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Aggregation of pigment granules in single cultured *Xenopus laevis* melanophores by melatonin analogues

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1 Isolated melanophores were differentiated from aggregates of neural crest obtained from neurula stage *Xenopus laevis* embryos after 2 days in culture.

2 Condensation of pigment granules in these cells by melatonin (5-methoxy N-acetyltryptamine, aMT) and various novel analogues was monitored with an image analysis system to quantitate the area occupied by pigment in individual cells.

3 Melanophores exposed to vehicle (a maximum of 0.1% MeOH) showed little (<5%) change in pigment area. aMT produced a dramatic condensation of pigment granules (EC_{50} = the concentration producing a half maximal condensation, 9 pM). The response was rapid, reached a maximum (~80% decrease in pigmented area) by 10 min, and was reversible after removal of aMT from the culture medium.

4 Aggregation to aMT was blocked by treating melanophores with pertussis toxin (1 $\mu g\,ml^{-1}$, 7 h) indicating a role for a guanosine 5' triphosphate (GTP)-binding protein in transducing the aMT receptor signal.

5 Structure-activity studies indicated that analogues of aMT lacking a side-chain N-acyl substituent (5-methoxytryptamine, MT) or a group at the 5-position of the indole ring (N-acetyltryptamine, aT) were unable to induce pigment aggregation ($EC_{50} > 10\,\mu M$).

6 Lengthening the side-chain N-acyl group (N-propionyl, N-butanoyl) was tolerated to some degree but eventually (N-valeroyl and larger) activity diminished. Of the 5-position analogues tested 5-methoxy (aMT) was by far the most potent.

7 Halogen substitution in the 6-position of the indole ring led to some loss of activity as did a 6-OH substitution. The 6-OCH_3 compound was inactive.

8 These studies demonstrate the utility of this model in investigations of structure-activity relationships at the aMT receptor and suggest that it may be a valuable system for determining the transduction mechanisms coupled to the aMT receptor.

Keywords: Melatonin; melatonin analogues; *Xenopus* melanophores; pigment aggregation; structure-activity relationships

Introduction

Melatonin (5-methoxy N-acetyltryptamine, aMT), synthesized by the vertebrate pineal gland and secreted into the general circulation, plays a fundamental role in regulating seasonal changes in the reproductive axis in photoperiodic species (Tamarkin *et al.*, 1985) and is involved in regulating circadian rhythms in birds, reptiles (Underwood, 1989) and, possibly, mammals (Armstrong, 1989). aMT, probably synthesized locally, also modulates various aspects of retinal physiology such as photoreceptor outer segment disc shedding and phagocytosis, melanosome aggregation in pigment epithelium, cone photoreceptor retinomotor movements and intraocular pressure (Besharse *et al.*, 1988). Recent evidence indicates that aMT exerts its biological effects through specific receptors in the brain and eye. The development of 2-[^{125}I]-iodomelatonin (2-[^{125}I]-aMT) initially for use in aMT radioimmunoassay (Vakkuri *et al.*, 1984), has enabled several groups to begin to identify and characterize high affinity aMT-binding sites in brains of various species including man (for review, see Morgan & Williams, 1989). Recent studies have also begun to focus on the transduction mechanisms utilized by the aMT receptor (Morgan *et al.*, 1989a; Vanecek & Vollrath, 1989). One of the few cellular models which exploits a known physiological response to aMT is the amphibian melanophore (for review see Rollag, 1988). In dermal melanophores aMT induces a condensation of pigment granules around the nucleus, producing a lightening of skin colour, a response which is part of the process adapting skin colour to external illumination. Pigment aggregation can also be produced in cultured melanophores *in vitro*. In the present study, melanophores obtained from the neural crest of *Xenopus laevis* embryos have been isolated and used to quantitate the pigment aggregation response to aMT and a number of novel analogues. These studies begin to allow an understanding of the structure-activity relationships at the amphibian aMT receptor and the transduction mechanisms triggered by aMT receptor activation.

Methods

Tissue culture

Xenopus laevis embryos were produced by adult frogs induced to lay by injection of human chorionic gonadotrophin (Chorulon, Intervet Laboratories Ltd., 400 iu/male, 600 iu/female). The embryos were reared in tap water until they reached stage 20, assessed from the normal table of *Xenopus* development (Nieuwkoop & Faber, 1956). The neural plate from about 20 embryos was dissected out and dispersed into small aggregates as described previously (Messenger & Warner, 1977). Tissue aggregates attached to the petri dish and after 2 to 3 days large pigmented cells, often at the edge of the aggregate, were readily visible among many nerve, muscle and undifferentiated cells. Cells were cultured in medium containing (mM): NaCl 100, KCl 2.5, $CaCl_2$ 2, $MgCl_2$ 2, $NaHCO_3$ 5 plus 10% foetal calf serum and penicillin 100 u ml^{-1} and streptomycin 100 $\mu g\,ml^{-1}$. All experiments were done between 7 and 12 days of culture.

