PHOTOAFFINITY LABELLING OF MSH RECEPTORS REVEALS A DUAL ROLE OF CALCIUM IN MELANOPHORE STIMULATION

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1. Introduction

UV irradiation of *Xenopus laevis* melanophores in the presence of photoreactive [Pap^{13}]-α-MSH induces irreversible pigment dispersion [1]. It appears that specific covalent labelling of MSH receptors results in the generation of a continuous signal lasting for several hours. This longlasting effect makes it possible to study the involvement of various factors, such as Ca^{2+}, at the different steps (see [2]) of hormonal signal transmission.

Ca^{2+} is well known to be indispensable for mediating the effect of α-MSH on melanophores of several species [3,4]. From earlier studies with melanophores of *Rana pipiens* [5] and *Xenopus laevis* [6] it was concluded that the interaction of α-MSH with its receptor leads to the activation of an adenylate cyclase system, as was shown with mouse melanoma cells [7]. The subsequent pigment dispersion is controlled by cAMP and does not depend on extracellular Ca^{2+}, since the dispersion can be brought about by db-cAMP in the absence of Ca^{2+} [3,8]. Thus the effect of Ca^{2+} seems to precede the action of cAMP; however, the precise site of Ca^{2+} action remained unclear. Photoaffinity labelling of MSH receptors enabled us to distinguish between receptor-associated and post-receptor Ca^{2+} requirement. Here we show that Ca^{2+} is essential for hormone–receptor binding, for coupling of the hormone receptor to adenylate cyclase and/or for activation of the enzyme.

Abbreviations: db-cAMP, dibutyryl cAMP; EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetracetic acid; MI, melanophore index; MSH, melanotropin (melanocyte-stimulating hormone); Pap, *p*-azidophenylalanine

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2. Materials and methods

α-MSH and [Pap^{13}]-α-MSH were prepared by a fragment condensation approach using the strategy in [9]. Racemization of α-MSH was performed in 0.1 N NaOH at 60°C for 30 min. Methoxyverapamil (D 600) was a gift of Knoll AG (Ludwigshafen).

Photolysis experiments were done as in [1], except for variations in the composition of the preincubation and wash media. Briefly, ventral tail-fin pieces (2 × 2 mm) of *Xenopus laevis* tadpoles were preincubated at 20°C for 20 min in assay media with or without Ca^{2+} (2×10^{-3} M) or with D 600 (8×10^{-5} M), and were then incubated in the peptide solutions for 20 min under normal lamp light. During the next 20 min the tail-fin pieces were exposed to UV irradiation (controls were kept under normal lamp light). The pieces were then washed continuously at 15°C in media with or without Ca^{2+} or with D 600. Ca^{2+}-free medium was prepared by adding 2×10^{-3} M Mg^{2+} to Ca^{2+}- and Mg^{2+}-free medium containing 10^{-4} M EGTA. Melanosome dispersion was quantified microscopically every 20 min using the melanophore index of [10].

3. Results and discussion

Pigment dispersion in ventral tail-fin melanophores of *Xenopus* tadpoles, induced in vitro by α-MSH or [Pap^{13}]-α-MSH, depends on the presence of extracellular Ca^{2+} (fig.1). In Ca^{2+}-free medium melanosome dispersion is completely abolished or readily reversed; readdition of Ca^{2+} to medium containing the hormone leads to normal dispersion. These results confirm that stimulation of tadpole melanophores by α-MSH requires Ca^{2+}, as was shown with melanophores of

