Assessment of TRH as a Potential MSH Release Stimulating Factor in *Xenopus laevis*

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Received 8 September 1986

VERBURG-VAN KEMENADE, B. M. L., B. G. JENKS, T. J. VISSER, M. C. TONON AND H. VAUDRY. Assessment of TRH as a potential MSH release stimulating factor in *Xenopus laevis*. PEPTIDES 8(1) 69-76, 1987.—This study considers the possible involvement of the tripeptide TRH (thyrotropin releasing hormone) in the physiological regulation of melanophore stimulating hormone (MSH) secretion from the pars intermedia of the toad, *Xenopus laevis*. TRH was shown to stimulate release of MSH from superfused neurointermediate lobes obtained from white-background adapted animals, but had no effect on secretion from lobes of black-background adapted animals. Immunohistochemical analysis revealed a rich TRH-containing neuronal network terminating in the neural lobe of the *Xenopus* pituitary. Plasma levels of TRH, determined with a specific radioimmunoassay, proved to be extremely high and no significant difference in this level could be found between white- and black-adapted animals. Plasma TRH probably originates from the skin, and our results show that its concentration is within the effective concentration range established for this peptide in stimulating MSH release from the pars intermedia. Therefore, while both our superfusion and immunohistochemical results argue favourably for a function of TRH in the regulation of MSH secretion, we conclude that, in any regulatory role, it would likely have to function within the pars intermedia at concentrations exceeding the high plasma values. While TRH could be involved in short-term activation of the secretory process in white-background adapted animals or in animals undergoing the initial stages of black background adaptation, our results indicate that this peptide may have no function in the maintenance of secretion from the pars intermedia of animals fully adapted to black background.

Pars intermedia MSH TRH Regulation of secretion

The pars intermedia of the pituitary gland synthesizes a precursor protein, proopiomelanocortin (POMC), from which melanophore stimulating hormone (MSH) and other peptides are derived (for reviews see [9,12]). In amphibians, MSH is released from the intermediate lobe of animals placed on a black background. This hormone stimulates the dispersion of pigment in dermal melanophores and consequently the skin displays a dark color. An important component in hypothalamic regulation of MSH secretion is an inhibitory mechanism, achieved by classical neurotransmitters such as dopamine [8, 9, 20, 24, 37, 39] and in the species *Xenopus laevis* at least, GABA [44]. Electronmicroscopical studies have revealed that the amphibian neurointermediate lobe is innervated by both classical neurons, and neurons of peptidergic nature [14,28]. In the frog, *Rana ridibunda*, the tripeptide pGlu-His-Pro(NH₂), which is the mammalian thyrotropin releasing hormone (TRH), has been shown to be very potent in stimulating MSH release from the pars intermedia [38]. In two species of *Rana* (*Rana catesbeiana* and *Rana ridibunda*), immunohistochemical studies indicate the presence of a TRH-containing neuronal network within the pars intermedia [27,32]. Taken together, the above studies indicate that TRH of hypothalamic origin could be a physiological factor involved in the stimulation of MSH secretion. Amphibians are known, however, to have high plasma levels of TRH, which probably originate from the skin [16]. Plasma TRH could conceivably also be involved in the regulation of MSH secretion. Therefore plasma values of TRH should be considered in evaluation of a possible physiological function for this neuropeptide in amphibians. The purpose of the present study was to examine if this peptide could represent a physiological MSH-releasing factor in the toad *Xenopus laevis*. For this purpose we have: (1) determined the effect of TRH on MSH release from superfused neurointermediate lobes, (2) conducted immunocytochemical analysis and radioimmunoassays (RIA) to determine if this tripeptide is...
FIG. 1. TRH immunocytochemistry of the pituitary gland of *Xenopus laevis*. (a) Anti-TRH staining in pituitary gland of a white-background adapted animal shown at 135× magnification and (b) shown at 430× magnification. The anti-TRH staining in the pituitary gland of a black-background adapted animal is shown in (c) at 190× magnification and (d) represents the immunoabsorbed control. pn, pars nervosa; pi, pars intermedia; pd, pars distalis; me, median eminence; po, preoptic nucleus.

