The melanocortin receptor in the rat lacrimal gland: a model system for the study of MSH (melanocyte stimulating hormone) as a potential neurotransmitter

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Received 4 January 1990, accepted 27 February 1990

The melanocortin receptors in intraorbital and extraorbital rat lacrimal glands were studied with \(^{125}\text{I}\)[Nle\(^4\),D-Phe\(^7\)]aMSH as radioligand and with several unlabeled melanocortin peptides. The pharmacological properties of the melanocortin receptor in both tissues appeared to be essentially identical. Receptor binding was studied in a membrane fraction sedimented at \(12000-100000 \times g\), establishing for \(^{125}\text{I}\)[Nle\(^4\),D-Phe\(^7\)]aMSH a \(K_o\) of 0.76 and 2.2 nM for the intra- and extraorbital glands, respectively. Binding of the radioligand was competitively inhibited by aMSH (\(\alpha\)-melanocyte stimulating hormone) and ACTH-(1-24) with \(IC_{50}\) values in the submicromolar range. MSH binding in both tissues was abolised by EGTA and was increased dose dependently with elevation of free \(Ca^{2+}\) ion concentration. The half-maximal effect on MSH binding was obtained around 200 nM \(Ca^{2+}\) and maximal binding was reached at nearly 2 mM free \(Ca^{2+}\) in membrane preparations from both tissues. The calmodulin-binding peptides, melittin, mastoparan and M5, the latter being the 18-amino acid synthetic analogue of the C-terminal calmodulin-binding domain of myosin light chain kinase, inhibited MSH binding in the concentration range of 1-20 \(\mu M\). Macroscopic autoradiographic analysis of cryosections prepared from either lacrimal gland to which \(^{125}\text{I}\)[Nle\(^4\),D-Phe\(^7\)]aMSH was subsequently bound, showed the melanocortin receptor to be uniformly distributed within the acinar lobes. At the microscopic level, MSH was found to be associated with the acinar cells, primarily at the basal perinuclear region. Peroxidase secretion from extraorbital lacrimal slices was stimulated by MSH, epinephrine and carbamylcholine to a similar extent. The response of the tissue to stimulation by MSH was however not blocked by \(\alpha/\beta\)-adrenoeceptor blockers or by atropine, suggesting that MSH acts as a primary secretagogue in this tissue. Thus, this system seems to be uniquely suited to serve as a model for the study of both the molecular and pharmacological details of the action of MSH and other melanocortins in a non-melanogenic tissue.

Melanocortins; MSH (melanocyte stimulating hormone) receptors; Lacrimal glands; Secretion; Neurotransmitters

1. Introduction

In recent years melanocortin peptides have emerged as regulators of an increasing number of processes that are unrelated to their classical role as pituitary hormones (reviewed by Eberle, 1988). Furthermore, the synthesis of pro-opiomelanocortin was shown to take place in several distinct regions of the central (Liotta et al., 1980) and peripheral nervous system (reviewed by Smith and Funder, 1988; Bardin et al., 1985), and not exclusively in the pituitary (Mains et al., 1977) as has been thought previously. When mice and rats were injected in vivo with \(^{125}\text{I}\)[Nle\(^4\),D-Phe\(^7\)]a-
melanocyte-stimulating hormone (αMSH), the peptide was shown to accumulate specifically in several glandular organs, white adipose tissue, bladder, duodenum, skin, spleen and hypothalamus. The highest specific binding was observed in the lacrimal and Harderian glands (Tatro and Reichlin, 1987). These observations suggested a functional role for MSH in these tissues. Furthermore, independent observations indicated that melanocortins act directly and influence respective target tissues in a way that is neither related to nor mediated by steroidogenesis or melanogenesis. Effects on animal and human behavior (De Wied and Ferrari, 1986; De Wied and Jolles, 1982), facilitation of nerve regeneration (Strand et al., 1981; Bijlsma et al., 1981) and recovery of motor performance following crush denervation (Saint-Come et al., 1982; Bijlsma et al., 1983), induction of long-lasting potentiation of neurotransmitter release (Johnston et al., 1983) and increase of miniature end-plate potentials in the neuromuscular junction (Strand et al., 1981), stimulation of lacrimal secretion (Jahn et al., 1982), acceleration of lipolysis (Tanaka et al., 1962) and stimulation of aromatase and plasminogen activator in rat Sertoli cells (Boitani et al., 1989) exemplify the diversity of melanocortin functions. The actual presence of specific melanocortin receptors in many of these tissues, their basic properties and mechanism of action, as well as the physiological significance of their effects have not been addressed.