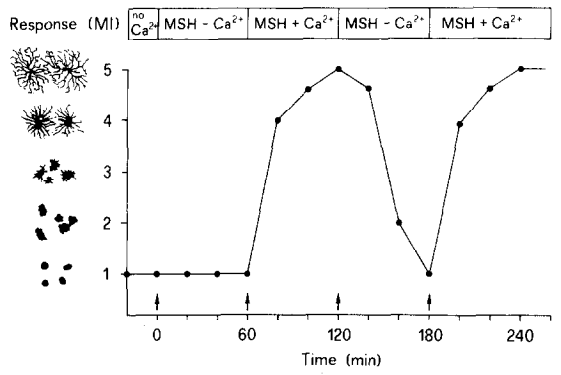

Fig.1. Effect of Ca^{2+} on pigment dispersion induced by α-MSH or [Pap^{13}]-α-MSH. After preincubation for 20 min in Ca^{2+}-free assay medium, the tail-fin pieces were incubated with 8×10^{-9} M α-MSH or with 1.2×10^{-8} M [Pap^{13}]-α-MSH, either in Ca^{2+}-free medium (0–60 and 120–180 min) or in normal medium containing 2×10^{-3} M Ca^{2+} (60–120 and 180–240 min). Each point represents the average of $n = 20$ measurements (animals). In all figures, SEM values are normally 0.1–0.5 *MI* unit; they are slightly higher during aggregation phases: (↑) buffer change; (*MI*) melanophore index as introduced in [10]; the drawings represent the degree of pigment dispersion of the 5 different stages. When 2×10^{-3} M Ca^{2+} was added to Ca^{2+}-free medium containing 10^{-4} M EGTA and the hormone, the inhibition of the MSH-induced pigment dispersion in the presence of EGTA was readily reversed and normal dispersion was observed.

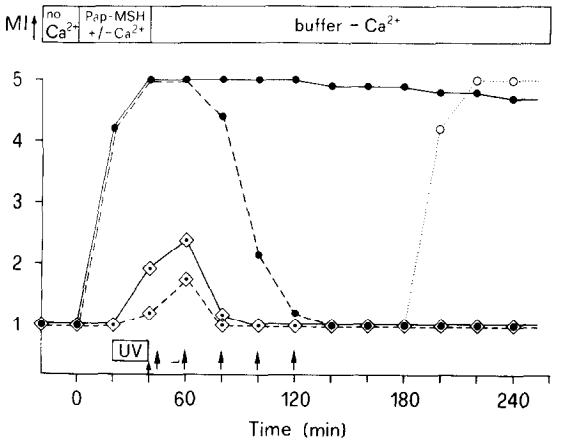

Fig.2. Role of Ca^{2+} in covalent labelling of MSH receptors with [Pap^{13}]-α-MSH. Tail-fin pieces were preincubated in Ca^{2+}-free medium and were further incubated with 1.2×10^{-8} M [Pap^{13}]-α-MSH in the presence (●, $n = 20$) or absence (◈, $n = 20$) of Ca^{2+} and were washed in normal assay medium containing 2×10^{-3} M Ca^{2+}. (UV) irradiation phase; incubation with (——) and without (– – –) UV irradiation; (↑) buffer change; restimulation of Ca^{2+}-free groups after 180 min with 8×10^{-9} M α-MSH in normal medium produced a normal response. Addition of 2×10^{-3} M Ca^{2+} to the Ca^{2+}-free medium containing 10^{-4} M EGTA prior to the experiment resulted in a time–response pattern identical with (●——●).

adult *Xenopus* [3]. Likewise, pigment dispersion in tadpole melanophores induced by db-cAMP (3×10^{-3} M) is Ca^{2+}-independent (unpublished). This indicates that Ca^{2+} plays a role in the transfer of the hormonal signal to the receptor and/or in the signal transduction through the membrane.

Tail-fin melanophores of *Xenopus* tadpoles occur only in small numbers, and though they can be isolated in a functional state [11], quantitative binding studies are not feasible. Nevertheless, it was possible to study the Ca^{2+} requirement for hormone–receptor binding by using photoreactive [Pap^{13}]-α-MSH on the one hand and racemized α-MSH on the other:

(1) Melanophores were incubated with [Pap^{13}]-α-MSH in Ca^{2+}-free medium and UV irradiated. Addition of Ca^{2+} and simultaneous removal of the hormone produced no longlasting effect (fig.2; a slight dispersion was readily reversed). This shows that Ca^{2+} must be present for specific covalent labelling and hence for binding of the hormone to the receptor.