TABLE 1

TRH VALUES IN NEUROINTERMEDIATE LOBE, PARS DISTALIS AND PLASMA OF BOTH BLACK- AND WHITE-BACKGROUND ADAPTED *Xenopus laevis*

<table>
<thead>
<tr>
<th></th>
<th>Black</th>
<th>White</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurointermediate lobe</td>
<td>ng/lobe 0.871 ± 0.063 (n= 7)</td>
<td>ng/lobe 0.800 ± 0.063 (n= 7)</td>
</tr>
<tr>
<td>Pars distalis</td>
<td>ng/lobe 0.104 ± 0.013 (n=41)</td>
<td>ng/lobe 0.041 ± 0.036 (n=16)</td>
</tr>
<tr>
<td>Plasma</td>
<td>ng/ml 42 ± 4.6 (n=47)</td>
<td>ng/ml 36 ± 8.2 (n=13)</td>
</tr>
</tbody>
</table>
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from white- and black-background adapted plasma extract were submitted to HPLC and analyzed by radioimmunoassay. Arrow indicates the elution position of synthetic pGlu-His-Pro-NH$_2$.

A nim a Is present in the neurointermediate lobe, and (3) analyzed plasma levels of this peptide in relation to the color of background to which the animals are adapted.

METHOD

Animals

Xenopus laevis were bred and reared in the aquatic facility of the Department of Zoology, Catholic University, Nijmegen, The Netherlands. Animals were adapted to a black or white background for four weeks under a constant illumination at 22°C. Degree of pigment dispersion was determined according to the melanophore index scale of Hogben and Slome [13].

Immunocytochemistry With Anti-TRH

Brain and pituitary tissue was fixed in Bouin-Holland, paraplast embedded and 5 µm sections were submitted to immunostaining with the peroxidase-anti-peroxidase method according to Sternberger [35] with 4-Cl-naphtol as oxygen acceptor. The antiserum 1:100 or 1:200 No. 4619 was characterized earlier [46,47]. Control experiments were performed after adsorption of the antiserum with TRH coupled to activated CNBr sepharose 4B [42].

TRH Extraction of Plasma and Pituitary Tissue

Plasma samples. To 1 volume of trunk blood, 4 volumes of cold 100% ethanol were immediately added. After vigorous shaking it was left overnight at 4°C, the supernatant was dried under nitrogen and redissolved in buffer in preparation for the radioimmunoassay (RIA) for TRH. The TRH immunoreactive material found in the plasma was analyzed by reverse phase HPLC using a 100×3 mm CP-Spher C$_{18}$ column (Chrompack, Middelburg, The Netherlands). Elution was performed with a linear gradient of 0–20% acetonitrile in 0.01 M sodium phosphate (pH 7.7). Solvent flow was 0.6 ml/min and 1 min fractions were collected. TRH content was determined in duplicate in 100 µl samples of each fraction.

Pituitary samples. The freshly dissected pituitary gland was separated into neurointermediate lobe and distal lobe. Each lobe was homogenized in 500 µl of cold 90% methanol in a glass potter, centrifuged for 2 min at 10,000 g, and the supernatant was dried under nitrogen. The dried extract was dissolved in buffer for RIA determination of TRH.

In vitro Superfusion of Neurointermediate Lobe Tissue

Superfusion was performed in a system containing four independent superfusion chambers (volume 10 µl each). Each neurointermediate lobe was placed in a chamber and incubation medium (112 mM NaCl, 2 mM KCl, 2 mM CaCl$_2$, 15 mM Hepes pH 7.38, 0.3 mg/ml BSA, 2 mg/ml glucose and carbogen aerated) was pumped through the chambers at a rate of 1.5 ml/h at 22°C. Fractions of 7.5 min were collected and stored at ~20°C until submission to radioimmunoassay for MSH. Experiments were conducted in two-, three- or four-fold. The results are expressed as a percentage of basal secretion; 100% basal secretion being defined as the average MSH value measured in several superfusion fractions before secretagogue addition. The fractions used in these calculations are given in the captions to the figures. Results of individual experiments were averaged and SEM values were calculated.

