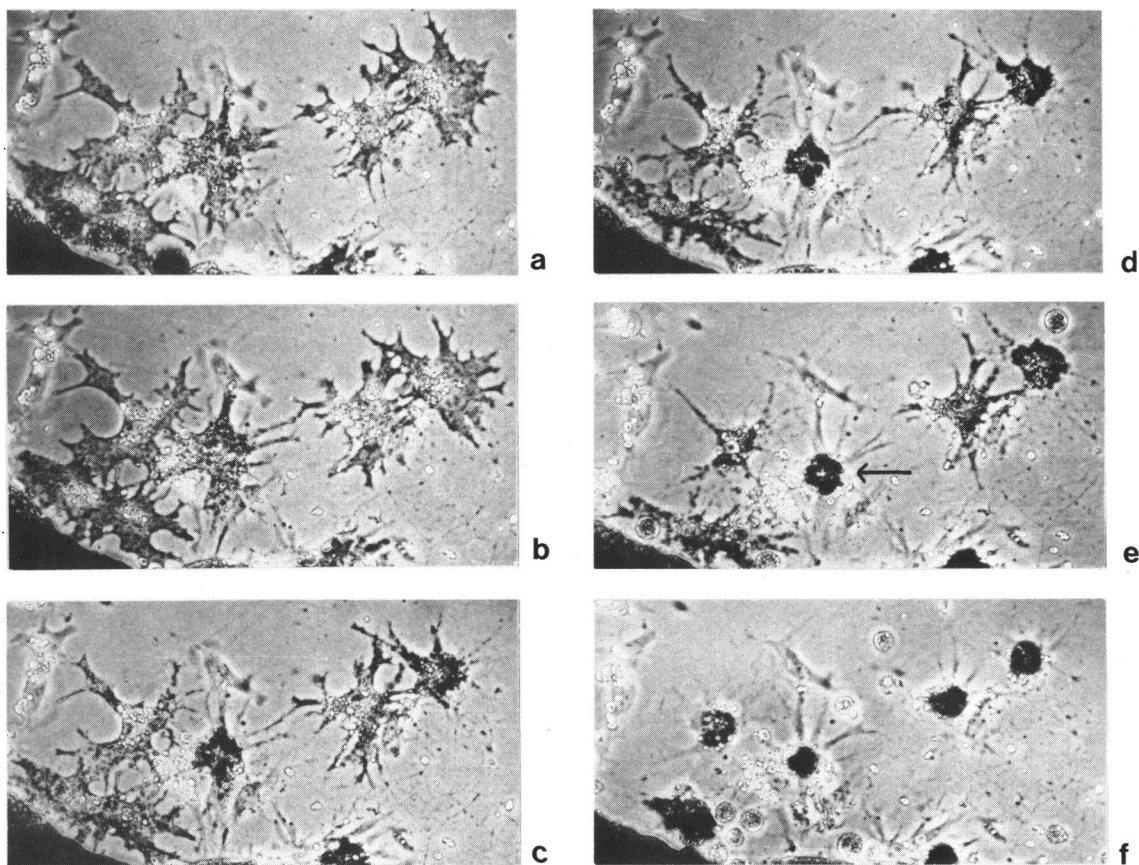


Figure 1 Photographs of a group of pigment cells to show gradual condensation of pigment as the concentration of melatonin is increased: (a) control; (b) 4.3×10^{-16} M; (c) 1.2×10^{-15} M; (d) 2.2×10^{-15} M; (e) 3.4×10^{-15} M (fully condensed cell arrowed); (f) 4.3×10^{-15} M; all cells fully condensed. ($\times 200$, phase contrast).

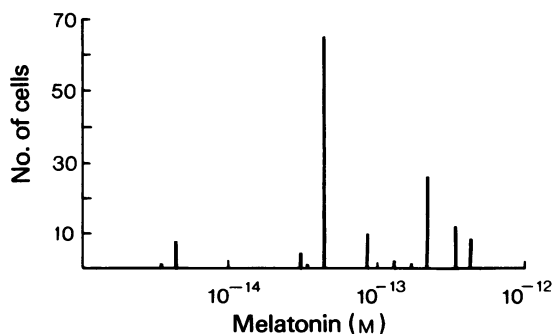


Figure 2 Histogram to show range of sensitivities to melatonin of individual pigment cells. Ordinate scale: no. of cells. Abscissa scale: concentration (M) of melatonin producing maximal condensation. Note logarithmic scale.

