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α-Melanotropin-induced changes in protein phosphorylation in melanophores *

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Summary

To investigate a possible role of protein phosphorylation in the mechanism of action of α-MSH, excised tail-fins of Xenopus tadpoles were incubated with or without α-MSH. After homogenization, in vitro endogenous protein phosphorylation was assayed using $[\gamma\text{-}^{32}P]$ATP. α-MSH treatment of intact tail-fins, producing full pigment dispersion, resulted in a 5-fold increase in ^{32}P-incorporation into a 53 kDa protein band. This increase in 53 kDa phosphorylation was completely reversible. The increase was not found in homogenates from the melanophore-free part of the α-MSH-treated tail-fins. Phosphorylation of the 53 kDa protein could be detected in homogenates of α-MSH-treated primary cultured melanophores. Incubation of tail-fins with $ACTH_{1-24}$, an α-MSH-like peptide producing full pigment dispersion, also induced an increase in 53 kDa phosphorylation. A structurally related peptide ($ACTH_{15-24}$) and an unrelated peptide (LH-RH), neither of which induced pigment dispersion, were ineffective in stimulating 53 kDa phosphorylation. Injection of white adapted tadpoles with 1 μg of α-MSH or adaptation of tadpoles to a black background also resulted in a significant increase in 53 kDa phosphorylation. α-MSH added to the homogenates did not affect 53 kDa phosphorylation, indicating that α-MSH acts through a receptor-mediated mechanism. The increase in 53 kDa phosphorylation measured in vitro (post hoc), most likely reflects an α-MSH-induced decrease in 53 kDa phosphorylation in vivo. Our results strongly suggest that a decrease in 53 kDa phosphorylation is involved in the mechanism of action of α-MSH on melanophores.

The peptide hormone α-melanotropin (α-MSH) plays an important role in the regulation of target cell function in a number of systems, including pigment migration in amphibian melanophores (Hadley and Bagnara, 1975), foetal development (Swaab and Martin, 1981), axonal regeneration (Strand and Smith, 1980; Bijlsma et al., 1983) and modulation of neuronal activity and behavior (De Wied and Jolles, 1982). Although the biological effects of α-MSH in most of these systems are well characterized, information on the molecular mechanism of α-MSH action is limited and mainly derived from studies with amphibian melanophores and melanoma.

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Most probably, in melanophores the binding of α-MSH to specific plasma membrane receptors activates an intracellular multistep mechanism eventually resulting in pigment dispersion (Eberle, 1980). The effects of α-MSH on melanophores are thought to be mediated by the second messengers cAMP (Abe et al., 1969; Van de Veerdonk and Konijn, 1970) and/or Ca^{2+} (Vesely and Hadley, 1971; Van de Veerdonk and Brouwer, 1973). By what mechanism these intracellular signals regulate pigment dispersion is completely unknown. Both second messengers are known to modulate the activity of specific protein kinases and hence the degree of phosphorylation of substrate proteins in response to hormones in a variety of cell systems (Rodnight, 1979).

Therefore, we were interested in a possible role of protein phosphorylation in the mechanism of α-MSH action on melanophores. Since cultured melanophores are not available in large quantities, we used melanophores in the ventral tail-fin of *Xenopus laevis* tadpoles in our studies (De Graan et al., 1983). In this system cAMP (De Graan et al., 1983, 1984) and Ca^{2+} (De Graan et al., 1982a, b) have been shown to be involved in the mechanism of action of α-MSH. Here we report that incubation of excised tail-fins of Xenopus tadpoles with α-MSH results in specific changes in the degree of phosphorylation of a 53 kDa melanophore protein.

Materials and methods

Animals

Tadpoles of *Xenopus laevis* were used at stage 51–53, according to the Normal Table of Nieuwkoop and Faber (1956). Details on standardized hatching and breeding conditions have been described earlier (De Graan et al., 1983). Prior to the experiments, tadpoles were adapted overnight to a black background (De Graan et al., 1983) unless otherwise stated.

Chemicals

Synthetic α-MSH was a gift from Dr. A. Eberle (Kantonsspital, Basel, Switzerland), LH-RH from Dr. H. Goos (Zoological Laboratory, State University of Utrecht, The Netherlands) and $ACTH_{1-24}$ and $ACTH_{15-24}$ from Dr. H. Greven (Organon Int., Oss, The Netherlands). [γ-^{32}P]ATP (specific activity 2900 Ci/mmol) and [α-^{32}P]ATP (specific activity 2900 Ci/mmol) were obtained from New England Nuclear (U.S.A.). All other chemicals employed were of the highest available commercial grade.