Radioimmunoassays

RIA for TRH. TRH was measured using antiserum produced against a TRH-dinitrophenylene-protein immuno- gen. Specificity characteristics have previously been described [46,47]. The antiserum was used at a final dilution of 1:20,000 and the assay was sensitive to 1 pg per tube. The RIA does not measure the major TRH-metabolite pGlu-His-Pro-OH nor the dipeptide fragment His-Pro-diketopiperazine. Precipitation of antibody-bound radioactivity was performed with second antibody in the presence of 2.5% polyethylene glycol. TRH was measured in duplicate.

RIA for αMSH. αMSH was determined with an antiserum produced and characterized by Vaudry et al. [43]. The antiserum was used at a final dilution of 1:40,000. It has equal sensitivity towards des-β-acetyl-αα-MSH and αα-MSH. Bound and unbound antiserum was separated with polyethylene glycol (final concentration: 7.5%), or with a second antibody. Each sample was measured in duplicate.

RESULTS

Immunocytochemistry With Anti-TRH

Brain and pituitary tissue of 6 black-background adapted animals and of 6 white-background adapted animals were submitted to immunocytochemical analysis with anti-TRH. This revealed positively reacting cells in the hypothalamus and a neuronal tract which terminated in the median eminence and neural lobe area (Fig. 1a). This positive reaction was visible in tissue from both black- and white-adapted animals (Fig. 1b,c). No immunoreaction was observed in the pars intermedia of black-background adapted animals (Fig. 1c). However, occasionally the pars intermedia of white-
adapted animals showed positive reaction in the intermediate lobe. An example of this is shown in Fig. 1a,b. This immunostaining was more diffuse than that observed in the neural lobe. No immunostaining was visible in the pars distalis of either black- or white-adapted animals.

Radioimmunoassays for TRH

The results of assays on plasma samples show a relatively high variation in TRH concentration in the blood of Xenopus (Table 1). With HPLC analysis, the TRH immunoreactive material in the plasma coeluted with synthetic TRH (Fig. 2). Plasma samples from black-background adapted animals contained a somewhat higher level of TRH than those of white-adapted animals, but these differences were not statistically significant. TRH levels in the pars distalis were very low, showing a higher content in lobes from black-background adapted animals. Neurointermediate lobes of both black- and white-adapted Xenopus laevis contained high levels of TRH. The levels found in black-adapted animals were slightly but not significantly higher than those measured for the lobes obtained from white-adapted animals.

Effect of TRH on In Vitro Secretion of MSH

The effects of 15-min pulses of TRH on MSH secretion from neurointermediate lobes of both black- and white-adapted animals were determined (Fig. 3). TRH appeared to be very effective in stimulating MSH release from lobes extripated from white-adapted animals. Table 2 gives the dose-response relationship for TRH stimulation of secretion. Due to the phenomenon of declining baselines this relationship was determined with data from the first pulse of TRH and averaged from a number of superfusion experiments. The stimulatory response to TRH, displayed by lobes of white-adapted animals, was of rather short duration (Fig. 3). Occasionally the stimulatory effect was already declining during the 15 minutes of TRH administration.

Lobes from black-background adapted animals were insenstive to the neuropeptide (Fig. 3). To determine whether the unresponsiveness of lobes of black-background adapted animals could be attributed to the fact that in vitro MSH secretion of these lobes was already at maximum level, secretion was partially inhibited by the addition of dopamine (10^{-5} M) prior to and during TRH administration. These lobes did not respond to TRH (Fig. 4).