0.8 fg/ml (3.4×10^{-15} M) produced complete condensation of pigment in one cell (arrowed in Figure 1e) and most cells in the group completely condensed with melatonin 4.3×10^{-15} M.

A frequency histogram of the dose of melatonin producing maximal condensation of pigment in 135 cells tested during this study is illustrated in Figure 2. The sensitivity lay between 3.4×10^{-15} M and 4.3×10^{-13} M, with the mode at 4.3×10^{-14} M. Although the range over the total population is widely spread, individual cells from the same culture were always very similar.

The degree of pigment condensation, determined with the microdensitometer, is plotted in Figure 3 against the concentration of melatonin on a logarithmic scale, for two cells responding in the modal range. The relationship was sigmoid with 50% condensation of pigment occurring at 3.3×10^{-15} M melatonin in Figure 3a and 1.4×10^{-14} M in Figure 3b.

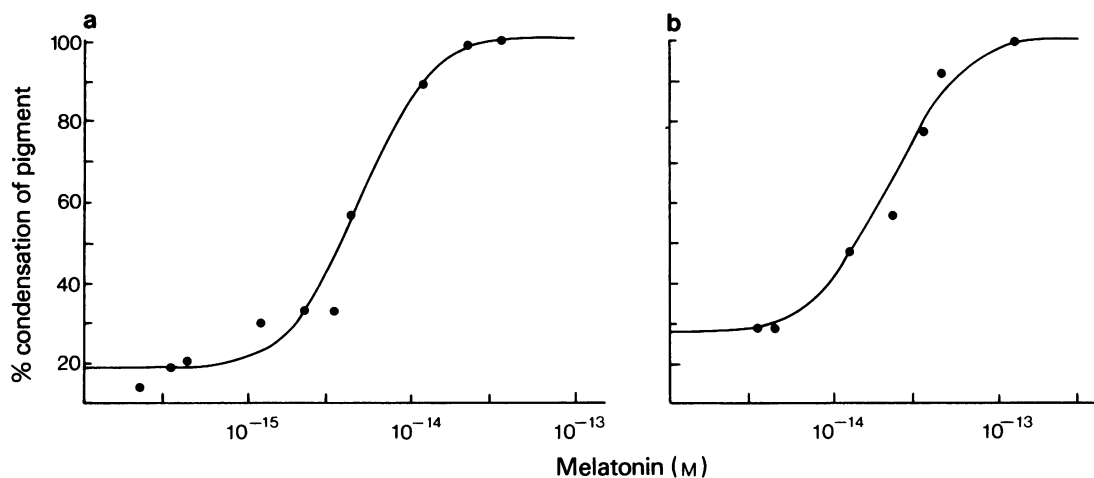


Figure 3 Dose-response curves for two pigment cells showing relation between melatonin concentration and degree of pigment condensation. Ordinate scale: condensation of pigment measured by densitometer. Abscissa scale: concentration of melatonin on logarithmic scale.

Table 1 Effect of blocking agents on melatonin response

<i>Likely action of drugs</i>	<i>Compound</i>	<i>Concⁿ (M)</i>	<i>Effect on melatonin response</i>	<i>Other observations</i>
β -Adrenoceptor agonist	Isoprenaline	10^{-6}	None	Condensation of pigment blocked by propranolol
β -Adrenoceptor blocker	Propranolol	10^{-6}	None	Blocks effect of isoprenaline
α -Adrenoceptor blocker	Methoxamine	2.5×10^{-5}		Full condensation of pigment
		10^{-6}		No effect
	Phentolamine	10^{-10}		Pigment condensation; blocked by propranolol
		10^{-13}	Blocked	
5-HT blocker	Thymoxamine	10^{-10}		Pigment condensation; blocked by propranolol
		10^{-10}	Blocked	In presence of propranolol
	BOL	10^{-5}		Pigment condensation
		10^{-7}	None	Blocks effect of 5-HT
Inhibitors of Ca^{2+} entry	Methysergide	3×10^{-6}	None	Blocks effect of 5-HT
		5×10^{-5}	Blocked	
	MnCl_2	2×10^{-3}	Blocked	
	$\text{La}(\text{NO}_3)_3$	10^{-3}	Blocked	

All compounds tested on at least 4 separate occasions.

