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Regulation of Melanotropin Release from the Pars Intermedia of the Amphibian *Xenopus laevis*: Evaluation of the Involvement of Serotonergic, Cholinergic, or Adrenergic Receptor Mechanisms

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Melanophore-stimulating hormone (MSH) release from the pars intermedia of the pituitary gland is probably regulated by multiple factors of hypothalamic origin. We have examined a number of potential regulatory factors for their effects on MSH release from the amphibian *Xenopus laevis*. Serotonin and acetylcholine have no effect on MSH release. Both adrenaline and noradrenaline inhibit release of MSH in a dose-dependent manner. Studies with specific receptor agonists and antagonists reveal that these neurotransmitters exert their in vitro effects primarily through a dopamine D-2 receptor, although an α-adrenergic receptor could not be excluded. We further conclude that the pars intermedia of *X. laevis* lacks a β-adrenergic receptor for the regulation of MSH secretion from the pars intermedia. In mammals, this receptor activates the adenylate cyclase system. Our studies reveal that despite the lack of β-adrenergic receptors, cyclic-AMP is likely an intracellular factor involved in the stimulation of MSH release.

tremely potent in stimulating release of MSH from *Xenopus* neurointermediate lobes (Jenks, 1977). In addition to the \(\beta\)-adrenergic receptor, the presence of \(\alpha\)-adrenergic receptors on melanotropes of both an amphibian (Bower *et al.*, 1974) and a mammalian species (Jackson and Lowry, 1983) has been proposed. In both cases this receptor is reported to be involved in inhibition of MSH release. We have undertaken a study to evaluate the possible involvement of various neurotransmitters in the control of MSH release from the pars intermedia of *X. laevis*. This study was accomplished through the analysis of release of immuno-reactive melanotropin from superfused neurointermediate lobes. By using specific receptor agonists and antagonists, an attempt was made to characterize the receptor mechanisms involved. As the physiological state of the animal (i.e., white- or black-background-adapted) may have some influence on melanotrope sensitivity to secretagogues, we tested each potential secretagogue on neurointermediate lobes of both white- and black-background-adapted animals.

**MATERIALS AND METHODS**

*Animals.* *X. laevis* were bred and reared in our aquatic facility. Animals were adapted to black or white background in black or white containers under constant illumination at 22°. Degree of pigment dispersion in dermal melanophores was determined by measurement of the melanophore index according to Hogben and Slome (1931).

*In vitro superfusion experiments.* Details concerning our method of superfusion of neurointermediate lobes are given elsewhere (Verburg-van Kemenade *et al.*, 1986). Briefly, superfusion was performed using small perifusion chambers (10 \(\mu\)l), each containing an individual lobe. The incubation medium contained 112 mM NaCl, 2 mM KCl, 2 mM CaCl\(_2\), 15 mM Heps, pH 7.38, 0.3 mg/ml BSA, 2 mg/ml glucose. Medium was pumped through each chamber (1.5 ml/hr) and fractions of 7.5 min were collected. Fractions were stored frozen (\(-20^\circ\)) before submitting them to radioimmunoassay for MSH determinations. As described earlier (Verburg-van Kemenade *et al.*, 1986), within an experiment the results of those lobes showing similar baseline patterns of release were grouped, percentage basal release was calculated for each lobe within the group, and these values were then averaged. Neurotransmitters, or their specific agonists or antagonists, were administered at various time periods during the superfusion. The following products were tested either individually or in various combinations: the neurotransmitters serotonin, acetylcholine, adrenaline, and noradrenaline; the \(\alpha\)-adrenergic receptor agonist phenylephrine and its antagonist phentolamine; the \(\beta\)-adrenergic receptor agonist isoproterenol and its antagonist propranolol; and the dopamine D-2 receptor antagonist sulpiride (all purchased from Sigma). Several experiments were conducted using the cyclic-AMP analog 8-bromo-adenosine 3',5'-monophosphate (8-Br-cAMP, Sigma).

**Radioimmunoassay.** The MSH concentration in the effluent was quantified by radioimmunoassay, using an antiserum produced and characterized by Vaudry *et al.* (1978). It shows equal cross-reactivity to desacetyl-\(\alpha\)-MSH and \(\alpha\)-MSH. Immunobound and unbound radioactive MSH were separated by precipitation with polyethylene glycol. Synthetic \(\alpha\)-MSH (Sigma) was used as a standard. All samples were measured in duplicate.

