IRREVERSIBLE STIMULATION OF *XENOPUS* MELANOPHORES BY PHOTOAFFINITY LABELLING WITH *p*-AZIDOPHENYLALANINE^{13}-α-MELANOTROPIN

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1. Introduction

α-Melanotropin induces pigment dispersion in melanophores and pigment formation in melanocytes and melanoma cells. The pigment-dispersing effect of MSH is completely reversible in vitro, as the melanosomes reaggregate readily upon removal of the hormone. The primary site of action of α-MSH appears to be a cell surface receptor [1], which can be stimulated by two different portions of the α-MSH molecule, namely the central (–Glu–His–Phe–Arg–Trp–) and the C-terminal (–Gly–Lys–Pro–Val · NH_2) message sequences [2]. The C-terminal residue is essential for the interaction with the receptor, since its absence reduces the biological activity considerably (in preparation); the structure of its side-chain, however, is less important, and it may therefore contain an affinity label.

Photoaffinity labelling is a promising approach for receptor localization and isolation (reviewed [3]). This technique was applied to study the action of peptides, using *p*-azidophenylalanine as photolabile residue [4–6]. Although a [Pap^2]-derivative of α-MSH had been prepared [7], amphibian pigment cells could not be labelled because of the lack of a suitable melanophore system, resistant to UV illumination but still transparent enough for an efficient photolysis of receptor-bound peptides. In the meantime, several other hormone receptors were successfully labelled using photolysable derivatives of, e.g., glucagon [8], insulin [9], oxytocin [10], angiotensin [11], enkephalin [12], or pentagastrin [13]. With pentagastrin, an irreversible stimulation upon photolysis was reported, whereas in the other cases either an irreversible block of the receptors was observed (oxytocin, angiotensin) or the incorporation was tested by irreversible binding to membrane components.

A new *Xenopus laevis* melanophore assay (in preparation) using tail-fin pieces of tadpoles has been adopted for labelling experiments with different photolabile α-MSH derivatives. Here we describe an irreversible stimulation of *Xenopus* melanophores by photoaffinity labelling with [Pap^{13}]-α-MSH, a potent analogue of α-MSH. The stimulation lasts for several hours, despite continuous washing. Control experiments indicate that the reaction is specific and due to labelling of MSH receptors. From variations of both the hormone concentration during the incubation/photolysis phase and of the temperature during the wash phase, the degree of receptor labelling and receptor turnover may be estimated.

Abbreviations: Amino acids and peptides, according to the tentative rules and recommendations of the IUPAC–IUB Commission on Biochemical Nomenclature, Eur. J. Biochem. (1972) 27, 201–207; α-MSH, α-melanotropin (α-melanocyte-stimulating hormone); Pap, *p*-azidophenylalanine; M.I., melanophore index

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2. Materials and methods

2.1. *Peptides*

Synthetic α-MSH [14] was used as a standard. [Pap^{13}]-α-MSH (Ac-Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Pap-NH_2) was prepared by a fragment condensation approach similar to that of a recent synthesis [15]; $[\alpha]_D^{25} = -56.0°$ (c = 0.48, 1% AcOH); UV (0.1 N NaOH): λ_{max} =

254 nm ($\epsilon = 2 \times 10^4$), 280 nm ($\epsilon = 6.8 \times 10^3$). All manipulations of peptides containing Pap were performed under sodium light or normal lamp light to avoid photolytic decomposition.

2.2. *Assay of melanotropic activity*

Tadpoles of *Xenopus laevis* (stage 51–53 according to [16]) were adapted to a white background for 18–24 h to obtain full aggregation of the melanosomes. Pieces of ~2 × 2 mm (containing ~100 melanophores) were excised from the ventral tail-fin and equilibrated in assay medium (Leibovitz L-15 [17]) without phenol red; diluted 1:1 with double distilled water; containing 2 mM $CaCl_2$, 0.1% bovine serum albumin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1.25 μg/ml amphotericin B; adjusted to 200 mOsm with NaCl; pH 7.6). The pieces were incubated in perspex wells containing different concentrations of α-MSH and [Pap^{13}]-α-MSH in assay medium. The melanosome dispersion was quantified microscopically after 15, 30, 45 and 60 min, using the melanophore index of [18].

The melanotropic activity of [Pap^{13}]-α-MSH was further determined with the modified [19] reflectometric test system [20] using the skin of *Rana pipiens*, and with a tyrosinase assay with *Cloudman* S-91 melanoma cells in monolayer culture [21], adapted from the method in [22].