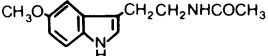
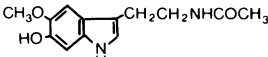
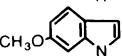
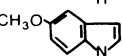
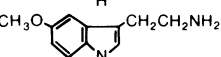
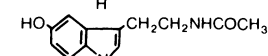
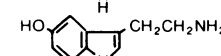
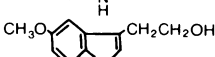
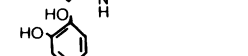
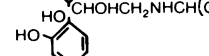
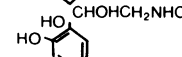
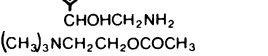
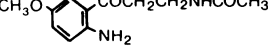
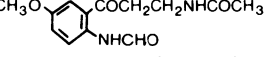
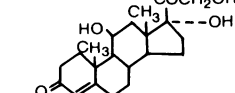
Compound	Structure	Relative potency
Melatonin		10^0
6-Hydroxy melatonin		$10^{-5.7}$
6-Methoxy indole		10^{-4}
5-Methoxy indole		10^{-5}
5-Methoxy tryptamine		10^{-7}
N-acetyl 5-hydroxytryptamine		10^{-8}
5-Hydroxytryptamine		10^{-8}
5-Methoxy tryptophol		$10^{-7.7}$
Isoprenaline		10^{-7}
Adrenaline		10^{-7}
Noradrenaline		10^{-5}
Acetylcholine		10^{-6}
N-acetyl-5-methoxy-kynuramine		10^{-8}
N-acetyl-5-methoxy formyl kynuramine		10^{-8}
Hydrocortisone		10^{-6}

Figure 4 Compounds tested for ability to induce pigment condensation.

Hill plots of the results illustrated in Figure 3 were linear (correlation coefficients (a) 0.94; (b) 0.94) with slopes which were not significantly different from 2, suggesting that two molecules of melatonin combine with each molecule activating the response.

Figure 4 lists 14 other compounds tested and found to produce pigment condensation, together with their structural formulae and their potency relative to that of melatonin (determined as described in Methods section). Two of the most potent compounds were 6-methoxyindole and 6-hydroxymelatonin, both metabolites of melatonin, but these were effective only at concentrations 4 orders of magnitude greater than

melatonin. Drugs lacking either the *N*-acetyl group (e.g. 5-methoxytryptamine) or the methoxy group (*N*-acetyl 5-hydroxytryptamine) also had low potencies relative to melatonin. The shape of the dose-response relation for these substances was similar to that of melatonin, except for *N*-acetyl 5-hydroxytryptamine where the response was spread over 2 or 3 log units. The reason for this exception is not clear. The low potency of 6-hydroxymelatonin found here contrasts with the results of Heward & Hadley (1975) who found 6-hydroxymelatonin to have a potency similar to that of melatonin itself. We were also unable to confirm their finding that *N*-acetyl 5-hydroxy-

tryptamine blocks the action of melatonin, so long as the cells were responding reproducibly to low concentrations of melatonin. Cells which required higher concentrations of melatonin to produce pigment condensation were also less responsive to other effective drugs; the relative potencies remained unchanged.

A number of agents were tested for the ability to inhibit the action of melatonin to try to shed light on the nature of the reactive site. The compounds, together with their effects, are listed in Table 1. The β -adrenoceptor blocking agent, propranolol (10^{-6} M) had no effect on the response to melatonin, yet it prevented isoprenaline, adrenaline and noradrenaline from producing their effect. The 5-hydroxytryptamine (5-HT) blocking agents BOL and methysergide similarly inhibited 5-HT, but not melatonin. 5-HT 10^{-6} M produced an irreversible decrease in sensitivity of the cells to melatonin.

Tests with drugs known to combine with α -adrenoceptors produced complex results. The α -adrenoceptor agonist, methoxamine (2.5×10^{-5} M), evoked complete pigment condensation, suggesting that melanocytes have membrane α -receptor sites. Two α -adrenoceptor blocking agents, phentolamine (10^{-11} M) and thymoxamine (10^{-10} M) themselves induced pigment condensation. This agonist effect was inhibited (up to 10^{-10} M α -blocker) by the β -blocker propranolol, suggesting that it is mediated through a β -receptor rather than an α -receptor. The agonist action of the α -adrenoceptor blocking agents could not be prevented in the μ M range necessary to test whether they inhibited the effect of methoxamine. Propranolol (up to 10^{-5} M) did not affect the pigment condensing action of methoxamine. The effect of methoxamine, and of μ M concentrations of phentolamine and thymoxamine was often irreversible. At low concentrations both α -blockers inhibited the action of melatonin; phentolamine at 10^{-13} to 10^{-12} M, and thymoxamine at 10^{-10} M, when its agonist action was inhibited by propranolol.