Stimulation of lacrimal secretion in the rat by the melanocortins, MSH and adrenocorticotropic hormone (ACTH) was first demonstrated in 1982 by Jahn et al. (1982). These investigators suggested that MSH-stimulated secretion was mediated by elevation of intracellular 3',5' cyclic AMP (cAMP) levels. However, it was not known whether MSH actually acts as a primary secretagogue, or through the release of endogenous neurotransmitters. The presence of MSH receptors in lacrimal tissue has been recently reported by us and others (Salomon et al., 1989; Hann et al., 1989).

Therefore, we first studied some of the pharmacological properties of the melanocortin receptors in the rat lacrimal intra- and extraorbital glands (Salomon et al., 1989). Using \([^{125}\text{I}]\text{Nle}^4,\text{D-Phe}^7\text{aMSH}\) as radioligand, we employed methodologies and compared aspects previously studied by us on a mouse melanoma cell MSH receptor, including peptide specificity, the effects of Ca\(^{2+}\) ions and calmodulin (CaM)-binding peptides (Gerst et al., 1986; 1987; Gerst and Salomon, 1987; 1988). Secondly, we determined the microscopic distribution of the MSH receptors in the lacrimal gland. Thirdly, we investigated whether MSH acts as a primary secretagogue in this tissue, independently of catecholamines and acetylcholine. The data indicate that lacrimal tissue is a good model system for the study of the biochemical mechanism of action of MSH in processes unrelated to its classical role as a melanocyte stimulating hormone.

2. Materials and methods

2.1. Materials

αMSH, porcine βMSH, (L-)epinephrine, carbamylcholine, dithiothreitol (DTT), atropine, propranolol, phentolamine, melittin, mastoparan, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and para-aminobenzamidine (PABA), bovine serum albumin (BSA) were from Sigma Chemical Co. Gonadotropin-releasing hormone (GnRH) and prolactin were from NIH. \([\text{Nle}^4,\text{D-Phe}^7]\)αMSH (Sawyer et al., 1980) was a gift from Dr. Lerner (Yale University). ACTH(1-24) was a gift from Organon (Oss, Holland). Lys-Arg-Arg-Trp-Lys-Asn-Phe-Ile-Ala-Val-Ser-Ala-Ala-Asn-Arg-Phe-Gly-NH\(_2\) (M5) peptide (Kennelly et al., 1987) and vasoactive intestinal peptide (VIP) were synthesized at the Weizmann Institute. Carrier-free iodine \(^{125}\text{I}\) was from Amersham. All other materials were of analytical grade.

2.2. Animals and surgical procedures

Male Wistar rats (200 ± 10 g) raised in the departmental facility were fed at libitum and kept at 25°C with 14 h light, 10 h dark. The intraorbital lacrimal gland as referred to in this study was excised along with the eyeball. The entire glandular tissue immediately surrounding the globe
including the Harderian gland was dissected. The extraorbital gland was removed by an external approach.