2.3. *Photoaffinity labelling*

Photolysis was carried out at 338 nm with a Xenon XBO 450 lamp (Zeiss) fitted with a water filter, an interference filter (338 nm; Balzers) and a UV-reflecting mirror (Zeiss), yielding an intensity incident on the tail-fin pieces of $\sim 10^{-3}$ W . cm^{-2}. These conditions give a complete photolysis of [Pap^{13}]-α-MSH in a 10^{-5} M solution within 20 min.

In each photolysis experiment, 6 equilibrated tail-fin pieces (2.2.), cut from 3 animals and divided into 2 wells, were incubated in the peptide solutions for 20 min under normal lamp light to induce melanosome dispersion. During the next 20 min one well was exposed to UV irradiation, while the other was kept under normal lamp light as a control. The pieces were then washed continuously with 4–6 changes of buffer in order to remove non-covalently bound MSH. The temperature during the wash phase was 15°C (exceptions see fig.3) and during the rest of the experiment 20°C.

3. Results

[Pap^{13}]-α-MSH is a full agonist of α-MSH with a relative activity of ~65% in both the *Rana* and the *Xenopus* melanophore assay, and of 50% in the tyrosinase assay. This indicates a similar recognition pattern of the receptors of all 3 cell types for this compound.

In the *Xenopus* assay [Pap^{13}]-α-MSH evokes full melanophore dispersion at 3×10^{-9} M. A time–response study comparing α-MSH with the [Pap^{13}]-derivative reveals an almost identical pattern during incubation and wash phases, when assayed under normal lamp light (fig.1). However, irradiation at 338 nm of tail-fin pieces, incubated with 1.2×10^{-8} M [Pap^{13}]-α-MSH, produces an irreversible melanophore-dispersion lasting >4 h. UV irradiated melanophores, stimulated with 8×10^{-9} M α-MSH, reaggregate within the same time as non-irradiated controls (fig.1). After irradiation the melanophores are not only capable of reaggregating completely, but they are still fully res-

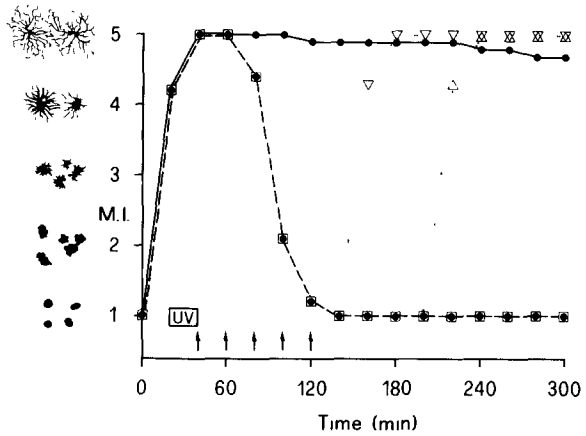

Fig.1. Irreversible stimulation of *Xenopus* tail-fin melanophores by photoaffinity labelling with 1.2×10^{-8} M [Pap^{13}]-α-MSH (●——●) and complete reaggregation of non-irradiated controls (▫– – –▫). Each point represents the average of $n = 23$ measurements (animals). The values for 8×10^{-9} M α-MSH (irradiated and non-irradiated tail-fin pieces; $n = 15$) both coincide with ▫ (within SEM) and are therefore not depicted separately. Restimulation of all control groups with 8×10^{-9} M α-MSH after 140 min (▽ . . . ▽) and 200 min (△ · · · △) gave identical results. In all figures, SEM values are normally 0.1–0.5 M.I. unit; they are slightly higher (0.6–0.8) during the aggregation phase (80–200 min) of non-irradiated pieces. (UV) irradiation phase; (↑) buffer change; (M.I.) melanophore index as introduced [18]; the drawings represent the degree of pigment dispersion of the 5 different stages.