Aggregation responses

On the day of the experiment, cells were washed twice with fresh culture medium. The number of melanophores in each dish was variable (between 10 and 100). Typically the response of 5 or 6 individual pigment cells per dish, chosen at random,

was assessed. Drugs were dissolved in MeOH at 10 mM and stored at −30°C. Drugs were diluted just before use with deionized water and added from 100× concentrated stock solutions. The maximal concentration of MeOH added (0.1%) did not induce pigment granule aggregation. For each individual cell, the area occupied by pigment was measured (generally 2000 to 10000 μm^2) and designated 100%. At rest, pigment occupied the full area of the cell. The area covered by pigment after drug addition was related to the initial pigment area. Cells were observed through a Leitz compound microscope (×100 magnification) attached to a See-Scan image analysis system (See-Scan, Cambridge, U.K.). Routinely drugs were added for 10 min, then the area of the cell covered by pigment determined. As it required approximately 0.5 min to 1 min to measure each cell and about 5 cells per dish were assayed, measurements were generally done between 10 and 15 min after drug addition. In this way cumulative concentration-response curves were constructed. When a cell was fully aggregated the area occupied by pigment was usually between 15 and 25% of the initial area. All cells responded equally well to melatonin, irrespective of their size. An EC_{50} value, calculated from each concentration-response curve, was defined as the concentration of agonist causing the area occupied by pigment granules to decrease by 50% relative to the maximum aggregation responses.

Drugs

The compounds used in these studies were obtained from the following sources: melatonin (aMT), 6-hydroxymelatonin (6-OHaMT), N-acetyl 5-hydroxytryptamine (OHaT), 5-methoxytryptamine (MT), 5-methyltryptamine (MeT), tryptamine (T) (Sigma Chemical Co., Poole, Dorset); 5-benzyloxytryptamine (BzOT), 4-methoxy-3-(2-aminoethyl) benzo[b]thiophene (4-MBTh), 6-methoxy-3-(2-aminoethyl) benzo[b]thiophene (6-MBTh) (Aldrich Chemical Co., Gillingham, Dorset); 2-iodomelatonin (2IaMT) (Research Biochemicals Inc., Mass., U.S.A.); 6-chloromelatonin (6-ClaMT), 6-fluoromelatonin (6-FaMT) and 6,7-dichloro 2-methylmelatonin (6,7-Cl2MeaMT) (Lilly Research Laboratories, Indianapolis, U.S.A.); 2-chloromelatonin (2-ClaMT) (Dr C.E. Smithen, Roche Products Ltd., Herts.); 5-methoxy N-hexanoyltryptamine (hMT) (Dr D.J. Skene, University of Surrey); 5-methoxy N-acetyl 3-(2-aminoethyl) benzo[b]thiophene (MaBTh) (Dr T.R. Bosin, Indiana University, U.S.A.); 6-methoxymelatonin (6-MaMT) (Dr D.C. Klein, NIH, Bethesda, U.S.A.). Several N-acylated compounds were synthesized: 5-methyl N-acetyltryptamine (MeaT); 5-benzyloxy N-acetyltryptamine (BzOaT); 5-methoxy N-formyltryptamine (fMT); 5-methoxy N-propionyltryptamine (pMT); 5-methoxy N-butanoyltryptamine (bMT); 5-methoxy N-isobutanoyltryptamine (ibMT); 5-methoxy N-valeroyltryptamine (vMT); 6-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (6-MaBTh); 4-methoxy N-acetyl 3-(2-aminoethyl)benzo[b] thiophene (4-MaBTh). The procedure used to synthesize all of these compounds was similar to that described by Ho *et al.* (1968) except that one equivalent of the appropriate anhydride was used. fMT was prepared by addition of acetic anhydride to 5-methoxytryptamine dissolved in formic acid. All products were characterized after recrystallization as single spots on two thin layer chromatography (t.l.c.) systems: (I) chloroform/methanol/acetic acid/water, 60:30:3:1; (II) ethyl acetate/butan-1-ol, 90:10 and structures were confirmed by nuclear magnetic resonance. The abbreviations used for melatonin analogues follow, as far as is possible, the code outlined previously (Smith, 1981).