α-MSH treatment

The melanophore-containing caudal part of the ventral tail-fins was excised and preincubated at room temperature for 60 min in assay medium (Leibovitz-L15, modified according to De Graan and Eberle, 1980) to obtain full melanosome aggregation. Subsequently, the tail-fin pieces were incubated for 60 min in assay medium with or without 5×10^{-6} M α-MSH or one of the following peptides: LH-RH, $ACTH_{1-24}$ and $ACTH_{15-24}$ (5×10^{-6} M each). At the end of the peptide treatment the degree of pigment migration was quantified microscopically, using the melanophore index (MI) of Hogben and Slome (1931); MI = 1 denoting complete aggregation and MI = 5 full dispersion. In one series of experiments the rostral (melanophore-free) part of the tail-fins was also excised and treated with or without α-MSH as described above.

To induce pigment dispersion in vivo, tadpoles which had been adapted overnight to a white background were injected into the ventral tail muscle with 5 μl 0.6% NaCl or 5 μl 0.6% NaCl containing 1 μg α-MSH. The tadpoles were allowed to swim on a white background and were killed after 30 min. The caudal part of the tail-fins was excised at 4°C and the MI was determined.

The endogenous MSH levels in the blood were manipulated using different background adaptation conditions. Tadpoles were adapted overnight to a white background to obtain full pigment aggregation (due to low endogenous MSH blood levels). Tadpoles were transferred to a black background and sacrificed after 30 or 60 min. Controls were transferred to a white background. The caudal part of the tail-fins was excised at 4°C.

Protein phosphorylation

Eight tail-fins were homogenized in 80 μl ice-cold phosphorylation buffer (10 mM sodium acetate, 10 mM magnesium acetate, 0.1 mM calcium acetate, pH 6.5) in a teflon on glass Potter

Elvehjem tube (clearance 0.125 mm, 10 up-and-down strokes at 800 rpm). Homogenates were diluted with phosphorylation buffer to a protein concentration of 1.5 mg/ml and kept on ice until assayed for protein phosphorylation. Protein was determined according to Lowry et al. (1951), using bovine serum albumin as a standard.

Endogenous protein phosphorylation was assayed using a previously published procedure (Zwiers et al., 1976) with slight modification. In short, 30 μg protein (in 20 μl phosphorylation buffer) was preincubated for 5 min at 24°C. The phosphorylation reaction was started by the addition of 3 μCi [γ-^{32}P]ATP to a final ATP concentration of 7.5 μM (total incubation volume 25 μl). Phosphorylation was stopped after 15 s by adding 12.5 μl of a denaturing solution. Proteins were separated on 11% SDS-polyacrylamide gels and stained with Fast Green; the gels were dried and subjected to autoradiography (Zwiers et al., 1976). ^{32}P-Incorporation into protein bands was quantified by densitometric scanning of the autoradiograms with a linear gel scanner (slit width 0.01 mm) and by measuring peak height above background (Ueda et al., 1973). Exposure times allowed a linear relationship between radioactivity and grain density per band. Comparisons were made only between samples on one gel. This quantification procedure was checked by counting 1 mm gel slices for radioactivity by liquid scintillation counting. Statistical analysis was performed with an analysis of variance (completely randomized design), followed by a supplemental *t*-test (Bruning and Kintz, 1977).

Results

Melanophores in tail-fins of *Xenopus laevis* tadpoles remain responsive to α-MSH treatment in vitro for several hours (De Graan et al., 1983). α-MSH treatment (5×10^{-6} M) did not affect the protein pattern of the tail-fins (Fig. 1). Endogenous phosphorylation of tail-fin homogenates using [γ-^{32}P]ATP resulted in ^{32}P-incorporation into at least 8 protein bands (Fig. 2); the predominant bands have apparent molecular weights of 58 kDa, 53 kDa and 50 kDa. A 60 min incubation of intact tail-fins with 5×10^{-6} M α-MSH resulted in a 5-fold increase in ^{32}P-incorporation into a protein

