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Calcium-Dependent Prolonged Effects on Melanophores of [4-Norleucine, 7-D-Phenylalanine]-α-Melanotropin

Abstract. *A single injection of the melanotropin analog [4-norleucine, 7-D-phenylalanine]-α-melanotropin into frogs* (Rana pipiens) *caused near maximum darkening of the skins of the frogs for at least 6 weeks. Injections of the natural hormone α-melanotropin or of the analog [Nle*^{4}*]-α-melanotropin also caused darkening, but this effect lasted only a few days. Morphological examination of the skins of frogs injected with [Nle*^{4}*, D-Phe*^{7}*]-α-melanotropin revealed that both dermal and epidermal melanophores were dispersed during the entire 6-week period. In vitro [Nle*^{4}*, D-Phe*^{7}*]-α-melanotropin also causes prolonged darkening of the skin of the lizard* Anolis carolinensis. *In the absence of the melanotropin, skins previously darkened with the analog could be lightened by removal of calcium from the incubation medium but could then be redarkened by adding calcium. The cycle could be repeated indefinitely without addition of melanotropin. These results demonstrate the role of calcium in receptor signal transduction and the prolonged biological effects of [Nle*^{4}*, D-Phe*^{7}*]-α-melanotropin long after its removal from the assay medium.*

α-Melanotropin (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2, α-MSH) is a tridecapeptide hormone that is synthesized in the pars intermedia of the vertebrate pituitary (*1*). It reversibly darkens amphibian skins by stimulating melanosome movement (dispersion) within melanophores. This melanotropin also affects mammalian melanocytes, both normal and transformed (melanoma) cells, by stimulating adenylate cyclase activity, tyrosinase activity, and melanin production. In addition, recent studies suggest that this hormone may have important functions in fetal development and in neural mechanisms related to learning and memory (*2*).

The amino acid residues that are important to the biological activity of α-melanotropin have been elucidated by systematic structure-function studies of α-MSH, α-MSH fragments, and related analogs on amphibian melanophores (*3, 4*) and, more recently, on mammalian melanoma cells (*5–8*). Treatment of melanotropins with heat and alkali leads to partial racemization of some amino acid residues within these peptides, and early investigators showed that these peptides darkened the skins of hypophysectomized frogs for at least 6 hours (*9*). However, the exact stereostructural changes responsible for these prolonged activities were not determined. Recently, we have investigated quantitatively the extent of racemization at individual amino acid residues of several melanotropins as a result of heat-alkali treatment (*8, 10*). On the basis of our results we synthesized [4-norleucine, 7-D-phenylalanine]-α-melanotropin ([Nle^4, D-Phe^7]-α-MSH) and demonstrated its prolonged (> 48 hours) melanosome-dispersing effect on frog (*Rana pipiens*) melanophores in vitro, high biological potency in stimulating mouse melanoma adenylate cyclase and tyrosinase activities, and resistance to degradation by serum enzymes that inactivate α-MSH (*8*).

The extraordinary potency and prolonged biological activity of this molecule in vitro led us to examine the biological effects of the peptide in vivo, using the frog (*Rana pipiens*) and the lizard (*Anolis carolinensis*). We report here that a single injection of [Nle^4, D-Phe^7]-α-MSH into a frog will darken its skin for periods up to 6 weeks, and that a similar injection into a lizard will darken the skin of this animal for several days. In addition, we have used the analog to investigate the mechanism of melanotropin receptor–mediated signal transduction on lizard melanophores in vitro and the role of calcium in the biological action of this peptide.

Frogs of both sexes were placed in white plastic containers with a small amount of water under overhead illumination. Under these conditions, the animals became light green in color, presumably because they were not releasing any endogenous MSH. Forty-eight hours later, light reflectance from the dorsal surface of the animals was measured with a Photovolt reflectometer (*11*). At this time, the frogs were injected subcutaneously with Ringer solution (controls) or Ringer containing α-MSH, [Nle^4]-α-MSH, or [Nle^4, D-Phe^7]-α-MSH (100 μl of a 10^{-4} *M* solution per 10 g of body weight) to provide a final body concentration of approximately 18 μg/10 g. Subsequent reflectance values were taken at 2- to 3-day intervals for 6 weeks (Fig. 1).