**RESULTS**

**Effects of serotonin and acetylcholine.** Serotonin in concentrations ranging from \(10^{-9}\) to \(10^{-5}\) \(M\) was unable to alter basal MSH secretion in lobes from black- or white-adapted animals. Figure 1b shows the results from superfusion experiments whereby \(10^{-8}\), \(10^{-6}\), and \(10^{-5}\) \(M\) of serotonin were tested. Similarly, basal secretion was unaffected by acetylcholine in concentrations ranging from \(10^{-9}\) to \(10^{-5}\) \(M\) (Fig. 1a).

**Involvement of \(\alpha\)-adrenergic and \(\beta\)-adrenergic receptor mechanisms.** The two neurotransmitters adrenaline and noradrenaline exhibited a dose-dependent inhibitory effect on secretion of immunoassayable \(\alpha\)-MSH (Fig. 2a and Table 1). Effective concentrations ranged from \(10^{-8}\) to \(10^{-5}\) \(M\) (Table 1). The lobes were slightly more sensitive to adrenaline than to noradrenaline. The inhibition of MSH secretion induced by adrenaline or by noradrenaline, both applied at a concentration of \(10^{-6}\) \(M\), was not affected by the \(\alpha\)-adrenergic receptor antagonist phenolamine (Figs. 2c
RECEPTOR MECHANISMS IN *Xenopus* MELANOTROPES

**Fig. 1.** Effects of acetylcholine and serotonin on MSH secretion from superfused neurointermediate lobes of black-background-adapted *Xenopus laevis*. In each experiment 7.5-min superfusion fractions were collected; secretagogues were given during three 15-min periods at the concentrations indicated. (a) Acetylcholine results show the mean of four individual experiments where 100% basal release for each lobe was 716, 880, 864, and 652 pg MSH/fraction. (b) Serotonin results show the mean of two individual experiments where 100% basal release for each lobe was 548 and 1160 pg MSH/fraction. Bars represent ±SEM.

and d) nor by the β-adrenergic receptor antagonist propranolol (Fig. 2b). Both antagonists were tested in concentrations of up to $10^{-4}$ M. When adrenaline was, however, used in concentrations of $10^{-7}$ M, we observed a partial blockage of the inhibition by phentolamine (Table 2). The inhibitory effect on secretion induced by $10^{-6}$ M adrenaline or $10^{-6}$ M noradrenaline could be blocked by the dopamine D-2 receptor antagonist sulpiride (Figs. 2e and f). The α-adrenergic agonist phenylephrine inhibited secretion when used at high concentrations of $10^{-5}$ M (Table 1). The inhibition of MSH secretion induced by $2 \times 10^{-5}$ M of phenylephrine was to a great extent antagonized by the administration of $10^{-4}$ M of phentolamine (Fig. 3).

In neurointermediate lobes of black-adapted animals, the β-adrenergic receptor agonist isoproterenol appeared to be unable to alter the basal level of MSH secretion when added in low concentrations of $10^{-10} - 10^{-7}$ M. At high concentrations ($10^{-5}$ M), the effect of isoproterenol on MSH secretion is clearly inhibitory (Fig. 4a). This inhibitory effect on melanotropin secretion could not be blocked with propranolol (Fig. 4c). Both sulpiride and phentolamine were, however, able to block the isoproterenol-induced inhibition (Fig. 4d and Table 2). An experiment with isoproterenol, but with lobes from white-adapted animals, did not show any stimulatory effect for this substance (Fig. 4b). Moreover, in an experiment with lobes from black-adapted animals that were maintained continuously under submaximum inhibition by dopamine ($10^{-6}$ M), isoproterenol again failed to induce stimulation of release. Also, we could not demonstrate any stimulation with isoproterenol in experiments that were conducted with the incubation medium at pH 6.8. Hadley et al. (1976) have reported that in *Rana*, lowering the pH of the incubation medium reduced basal levels of melanotropin secretion, thus increasing sensitivity in detecting stimulatory effects on secretion. In *Xenopus* we found no reduction in basal secretion after lowering the pH from 7.38 to 6.80.