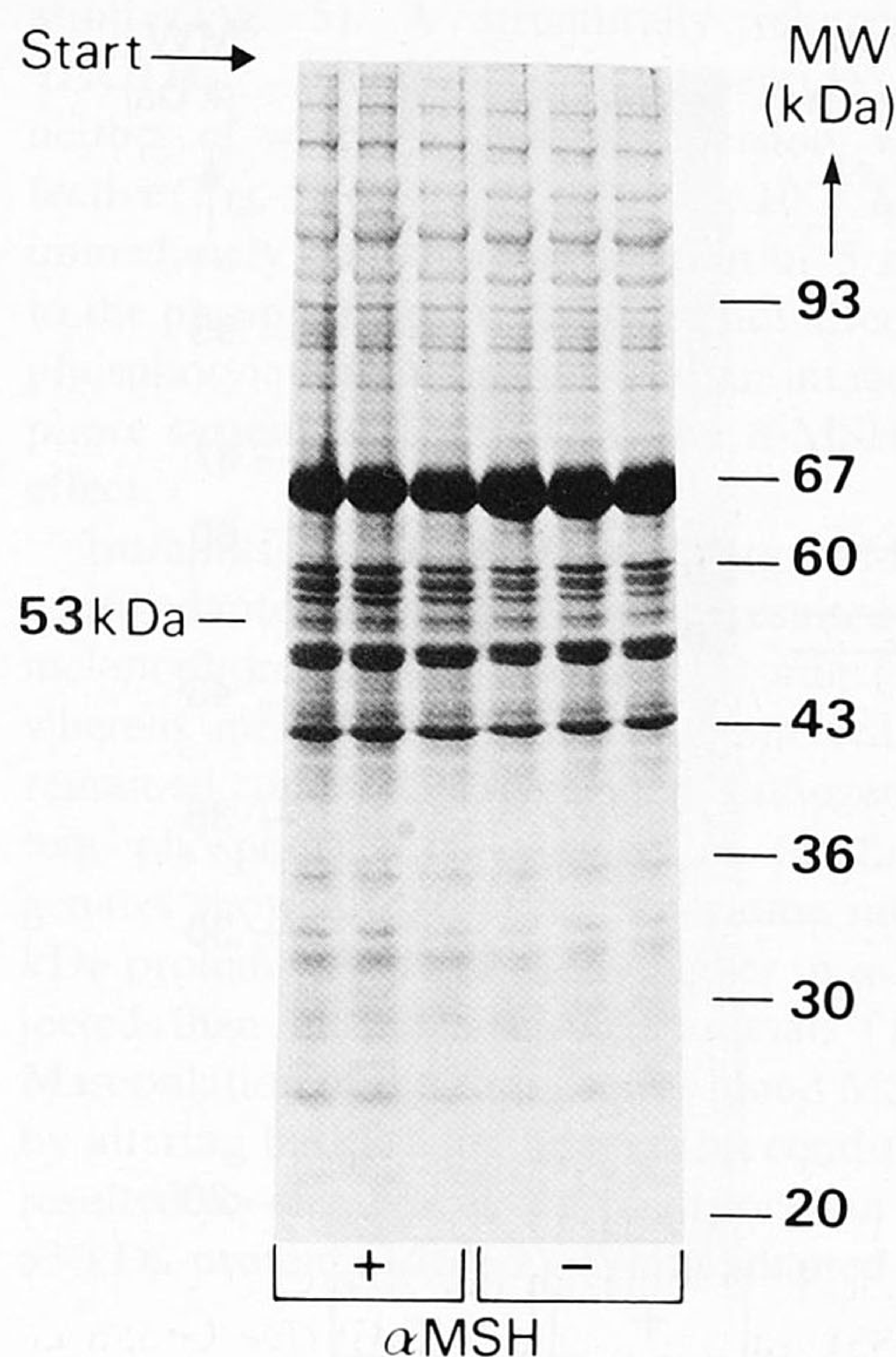

Fig. 1. Effect of α-MSH treatment of excised intact tail-fins on the protein pattern of tail-fin homogenates. Tail-fins were incubated with or without 5×10^{-6} M α-MSH for 60 min, homogenized and assayed for endogenous protein phosphorylation. Proteins were separated on 11% SDS-polyacrylamide gels and stained with Fast Green. The molecular weights of reference proteins are indicated on the right. For details see Materials and methods.

band with an apparent molecular weight of 53 kDa (Fig. 2). ^{32}P-Incorporation into the other phosphoprotein bands did not change significantly upon α-MSH treatment of the intact tail-fins (Fig. 3). The increase in 53 kDa phosphorylation could be reversed by incubating the α-MSH-treated tail-fins for 30 min in assay medium prior to the homogenization.

The nature of the ^{32}P-incorporation into the 53 kDa band was studied using [α-^{32}P]ATP instead of [γ-^{32}P]ATP (with identical specific and total activity) in the phosphorylation assay. Under these conditions no label was incorporated into any of the protein bands. This rules out the possibility of (non)specific ATP binding to the 53 kDa band and indicates the ^{32}P-incorporation to be a kinase-mediated reaction. The involvement of an