Maximum darkening of the frogs was obtained with [Nle^4, D-Phe^7]-α-MSH; at

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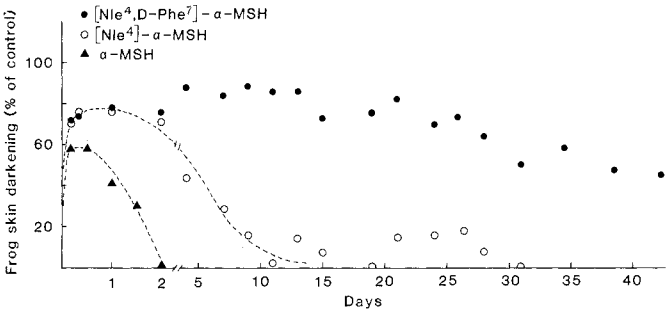

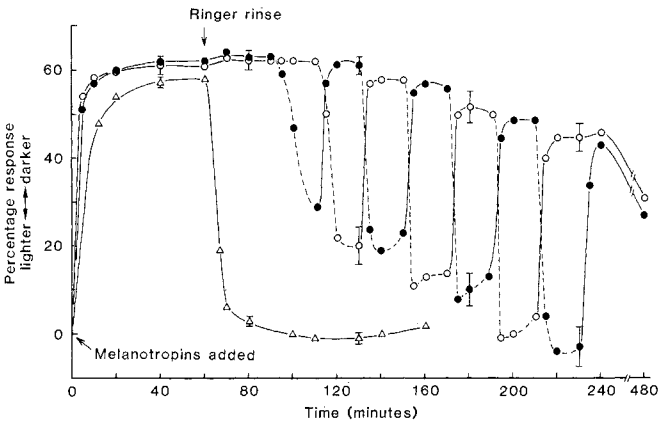

Fig. 1 (left). The long-term effect of [Nle^4, D-Phe^7]-α-MSH (●) on darkening of the frog *R. pipiens*. The transient darkening effects of α-MSH (▲) and [Nle^4]-α-MSH (○) are also shown. Values represent the mean darkening response of melanotropin-injected frogs (18 μg/10 g, final body concentration; four frogs per experimental group) compared to that of control frogs (taken as the zero or base response). Fig. 2 (right). At time zero, [Nle^4]-α-MSH ($5 \times 10^{-9}M$) was added to one group of lizard (*A. carolinensis*) skins (△) and [Nle^4, D-Phe^7]-α-MSH ($5 \times 10^{-9}M$) was added to the skins in two other experimental groups (●, ○). At 60 minutes, the skins (six per group) were transferred to Ringer solution in the absence of the melanotropins. At subsequent time periods the skins in these last two groups were alternatively transferred to Ringer solution in the absence (------) and presence (——) of calcium ion ($10^{-3}M$). The mean ± standard error values of selected data points are provided.

6 weeks the frogs injected with this diastereoisomeric analog of melanotropin still showed half-maximum darkening (Fig. 1). In contrast, the darkening effect of α-MSH was transient, being totally lost by 2 days after injection. [Nle^4]-α-MSH was effective for only a slightly longer period than α-MSH. A single injection of a lower concentration (1.8 or 0.18 μg/10 g, final body concentration) of [Nle^4, D-Phe^7]-α-MSH was nearly as effective as the higher dose (18 μg/10 g).

A single injection of [Nle^4, D-Phe^7]-α-MSH (18 μg/10 g, final body concentration) into the lizard (*A. carolinensis*) also caused a prolonged darkening (about 3 days) of the skin compared to the effect of either α-MSH or [Nle^4]-α-MSH.

When frog skin darkens in response to melanotropin treatment, there is an initial rapid physiological color change that involves melanosome dispersion within dermal melanophores. Morphological color change, however, results from a slower but long-term increase in melanin synthesis and secretion by epidermal melanophores (*1*). Cytological observations of skin taken from frogs 2 weeks, 4 weeks, and 6 weeks after injection of [Nle^4, D-Phe^7]-α-MSH revealed that both dermal and epidermal melanocytes were near maximally dispersed. Increased amounts of melanin were also present within the epidermis. Melanophores of control or [Nle^4]-α-MSH injected animals were unstimulated at these time intervals and the amount of epidermal melanin was minimal.