**Effects of cyclic-AMP.** Lobes originating from animals adapted to a black background appeared to be insensitive to 8-Br-cyclic-AMP (Fig. 5a). Additional experiments, where 8-Br-cyclic-AMP was given for 50-min periods, also showed no effect of this substance on MSH release. In comparison to lobes from black-adapted animals, lobes from white-adapted animals showed considerably more variation in their baseline pattern of MSH release, which generally tended to decline much more rapidly. Figures 5b, c, and d give re-
FIG. 2. Effects of noradrenaline, adrenaline, and receptor antagonists (propranolol, β-adrenergic receptor antagonist; phentolamine, α-adrenergic receptor antagonist; sulpiride, dopamine D-2 receptor antagonist) on MSH secretion from superfused neurointermediate lobes of black-background-adapted *Xenopus laevis*. In each experiment noradrenaline or adrenaline was given during three 15-min periods; administration of the receptor antagonist was started several fractions before the second pulse of noradrenaline or adrenaline and continued for several fractions after this pulse, as indicated in the figure. All graphs represent the mean of two to four experiments and the bars represent ± SEM. Basal levels of release for the individual lobes within each experiment were (a) noradrenaline, 1344 and 3060 pg MSH/fraction; (b) noradrenaline/propranolol, 770 and 1888 pg MSH/fraction; (c) noradrenaline/phentolamine, 1464, 1756, 2120, and 2948 pg MSH/fraction; (d) adrenaline/phentolamine, 1000, 1512, and 756 pg MSH/fraction; (e) noradrenaline/sulpiride, 1552, 2360, 2938, and 1640 pg MSH/fraction; and (f) adrenaline/sulpiride, 276 and 1560 pg MSH/fraction.

**DISCUSSION**

Previous studies have shown that the physiological state of the neurointermediate lobe tissue remains viable during a 6-h superfusion period (Martens et al., 1981; Verburg-van Kemenade et al., 1986). Following *in vitro* superfusion we have found...
TABLE 1

Dose–Response Relationship for the Inhibition of MSH Secretion from Superfused Neurointermediate Lobes of *Xenopus laevis* by Adrenaline and Noradrenaline and by the Adrenergic Receptor Agonists Isoproterenol (β-Receptor) and Phenylephrine (α-Receptor)

<table>
<thead>
<tr>
<th>Secretagoge</th>
<th>10⁻⁹ M</th>
<th>10⁻⁸ M</th>
<th>10⁻⁷ M</th>
<th>10⁻⁶ M</th>
<th>10⁻⁵ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noradrenaline</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>32 ± 5.8 (5)</td>
<td>83 ± 3.3 (20)</td>
<td>100 ± 0.0 (2)</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>68 ± 5.5 (11)</td>
<td>86 ± 2.6 (19)</td>
<td>100 ± 1.0 (5)</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>0 (3)</td>
<td>28 ± 1.4 (2)</td>
<td>66 ± 4.4 (23)</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>0 (2)</td>
<td>68 ± 3.5 (2)</td>
</tr>
</tbody>
</table>

Note. Secretagogues were added during 15-min periods to superfused lobes; 7.5-min fractions were collected and the amount of MSH in each fraction determined with a radioimmunoassay. Average value of MSH in the three fractions before addition of secretagogue was defined as basal level and the decrease in MSH value observed for the second 7.5-min fraction of secretagogue addition was expressed as a percentage of the basal value. Percentage inhibition is given ± SEM and the numbers in parentheses indicate the number of independent determinations for each concentration of secretagogue tested.

that biosynthesis and release of pro-opiomelanocortin-related peptides are essentially normal, and that the ultrastructure of the tissue compared favorably to freshly dissected tissue (unpublished results).

Biochemical (Loh and Gainer, 1977; Martens et al., 1982) and morphological studies (Jenks et al., 1977; Weatherhead and Whur, 1972) have shown that the cells of the pars intermedia of black-background-adapted toads are extremely active in biosynthesis and release of melanotropins. In contrast, white-background-adapted animals have low biosynthetic rates in these cells (Jenks et al., 1977) and, in vivo, release of melanotropins is under tonic inhibition. During in vitro superfusion, neurointermediate lobes from black-background-adapted animals gave relatively stable baselines of MSH release, while lobes from white-background-adapted animals tended to give declining

TABLE 2

Summary of the Effects of Specific Receptor Antagonists on Secretagogue-Induced Inhibition of MSH Release from Superfused Neurointermediate Lobes of *Xenopus laevis*

<table>
<thead>
<tr>
<th>Secretagogues</th>
<th>Propranolol (β-receptor)</th>
<th>Phentolamine (α-receptor)</th>
<th>Sulpiride (D-2 receptor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrenaline</td>
<td>n.d.</td>
<td>±</td>
<td>n.d.</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>—</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>—</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>n.d.</td>
<td>+</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Note. Each secretagogue was administered during a 15-min period to superfused lobes and the percentage inhibition of MSH secretion determined as described in Table 1. Administration of receptor antagonists (receptor specificity indicated in parentheses) was started several fractions before a second 15-min pulse of secretagogue and continued for several fractions after this pulse. Inhibition of release by the secretagogue during this second pulse was compared to that obtained during the first pulse. Symbols: (+) complete or almost complete antagonism of inhibitory effect of secretagogue; (±) partial antagonism of inhibitory effect of secretagogue; (−) no detectable antagonism of secretagogue; n.d., not determined.
VERBURG-VAN KEMENADE, JENKS, AND VAN OVERBEEKE

% basal level (MSH)

phenylephrine $10^{-5} M$

phenylephrine $10^{-5} M$

0 5 10 15 20 25 30 35

fraction no.