Pertussis toxin was obtained from Porton Products (Porton Down, Wilts).

Results

Large numbers of nerve and muscle cells differentiated from the fragments of neural crest placed in each culture dish as was seen previously (Jackson *et al.*, 1974; Messenger & Warner, 1977). Among these were smaller numbers of melanophores (10–100) easily recognised by their large size (50–100 μm diameter), distinctive shape and dark appearance due to the presence of many melanin-containing pigment granules (Figure 1a). Addition of aMT to these cultures resulted in a dramatic condensation of pigment granules in the melanophores (Figure 1b). The response was readily quantitated by determining the area of the cell occupied by pigment (Figure 1c, d) and could be detected within 1–2 min and had reached a maximum by 10 min (Figure 2). The continued presence of aMT in the culture medium maintained the pigment granules of melanophores in an aggregated state. Removal of aMT by washing the cells with fresh aMT-free medium resulted in a dispersal of pigment and a return, after about 20 min, of pigmented cell area to values very similar to those seen initially. Treatment of melanophores with vehicle (0.1% v/v MeOH) did not alter the mean pigmented cell area (Figure 2). In some cells there were movements of pigment granules into and out of projections as the shape of the cell slowly changed during the course of the experiment. Such changes produced only minor alterations in the area occupied by pigment.

Treatment of cultures with pertussis toxin (1 $\mu g\,ml^{-1}$, 7 h) completely prevented the aggregating effect of aMT (10 nM) (Figure 3). Removal of Ca^{2+} from the culture medium by chelation with EGTA (2.3 mM, calculated free Ca^{2+} 0.2 μM) prevented the aggregation response to aMT (10 nM) but the response returned when Ca^{2+} was restored to the culture medium (Figure 4).

aMT produced a concentration-related aggregation of pigment granules in melanophores (EC_{50} 9 pM). N-acetyl-5-hydroxytryptamine (OHaT), the immediate precursor of aMT in the pineal gland, did not aggregate pigment granules in melanophores even at a high concentration (10 μM). The major metabolite of aMT, 6-OHaMT was a weak agonist and induced pigment aggregation with an EC_{50} of 63 nM. Other endogenous indoles related to aMT were inactive (no aggregation at 10 μM with 5-hydroxytryptamine, tryptamine and MT).

All of the aMT analogues tested, with the exception of fMT, gave concentration-response curves parallel to that for aMT (Figure 5). The fMT concentration-response curve was shallow. Several compounds were tested in which the N-acetyl group on the side chain of aMT was changed. A smaller group (N-formyl, fMT) led to a marked loss of activity (EC_{50} 296 nM) but compounds with larger substitutions (N-propionyl, pMT; N-butanoyl, bMT) were recognised by the aMT receptor (EC_{50} 11 pM and 60 pM respectively). Substitution of a larger group in the indole side-chain led to a loss of activity (N-isobutanoyl, ibMT EC_{50} 2.4 nM; N-valeroyl, vMT EC_{50} 14.1 nM; N-hexanoyl, hMT $EC_{50} > 10\,\mu M$).

Analogues with substitutions other than methoxy on the 5-position of the indole ring were inactive ($EC_{50} > 1\,\mu M$ for 5-methyl, 5-benzyloxy, 5-H and 5-hydroxy analogues; Figure 6).

Analogues of aMT with a substituent in the 6-position of the indole ring retained agonist activity but were less potent than aMT itself (6-fluoro, EC_{50} 2.5 nM; 6-chloro, EC_{50} 5.3 nM; 6,7-dichloro 2-methyl, EC_{50} 52 nM; 6-methoxy, $EC_{50} > 1\,\mu M$; Figure 7).

A series of analogues in which the indole nucleus of aMT was replaced by a benzo[b]thiophene ring were studied (Figure 8). The direct benzo[b]thiophene analogue of aMT (MaBTh) was an agonist at the aMT receptor in melanophores (EC_{50} 1.5 nM) although much less potent than aMT itself. The effect of the position of the 5-methoxy group on the ring was examined in this series. The 4-methoxy derivative retained some potency (EC_{50} 41 nM) but the 6-methoxy derivative was completely inactive ($EC_{50} > 1\,\mu M$).