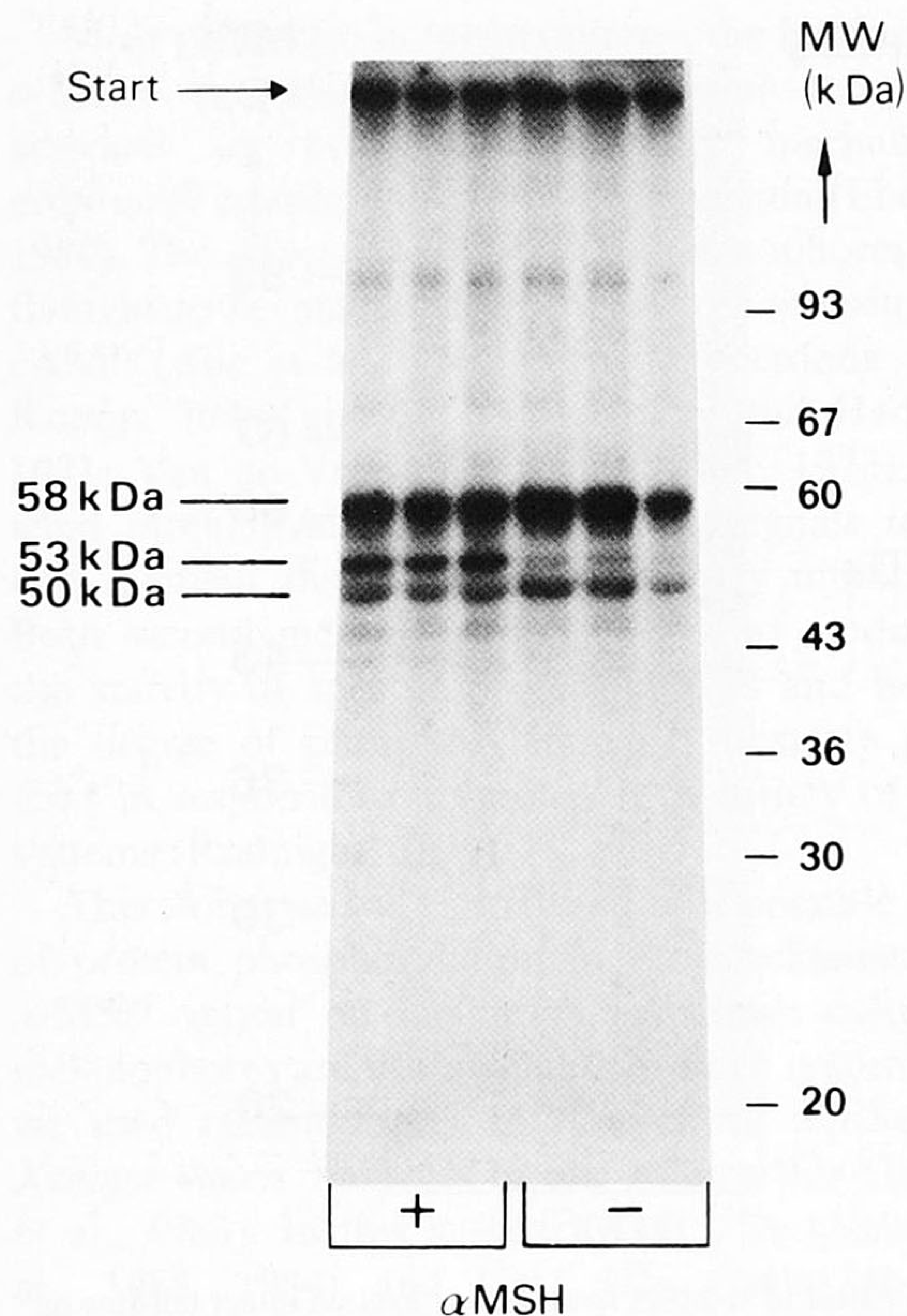

Fig. 2. Effect of α-MSH treatment of intact tail-fins on ^{32}P-incorporation into protein bands. Tail-fins were treated and processed as described in Fig. 1. After protein staining, gels were dried and subjected to autoradiography.

enzyme is supported by the fact that boiling of the homogenate prior to the phosphorylation assay for 2 min completely abolished ^{32}P-incorporation. No loss of label from the 53 kDa protein could be detected during a 10 min incubation of a phosphorylated homogenate at 24°C. Thus, phosphatase activity in the homogenates is negligible under the phosphorylation conditions used. Addition of a phosphorylated homogenate from α-MSH-treated tail-fins back to a new extract during homogenization did not alter ^{32}P-incorporation into the 53 kDa band. This indicates that the phosphoprotein is stable during processing.