An antibody to melatonin (titre 1:20,000) produced for radio-immunoassay (Arendt, Paunier & Sizonenko, 1975) and kindly donated by Dr J. Arendt, was tested for the ability to prevent melatonin from activating the condensation of pigment. As expected the antibody rendered the melatonin inactive.

The role of the membrane potential and calcium ions

Martin & Snell (1968) showed amphibian skin pigment cells to have resting membrane potentials close to -90 mV. These authors considered whether depolarization might possibly form part of the mechanism controlling the movement of pigment granules, but were unable to demonstrate any depolarizing effect of melanocyte stimulating hormone (MSH), which causes pigment granule dispersion. In

accord with these results they found that raising the external potassium concentration produced no pigment dispersion in cells which started with their pigment fully condensed. We found that raising the extracellular potassium concentration to 10 mM, which should reduce the membrane potential to about -60 mV, consistently produced condensation in cells whose pigment granules were initially dispersed. Any depolarizing response is most likely to involve an increase in permeability to Na^+ ions; in keeping with this, melatonin did not produce pigment condensation when Na^+ ions in the bathing solution were replaced with equimolar concentrations of choline or Tris. The present results, taken together with those of Martin & Snell (1968), are consistent with the view that melatonin, but not MSH, acts by depolarizing the pigment cell membrane.

Many hormones, particularly those whose effects are accompanied by depolarization, produce an increase in entry of calcium into the cell. Three agents known to block calcium entry: α -isopropyl- α -[(*N*-methyl-*N*-homoveratryl)- α -aminopropyl]-3,4,5-trimethoxyphenylacetone nitrile (D600), Mn^{2+} and La^{3+} , were tested for the ability to block the action of melatonin (see Table 1). All three prevented melatonin-induced pigment granule condensation: melatonin was without effect in calcium-free solutions. Tests using the calcium ionophore, A23187, proved equivocal; the ionophore caused slight pigment condensation, but this was rapidly superseded by the death of all cells in the culture.

The action of cyclic nucleotides

MSH and adrenocorticotrophic hormone (ACTH) both cause dispersion of pigment granules; this effect can be mimicked by treatment with between 0.1 and 10 mM cyclic adenosine 3',5'-monophosphate (cyclic AMP) (Lyerla & Novales, 1972). We confirmed Lyerla & Novales' results with cyclic AMP on our preparation of cultured pigment cells, and also found that cyclic GMP, which has been suggested to act antagonistically to cyclic AMP, produced pigment condensation, i.e. it mimicked the action of melatonin. The effective concentration range lay between 0.1 and 10 mM, similar to that found previously for dispersion of granules by cyclic AMP (Lyerla & Novales, 1972). The dibutyryl derivative of cyclic GMP was not noticeably more effective.

Both nucleotides acted relatively slowly, taking 20 to 25 min to have their full effect, in contrast to melatonin where noticeable condensation occurred within 2–3 min, the reaction being complete within 10 minutes. The effect of the two nucleotides appeared to be relatively independent since the same concentration of cyclic GMP was effective on cells where the pigment was initially dispersed and on cells where granule dispersion had previously been achieved by cyclic AMP.

Discussion

Tissue cultured pigment cells are 100 times more sensitive to the pigment condensing hormone, melatonin, than cells in intact frog skin (Lerner & Wright, 1960). The site activating condensation of the pigment granules is relatively specific; all other substances able to induce pigment condensation were at least 10,000 times less effective than melatonin. None of the drugs tested was without pigment condensing action, although μM concentrations were necessary for the most ineffective. This is in contrast to the results of Heward & Hadley (1975), who found a number of compounds that were inactive on intact frog skin up to $100\ \mu\text{M}$. The difference probably arises because of the much greater sensitivity of the system described here ($10^{-13}\ \text{M}$ melatonin for full condensation compared to $10^{-9}\ \text{M}$ in Heward & Hadley's experiments).