(2) Racemized α-MSH produces (in the presence of Ca^{2+}) a longlasting stimulation of melanophores of *Rana pipiens* [12] and *Xenopus* tadpoles (not shown) without photoreaction, most probably because of a 'tighter' binding of that part of α-MSH molecules which contains D-Phe^{7} [13]. However, like α-MSH, racemized hormone does not induce pigment dispersion in the absence of Ca^{2+}. When melanophores were stimulated by racemized α-MSH in the presence of Ca^{2+}, and when Ca^{2+} was then temporarily removed and re-added, the irreversible stimulation was completely abolished (fig.3). This means that receptor-bound racemized α-MSH was released upon removal of Ca^{2+}; thereby the receptors remained fully functional since the melanophores could be restimulated by addition of α-MSH (fig.3). We conclude that there is a Ca^{2+} site associated with hormone binding, possibly on the receptor. A similar Ca^{2+} requirement has been found for the agonist-binding of catecholamines [14]. However, in many other systems, including ACTH [15,16], Ca^{2+} may not be essential for hormone–receptor binding (reviewed in [17]).

Ca^{2+} is not only essential for hormone–receptor

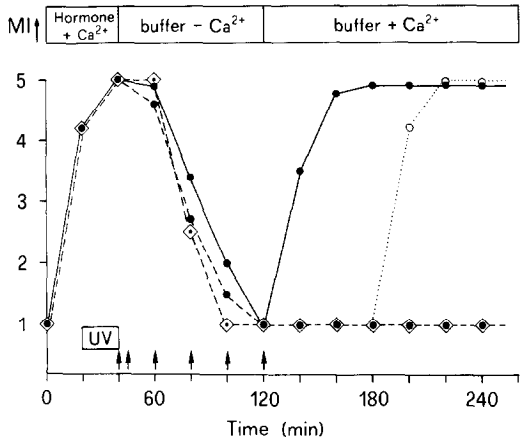

Fig.3. Role of Ca^{2+} in maintaining the irreversible stimulation after photoaffinity labelling with 1.2×10^{-8} M [Pap^{13}]-α-MSH (●, $n = 20$) or after incubation with 5.3×10^{-9} M racemized α-MSH (◈, $n = 19$). The tail-fin pieces were incubated for 40 min in normal assay medium containing the peptides, washed in Ca^{2+}-free medium for 80 min, and were then transferred back to normal medium. (UV) irradiation phase; incubation with (——) and without (– – –) UV irradiation; (↑) buffer change. Restimulation of ◈ with 8×10^{-9} M α-MSH after 180 min produced a normal response (○···○). Addition of 2×10^{-3} M Ca^{2+} to the Ca^{2+}-free, EGTA-containing medium of the last wash resulted in a dispersion pattern identical with (●——●).

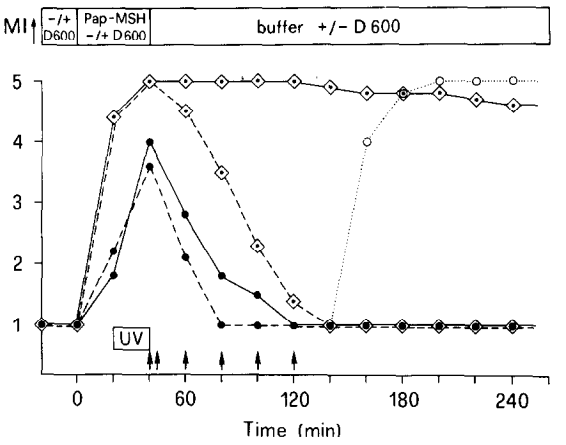

Fig.4. Effect of the Ca^{2+}-antagonist methoxyverapamil (D 600) on pigment dispersion induced by [Pap^{13}]-α-MSH. One group of tail-fin pieces (●, $n = 20$) was preincubated in normal medium containing 8×10^{-5} M D 600, further incubated in the same medium with 1.2×10^{-8} M [Pap^{13}]-α-MSH, UV irradiated and then washed in normal medium. The other group (◈, $n = 22$) was preincubated in normal medium, further incubated in normal medium with 1.2×10^{-8} M [Pap^{13}]-α-MSH, UV irradiated and then washed in medium containing 8×10^{-5} M D 600. (UV) irradiation phase; incubation with (——) and without (– – –) UV irradiation; (↑) buffer change; (○···○) restimulation of (●) with 8×10^{-9} M α-MSH after 140 min produced a normal response.