To investigate the specificity of the α-MSH effect on 53 kDa phosphorylation, a number of

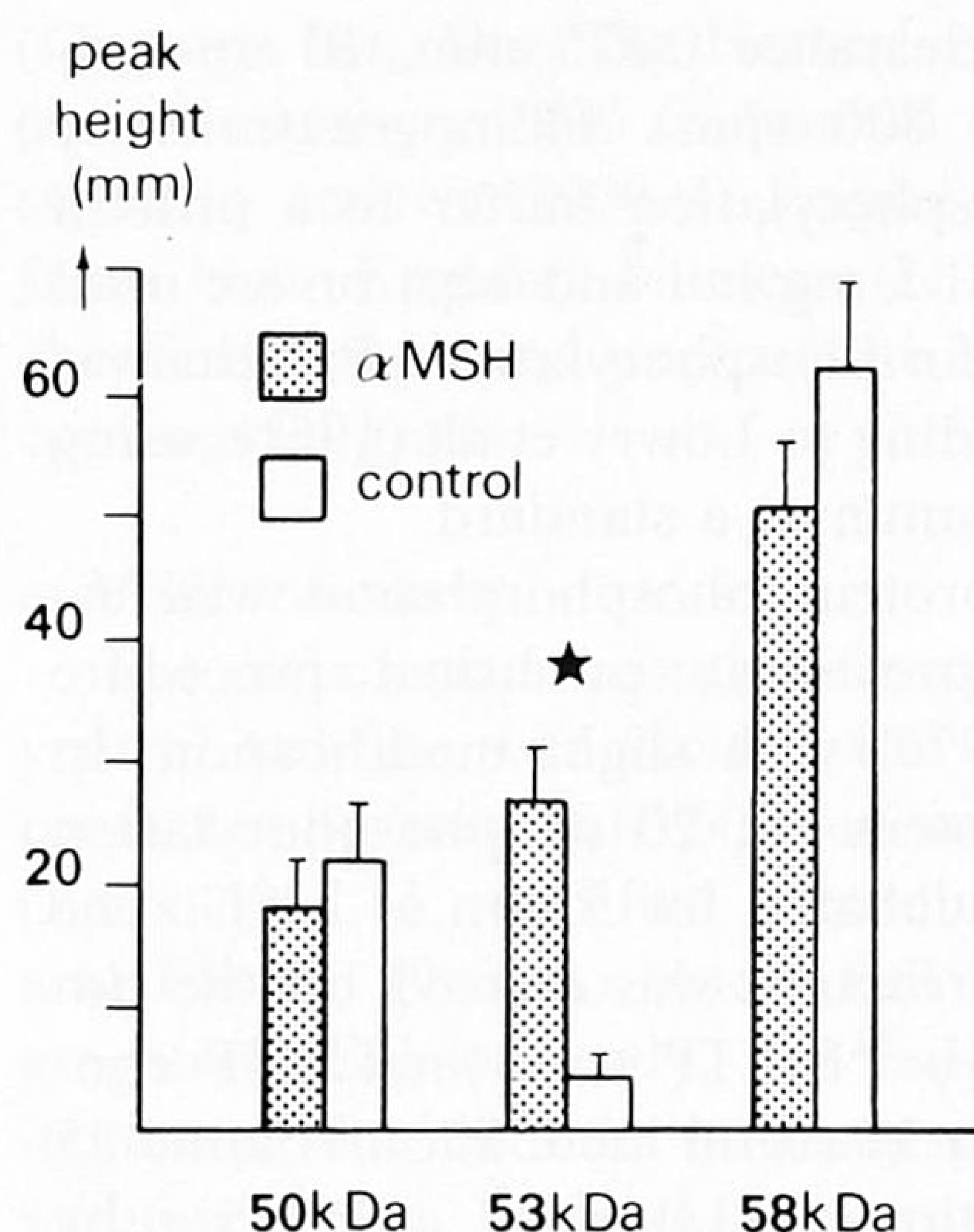

Fig. 3. Quantification of the effect of α-MSH treatment on ^{32}P-incorporation into 3 major phosphoproteins. ^{32}P-incorporation was quantified by densitometric scanning of the autoradiogram. For details see Materials and methods.★ Significantly different from control, $P < 0.001$, $n = 6$; bars indicate SEM.

TABLE 1

EFFECT OF α-MSH INJECTION ON 53 kDa PHOSPHORYLATION

Injection	53 kDa phosphorylation (peak height in mm)	Degree of pigment dispersion (melanophore index)
Saline	13.8 ± 1.4	1.0
α-MSH	41.2 ± 1.0 ★	4.5

★ Significantly different from control, $P < 0.001$, $n = 6$.

White adapted tadpoles were injected with 5 μl 0.6% NaCl with or without 1 μg α-MSH. After 30 min the MI was determined and the tail-fins were homogenized and assayed for endogenous protein phosphorylation as described in Materials and methods.

TABLE 2

EFFECT OF BACKGROUND ADAPTATION ON 53 kDa PHOSPHORYLATION

Adaptation	53 kDa phosphorylation (peak height in mm)	Degree of pigment dispersion (melanophore index)
White (60 min)	4.8 ± 0.1 ($n = 5$)	1.0
Black (30 min)	8.9 ± 0.4 ★ ($n = 4$)	3.2
Black (60 min)	9.1 ± 0.6 ★ ($n = 4$)	4.0

★ $P < 0.01$.