Discussion

The ability of aMT to induce aggregation of pigment granules in amphibian melanophores has long been recognized (Rollag,

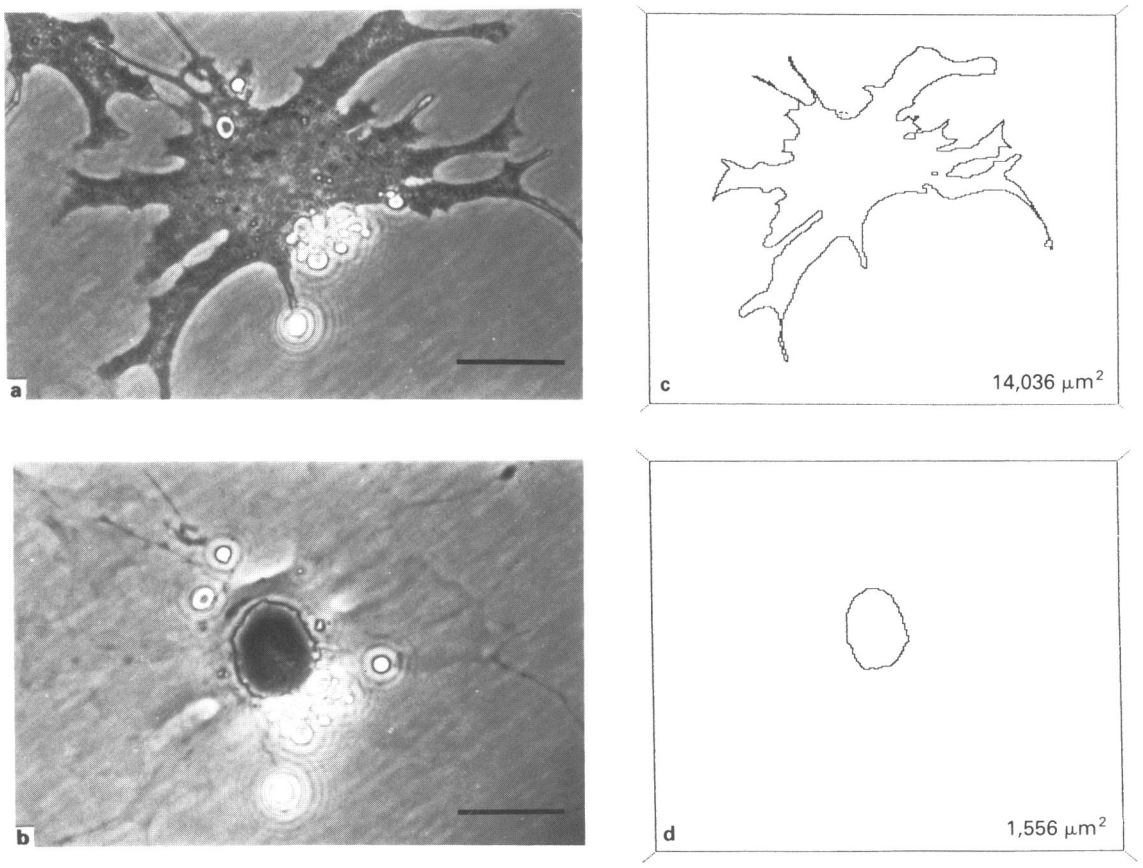

Figure 1 Photographs (a, b) and digitized processed images (c, d) of a typical melanophore before (a, c) and after (b, d) addition of melatonin (1 nM, 10 min). The horizontal bar represents 50 μm. The area occupied by pigment in (c) is 14036 μm^2 and in (d) 1556 μm^2.

1988). Indeed, this sensitive and specific response played an essential part in the original isolation and chemical characterization of aMT over 30 years ago as the skin-lightening factor present in pineal gland extracts (Lerner *et al.*, 1958). Since that time it has been recognised that aMT participates in regulating physiological colour changes in amphibians such as *Xenopus* (Bagnara, 1960). The response to aMT which occurs in melanophores *in vivo* can also be readily demonstrated in isolated melanophores prepared either from *Xenopus* embryos (Messenger & Warner, 1977) or tadpole skin (Fukuzawa & Ide, 1983; Rollag, 1988). In the present study, *Xenopus* melanophores have been used as a model system to study the