2.3. Preparation of rat lacrimal cell membranes

The rats were killed by cervical dislocation (experiments 1-7) or by ether anaesthesia (experiment 8), and the glands were excised, collected and homogenized in 10 volumes of ice-cold homogenization medium (0.3 M sucrose, Tris acetate 20 mM, pH 7.6, 0.1 mM EDTA, 1 mM PABA), using a loosely fitted Dounce homogenizer. The homogenate was filtered through a No. 130 nylon mesh and centrifuged for 10 min at 12,000 × g. The pellet was discarded and the supernatant was centrifuged further for 60 min at 105,000 × g. The resulting pellet was resuspended in 10 mM Tris acetate pH 7.4 1 mM DTT and frozen in liquid nitrogen in aliquots that were thawed once shortly before use.

2.4. Iodination of [Nle₄, D-Phe⁷]aMSH

[Nle₄, D-Phe⁷]aMSH (5 µg) was iodinated with chloramine-T, as previously described for iodination of βMSH (Gerst et al., 1986), but using 0.025% chloramine-T and incubating at room temperature for 45 s only. After addition of DTT to 0.75 M, and KI to 36 mM, the peptide was imme- diately separated from free iodine by gel filtration on Biogel P-2. The [¹²⁵I]iodo-[Nle₄, D-Phe⁷]aMSH concentration was determined by its ability to activate MSH-sensitive adenylate cyclase in M2R mouse melanoma cells, as previously described (Gerst et al., 1986) using a calibration curve made with authentic [Nle₄, D-Phe⁷]aMSH. The specific radioactivity of the labeled peptide was 1000-3000 c.p.m./fmol.

2.5. [¹²⁵I]Iodo-[Nle₄, D-Phe⁷]aMSH binding to rat lacrimal membranes

Binding of hormone to lacrimal gland membranes was carried out essentially as described by us for [¹²⁵I]iodo-βMSH binding to M2R mouse melanoma cell membranes (Gerst et al., 1987). The incubation medium in phosphate-buffered saline (PBS) contained [¹²⁵I]iodo-[Nle₄, D-Phe⁷]aMSH (0.4-2.0 × 10⁵ c.p.m.), 1 mg/ml BSA, 10 µg/ml leupeptin, 1 mM PABA, 10 µg/ml soya bean trypsin inhibitor, 1 mM CaCl₂ and 1 mM MgCl₂ (binding incubation cocktail) and test substances or varying concentrations of unlabeled [Nle₄, D-Phe⁷]aMSH as specified in the individual experiments. Binding was initiated by the addition of 20-90 µg of lacrimal cell membrane protein to a final volume of 100 µl. Binding was linear with membrane concentration in the range tested and incubation was for 20 min at 30°C, conditions under which equilibrium binding was attained. The binding reaction was terminated by the addition of 2 ml ice-cold PBS containing 1.0 mg/ml BSA, 1 mM CaCl₂ and 1 mM MgCl₂. The membrane suspension was filtered through 0.22 µm OE-66 Millipore filters (2.5 cm diameter) pre-soaked in PBS containing 1 mg/ml BSA, and washed twice with 2 ml of ice-cold PBS containing 1 mg/ml BSA. The filters were counted in an autogamma spectrometer. Non-specific binding was determined in the presence of 1 µM unlabeled [Nle₄, D-Phe⁷]aMSH and subtracted from the values for total binding. Specific binding (> 80% of total binding) is expressed as fmole of [Nle₄, D-Phe⁷]aMSH bound/mg membrane protein, means ± S.E.M. of triplicate determinations.

2.6. Calculation of free Ca²⁺ concentrations

The free calcium concentrations were set with an EGTA buffer system, as described by us earlier (Gerst et al., 1987), using a computer program kindly made available to us by Dr. T. Abrams (constants derived from Tsien and Rink (1980)).

2.7. [¹²⁵I]Iodo[Nle₄, D-Phe⁷]aMSH binding to rat lacrimal tissue slices for autoradiographic analysis

Freshly excised rat lacrimal glands were quickly frozen on aluminum foil placed on dry ice and stored at -70°C until sectioned. For sectioning, the frozen tissue was equilibrated to -20°C, and mounted onto the cryostat stage. Sections, 10 µm were prepared using a Reichert-Jung Frigocut N-2800 cryostat. Sections placed on gelatin-coated
microscope slides were stored at −70°C until further processed.