White adapted tadpoles were transferred to a black background. After 30 or 60 min the animals were sacrificed and the tail-fins assayed for endogenous protein phosphorylation as described in Materials and methods. Controls were kept on a white background for 60 min.

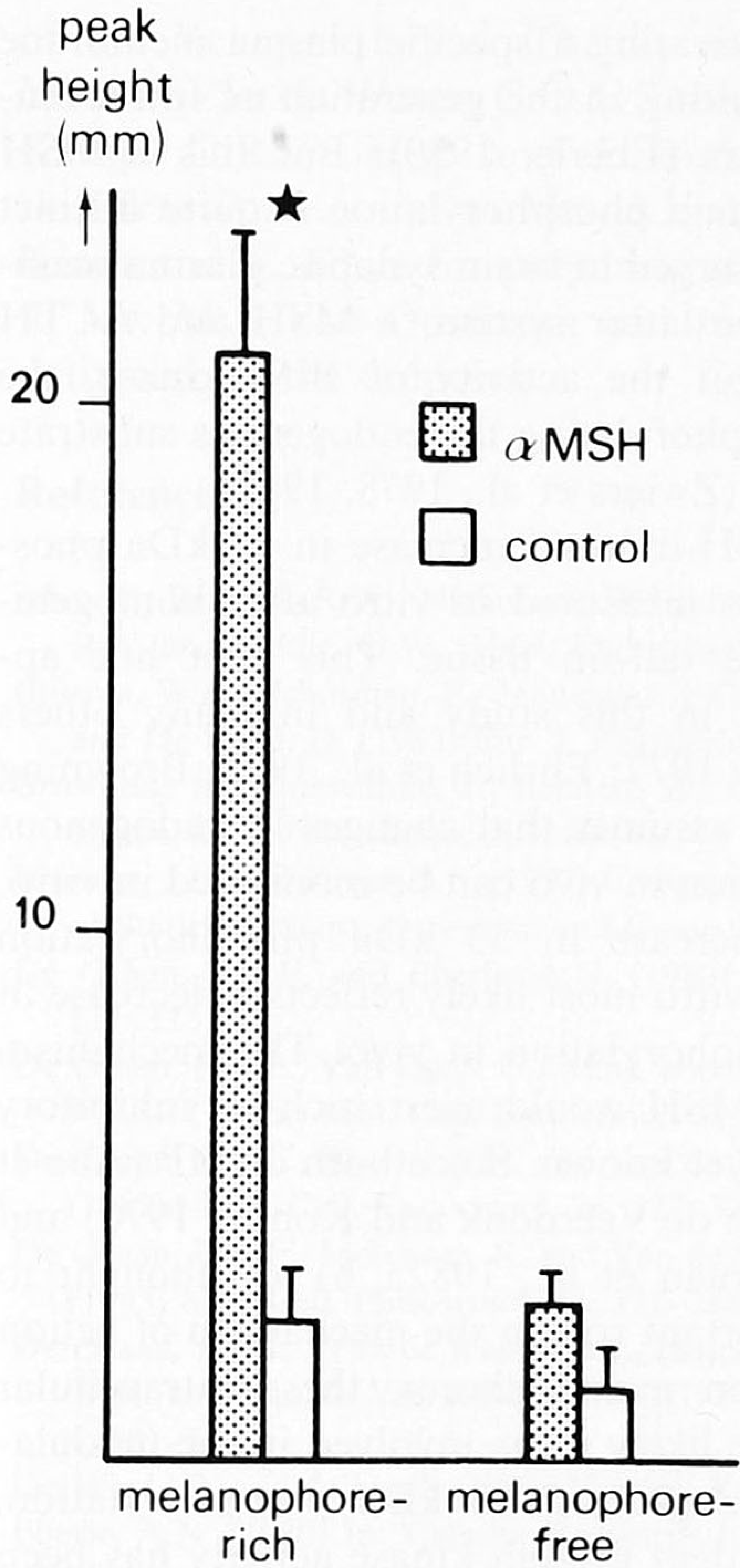

Fig. 4. Effect of α-MSH treatment of intact tail-fins on 53 kDa phosphorylation in homogenates from the melanophore-rich and melanophore-free part of the tail-fin. ★ Significantly different from control, $P < 0.001$, $n = 8$; bars indicate SEM.

control experiments were performed; (i) the melanophore-rich and melanophore-free part of the tail-fins were incubated separately with 5×10^{-6} M α-MSH and assayed for endogenous protein phosphorylation. The significant increase in 53 kDa phosphorylation was found only in the melanophore-rich tissue (Fig. 4). Moreover, the 53 kDa phosphoprotein could be demonstrated in phosphorylated homogenates of α-MSH-treated primary cultured melanophores isolated (Seldenrijk et al., 1979) from ventral tail-fins of *Xenopus laevis* tadpoles (results not shown), (ii) incubation of tail-fins with $ACTH_{1-24}$, an α-MSH-like peptide producing complete melanosome dispersion, also induced an increase in 53 kDa phosphorylation (Fig. 5). A structurally related peptide ($ACTH_{15-24}$) and an unrelated peptide (LH-RH), neither of which produced dispersion, were ineffective (Fig. 5), (iii) addition of 5×10^{-6} M α-MSH immediately after homogenization or 5 min prior to the phosphorylation assay did not affect 53 kDa phosphorylation, indicating that an intact melanophore system is required for the α-MSH-induced effect.