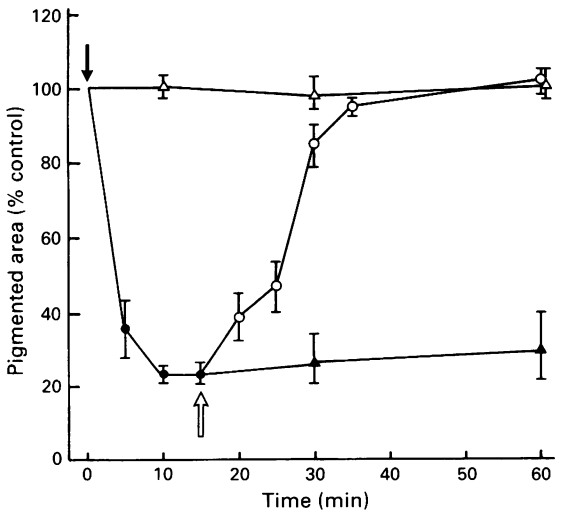

Figure 2 Time-course of pigment aggregation. Melanophores were treated with vehicle (△, 0.1% MeOH, $n = 10$) or 5-methoxy N-acetyltryptamine, (aMT, ●, 1 nM) at the arrow (↓). aMT-treated cells were washed with fresh medium (○, $n = 5$) or aMT-containing medium (▲, $n = 5$) at the second arrow (⇧). The area of each cell occupied by pigment granules was determined as described in Methods. All area measurements were related to the initial pigmented area of each cell. Each point represents the mean with s.e.mean shown by vertical bars.

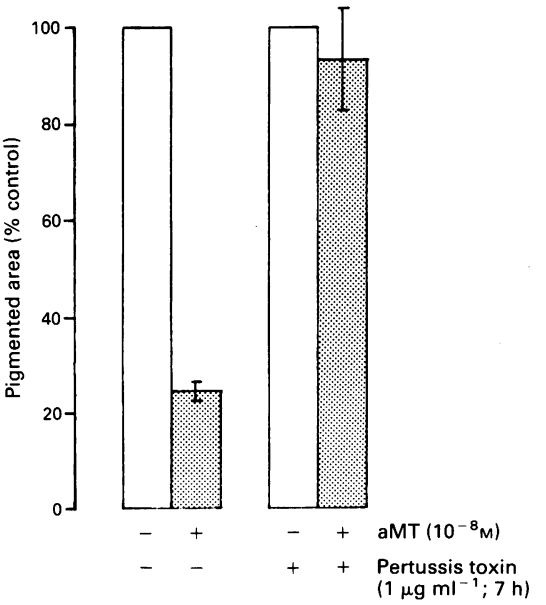

Figure 3 Effect of pertussis toxin on the pigment aggregation response to 5-methoxy N-acetyltryptamine (aMT). Cultures were treated with pertussis toxin (1 $\mu g\,ml^{-1}$, 7 h) or vehicle (0.25% glycerol/0.25 mM sodium phosphate/2.5 mM sodium chloride) then the response to aMT (10 nM) tested. Each column represents the mean of 6 cells; vertical bars show s.e.mean.

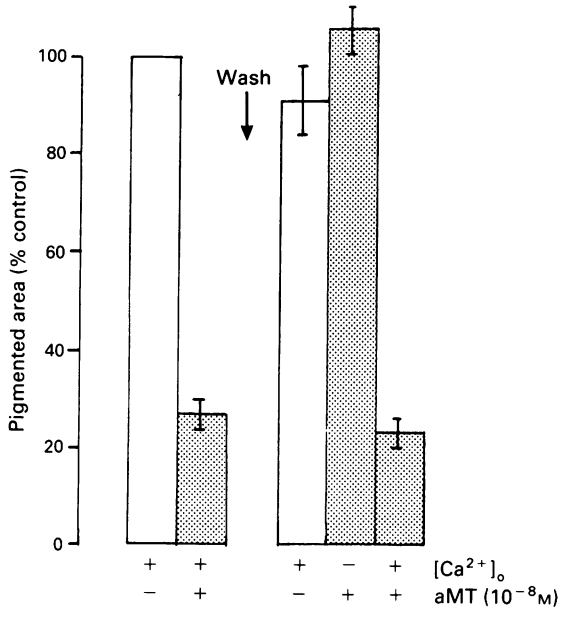

Figure 4 Effect of low external Ca^{2+} $[Ca^{2+}]_0$ on the pigment aggregation response to 5-methoxy N-acetyltryptamine (aMT). Six melanophores in a single culture were exposed to the following treatments: aMT (10 nM), which aggregated pigment granules as expected; fresh aMT-free medium, which reversed the aggregation produced by aMT; aMT (10 nM) plus EGTA (2.3 mM) to chelate extracellular Ca^{2+} (calculated free $Ca^{2+} = 0.2\,\mu M$), which prevented the expected response to aMT; restoration of extracellular Ca^{2+} (2 mM), by adding fresh medium, in the presence of aMT (10 nM) which resulted in pigment aggregation.

structure-activity relationships at the aMT receptor and to begin to examine the molecular mechanisms involved in aMT action.