The pigment cells described here possess a number of separate membrane receptor sites whose activation leads to condensation of pigment granules. Thus the pigment condensing action of the β -adrenoceptor agonist, isoprenaline, is prevented by the β -blocker propranolol, that of 5-HT is inhibited by BOL and methysergide; in both cases the blocking agents leave the efficacy of melatonin unimpaired. The possibility that the cells also possess pure α -adrenoceptors remains open since an agonist action, at low concentrations of the α -blockers made it impossible to test for inhibition of the action of methoxamine. The pigment condensation produced by phentolamine and thymoxamine seems likely to be mediated via a β -receptor since it was blocked by propranolol. This finding may be relevant to experiments on heart muscle, where phentolamine has been shown to have effects similar to that of sympathetic stimulation (Das & Parratt, 1971). In the presence of propranolol, both α -blockers inhibited the effect of melatonin, suggesting that the melatonin site may share some properties with the α -receptor. Such findings make firm conclusions about the structure-activity relation of the melatonin site possible only when these other receptor sites have been blocked with the appropriate drugs. Heward & Hadley (1975) concluded that the *N*-acetyl group of melatonin binds to the receptor and the methoxy group confers activity. They based this conclusion on their finding that drugs lacking one or other of these groups were much less active, together with the finding that melatonin and 6-hydroxymelatonin had similar potencies. Our results do not allow this conclusion to be drawn because 6-hydroxymelatonin, which contains both of the suggested active groups, was nevertheless much less effective than melatonin in causing the condensation of pigment granules.

It seems likely that melatonin increases the sodium permeability of the cell membrane, so causing depolarization, accompanied or followed by calcium

entry, as suggested for many other hormones. The increased intracellular calcium ion concentration could either cause granule movement directly or bring about some other change, which is then responsible for the effect. Although concentrations of melatonin producing maximal condensation initiated granule movement within 2–3 min, the effect usually took 10 min to come to completion. The finding that the effect of melatonin is mimicked by cyclic GMP, whereas that of MSH is mimicked by cyclic AMP, raises the possibility that one of the steps following binding is an alteration in the levels of cyclic nucleotides. The slow rate of action of the cyclic nucleotides when compared with that of melatonin may reflect a low nucleotide permeability of the membrane, the effect being exerted gradually as the nucleotide enters the cell. There are at least three possible steps common to the action of melatonin and drugs acting at other receptor sites: (i) membrane depolarization; (ii) an increase in membrane permeability to calcium ions; (iii) alterations in the concentration of either cyclic AMP or GMP.

The melanocytes produced by the present *in vitro* technique have a surprisingly wide range of membrane receptor sites. It is not known whether pigment cells *in vivo* are similarly well endowed. The cells under study here are produced as a result of primary differentiation *in vitro*. Little is known about the factors controlling the kinds of membrane receptor inserted during differentiation, but it seems plausible that cells which undergo primary differentiation in the absence of normal, local cues might develop a much wider range of potential membrane responses than found *in vivo*. It may be that in the course of normal differentiation a complete range of receptor molecules is initially inserted into the membrane and selective inhibition brings about the final membrane complement of receptors. The diversity of receptor sites in the cultured pigment cell may offer a useful preparation for the study of interactions between different membrane components in a single cell.

The exceptional sensitivity to melatonin of the cells described here deserves comment. This could be a consequence of the isolation of pigment cells from other cells normally found in the frog or *Xenopus* tadpole epithelium. This seems likely since we consistently observed that highly sensitive cells were always found in isolation; pigment cells which differentiated within a sheet or aggregate had sensitivities more closely allied to that found by others. Since there is now increasing evidence for interaction between melatonin and other endocrine secretions (Cardinali, 1974; Minneman & Wurtman, 1975) it seems possible that the sensitivity of the receptors of pigment cells in sheets is controlled either by local levels of other hormones or cyclic nucleotides. Alternatively, pigment cells in contact could be electrically coupled to each other and to other cells in the sheet. In that case, intracellular changes induced

by melatonin might be smoothed out by diffusion through the intercellular junction. The basis of the variation in sensitivity to melatonin from one isolated cell to the next is not clear, but it could be related to the prevailing intracellular level of the cyclic nucleotides.

It seems worth considering whether cultured pigment cells might provide a useful bioassay for melatonin since the preparation is simple and convenient and the cells respond relatively specifically in the 10^{-15} to 10^{-14} M concentration range. The use of appropriate blocking agents (see Table 1) would eliminate responses from interfering substances, such as 5-HT, likely to be present in biological samples such as pineal homogenates. The variability in pigment cell sensitivity would not detract from the quantitative value of the method since an unknown solution may be compared with a standard by densitometry on a single sensitive pigment cell.

Preliminary experiments, done in collaboration with Dr J. Arendt, in which the same pineal extract was assayed visually on isolated pigment cells, by the tadpole bioassay method of Ralph & Lynch (1970) and by radio-immunoassay (Arendt *et al.*, 1975) showed the present preparation to give results similar to those obtained by the two established assay methods. The potential usefulness of the isolated pigment cell for the study of the interactions between melatonin and the steroid hormones (Cardinali, Nagle & Rosner, 1975) should also not be overlooked.

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