Intramuscular injection of 1 μg α-MSH into white-adapted Xenopus tadpoles resulted in a full melanophore dispersion within 30 min (Table 1), whereas melanophores of saline-injected animals remained completely aggregated. Endogenous protein phosphorylation assayed in tail-fin homogenates showed that ^{32}P-incorporation into the 53 kDa protein was significantly higher in α-MSH-injected than in saline-injected animals (Table 1). Manipulation of the endogenous blood MSH-levels by altering background adaptation conditions also resulted in changes in ^{32}P-incorporation into the 53 kDa protein (Table 2). White-adapted animals,

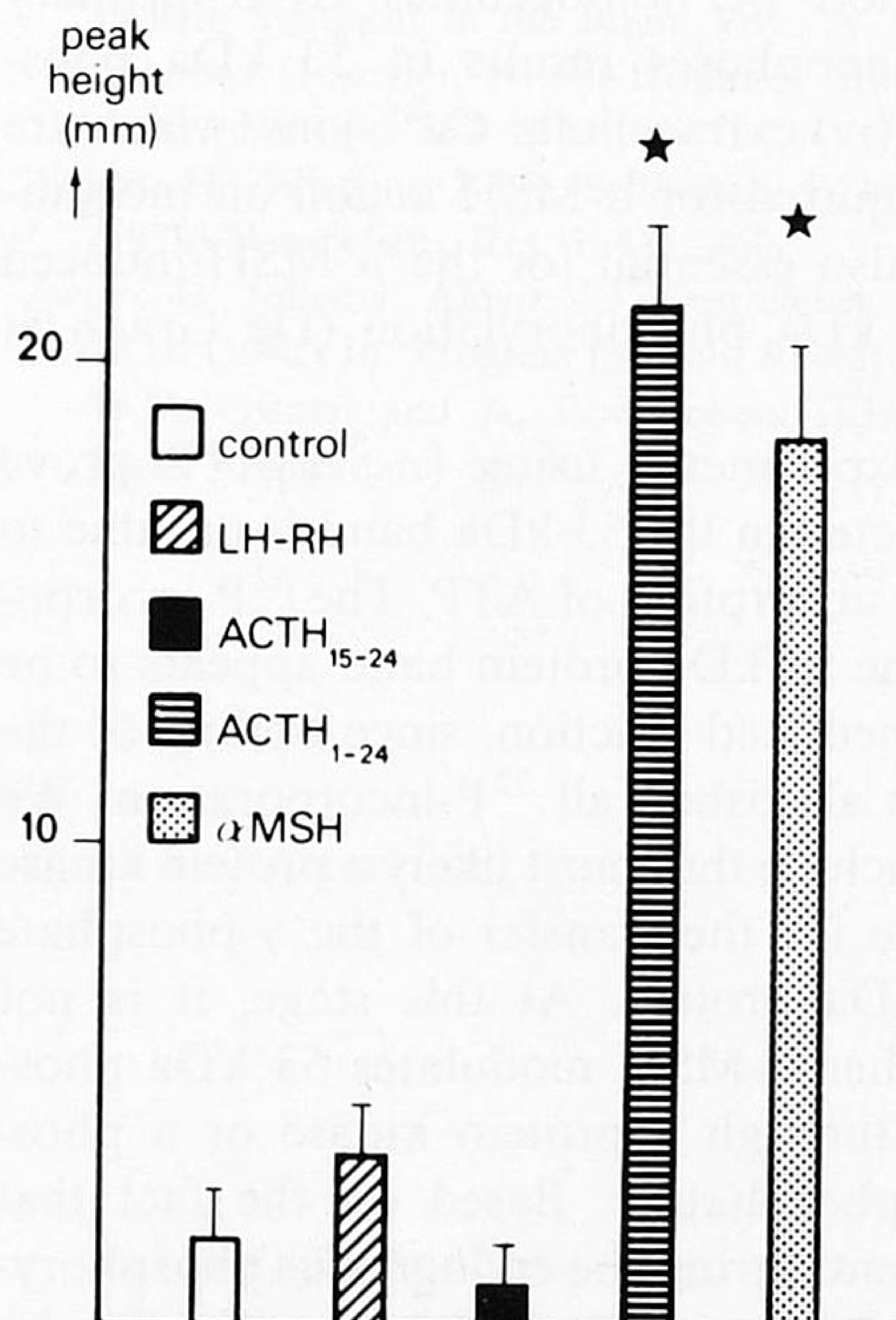

Fig. 5. Effect of incubating tail-fins with melanotropic and non-melanotropic peptides on 53 kDa phosphorylation. The melanotropic peptides ($ACTH_{1-24}$ and α-MSH) and the non-melanotropic ones ($ACTH_{15-24}$, and LH-RH) were all tested at 5×10^{-6} M. ★ Significantly different from control, $P < 0.001$, $n = 6$; bars indicate SEM.

which were transferred to a black background 30 or 60 min prior to the tail-fin excision, had a level of ^{32}P-incorporation into the 53 kDa protein which was significantly higher than that of tail-fins from animals kept on a white background.

Discussion

Incubation of excised tail-fins with 5×10^{-6} M α-MSH induces a full melanosome dispersion. Endogenous protein phosphorylation in homogenates from these α-MSH-treated tail-fins reveals a 5-fold increase in ^{32}P-incorporation into a 53 kDa protein band. Moreover, under physiological conditions known to increase blood MSH levels, a significant increase in 53 kDa phosphorylation could be detected. Although melanophores constitute only a low percentage of the tail-fin cells, there is strong evidence indicating the effect of α-MSH on 53 kDa phosphorylation to be localized in melanophores: (i) the effect is not found in the melanophore-free part of the tail-fin; (ii) only melanotropic peptides induce the effect; (iii) endogenous phosphorylation of homogenates from primary cultured melanophores results in 53 kDa phosphorylation; (iv) extracellular Ca^{2+} ions, which are absolutely required for α-MSH action on melanophores, are also essential for the α-MSH-induced effect on 53 kDa phosphorylation (De Graan et al., 1984).