binding, but also for maintaining the irreversible stimulation produced by covalent MSH–receptor complexes. When MSH receptors were labelled with [Pap^{13}]-α-MSH in the presence of Ca^{2+} and when Ca^{2+} was then removed, the pigment aggregated completely (fig.3). Readdition of Ca^{2+} (without any peptide) brought about a full restimulation. This shows that, in contrast to racemized α-MSH, [Pap^{13}]-α-MSH is not removed from the receptor, and that the covalent MSH–receptor complexes are not inactivated by a temporary lack of Ca^{2+} in the medium. Whether these complexes are inactive in the absence of Ca^{2+} and hence cannot activate adenylate cyclase (→ hormone loosely bound to the receptor) or whether a second Ca^{2+} site needs to be occupied for the activation of the enzyme cannot be finally concluded from this experiment. It is very unlikely that EGTA, which is present in the Ca^{2+}-free medium, interferes with melanophore dispersion (see fig.1–3).

To investigate further the presence of a second Ca^{2+} site in the action of α-MSH, the Ca^{2+} antagonists D 600 and La^{3+} were used (D 600 blocks Ca^{2+} influx into cells [18] and La^{3+} is a powerful competitor for Ca^{2+} binding sites on membranes [19]). Melanophores were preincubated in normal medium containing D 600, further incubated in the same medium containing D 600 and [Pap^{13}]-α-MSH, and UV irradiated. Upon removal of D 600 and non-bound hormone, a partial dispersion was readily reversed and no long-lasting stimulation was observed (fig.4). This indicates that 8×10^{-5} M D 600 almost entirely inhibits photoaffinity labelling by [Pap^{13}]-α-MSH and hence hormone–receptor binding. Addition of D 600 after the formation of the covalent MSH–receptor complex, however, did not at all affect the irreversible stimulation (fig.4). Similar results were obtained with 7×10^{-4} M La^{3+}: ~70% inhibition of hormone binding, but no effect on maintaining the irreversible stimulation (not shown). This leads to the conclusion that apart from the Ca^{2+} site associated with the receptor there must be another Ca^{2+} site involved in the signal transduction through the membrane which is not accessible to the Ca^{2+}-antagonists.

The second Ca^{2+} site appears to be important for

coupling the hormone receptor to the adenylate cyclase, though Ca^{2+} may not be the only factor involved. Extracellular Mg^{2+} seems not to be essential (unpublished). From numerous studies with mammalian cell and membrane systems it has become evident that hormone–receptor binding and receptor–adenylate cyclase coupling is regulated by GTP and the GTP binding protein as well as by divalent ions (reviewed in [20–22]). To what extent the signal transduction in amphibian melanophores corresponds to the mammalian systems is not yet known. Unpublished experiments studying the action of cholera toxin and fluoride ions on tail-fin melanophores have so far given no clue on the involvement of a GTP binding protein in adenylate cyclase regulation. On the other hand, the stimulatory role of Ca^{2+} on adenylate cyclase activity has been described for several systems, including brain tissue and ACTH-stimulated adrenal cells and adipocytes (reviewed in [23]). We conclude that *Xenopus* melanophores have a specific requirement for Ca^{2+} at $\geqslant 2$ distinct sites during stimulation by α-MSH.

Irreversible stimulation by covalent labelling of hormone receptors represents a useful approach for studying stimulus–response coupling in intact cells. Only there are the receptor and the adenylate cyclase located in their different ionic environments and only there is an electrical potential maintained across the plasma membrane. Thus, our system offers the possibility of studying under almost physiological conditions the involvement of various factors (such as divalent ions) in receptor binding and membrane signal transduction, even though only a limited number of cells is available.

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