For [125I]iodo-[Nle⁴,D-Phe⁷]αMSH binding to the tissue sections, the slides were brought to room temperature and the sections were covered with a sufficient volume of binding incubation cocktail (~100,000 c.p.m./100 μl). Incubation was for 30 min at 37°C at saturated humidity. Subsequently, the slices were washed twice (15 min each) with ice-cold PBS containing 5 mg/ml BSA.

Further treatment of the sections was as follows. (a) Autoradiography for macroscopic analysis: the sections were dried and exposed to Kodak XAR-5 X-ray film with intensifier screen for the period of time indicated and developed. (b) Autoradiography for microscopic analysis: the wet sections were washed twice with ice-cold PBS and post-fixed with 2% glutaraldehyde. The specimens were further washed with PBS, dehydrated with ethanol, dried and coated with photographic emulsion (Ilford, K-5) and exposed for the period of time indicated. After development of the photographic layer, the sections were stained with Toluidine blue, dried and covered with coverslips. Light microscopy was performed with a Zeiss photomicroscope III.

2.8. Determination of protein secretion from lacrimal gland slices

Protein secretion from rat extraorbital lacrimal gland slices was determined as described by Friedman et al. (1981). Fresh extraorbital glands were collected in Krebs Ringer Bicarbonate (KRB) supplemented with 25 mM HEPES, pH 7.4, and 5 mM β-hydroxybutyric acid (HβKRB) and gassed with 95% O₂-5% CO₂ mixture at 37°C. The glands were then collected and sliced (450 μm) with a Mickel tissue slicer. The slices were incubated in fresh HβKRB for 10 min, collected on a nylon mesh No. 130 and washed extensively with the same solution. Duplicate vials containing tissue-slice equivalents of about 0.75-1 gland were incubated with test substances in a final volume of 2 ml HβKRB, briefly gassed and stoppered. Incubation (60 min at 37°C) was carried out with shaking, and gassing was repeated every 15 min. Under these conditions, secretion is progressive and has not leveled off within 60 min. Samples of the incubation medium (0.1 ml) were removed at zero time and 60 min and stored in the cold. The remaining tissue was homogenized and kept on ice. Peroxidase activity released during the incubation was taken as a measure of protein secretion and was expressed as percentage of the total activity of enzyme in each vial. The total amount of peroxidase in each vial was taken as 100% and calculated from the sum of peroxidase activity secreted at the end of the experiment plus the remaining activity determined in the tissue homogenate.

2.9. Peroxidase assay

Peroxidase was determined under conditions modified from those described by Engvall (1980). Enzyme activity was assayed in a final volume of 0.2 ml, in the presence of 25 mM citric acid, 1.46 mM H₂O₂, 50 mM sodium phosphate and 0.45 mM 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) at pH 4.6. Assays were performed in duplicate with incubation at room temperature; optical density at 600 nm was recorded at 15 min using an ELIZA reader (Bio-Tek Instruments).

2.10. Protein determination

Protein was determined according to Bradford (1976).

3. Results

3.1. [125I][Nle⁴,D-Phe⁷]αMSH binding to rat lacrimal cell membranes

We measured MSH binding to cell membranes prepared from both intra- and extraorbital lacrimal glands of the rat. In a representative experiment (fig. 1), binding was shown to be dose-dependent, reaching saturation at 10 nM [125I][Nle⁴,D-Phe⁷]αMSH. The dissociation constant, Kᵦ, for the MSH receptor as calculated from a double reciprocal plot was very similar, 2.0 and 1.3 nM for the intra- and extraorbital gland membranes, respectively (fig. 1). The average values obtained
Fig. 1. Binding of $[\text{125I}]$iodo-$[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$ ($[\text{125I}]$iodo-4,7-\text{aMSH}) to rat intraorbital lacrimal gland membranes. Rat intraorbital lacrimal gland membranes ($\bullet$) (60 nM protein/assay) or extraorbital gland membranes ($\bigcirc$) (50 nM protein) were incubated with $[\text{125I}]$iodo-$[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$ (0.2-10 nM). The $K_D$ in a representative experiment performed with each tissue was calculated from the double reciprocal plot, as indicated on the figure. Binding was determined as described in Methods.