Cultures pretreated for several hours with pertussis toxin failed to show aggregation in response to a high concentration (10 nM) of aMT (Figure 2). This finding suggests that the action of aMT is mediated by a guanine nucleotide-binding protein which is a pertussis toxin substrate, such as G_i. This idea is consistent with the finding that aMT inhibits the MSH-induced increase in adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in melanophore-rich skin of *Xenopus* (Van de Veerdonk & Konijin, 1970) and *Rana pipiens* (Abe *et*

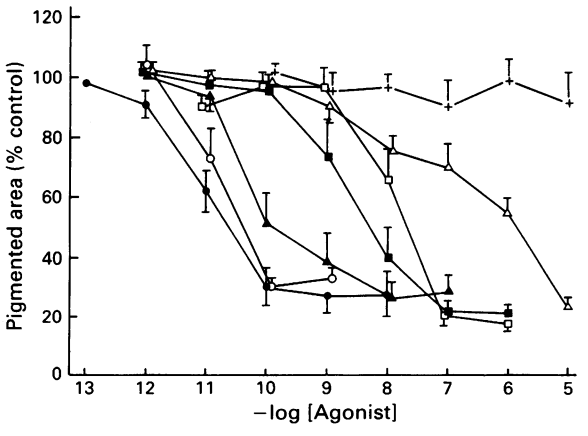

Figure 5 Pigment aggregation in response to N-acyl 5-methoxytryptamine derivatives. Concentration-response curves were obtained as described in Methods. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: N-formyl 5-methoxytryptamine (△, $n = 7$); aMT (●, $n = 18$); N-propionyl 5-methoxytryptamine (○, $n = 6$); N-butanoyl 5-methoxytryptamine (▲, $n = 6$); N-isobutanoyl 5-methoxytryptamine (■, $n = 6$); N-valeroyl 5-methoxytryptamine (□, $n = 8$); N-hexanoyl 5-methoxytryptamine (+, $n = 5$).

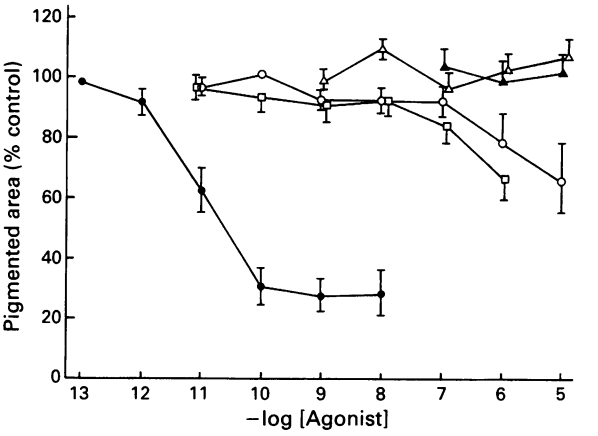

Figure 6 Pigment aggregation responses to 5-substituted N-acetyltryptamines. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: 5-methyl N-acetyltryptamine (○, $n = 7$); N-acetyltryptamine (▲, $n = 5$); 5-hydroxy N-acetyltryptamine (△, $n = 6$); 5-benzyloxy N-acetyltryptamine (□, $n = 9$).

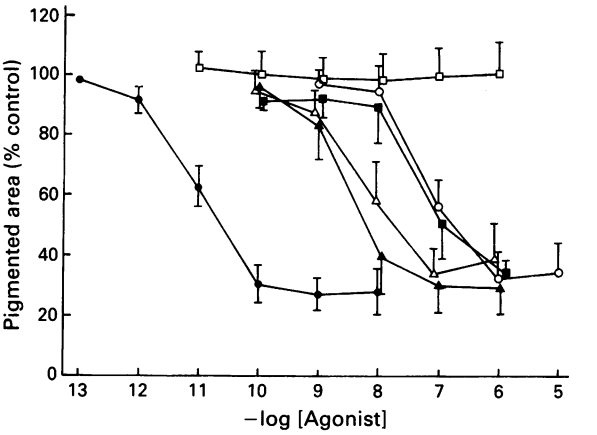

Figure 7 Pigment aggregation responses to 6-substituted melatonin (aMT) analogues. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: aMT (●, $n = 18$); 6-hydroxymelatonin (○, $n = 8$); 6-fluoromelatonin (▲, $n = 6$); 6-chloromelatonin (△, $n = 8$); 6-methoxymelatonin (□, $n = 6$); 6,7-dichloro 2-methylmelatonin (■, $n = 6$).