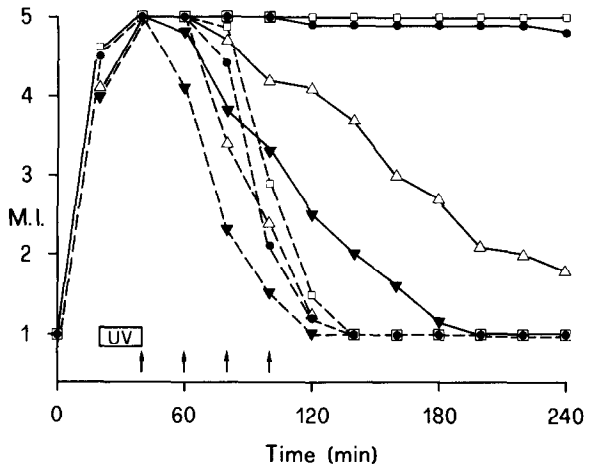

Fig.2. Dose dependence of [Pap^{13}]-α-MSH induced irreversible melanophore dispersion: 3×10^{-9} M (▽, $n = 6$); 6×10^{-9} M (△, $n = 6$); 1.2×10^{-8} M (●, $n = 23$); 1.8×10^{-8} M (□, $n = 9$); incubation with (——) and without (– – –) UV irradiation.

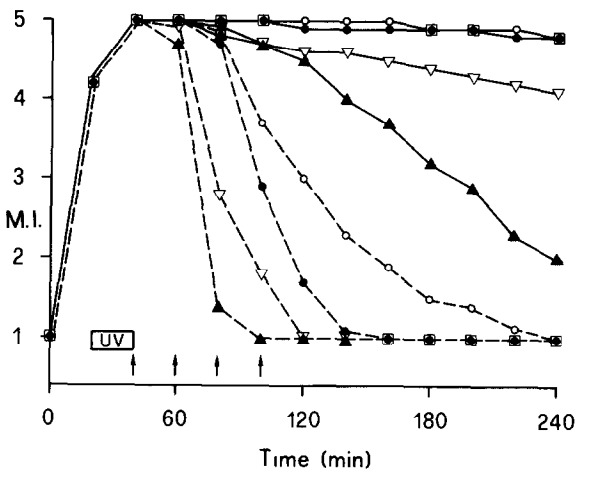

Fig.3. Temperature dependence of the irreversible melanophore dispersion during the wash phase. Tail-fin pieces were incubated with 1.2×10^{-8} M [Pap^{13}]-α-MSH at 20°C and then washed at 26°C (▲, $n = 6$); 20°C (▽, $n = 6$); 15°C (●, $n = 23$); or 12°C (○, $n = 6$); incubation with (–○–) and without (– – –) UV irradiation.

ponsive to restimulation by α-MSH (fig.1). This proves that pigment migration is not influenced by the UV irradiation employed.

The persistence of the melanophore stimulation upon irradiation in the presence of [Pap^{13}]-α-MSH depends on the concentration of the hormone during the photolysis. The minimal dose which produces full melanosome dispersion (3×10^{-9} M) leads to a complete reaggregation of the melanosomes after a 160 min wash phase, although the aggregation kinetics are significantly different from non-irradiated controls (fig.2). At 6×10^{-9} M [Pap^{13}]-α-MSH the effect is still reversible; it becomes irreversible at $\geqslant 1.2 \times 10^{-8}$ M.

The duration of the dispersion is closely related to the temperature of the wash phase: at 20°C the stimulation is slightly reversible and markedly so at 26°C, whereas at 15°C and 12°C the melanophores remain dispersed (fig.3). Although the mechanism of aggregation itself is temperature-dependent (see non-irradiated controls in fig.3), the considerable differences can only be explained with a better persistence of the intracellular signal for melanosome dispersion at $\leqslant$15°C.

When a 50-fold excess of α-MSH (6×10^{-7} M) is simultaneously present in the incubation medium, the irreversible stimulation by 1.2×10^{-8} M [Pap^{13}]-α-MSH is completely abolished: irradiated tail-fin pieces