Control experiments using [α-^{32}P]ATP prove that ^{32}P detected in the 53 kDa band is not due to (non)specific absorption of ATP. The ^{32}P-incorporation into the 53 kDa protein band appears to be an enzyme-mediated reaction, since boiling of the homogenates abolished all ^{32}P-incorporation. We therefore conclude that most likely a protein kinase is responsible for the transfer of the γ-phosphate to the 53 kDa protein. At this stage, it is not known whether α-MSH modulates 53 kDa phosphorylation through a protein kinase or a phosphoprotein phosphatase. Based on the fact that α-MSH present during the endogenous phosphorylation assay does not affect 53 kDa phosphorylation, it appears that α-MSH elicits its effect through a receptor-mediated mechanism, rather than through a direct interaction with a kinase or phosphatase. These data are in line with the hypothesis that α-MSH elicits its effect on melanophores by activating a specific plasma membrane receptor, resulting in the generation of intracellular messengers (Eberle, 1980). But this α-MSH effect on protein phosphorylation is quite distinct from that observed in brain synaptic plasma membranes. In the latter system, α-MSH and ACTH directly inhibit the activity of B-50 kinase, the enzyme phosphorylating the endogenous substrate protein B-50 (Zwiers et al., 1978, 1982).

The α-MSH-induced increase in 53 kDa phosphorylation is measured in vitro after homogenization of the tail-fin tissue. This post hoc approach, used in this study and in many others (Zwiers et al., 1977; Ehrlich et al., 1978; Browning et al., 1979), assumes that changes in endogenous phosphorylation in vivo can be monitored in vitro. Thus, the increase in 53 kDa phosphorylation measured in vitro most likely reflects a decrease in 53 kDa phosphorylation in vivo. The mechanism by which α-MSH would exert such an inhibitory effect is not yet known. Since both cAMP (Abe et al., 1969; Van de Veerdonk and Konijn, 1970) and Ca^{2+} (De Graan et al., 1982a, b) are thought to play an important role in the mechanism of action of α-MSH on melanophores, these intracellular mediators are likely to be involved in the modulation of the degree of 53 kDa phosphorylation. cAMP-dependent protein kinase activity has been shown in melanoma cells (Niles and Logue, 1979). Moreover, forskolin, a diterpene known to increase intracellular cAMP levels in many intact cell systems, stimulates post hoc 53 kDa phosphorylation in a concentration-dependent manner (De Graan et al., 1984).

The above results suggest that phosphorylation of the 53 kDa protein is related to α-MSH-induced pigment migration. Presumably, peptide-receptor activation is followed by a decrease in the degree of phosphorylation of the 53 kDa protein. Since pigment translocation is a major event in the melanophore response to α-MSH, this 53 kDa protein may play a role in melanosome migration.

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