GABA and Dopamine Act Directly on Melanotropes of *Xenopus* to Inhibit MSH Secretion

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VERBURG-VAN KEMENADE, B. M. L., B. G. JENKS AND A. G. J. DRIESSEN. GABA and dopamine act directly on melanotropes of *Xenopus* to inhibit MSH secretion. BRAIN RES BULL 17(5) 697-704, 1986.-The release of melanophore stimulating hormone (MSH) from the pars intermedia of the amphibian *Xenopus laevis* is regulated by multiple factors of hypothalamic origin. The aim of this study was to determine if potential secretagogues function through a direct action on the melanotrope cell. For this purpose an in vitro superfusion system containing isolated melanotropes (cell suspension) was utilized. The viability of the cells in suspension was tested by examining their ability to synthesize, process and release pro-opiomelanocortin (POMC) related peptides. All biosynthetic functions appeared normal, with the exception that the isolated melanotropes are unable to N-terminally acetylate MSH. Release of immunoreactive MSH from these cells was shown to be Ca²⁺-dependent, and high K⁺ stimulated release. Both the neurotransmitters dopamine and γ-aminobutyric acid (GABA), which are thought to be physiologically important MSH-release inhibiting factors, were shown to inhibit MSH release from isolated melanotropes. Dopamine appeared to function through a dopamine D₂ type receptor mechanism while for GABA, both a GABAₐ and GABAₐ receptor mechanism are involved.

**THE regulation of dispersion of the black pigment melanin in dermal melanophores of amphibians is an example of a neuroendocrine reflex [1]. Most amphibians, when placed on a black background, release melanophore stimulating hormone (MSH) from the pars intermedia of the pituitary gland and consequently there is a darkening of the skin. While it is thought that central integration of the environmental input (color of background) would play an important role in determining the rate of MSH secretion, a consideration of the complexity of the innervation of the pars intermediate lobe itself may be an important factor in this integration. For example, in the aquatic toad *Xenopus laevis*, a dopaminergic system [24,25], an adrenergic system [9] and a GABAergic system [29] have been described within the pars intermedia. Moreover it has been shown that both dopamine [10,14] and α-adrenergic receptor agonists [28] as well as GABA [29] inhibit MSH release from incubated neurointermediate lobes of this species. In studies with intact neurointermediate lobe tissue the question arises if the secretagogues affect MSH release through an action directly on the melanotrope cells. The alternative is an indirect action, such as GABA induced release of dopamine, a phenomenon known to occur in the central nervous system [23]. To determine to what extent the pars intermedia melanotrope cell might be involved in integrating signals from the diverse neuronal systems within the neurointermediate lobe tissue, we are examining the effects of secretagogues on the release of immunoreactive MSH from isolated superfused melanotrope cells. The present study describes our preparation of melanotrope cell suspension and shows that both dopamine and GABA act directly on these cells.**

**METHOD**

**Animals**

*Xenopus laevis* were bred and reared in our aquatic facility. Prior to the experiment the animals were kept for four weeks on a black background under constant illumination at 22°C.

**Preparation of Cell Suspension**

Neurointermediate lobes were extirpated, rinsed in incubation medium (IM) containing 112 mM NaCl, 2 mM KCl, 2
FIG. 1. Comparison of viability of neurointermediate lobes and isolated intermediate lobe melanotropes of Xenopus laevis. Both intact tissue and cell suspension were given a 1 hr pulse-incubation in [3H]lysine followed by a 3 hr chase incubation in medium containing L-lysine. Tissue extract, cell extract and chase incubation media were submitted to HPLC. The elution gradient (% secondary solvent, B) is given. Characterization of the newly synthesized peptides (numbered I-VIII) has been reported earlier [19] and is summarized in the Results section. Arrows indicate a major difference between intact tissue and cell suspension, namely the relatively high contribution of product II to the incubation medium of the cell suspension. This product has been previously identified as desacetyl-oMSH.