We previously documented an acute calcium ion requirement for α-MSH activation of melanophores of *A. carolinensis* (*12*). Using [Nle^4, D-Phe^7]-α-MSH as a molecular probe, we investigated in the present experiments the functional role of calcium ion in relation to the prolonged melanosome dispersion in vitro effected by this analog. Lizard skins darkened by α-MSH or [Nle^4]-α-MSH lightened after transfer to Ringer solution in the absence of these peptides. Skins darkened by [Nle^4, D-Phe^7]-α-MSH, in contrast, remained darkened after transfer to Ringer solution in the absence of the melanotropin; however, transfer of these skins to Ringer lacking calcium ion caused them immediately to lighten, although they subsequently redarkened on addition of calcium ion (1 mM) to the Ringer solution (Fig. 2). These results suggest that calcium reversibly modulates signal transduction effected by [Nle^4, D-Phe^7]-α-MSH.

Melanotropin-induced melanosome dispersion within melanophores involves the activation of membrane-bound adenylate cyclase and the intracellular production of adenosine 3′,5′-monophosphate (cyclic AMP) (*13, 14*). Our results clearly document the potent biological activity of [Nle^4, D-Phe]-α-MSH on integumental melanophores of the frog and the lizard both in vivo and in vitro. It is conceivable that [Nle^4, D-Phe^7]-α-MSH effects a noncovalent but apparently irreversibly bound melanotropin-receptor complex in these species. In this regard, DeGraan and Eberle (*15*) reported that photoaffinity labeling of tadpole (*Xenopus laevis*) melanophores in vitro with [*p*-azido-Phe^{13}]-α-MSH led to melanosome dispersion that persisted for several hours. In this case, photolysis (which presumably affected a covalent attachment of the [*p*-azido-Phe^{13}]-α-MSH to the receptor) was a prerequisite to the prolonged effect in vitro, since in the absence of photolysis a normal, short-term, reversible skin darkening occurred. However, the prolonged activity of [Nle^4, D-Phe^7]-α-MSH in vivo and in vitro appears to be the result of the conformational and dynamic properties of the compound on interaction with the melanotropin receptor system, or a result of its metabolic "stability" at the receptor. An alternative hypothesis might be that the [Nle^4, D-Phe^7]-α-MSH in some manner irreversibly activates signal transduction leading to continued activation of adenylate cyclase and cyclic AMP production.

Ketelslegers *et al.* (*16*) have presented evidence from kinetic studies that a prolonged phase of slow and incomplete reversal can occur for hormone-receptor complexes following an initial rapid phase of dissociation. Catt *et al.* (*17*) have further suggested that initial hormone interaction with receptor can effect a conformational change at the receptor which can result in tighter binding of the hormone to the receptor. Such an effect is consistent with thermodynamic considerations and the "zipper" model of hormone action (*18, 19*). Interaction of hormone-occupied receptor complexes with adenylate cyclase has been implicated as effecting such a higher-affinity, hormone-binding site (*20*). Possibly, as a result of its intrinsic physicochemical properties, [Nle^4, D-Phe^7]-α-MSH may similarly induce tighter (apparently irreversible) binding to its receptor, leading to a sustained, biologically active hormone-receptor–adenylate cyclase complex.

This synthetic melanotropin should prove useful for a number of studies. Methods are available for the preparation of biologically active radioiodinated melanotropins (*21, 22*) and, as previously mentioned, melanotropins interact with receptors of mammalian melanoma membrane preparations to activate melanoma adenylate cyclase in a dose-related

manner (*5, 8, 14*). Thus, design of a radioactive receptor assay with this system should permit an examination of the molecular basis for interaction of [Nle^4, D-Phe^7]-α-MSH and other melanotropins with its receptor. It may also be possible to use [Nle^4, D-Phe^7]-α-MSH in the detection of human melanoma. Since the labeled melanotropin would presumably bind preferentially to melanoma membrane receptors, radioisotope imaging methods could be used to detect melanoma tumors in skin. It might also be possible, by conjugating adriamycin or other cancer therapeutic agents to [Nle^4, D-Phe^7]-α-MSH, to develop a cell-specific (melanoma cell) drug delivery system; we found previously (*8*) that [Nle^4, D-Phe^7]-α-MSH is nonbiodegradable in serum.