FIG. 3. Effect of $\alpha$-adrenergic receptor agonist phenylephrine and $\alpha$-adrenergic receptor antagonist phentolamine on MSH secretion from superfused neurointermediate lobes of black-background-adapted Xenopus laevis. Phenylephrine ($2 \times 10^{-5} M$) was added during two 15-min periods; phentolamine administration was started several fractions before the second pulse of phenylephrine and continued for several fractions after this pulse, as indicated in the figure. Figure represents the mean of four experiments. Bars represent $\pm$ SEM and basal levels of release from the individual lobes were 1816, 3024, 2648, and 2008 pg MSH/fraction.

baselines. In our opinion this latter observation is indicative of hormone depletion and reflects the low biosynthetic capacity of the melanotropes of white-adapted animals. In lobes of black-adapted animals the high level of hormone production can apparently maintain a high rate of in vitro release.

The results of our superfusion experiments clearly show that the indolamine serotonin has no effect on the rate of MSH release from the pars intermedia of either white- or black-background adapted Xenopus. Immunocytochemically, we have found that in X. laevis, serotonergic fibers are restricted to the neural lobe, and no evidence for the presence of serotonin in the pars intermedia was found (unpublished results). Thus it would seem unlikely that serotonin plays a direct role in the regulation of peptide release from the pars intermedia of Xenopus. In this respect, there could be species differences in the regulation of melanotropin release. Thornton and Geschwind (1975) found a dose-dependent potent stimulation of MSH release in the lizard Anolis carolinensis. Ueda et al. (1984) and Kondo et al. (1983) demonstrated the presence of serotonergic fibers in the pars intermedia of Rana catesbeiana. That serotonergic innervation of the pars intermedia might be restricted to lower vertebrate species is suggested by the studies of Kondo and coworkers, who showed that the pars intermedia of the rat, hamster, and dog were serotonin negative.

Electron microscopic studies have indicated the existence of cholinergic axon vesicles in the pars intermedia of Rana (Nakai and Gorbman, 1969) and the neurotransmitter acetylcholine has been shown to stimulate MSH secretion (Davis and Hadley, 1978). Hopkins (1971), in characterizing neurons of the pars intermedia of X. laevis, concluded that cholinergic innervation is absent from the intermediate lobe of this species. The results of our superfusion studies, showing that acetylcholine has no influence on MSH secretion, are in agreement with Hopkins’ conclusion that acetylcholine has no role, at the level of the Xenopus pars intermedia, in regulating melanotropin release.

The observation that both adrenaline and noradrenaline were very effective in inducing inhibition of MSH release in a dose-dependent manner suggests the involvement of an $\alpha$-adrenergic receptor mechanism. This suggestion is supported by the fact that the $\alpha$-adrenergic receptor agonist phenylephrine inhibited hormone release, a response that could be antagonized by phentolamine. However, high concentrations of phenylephrine were needed and in antagonizing the adrenaline- and noradrenaline-induced inhibition of secretion, the dopamine D-2 receptor antagonist sulpiride proved to be much more potent than the $\alpha$-adrenergic receptor antagonist phentolamine. This indicates that, in vitro at least, adrenaline and noradrenaline inhibit MSH release primarily through a dopamine re-
RECEPTOR MECHANISMS IN Xenopus MELANOTROPES

Fig. 4. Effect of β-adrenergic receptor antagonist isoproterenol on MSH secretion from superfused neurointermediate lobes of black-background-adapted Xenopus laevis. (a) Effect on secretion from lobes of black-background-adapted animals; (b) effect on secretion from lobes of white-background-adapted animals; (c) and (d) effects of β-adrenergic receptor antagonist propranolol and dopamine D-2 receptor antagonist sulpiride on isoproterenol-induced inhibition of MSH release. The concentrations of isoproterenol and receptor antagonists used are indicated in the figures. Administration of the receptor antagonist was started several fractions before the second pulse of isoproterenol and continued for several fractions after this pulse. All graphs represent the mean of at least two individual experiments and bars represent SEM. Basal levels of release for individual lobes within each experiment were: isoproterenol black, 2608 and 2312 pg MSH/fraction; isoproterenol white, 1424 and 948 pg MSH/fraction; isoproterenol/propranolol, 2408 and 872 pg MSH/fraction; isoproterenol/sulpiride, 1600, 1128, 1868, and 1336 pg MSH/fraction.

ceptor mechanism, similar to the situation reported for the rat pars intermedia (Munemura et al., 1980). In in vitro experiments the tissue is, of necessity, flooded with potential secretagogue. In this situation adrenaline or noradrenaline can inhibit MSH release through activation of either abundantly available dopamine-type receptors (a manuscript concerning their characteristics is in preparation), or less abundant α-adrenergic receptors. If in vivo, however, the neurotransmitter was to be delivered to the tissue in highly directed synapses between adrenergic nerve terminals and the melanotropes; then it cannot be excluded that an α-adrenergic receptor mechanism plays a role in regulating the pars intermedia function.