One experiment was performed whereby the TRH-induced secretory response of neurointermediate lobe tissue of Rana ridibunda was tested to compare this response to that registered for Xenopus. The frogs were taken from a neutral grey background. Neurointermediate lobes of Rana could be stimulated by administration of TRH (Fig. 5). Already at 10^{-5} M of TRH, 140% of the basal level of MSH secretion was observed. With increasing concentrations of TRH, this level increased to a maximum of 160% of basal secretion. Similar to Xenopus tissue, a rapid secretory re-
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TRH, A POTENTIAL MSH RELEASE STIMULATING FACTOR 73

FIG. 4. Effect of TRH on secretion of MSH during in vitro superfusion of neurointermediate lobes of black-background adapted Xenopus laevis under basal and dopamine inhibited conditions. Fifteen-minute pulses of $10^{-6}$ M TRH were given. Figure represents the average of three individual lobes. The 100% values of basal secretion were defined from the average MSH-value in fractions 16-19; these were 462, 549, 975 pg MSH/7.5 min fraction. Bars indicate ± SEM.

FIG. 5. Effect of TRH on basal secretion of MSH from neurointermediate lobes of Rana ridibunda, during in vitro superfusion. Fifteen-min pulses of $10^{-8}$, $10^{-7}$, $10^{-6}$ and $10^{-5}$ M were given as indicated in the figure. Figure represents the average of two individual experiments. One hundred percent values of basal secretion were calculated from the average MSH-value in fractions 8-10; these were 180 and 116 pg MSH/7.5 min fraction.

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The tripeptide pGlu-His-Pro-NH$_2$ received the name thyrotropin releasing hormone, TRH, for its stimulatory effect on secretion of thyrotropin from the pituitary gland of mammals. Indeed, in mammals it is probably the most important hypothalamic factor for regulation of thyrotropin release. In rat, TRH could not affect MSH secretion [21]. In cold-blooded vertebrates, TRH has no effect on the pituitary-thyroid axis [11, 36, 41]. It has, however, been shown to stimulate in vitro release of MSH from lobes of white-adapted Xenopus (Table 2). Like TRH, MK771 caused a rapid increase in MSH release which was of short duration.

**DISCUSSION**

The tripeptide pGlu-His-Pro-NH$_2$, also known as TRH, is a potential MSH release stimulating factor in this species. A unique characteristic of the TRH-induced release of MSH secretion from the neurointermediate lobe of Xenopus is the observation that this tripeptide is effective in stimulating release from lobes of white-background but not black-background adapted animals. The simplest explanation for this phenomenon would be that melanotropes of black-background adapted animals are already releasing MSH at the maximum obtainable rate. We have found, however, that such lobes can be induced to higher levels of secretion through the use of a calcium inophore (unpublished observations), indicating that the release from these lobes is not at its maximum level. Moreover, the observation that TRH failed to stimulate secretion from dopamine-inhibited lobes of black-background adapted animals would also argue against this simple explanation. TRH receptors have been reported to undergo a very rapid ligand-induced desensitization [4, 29, 33]. Therefore, another possibility worth considering is that the TRH receptors in neurointermediate lobes of black-background adapted Xenopus are in a desensitized state.

The response of Xenopus neurointermediate lobe tissue to TRH was very short duration. Indeed, MSH release was often returning to basal level before TRH administration had even been terminated, a phenomenon not observed in earlier studies with Rana [38]. In view of this apparent difference in response between lobes of the two amphibian species, we decided to test lobes of Rana ridibunda under identical superfusion conditions as those for Xenopus. This study confirmed that the response of Rana lobes was indeed of much longer duration. In mammalian studies, TRH stimulation of thyrotropin release [7,31] and prolactin release [2,26] have been described as biphasic, consisting of a very rapid initial phase of hormone secretion, followed by a plateau of elevated secretion. It has been suggested that different intracellular mechanisms are responsible for the two phases of TRH-induced stimulation [2,26,34]. Interestingly, studies with rat thyrotropes [2] and GH3 tumor cells [34] indicate that the second phase of the response to TRH is dependent

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on protein synthesis. The melanotropes of white-background adapted *Xenopus* are known to have an extremely low level of biosynthetic activity [19, 24, 48]. This low biosynthetic capacity is probably responsible for the declining baseline of biosynthesis of the peptide. This point has been discussed in detail elsewhere [45]. Possibly, the observed absence of a sustained biphasic response to TRH by *Xenopus* lobes reflects their low biosynthetic capacity.