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Myeloma Neuropathy: Passive Transfer from Man to Mouse

Abstract. *Mice were injected daily, for up to 10 weeks, with purified monoclonal immunoglobulin G from patients with myelomatous polyneuropathy or benign gammopathy. The animals developed a demyelinating polyneuropathy with slowed nerve conduction velocities. The putative antinerve factor may be an antibody since injection of Fab fragments from the monoclonal immunoglobulin G produced a similar demyelination. This provides evidence of a circulating factor in the serum of myeloma patients with polyneuropathy that reproduces typical features of the human disease on passive transfer. This disorder is thus distinguished from other neuropathies that occur as remote effects of malignant disease but have no identified pathogenic factors associated with them.*

Peripheral neuropathies (PNP's) have been described in patients with solitary and multiple myeloma (*1*). In this malignant disease of immunocytic origin as well as in other neoplastic conditions the "paraneoplastic" PNP is characterized by demyelination (*2, 3*).

The pathogenesis of myeloma-PNP has long been debated. The finding of immunoglobulins G and M (IgG, IgM) and complement in sural nerve specimens from patients with myeloma-PNP led to the hypothesis that monoclonal immunoglobulins (m-Ig) may play an important pathogenic role (*4, 5*). More recently it was argued that binding of m-IgG to nerves might be an unspecific epiphenomenon since IgG deposition can also be seen in other neuropathies (*6, 7*). Furthermore, it has been speculated that the demyelinating m-Ig could be an antibody against peripheral nerve components (*8*), since (i) myeloma is associated with autoimmune diseases (*9*) and (ii) myeloma cells occasionally produce m-Ig with distinct antibody specificities (*10*).

In this report we demonstrate that m-IgG plays a part in the pathogenesis of PNP in m-IgG–secreting myeloma and we present preliminary evidence of an immunopathologic mechanism. In order to test the direct pathogenic effect of m-IgG we passively transferred human m-IgG to mice. This model system was originally devised for studies on the immune mechanism in myasthenia gravis (*11*). Plasma was obtained from seven patients, six with severe m-IgG–secreting myeloma who had undergone therapeutic plasmapheresis, and one with benign monoclonal gammopathy. Two of the myeloma patients and the one with monoclonal gammopathy had clinical signs of a PNP, whereas three had no clinical evidence of nerve involvement. One patient had died before this study was started and his neurological status was not recorded. Pure IgG was prepared by ion exchange chromatography on DEAE-Sephacel (Pharmacia) as previously described (*12*). The eluates were reduced to a final concentration of 20 mg of IgG per milliliter (Amicon-XM 50), dialyzed against physiological saline, and stored at −70°C until use. All IgG fractions were free of other serum proteins as assessed by standard immunoelectrophoresis with rabbit polyvalent antiserum against such human proteins (Behring-Werke). Polyclonal IgG (p-IgG) from a serum pool of more than ten healthy donors was prepared in the same manner. Immediately before use the material was thawed and sterilized through membrane filtration (Minisart, Sartorius; pore size, 0.2 μm).

Thirty-four female B6D2F1/J inbred mice (Jackson Laboratory) 8 to 11 weeks of age, 20 to 22 g in body weight, were used in these experiments. Each mouse received daily intraperitoneal injections of about 20 mg of m-IgG or p-IgG for 8 to 10 weeks in the initial set of experiments and for 10 to 14 days in the later part of the study.

This dose amounted to IgG concentrations of 15 to 20 g per liter in the mouse plasma and was therefore roughly equivalent to the IgG concentrations in the patients from whom IgG was obtained. Tolerance to human proteins was induced by a single dose of cyclophosphamide 24 hours after the first injection (300 mg per kilogram of body weight) (*13*).

In 24 mice we measured nerve conduction velocities in vivo at the mouse tail nerve. Nerve conduction velocities were examined at weekly intervals in the long-term experiments to detect the first signs of nerve damage. In the short-term study at least two measurements were carried out before and after the injection

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