With respect to possible β-adrenergic receptor mechanisms, we have shown that for Xenopus, low concentrations of the β-adrenergic receptor agonist isoproterenol have no effect on MSH release and that high concentrations actually inhibit release. The fact that this inhibition could not be blocked by the β-receptor antagonist propranolol, but was blocked by sulpiride or phentolamine, indicates that it does not involve activation of a β-adrenergic receptor, and we therefore conclude that Xenopus
melanotropes are not regulated through a β-adrenergic mechanism. In contrast, it has been demonstrated for both *R. pipiens* (Bower *et al.*, 1974) and *R. ridibunda* (Tonon *et al.*, 1983) that a β-receptor mechanism stimulates release of MSH from the pars intermedia. It is interesting to note that with respect to the β-adrenergic receptor, a similar discrepancy between the frog and *Xenopus* has been reported for the receptor profile of their erythrocytes. Frog erythrocytes contain a high number of β-adrenergic receptors, whereas the erythrocytes of *Xenopus* lack this type of receptor (Cerione *et al.*, 1983).

β-adrenergic receptors generally activate the adenylate cyclase system (Cote *et al.*, 1980; Munemura *et al.*, 1980). The lack of this receptor on MSH cells of *X. laevis* raises questions concerning the involvement of cyclic-AMP as an intracellular factor for stimulation of hormone release. In both our present experiments and those reported earlier (Jenks *et al.*, 1977), we found a clear stimulation of MSH release, in contrast to Loh *et al.* (1981) who conclude from their results that cyclic-AMP inhibits release of MSH. In the present investigation the superfusion system has allowed us to follow the time course of cyclic-AMP-induced stimulation. These studies have revealed that a pulse of 8-Br-cyclic-AMP is so effective in stimulating the secretory process, that in many cases the lobes became almost depleted of MSH and thus could no longer maintain release.

**FIG. 5.** Effects of 8-bromo-adenosine 3',5'-cyclic monophosphate (cAMP) on MSH secretion from superfused neurointermediate lobes obtained from black- and white-background-adapted *Xenopus laevis*. The concentrations and times of cAMP administration are indicated in the figure. All graphs represent the means of at least two experiments and bars represent -SEM. Basal levels of release for the individual lobes within each experiment were (a) cAMP black, 264 and 280 pg MSH/fraction; (b) cAMP white, 300 and 972 pg MSH/fraction; (c) cAMP white, 356 and 388 pg MSH/fraction; (d) cAMP white, 1104, 1864, and 1760 pg MSH/fraction.
and were hardly sensitive to subsequent pulses of cyclic-AMP. In their studies, Loh et al. (1981) preincubated lobes of white-background-adapted animals for 30 min prior to the start of the release experiment, which proceeded for an additional 3 hr of in vitro incubation. In the case of the cyclic-AMP treated group, cyclic-AMP was present during both the preincubation and experimental periods. Possibly the pretreatment with cyclic-AMP depleted the lobes of MSH.

Our results with 8-Br-cAMP illustrate the importance of considering responses of lobes from both white-adapted and black-adapted animals. The reason for the failure of lobes from black-adapted animals to respond, or to respond very poorly, to cyclic-AMP treatment remains to be clarified. Possibly the endogenous cyclic-AMP levels in the cells of the pars intermedia of black-adapted animals were already elevated, reflecting the physiological state of the animals prior to conducting the experiments. In that Xenopus lacks a $\beta$-adrenergic receptor, still to be resolved are questions concerning which receptor systems might be involved in activation of the adenylate cyclase system in the melanotropes of this species. In recent experiments we have found that both the neuropeptides thyrotropin-releasing hormone and corticotropin-releasing hormone show a similar phenomenon as that displayed by cyclic-AMP, namely, that they stimulate release from lobes of white-adapted but not black-adapted animals (manuscript in preparation). We are investigating whether one of these neuropeptides could be an MSH release stimulating factor in X. laevis.

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REFERENCES