3.2. Binding specificity of the lacrimal MSH receptor

The specificity of $[\text{125I}]$iodo-$[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$ binding to the MSH receptor in both glands was tested next (fig. 2). We used unlabeled $[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$, $\text{aMSH}$, ACTH-(1-24) and porcine $\beta\text{MSH}$ to compete for receptor binding to membranes derived from the intraorbital and extraorbital gland, respectively. At saturating concentrations of peptide, the maximal binding fmol/mg protein was 195 ± 45 (n = 5) and 395 ± 100 (n = 3) for membranes derived from these glands, respectively.

3.3. $\text{Ca}^{2+}$ ion requirement of MSH binding

$[\text{125I}]$iodo-$[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$ binding to lacrimal cell membranes was undetectable if $\text{Ca}^{2+}$ ions

\[\alpha\text{MSH} \ (IC_{50} = 200 \text{ nM}) \text{ in the intraorbital gland.} \]

The $IC_{50}$ values for $\alpha\text{MSH}$ and ACTH-(1-24) in the extraorbital gland were 80 and 300 nM, respectively. $\beta\text{MSH}$ showed the lowest receptor affinity (> 300 nM) in this group of peptides in both tissues.

Unrelated peptides, VIP and GnRH in the case of the intraorbital gland, and GnRH and PRL in the extraorbital gland, were totally ineffective (fig. 2).

Fig. 2. Binding of $[\text{125I}]$iodo-$[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$ to rat intraorbital lacrimal gland membranes: competition by various peptides. Rat intraorbital gland membranes (60 nM protein) (A) or extraorbital gland membranes (50 nM protein/assay) (B) were incubated with $[\text{125I}]$iodo-$[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$ (85 pM, A) (62 pM, B) and the indicated concentrations of $[\text{Nle}^4,\text{D-Phe}^7]\text{aMSH}$, $\text{aMSH}$, ACTH-(1-24), porcine $\beta\text{MSH}$, GnRH, PRL or VIP. Binding of the radioactive peptide and all other details were as described under Methods.
were omitted and EGTA (10 mM) was included in the incubation medium. The dependence of $[^{125}\text{I}]	ext{iodo-[Nle}^4,\text{D-Phe}^7\text{]}\text{oMSH}$ binding to lacrimal cell membranes of both sources on ambient free Ca$^{2+}$ was therefore determined (fig. 3). We found that $[^{125}\text{I}]	ext{iodo-[Nle}^4,\text{D-Phe}^7\text{]}\text{oMSH}$ binding increased over 20-fold in the range of 0.1-2000 μM free Ca$^{2+}$. The major positive effect (~5-fold increase in MSH binding capacity) was seen in the range of 10 to 2000 μM free Ca$^{2+}$, and comprised approximately 80% of the total binding obtained at the saturating Ca$^{2+}$ concentration. The half-maximal Ca$^{2+}$ ion concentration was 0.21 mM ± 0.02 (n = 4) in the intraorbital and 0.17 mM ± 0.01 (n = 3) in the extraorbital gland. A smaller effect, comprising only ~20% of the total binding, was seen in the range of 0.1-10 μM free Ca$^{2+}$ and may represent an additional Ca$^{2+}$ dependence site with higher affinity.