The percentage of cell death was established with trypan blue and cell yield was estimated with a haemocytometer. Pans nervosa remnants were traced by cytochemical staining with anti-oxytocin.

**Pulse-Chase Incubation of Neurointermediate Lobe Tissue or Isolated Melanotropes**

Neurointermediate lobe tissue was extirpated, rinsed in IM and given a pulse incubation in 200 μl IM containing 40 μCi [3H]lysine (75 Ci/mmol, Amersham), on a shaking water bath at 22°C. Tissue was rinsed in IM and chase incubated in 200 μl medium containing L-lysine (2 mM). It was then homogenized in 500 μl ice-cold 0.1 N HCl in a glass homogenizer and centrifuged (10 min, 10,000 g, 4°C). The supernatant was stored at −20°C before it was submitted to high performance liquid chromatography (HPLC) for separation of peptides. The incubation medium was acidified with 20 μl 1 N HCl and kept at −20°C before chromatography.

For pulse-chase analysis of cell suspensions, the cell pellets were resuspended in 200 μl IM. Pulse incubation was started by addition of 100 μl IM, containing 40 μCi
HPLC Analysis of Radioactive Peptides

Peptides were separated on a Spherisorb 10 ODS column (Chrompack, Middelburg, the Netherlands). The primary solvent was 0.5 M formic acid/0.14 M pyridine, pH 3.0 (A) and elution was accomplished with a gradient of n-propanol (B). The elution profile is indicated in Fig. 1. Fractions of 0.5 min were collected, 4 ml of scintillation fluid (Packard 199) were added for subsequent determination of radioactivity in a liquid scintillation counter.

Radioimmunoassay of αMSH

The C-terminal directed antiserum to αMSH was produced and characterized by Vaudry et al. [27]. It has equal reactivity to αMSH and desacetyl-αMSH. Bound and unbound MSH were separated by precipitation with polyethylene glycol (7.5%). Sensitivity threshold of the assay is 5 pg.

Superfusion of Isolated Melanotropes

Cells were suspended in a small volume of incubation medium and loaded in a 50 μl superfusion chamber with a millipore 0.45 μm filter (type HA) at the outlet to support a layer of biogel P2 (BioRad). Following loading the chamber was filled with biogel. Medium was pumped with a peristaltic pump at a rate of 1.5 ml/hr and 7.5 min fractions were collected in 100 μl ice-cold 0.1 N HCl. Fractions were stored at -20°C before submission to radioimmunoassay. After an equilibration period of approximately 1 hr the effect of calcium-free medium with 0.5 M EGTA, or high K⁺ (osmolality maintained by addition of NaCl) was established by giving 15 min pulses of the modified media. For comparative purposes the effect of high K⁺ on MSH release from intact neurointermediate lobe tissue was investigated. Lobes were placed in a 10 μl superfusion chamber and superfusion was performed as described earlier [28,29].

The effect of dopamine and/or GABA was investigated by giving 15 min pulses of these neurotransmitters to the superfused melanotropes. In additional experiments the dopamine D2-receptor antagonist sulpiride (Delagrange), the GABAA agonists homotaurine (kindly provided by Dr. Maggi, Italy) and isoguvacine (Cambridge Research Biochemicals) and the GABAB agonist baclofen (Ciba Geigy) were used.

RESULTS

Analysis of Cell Suspension Viability

Average yield of cells was 18,000 per neurointermediate lobe. Microscopical analysis showed that the suspension consisted for the most part of individual cells with occasional cohesion of two or three cells. Percentage of cell death was estimated to be 12%. As judged from the anti-oxytocin cytochemical staining, the cell suspension was free of neural lobe fragments. The oxytocin positive material remained as intact tissue on the top of the nylon filter.