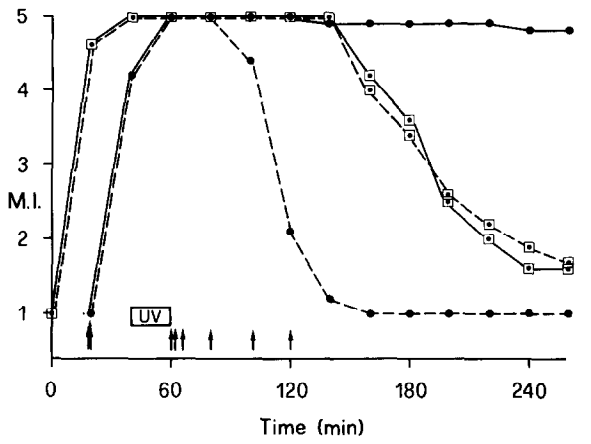

Fig.4. Protection from [Pap^{13}]-α-MSH induced irreversible melanophore dispersion by a 50-fold excess of α-MSH. After preincubation for 20 min with 6×10^{-7} M α-MSH, the tail-fin pieces were further incubated with 6×10^{-7} M α-MSH plus 1.2×10^{-8} M [Pap^{13}]-α-MSH (▣, $n = 6$), and the experiment was continued as in fig.1, with the exception of two additional buffer changes during the first 20 min of the wash phase. Control groups (●, $n = 15$) were incubated with 1.2×10^{-8} M [Pap^{13}]-α-MSH. Incubation with (——) and without (– – –) UV-irradiation. (⇑) Addition of [Pap^{13}]-α-MSH. Note that after stimulation with a large excess of α-MSH the aggregation per se is considerably retarded; this effect, which is also observed with *Rana* melanophores, may be due to the longer duration needed for complete removal/inactivation of the hormone and (probably more important) of the intracellular second messenger.

and non-irradiated controls show an almost identical aggregation pattern (fig.4). *p*-Amino benzoic acid (2×10^{-5} M), present during incubation and photolysis as a scavanger to prevent non-specific labelling, does not alter the irreversible stimulation by 1.2×10^{-8} M [Pap^{13}]-α-MSH. The reagent itself has no effect on pigment migration. Finally, short biologically inactive peptides containing Pap, such as Z-Ala–Ala–Pap-OH [23] or Ac-D-Ala–Pap-OH [7], were without any effect upon irradiation at $\leqslant 2 \times 10^{-3}$ M.

4. Discussion

Tail-fin melanophores of *Xenopus laevis* tadpoles are irreversibly stimulated by photolysis of [Pap^{13}]-α-MSH during incubation with the hormone. The effect appears to be due to a specific labelling of MSH receptors, since the dispersion is neither diminished by a scavanger in a large excess nor produced by photolysable non-melanotropic peptides. Furthermore, the MSH receptors were fully protected from the irreversible stimulation by [Pap^{13}]-α-MSH by a 50-fold excess of α-MSH; therefore, both peptides seem to act via the same receptor. This is a major difference from the report [13] of an irreversible stimulation, where pentagastrin did not protect pancreatic lobules from irreversible protein discharge upon photolysis of 2-nitro-5-azidobenzoyl-pentagastrin, and therefore two types of receptors had to be postulated.

Potent MSH antagonists are still lacking, and therefore MSH receptors cannot be labelled without concomitant stimulation, nor can they be protected from photolytic incorporation with unlabelled peptide without temporary pigment dispersion. New analogues with modifications in position 9 [24] with and without photolabile groups may be more suitable for such experiments.

The molecular mechanism of the irreversible stimulation is unclear. Most probably the labelled MSH receptors remain activated for several hours, generating a constant intracellular signal. Covalent MSH/receptor complexes appear to be rather stable at $\leqslant 15°C$; at 20°C receptor inactivation may range from 20–30%, and at 26°C from 40–60% per hour. It is however too early to give more precise figures since the number of receptors per cell and any of their properties are not known at present.

It can be concluded from the wash-out characteristics of large amounts of MSH (fig.4) that only part of the receptors of each melanophore must be occupied for full stimulation (→ spare receptors). As the incubation with 3×10^{-9} M [Pap^{13}]-α-MSH leads to a complete pigment dispersion, but 1.2×10^{-8} M is needed for full irreversible stimulation by irradiation, we assume that about 25% of the occupied receptors are covalently labelled by the photolysis step (provided that hormone–receptor binding is linear in this concentration range). With a lower receptor occupancy the intracellular signal decreases soon after the wash phase is started.

With respect to the position of the photoaffinity label within the α-MSH molecule, [Pap^{13}]-α-MSH has been regarded as ideal since the label forms part of the C-terminal message sequence of the hormone, ensuring a close contact with the receptor. However, preliminary studies with other photolysable MSH derivatives have shown that the label can be located, e.g., in position 9 or 1, without markedly changing the results (in preparation). Thus, we expect such experiments to lead to a more detailed understanding of the interaction of the different MSH message sequences with the receptor and of the signal generation by the hormone–receptor complex.

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