3.4. Inhibition of MSH binding by calmodulin-binding peptides

The effect of several CaM-binding peptides on MSH binding was tested next. It was found that M5 (IC$_{50}$ = μM 1.5 ± 0.1, n = 4), melittin (IC$_{50}$ = μM 3.4 ± 0.4, n = 3) and mastoparan (IC$_{50}$ = μM 12 ± 2, n = 3), all inhibited and at 50 μM nearly abolished MSH binding to intraorbital gland membranes as shown in a representative experiment (fig. 4A). Similar results were observed with the extraorbital gland, but at higher peptide concentrations, M5 (IC$_{50}$ μM = 13 ± 6, n = 3) and melittin (IC$_{50}$ μM = 22 ± 3, n = 3) as shown in a representative experiment (fig. 4B). As in the case of the melanoma MSH receptor, M5 was found to be the most potent inhibitor in this group of peptides.

3.5. Autoradiographic analysis of MSH-receptor distribution in rat lacrimal glands

Freshly frozen tissue was cryosectioned in order to examine the distribution of MSH-receptors in the rat intraorbital lacrimal gland. The 10-μm-thick slices were collected on microscope slides and incubated for 30 min at 37°C with $[^{125}\text{I}]	ext{iodo-[Nle}^4,\text{D-Phe}^7\text{]}\text{oMSH}$ under conditions otherwise identical to those used in the binding assay. After washing, the dried slices were exposed to X-ray film (fig. 5). Macroscopic examination of the densitogram revealed that MSH binding sites were
are homogeneously distributed on the entire slice with clear imprints of the boundaries of the acinar lobes (fig. 5a). The presence of excess (1 μM) unlabeled [Nle⁴,D-Phe⁷]αMSH reduced the binding of the radiolabeled peptide substantially (fig. 5b).

Binding of MSH to the tissue slices was Ca²⁺-dependent and reduced to control levels by inclusion of 10 mM EGTA in the binding step (fig. 5c), in agreement with the results shown in fig. 3. Similarly, [125I][Nle⁴,D-Phe⁷]αMSH binding to the tissue slices was found to be inhibited to near the control levels by 10 μM melittin (fig. 5d) or 10 μM M5 (fig. 5e), in agreement with the results shown in fig. 4A. Thus, we took it that the MSH receptor distribution in the slice is uniform. Furthermore, the basic pharmacology of the MSH receptor, as studied in tissue slices, reflects results obtained in the standard binding assays using cell membrane preparations.

For quantitative evaluation of these experiments, the specific radioactivity bound to the sections was also counted directly. In early experiments, groups of 10 slices each were incubated in test tubes under conditions otherwise used in the direct binding assays. Incubations were in the presence or absence of 1 μM unlabeled [Nle⁴,D-Phe⁷]αMSH. The amount of bound radioactivity was determined by Millipore filtration as described for binding assays with lacrimal cell membranes. We found that the ratio between total [125I][Nle⁴,D-Phe⁷]αMSH binding to non-specific binding using such material was ~10, with 700 and 900 c.p.m. specifically bound per slice in two experiments. Consequently, in each of the experiments carried out with intraorbital gland sections

![Image](image-url)
in the first phase of the experiments, three slices/group/experiment were scraped off the microscope slide at the end of the washing step. The radioactivity in this material was counted directly with no further processing, to determine the amount of total and non-specifically bound \([^{125}\text{I}][\text{Nle}^4,\text{D-Phe}^7]\alpha\text{MSH}\). The ratio of specific to non-specific binding obtained in these studies with intraorbital gland slices was \(7.2 \pm 1.0\) \((n = 8)\) with 385–1676 c.p.m./slice specifically bound in the different experiments. The total amount of radioactivity bound/slice varied with the surface area of the slices and the amount of radioactivity used in each experiment but varied little within each experiment.