Radioabeled lysine was a convenient label for our initial biosynthetic analysis because this amino acid is present in all POMC-related peptides products of Xenopus melanotropes [16,19]. Shown in Fig. 1 are the results of pulse-chase analysis of intact neurointermediate lobe tissue and the cell suspension. The HPLC resolved peptides have been characterized earlier [19] to be: I, γMSH; II, desacetyl-αMSH; IV, co-eluting peptides αMSH and a form of corticotropin-like intermediate peptide (CLIP), designated CLIP I; V, a second form of CLIP, designated CLIP II; VI, unidentified; VII/VIII, peptides immunologically related to mammalian endorphin and designated endorphin-like peptides. These earlier studies failed to identify βMSH. Sequence determination of Xenopus POMC-cDNA has revealed that βMSH is a potential biosynthetic product of Xenopus neurointermediate lobes and isolated intermediate lobe melanotropes of Xenopus laevis. Neurointermediate lobes or the cell suspension were given a pulse incubation in [3H]tryptophan and a 3.5 hr chase incubation in medium containing L-tryptophan. Lobe extracts, cell extracts, and chase incubation media were then submitted to HPLC. The HPLC elution gradient is identical to that given in Fig. 1. Tissue and cell analyses are given in the upper profiles; analyses of incubation media are given in the lower profiles. Tryptophan labeled product II is desacetyl-αMSH, product IV is αMSH and product I represents the co-eluting peptides αMSH and βMSH.
mediate lobes [16], and indeed our subsequent analysis has shown that newly synthesized aMSH co-elutes with yMSH in a peak I (manuscript in preparation). Co-elution of two peptides may account for the relatively high CPM value associated with peak I.

The HPLC profile of newly synthesized lysine labeled peptides produced by and released from cell suspensions is very similar to that displayed by intact tissue with the notable exception of the relatively high contribution of product II (desacetyl-aMSH) to the incubation medium of the cell suspension (Fig. 1). To examine the biosynthesis of aMSH this experiment was repeated with [3H]-tryptophan as label (the CLIP peptide co-eluting with aMSH lacks tryptophan). The major tissue form of the newly synthesized aMSH related peptides is desacetyl-aMSH, although some aMSH is associated with the tissue (Fig. 2). The intact tissue secretes both desacetyl-aMSH and aMSH, but aMSH now makes a major contribution to the HPLC profile. For the cell suspension newly synthesized desacetyl-aMSH is both the major cellular and secretory form of aMSH related peptides. There is virtually no newly synthesized aMSH present (Fig. 2).

Analysis of Immunoreactive aMSH Related Peptides

Desacetyl-aMSH was the major form of immunoreactive MSH associated with both tissue (Fig. 3, left) and isolated cells (Fig. 3, right). Separate experiments (n=3) showed that the percentage of non-acetylated aMSH was 77±1.7% in intact tissue and 88±3.0% in isolated melanotropes.

HPLC analysis of immunoreactive MSH from incubation medium of intact tissue shows that desacetyl-aMSH and aMSH make an approximately equal contribution to the medium (Fig. 3, left). There is apparently a reduction of tissue stores of MSH during in vitro incubation, indicated by the fact that the amount of MSH in the tissue of the incubated lobe is only about 10% of that found in an unincubated neurointermediate lobe (Fig. 3, insert). Relative to intact tissue, the cell suspension had a low level of immunoreactive MSH (Fig. 3, right). These cells released predominantly the non-acetylated form of aMSH (Fig. 3).

MSH Release From Superfused Melanotropes

Superfused melanotropes had a tendency to show a slo-
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FIG. 4. The release of immunoreactive MSH from superfused melanotropes of *Xenopus laevis*. Superfusion fractions were submitted to radioimmunoassay for α-MSH. Short pulses (15 min) of Ca-free medium (containing 0.5 mM EGTA), medium containing 60 mM K⁺, or a combination of the two treatments were given as indicated in the figure. Insert: The effect of 60 mM K⁺ on the release of MSH from intact neurointermediate lobes. Black bar indicates those fractions where sulpiride (10⁻⁶ M) was included in the superfusion medium. Results are expressed as percent basal release (±SEM, n=4); 100% basal release was defined in each experiment as the average amount of MSH in the three fractions preceding the first pulse of K⁺ (indicated by open circles).