The results of the immunocytochemical analysis of TRH distribution in the hypothalmo-hypophyseal complex of *Xenopus* give an indication for a morphological basis for TRH regulation of the intermediate lobe. As the majority of the TRH positive neurons innervating the pituitary gland seems to terminate in varicosities of the neural lobe, TRH would presumably reach the melanotropes of the pars intermedia through extracellular diffusion from the pars nervosa, or alternatively, via the blood. Since the intermediate lobe is closely associated with the neural lobe tissue and, moreover, it receives its blood supply from this latter tissue, both mechanisms seem possible. In the pituitary gland of the frog *Rana catesbeiana*, Seki et al. [32] have found a similar distribution of TRH-immunoreactivity to that we now report for *Xenopus*. In addition, they describe a very scarce granular network of positive staining in the pars intermedia which demonstrates that at least some TRH-positive fibers are reaching the intermediate lobe. Occasionally, we observed a positive immunoreaction in the pars intermedia of *Xenopus laevis* but, from its very diffuse nature, we feel that it is unlikely that this staining represents nerve terminals. It could well be similar to the intracellular location of TRH that has been reported in the frog [27]. It is worth mentioning that the immunostaining of the pars intermedia, when observed, is not reflected in tissue levels of the peptide. Thus these results of our superfusion experiments suggest that only the former lobes contain functional TRH-receptors.

The difference in neurointermediate lobe sensitivity to TRH between white- and black-background adapted animals and the differences in the immunocytochemical staining characteristics of their neurointermediate lobe tissue, raise the important question: is there a difference in secretory activity of TRH-neurons innervating lobes of white- and black-background adapted *Xenopus*? In the expectation that a high rate of secretion might lead to an altered tissue content of TRH, we decided to quantify tissue levels of the peptide. These results, showing no significant difference in neurointermediate lobe levels of TRH between white- and black-background adapted *Xenopus*, indicate that if differences in secretory activity exist in response to background color changes, this is not reflected in tissue levels of the peptide. Thus these data argue neither for, nor against, a physiological role for the TRH-neurons in the process of background adaptation.

Of critical importance in our evaluation of TRH as a potential regulatory factor of pars intermedia function, is the fact that amphibians are known to have high levels of this peptide in their skin and plasma [6,30]. The poison glands of the skin of *Xenopus laevis* have been shown to synthesize TRH [30]. In this species, the extremely high level of TRH in the skin, in combination with the observation that brain tissue contains a very low amount of this peptide, has led to the conclusion that plasma TRH is derived from the skin [16,30]. We too find high plasma values for TRH, but could find no significant difference in plasma TRH level between a black- and white-adapted animal. The observation that this plasma level is approximately $10^{-7}$ M, a concentration which is within the range of the dose-response relationship we established for this peptide, would indicate that for any regulatory role in the intermediate lobe, it would have to function at a relatively high concentration (above the plasma value of $10^{-7}$ M). Alternatively, the tissue might possess an enzymatic system for degradation of TRH so that melanotropes are not continuously exposed to the high circulating levels of TRH. In this respect it should be pointed out that intermediate lobe tissue is poorly vascularized [22], and thus one might expect a slow delivery of TRH from the blood.

In conclusion, while the presence of a rich TRH neuronal network within the neural lobe, and the demonstrated stimulatory effect this peptide has on MSH secretion, argue favorably for a physiological role of TRH in regulating the pars intermedia, some caution is necessary in defining the physiological role of TRH in *Xenopus laevis*. The peptide is capable of giving activation of MSH secretion in lobes of white-background adapted animals and thus, in this physiological state, or during the initial stages of adaptation to black background, it could be involved in adjusting the level of secretion. From our results it would seem doubtful that TRH has any regulatory role in the pars intermedia of fully black-background adapted *Xenopus laevis*.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. P. M. J. M. Cruissen, Mr. Th. A. J. M. Coenen, Mr. W. Klootwijk and Mr. R. F. Engels for excellent technical assistance, and Mrs. E. M. Jansen-Hoover for her secretarial assistance. This work was supported by EEG grant No. STI-084-5C.

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