In order to examine the subacinar and cellular MSH-receptor distribution, tissue slices were processed as described above but were post-fixed with glutaraldehyde, covered with photographic emulsion and subjected to microscopic analysis following exposure for 9 days. Autoradiography showed that the radioactive hormone as revealed by silver grains on the photographic plate is distributed over all acinar cells in both the intraorbital (fig. 6A) and extraorbital (fig. 7A) glands. Grain density was significantly lower in control slices in which incubation with \([^{125}\text{I}][\text{Nle}^4,\text{D-Phe}^7]\alpha\text{MSH}\) was carried out in the presence of excess \((1 \mu\text{M})\) unlabeled \([\text{Nle}^4,\text{D-Phe}^7]\alpha\text{MSH}\), in the intraorbital (fig. 6B) and extraorbital (fig. 7B) gland slices. The acinar regions can be identified by the concentric array of individual cell nuclei (Nu). Furthermore, in regions where a clear lumen (L) can be seen the receptors seem to be associated with the perinuclear regions, i.e. at the basal aspect of the acinar cell. These experiments show that MSH receptors are present in both glands in high concentrations and, therefore, are likely to play an important role in lacrimal physiology.

3.6. Stimulation of protein secretion in lacrimal gland slices by MSH

We next examined whether the stimulatory action of MSH on protein secretion in the rat lacrimal gland results from direct stimulation of the acinar cells by MSH or whether stimulation by the peptide could be mediated by the release of acetylcholine or catecholamines endogenously present in nerve endings. In such a situation, the MSH receptors would have been expected to be distributed on neuronal components within the gland. We stimulated peroxidase secretion in extraorbital gland slices by using supramaximal concentrations of epinephrine \((10 \mu\text{M})\), carbamylcholine \((10 \mu\text{M})\) or \([\text{Nle}^4,\text{D-Phe}^7]\alpha\text{MSH} \((1 \mu\text{M})\) (fig. 8). The three stimulants increased protein dis-
charge 9-, 11- and 7-fold, respectively, as compared to the unstimulated control (basal). Propranolol (10 μM) and phentolamine (10 μM), blocking β and α actions of epinephrine, respectively, and atropine (10 μM), blocking the actions of carbachol, had no effect on protein secretion when added alone, but effectively blocked the stimulatory activities of the neurotransmitters. However, these compounds failed to affect the secretion stimulated by [Nle₄,D-Phe⁷]αMSH. These results therefore suggested that the action of MSH as a secretagogue in this tissue is exerted directly on the secreting cells and is not likely to be mediated by the release of these endogenous classical neurotransmitters.

4. Discussion

Our results show that the MSH-receptor in both rat lacrimal glands exhibits similar pharmacological properties. Furthermore, these properties are basically similar with respect to receptor affinities to what we (Salomon et al., 1989; Gerst et al., 1986; 1987) and others (Eberle, 1988; Sawyer et al., 1980; Scimonelli and Eberle, 1987) reported for melanoma cells.

While αMSH and ACTH-(1-24) have basically similar IC₅₀s in these glands, βMSH seems to
have the lowest relative affinity in this group, being essentially unable to compete with [Nle⁴,D-Phe⁷]αMSH in the case of the intraorbital gland. This is consistent with the inability of βMSH (1 μM) to stimulate cAMP production in intraorbital gland slices (data not shown). There was a previous report (Gerst et al., 1986) that ACTH-(1-24), βMSH and αMSH were essentially equal in their affinity for the M2R melanoma cell receptor. The question of whether this difference qualifies for a separate classification of the glandular MSH-receptor vis-à-vis the melanoma-receptor remains to be tested. The generally similar response of the lacrimal tissue receptor system towards native αMSH and ACTH-(1-24) warrants its designation as a melanocortin receptor. Due to operational considerations, we refer to this receptor as an MSH receptor also even though this semantic choice may be a matter for some debate.