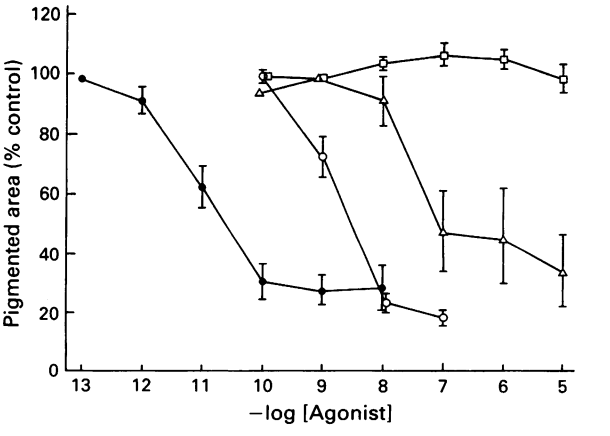

Figure 8 Pigment aggregation in response to methoxy substituted N-acetylaminoethyl benzo[b]thiophenes. Each point represents the mean of the number of cells indicated, s.e.mean shown by vertical bars: 5-methoxy N-acetyltryptamine (●, $n = 18$); 5-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (○, $n = 7$); 4-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (△, $n = 5$); 6-methoxy N-acetyl 3-(2-aminoethyl)benzo[b]thiophene (□, $n = 5$).

al., 1969). Furthermore, agents elevating intracellular cyclic AMP such as forskolin, MSH and cell-permeable analogues of cyclic AMP induce an opposite response to aMT, namely pigment granule dispersal (Seldenrijk *et al.*, 1979). Pertussis toxin treatment of meningeal explants blocks the ability of aMT to reverse pigment granule dispersal induced by forskolin in melanophores in this tissue (White *et al.*, 1987), again suggesting that aMT may initiate aggregation by inhibiting adenylate cyclase activity via G_i.

The aggregation response to aMT was also absent when Ca^{2+} was removed from the incubation medium by addition of EGTA but was rapidly restored when extracellular Ca^{2+} was replenished (Figure 3). The inhibition of the aMT response in the presence of low (0.2 μM) extracellular Ca^{2+} may reflect a requirement for Ca^{2+} of the cellular machinery controlling the physical movement of pigment granules. Alternatively, extracellular Ca^{2+} may be involved in the aMT receptor transduction mechanism. However, an increase in extracellular K^+ did not induce pigment aggregation (data not shown), and an earlier study on pigment granule movement in melanophores of *Rana* skin (Wright & Lerner, 1960) showed that extracellular Ca^{2+} was not required for the aggregating action of aMT.

In agreement with earlier studies using either an indirect photometric method (Heward & Hadley, 1975) or the semi-quantitative 'melanophore index' (Quay, 1968), the present studies indicate that the 5-methoxy group on the indole nucleus and the N-acetyl side chain of aMT are important determinants of agonist activity at the melanophore aMT receptor. However, examination of several novel aMT analogues on the melanophore response has allowed a finer characterization of structure-activity relationships at the aMT receptor (Table 1). In particular, it is apparent that larger N-acyl groups on the indole side-chain are recognized by the receptor almost as well as the N-acetyl group of aMT. The EC_{50} value for the N-propionyl substituted compound is similar to aMT (11 pM) while that for the N-butanoyl analogue is reduced only slightly (60 pM); larger side-chain substitutions lead to a marked, progressive loss of activity as the length of the side-chain increases. An N-formyl substitution is too small to be recognised by the receptor. Broadly this agrees with recent data from radioligand binding experiments in which 2-[^{125}I]-iodomelatonin (2-[^{125}I]-aMT) was used to identify aMT binding sites; in membranes prepared from both whole chick brain and sheep pars tuberalis, N-propionyl and N-butanoyl analogues displace 2-[^{125}I]-aMT binding with affinity constants (K_i) actually slightly higher ($\sim$10 fold) than aMT itself (Sugden & Chong, 1991). Radioligand binding experiments on *Xenopus* tissue with the use of a wide range of aMT analogues will be needed to determine if the *Xenopus* aMT receptor is identical to the high affinity site characterized in chick brain and retina, sheep pars tuberalis and some specific nuclei of rat brain (Dubocovich, 1989; Morgan *et al.*, 1989b; Laitinen *et al.*, 1990; Sugden & Chong, 1991).