FIG. 5. The effect of dopamine and GABA on the release of immunoreactive MSH from superfused melanotropes of *Xenopus laevis*. Results are expressed as percent basal release (±SEM, n=4). Open circles indicate those fractions used to determine 100% basal release. Superfusion conditions are described in Fig. 4.

FIG. 6. Effect of dopamine and the dopamine D₂-receptor antagonist sulpiride on the release of immunoreactive MSH from superfused melanotropes of *Xenopus laevis*. Superfusion conditions are described in Fig. 4.
results from more than one experiment, they are expressed relative to this basal value. Superfusion conditions were then expressed relative to their contribution to the corresponding tissue profile. The results indicate that while a small amount of intracellular acetylation is occurring, the non-acetylated peptide remains the major newly synthesized and immunoreactive tissue form of αMSH. In contrast, pulse-chase analysis with rat and mouse intermediate lobe tissue or cells show that desacetyl-αMSH is acetylated immediately after its biosynthesis [4, 5, 8, 11, 15]; not surprisingly, radioimmunoassay analysis of neurointermediate lobes of these species show that desacetyl-αMSH makes only a very minor contribution of acetylated forms of this peptide [2, 6, 11, 12, 22]. Goldman and Loh [7] have recently suggested that the major intracellular form of MSH in Xenopus makes such a comparison untenable.

As expected for a secretory process, the release of immunoreactive MSH from superfused melanotropes was found to be Ca**+-dependent. The finding that high K+ in the superfusion medium leads to a stimulation of the release of MSH was to be expected in that K+ depolarizes cell membranes. More interesting is the response of intact tissue to high K+, namely a complete inhibition of the secretion process. In that intact neurointermediate lobe tissue is rich in nerve terminals [3, 9, 20, 24, 25, 29], a good assumption is that high K+ induces secretion of a factor or factors from these terminals which subsequently inhibit MSH secretion from the melanotrope. Indeed, we have found that high K+ stimulates release of both dopamine and GABA from nerve terminals within the neurointermediate lobe tissue (unpublished observation). In view of the presence of at least these two inhibitory neurotransmitter systems within this tissue, it is not surprising that the dopamine receptor antagonist sulpiride failed to antagonize the K+ induced inhibition of MSH secretion. An important conclusion we can draw from the results with high K+ is that the isolated melanotropes appear to be free of any interfering nerve terminals. Therefore, in our subsequent studies with these cells we consider that any effect of secretagogues on release of MSH from the superfused melanotropes is due to a direct action of the secretagogues on the melanotropes themselves.

Dopamine is an important MSH-inhibiting factor in both mammals and sub-mammalian species [1]. For Xenopus laevis it has been shown that this neurotransmitter is extremely potent in inhibiting release of both MSH [10,14] and other POMC-related peptides [13,17]. All these studies have been conducted with intact neurointermediate lobe tissue. The present results establish that, in inducing inhibition of MSH release, dopamine acts directly on the melanotrope.
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The observation that sulpiride, a specific D2 receptor antagonist, could reverse the dopamine induced inhibition indicates that Xenopus melanotropes possess dopamine receptors of the D2 type.

There are recent indications that the neurotransmitter GABA may also be of general importance as an MSH secretagogue. A GABAergic fiber network has been found throughout the pars intermedia of both the rat [21,31] and toad Xenopus laevis [29] and GABA has been shown to affect secretion of MSH in these species [26,29]. The response of Xenopus neurointermediate lobes to exogenous GABA is a very rapid inhibition of secretion of immunoreactive MSH [29]. Subsequent receptor characterization has shown that the Xenopus neurointermediate lobe tissue contains both GABA and GABAB receptors. Activation of either of these receptors induces inhibition of in vitro MSH secretion [30]. It is apparent from the present results that the Xenopus laevis melanotrope cell itself possesses both the GABAAs and the GABAB receptor. Receptor localization at the ultrastructural level will have to resolve whether independent GABAergic neuronal systems exist to independently utilize these two receptor mechanisms.

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