The interesting Ca²⁺ requirement for MSH binding, as first reported by us for M2R melanoma cells (Gerst et al., 1987) with porcine βMSH, seems to hold equally well in the case of the lacrimal cell MSH-receptors and to be independent of the type of peptide used, since [Nle⁴,D-Phe⁷]αMSH was used in the present study (fig. 3). Ca²⁺ was found to increase the affinity of MSH for receptor binding in melanoma cells 20-fold. This effect was reversible since the peptide-receptor complex dissociated readily upon addition of EGTA and re-associated following replenishment of free Ca²⁺ ion to millimolar concentrations (Gerst et al., 1987). While the molecular basis for this requirement is not yet clear, it appears to be a general property of this class of receptors. The possible physiological significance of this peculiar ionic requirement may indeed be more relevant to the acute and dynamic control of protein secretion, which itself is a Ca²⁺-requiring process, than in the case of the long-lasting trophic control of melanogenesis. Also, in the case of the M2R cell MSH receptors, we observed two Ca²⁺-dependence sites saturating in the micromolar and sub-millimolar ranges, respectively. This may implicate two independent Ca²⁺ binding sites in the control of MSH receptor activity. The similarity continues with respect to the inhibition of receptor binding activity by various CaM-binding peptides (fig. 4) which we have described previously for the melanoma cell MSH-receptor (Gerst and Salomon, 1987; 1988). Even though the effect of these peptides implies the possible involvement of CaM or a similar calcium-binding protein in MSH-receptor function, no direct link has been established between this line of evidence and the Ca²⁺ requirement of receptor activity. It is logical to assume that melanocortins regulate lacrimal function in the rat in vivo, although direct evidence in proof of this assumption is lacking. In this regard, the observation made by Tatro and Reichlin (1987), that [Nle⁴,D-Phe⁷]αMSH uptake by lacrimal tissue of the rat and mouse was the highest as compared to that of other organs, is most interesting and rather surprising if viewed according to classical concepts and terminology that earmark MSH peptides for the control of pigmentation. Whether MSH acts in lacrimal tissue as a hormone or may be released from nerve endings as a neurotransmitter also remains to be established. It should be mentioned that VIP has been shown to be present in nerve endings in the cat lacrimal gland (Uddman et al., 1980) and to act as secretagogue in the rabbit lacrimal gland (Dartt et al., 1988). Moreover, the involvement of several peptides/neuropeptides in the regulation of exocrine glands, such as salivary glands (VIP and substance P) (Ekstrom et al., 1983; Martinez and Martinez, 1981) and the exocrine pancreas (cholecystokinin, gastrin, substance P and bombesin, VIP and PHI) (Dimaline and Dockray, 1980; Gardner and Jensen, 1980), has been described. Their coexistence along with classical neurotransmitters and their exact role in controlling exocrine function has to be evaluated further.

We have now shown MSH to be a primary secretagogue in the rat lacrimal gland, as its activity was unaffected by the presence of specific blockers (phentolamine, propranolol and atropine) of the classical neurotransmitters (fig. 8). This result eliminated the possibility that MSH stimulates secretion indirectly by release of endogenous neurotransmitters. Our study also showed for the first time that MSH-receptors heavily populate the lacrimal gland in its entirety (fig. 5), and that MSH receptors are located on practically all acinar cells and are not confined to a selected population
or group of cells within the gland. The association of the MSH receptors with the basal aspects of the acinar cells is suggested from observations of regions in the tissue section in which a clear lumen, limited by the apical side of the acinar cells can be seen (figs. 6, 7). A distinct conclusion in this regard, however, must await the development of techniques permitting receptor imaging in much thinner tissue sections or, alternatively, the comparison of MSH-receptor content on basal and apical acinar membranes prepared differentially, as proposed by Mircheff et al. (Mircheff et al., 1983). We believe that the instant and acute secretory response of lacrimal tissue to MSH, presents a highly appropriate model for the study of the dynamic aspects of MSH action in non-melanogenic tissue which seems to resemble those of neuropeptides acting as neurotransmitters rather than hormones.

Acknowledgements

We wish to thank Ms. Rachel Benjamin for her devoted secretarial assistance, and M. Tosky and J. Schultz for their critical review of the manuscript. Y.S. is the incumbent of the Charles W. and Tillie K. Lubin Professorship of Hormone Research.

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