Table 1 Potency of 5-methoxy N-acetyltryptamine (aMT) and some analogues in producing pigment aggregation in isolated *Xenupus laevis* melanophores

Compound	EC_{50} (pM)
N-acyl substitution	
aMT	9
pMT	11
bMT	60
ibMT	2424
vMT	14125
fMT	296000
hMT	>10000000
6-substitution	
6FaMT	2540
6ClaMT	5280
6,7diCl2MeaMT	52000
6OHaMT	63400
6MaMT	>1000000
5-substitution	
BzOaT	1000000
MeaT	10000000
aT	>10000000
OHaT	>10000000
Benzo[b]thiophenes	
5MaBTh	1560
4MaBTh	41300
6MaBTh	>1000000

EC_{50} values were determined from Figures 5 to 8 using a weighted log-logit equation.
For abbreviations, see *Drugs* section of text.

None of the substitutions at the 5-position of the indole nucleus which were tested (-H, -OH, -CH_3, -benzyloxy), other than the -OCH_3 of aMT, gave compounds which aggregated melanophore pigment granules ($EC_{50} > 1\,\mu$M) suggesting that the size and/or electronic properties of the substituent at this 5-position is critical for agonist activity. Previous work on pigment aggregation responses (Quay, 1968; Heward & Hadley, 1975) supported the idea that the substituent at the 5-position of the indole ring was essential for agonist activity and that the side-chain N-acetyl substituent determined the affinity of binding to the receptor. Clearly the 5-OCH_3 substituent is vital for agonist activity but this is also influenced by the N-acyl group. Furthermore, radioligand binding studies have shown that substitutions at both positions can markedly affect binding affinity (Sugden & Chong, 1991).

The indole nucleus is not essential for aMT activity in that a benzo[b]thiophene analogue with a -OCH_3 substitution at the appropriate position aggregated melanophore pigment granules although this compound was considerably less potent (EC_{50} 1560 pM) than aMT itself. Interestingly, a comparison of -OCH_3 substitution at different positions on the benzo[b]thiophene ring showed that the 6-OCH_3 compound was inactive ($EC_{50} > 1\,\mu$M) yet the 4-OCH_3 derivative retained some activity (EC_{50} 41 nM). A similar order of potency has been seen with these analogues in radioligand binding experiments on chick brain and sheep pars tuberalis membranes (Sugden & Chong, unpublished) again suggesting a close similarity of the amphibian, avian and mammalian aMT receptors.

Some analogues of aMT with 6-position substituents retained some activity at the melanophore receptor (e.g. -F, -Cl, -OH). However, a 6-OCH_3 substitution leads to a loss of activity (EC_{50} >1000 nM). This finding clearly distinguishes the amphibian aMT receptor from a low affinity (K_d 1.5–3 nM) 2-[^{125}I]-aMT binding site reported to be widely distributed in the brain of hamsters (Duncan *et al.*, 1988; 1989) which recognizes 6-MaMT as readily as aMT itself.

It appears that the aMT receptor in *Xenopus* melanophores is similar to the high affinity aMT receptor, now well-characterized pharmacologically in 2-[^{125}I]-aMT radioligand binding assays in a mammal (sheep pars tuberalis), bird (chick brain and retina) and a marsupial (brain of the Bennett's wallaby) (Sugden & Chong, 1991; Dubocovich, 1989; Sugden *et al.*, unpublished). The structural requirements for pigment aggregating activity seem to be more strict than for binding to the high affinity 2-[^{125}I]-aMT binding site identified in membrane assays. For example, while 5-substitutions other than 5-OCH_3 certainly reduce binding affinity they lead to a complete loss of agonist activity on melanophores. Similarly 6-substitutions are better tolerated by the site detected by 2-[^{125}I]-aMT than by the melanophore receptor. Conceivably this may represent a 'relaxation' of the aMT receptor, in terms of the structures it will accommodate, during evolution. The validity of such a speculation awaits binding data from *Xenopus* which can be directly compared with that available in higher vertebrates and the development of simple cellular models in these vertebrates which can compare the biological activity of aMT and its analogues. It must be remembered that of the 2-[^{125}I]-aMT binding sites detected in vertebrates, only that found in the neuronal retina has clearly been shown

to mediate a biological response, namely aMT-induced inhibition of Ca^{2+}-dependent [^{3}H]-dopamine release (Dubocovich, 1988). Indeed, few *in vitro* models exist in which a response to aMT can be quantitated in order to study either structure-activity relationships or aMT receptor transduction mechanisms (Morgan *et al.*, 1989a; Vanecek & Vollrath, 1989). The melanophore model described here will continue to be useful for such studies.

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