Evaluation of ovarian POMC mRNA through quantitative RT-PCR analysis in *Rana esculenta*

M. NABISSI,1 L. SOVERCHIA,1 I. LIHRMANN,2 H. VAUDRY,2 G. MOSCONI,1 AND A. M. POLZONETTI-MAGNI1

1Dipartimento di Scienze Morfologiche e Biochimiche Comparate, Università degli Studi di Camerino, 62032 Camerino, Italia; and 2Laboratory of Cellular and Molecular Endocrinology, European Institute for Peptide Research, Institut National de la Santé et de la Recherche Médicale U413, UA CNRS, University of Rouen, France

Received 5 May 1999; accepted in final form 15 December 2000

Nabissi, M., L. Soverchia, I. Lihrmann, H. Vaudry, G. Mosconi, and A. M. Polzonetti-Magni. Evaluation of ovarian POMC mRNA through quantitative RT-PCR analysis in *Rana esculenta*. Am J Physiol Cell Physiol 280: C1038–C1044, 2001.—The evaluation of changes in the expression of specific genes requires accurate measurement of the corresponding mRNA concentration, especially when the gene is expressed at a very low level. We previously showed that the proopiomelanocortin (POMC) gene is expressed in the ovary of the frog *Rana esculenta*, and, to evaluate its mRNA content in frog ovary, we have now developed a sensitive quantitative RT-PCR method. This study provides evidence for the validation of this method and for the effects of captivity and hypophysectomy on POMC gene expression in the ovary of this anuran. Our data indicate that ovarian POMC gene is involved in short-term captivity stress response and seems not influenced by pituitary. These results are discussed taking into account the knowledge of the role played by opioids in stress response; moreover, a local control of POMC gene expression is also suggested.

**MATERIALS AND METHODS**

**Animals.** Adult female frogs were collected during the postreproductive period in a mountain pond (Colfiorito, Umbria). The animals were maintained in deep water tanks at 18°C under natural photoperiod and fed with fly larvae.

**In vivo experiments.** After 1 day of captivity, 10 frogs were hypophysectomized, and the animals were killed either after 2 days (Hd; 5 frogs) or after 2 wk (Hw; 5 frogs). Fifteen animals were used as controls and killed the day after capture (Co; 5 frogs), after 3 days of captivity (Cd; 5 frogs), or after 16 days of captivity (Cw; 5 frogs). Hypophysectomy was carried out in the 16-day captive frogs, which were killed 2 days after the operation (Hdr; 5 frogs). The ovarian tissues from the five frogs from each of the six groups were immediately processed for RT-PCR. Animal manipulations were performed according to the recommendations of the ethical committees at our institutions and under the supervision of authorized investigators.

**In vitro experiments.** Five female frogs were anesthetized with ice and killed. The ovaries were removed and washed with culture medium (Dulbecco’s modified Eagle’s medium; Sigma) containing glutamine (GIBCO, Netherlands), 10 mM HEPES (Sigma), and 45 mM NaHCO3.

**RESULTS**

Proopiomelanocortin (POMC) is a multifunctional precursor protein that generates, through tissue-specific proteolytic processing, a number of biologically active peptides, including adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), and β-endorphin (7). Molecular cloning of the POMC cDNAs from various representative species has revealed that the POMC structure has been highly preserved during evolution (15, 23, 28). The POMC gene is primarily expressed in corticotrope cells of the pars distalis and in melanotrope cells of the pars intermedia of the pituitary as well as in discrete neuronal populations in the central nervous system (12, 17). The occurrence of POMC-derived peptides and POMC mRNA has also been detected in various peripheral organs such as the heart (6, 27), pancreas (29), adrenal medulla (6, 8), gastrointestinal tract (24), and skin (11, 31). Moreover, the expression of the POMC gene has also been demonstrated in the male and female genital tract in various mammalian species; in particular, the presence of a POMC mRNA shorter than the pituitary transcript has been observed in testis (3, 25) and ovary (2).

In *Rana esculenta* ovary, previous studies demonstrated the presence of both POMC transcript (21) and POMC-derived peptide, β-endorphin; the involvement of this opioid in the control of ovarian function was demonstrated (1).

In the present study, a competitive RT-PCR technique for quantifying POMC gene expression in frog ovary was developed. With this technique, the effects of captivity and hypophysectomy on ovarian POMC gene expression were evaluated to determine whether ovarian POMC gene is involved in captivity stress response and whether pituitary regulates its expression.

**ACKNOWLEDGMENTS**

Address for reprint requests and other correspondence: A. M. Polzonetti-Magni, Dipartimento di Scienze Morfologiche e Biochimiche Comparate, via Camerini 2, 62032 Camerino (MC), Italia (E-mail: alberta@camserv.unicam.it).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Four pieces of tissue (100 mg each) from individual animals were placed on a sterile plate and incubated for 6 h in a thermostat incubator (18°C) with 1 ml of culture medium supplemented with homologous pituitary homogenate (HPH; [1/10] eq/ml), bullfrog luteinizing hormone (fLH; 100 ng/ml), and bullfrog follicle-stimulating hormone (fFSH; 100 ng/ml). The control contained culture medium alone. Gonadotropins of amphibian origin were applied, i.e., *Rana catesbeiana* FSH and LH (fFSH, fLH); the doses employed came from previous data obtained when fFSH and fLH were applied in vitro in the *R. esculenta* ovarian tissue (26). From the same five frogs, pituitaries were removed for preparing HPH.

fLH and fFSH were provided by Hayashi et al. (13). After 6 h, the incubation was stopped by tissue freezing in liquid nitrogen. At this point, the tissues were ready for total RNA extraction (TRIZol RNA isolation reagent; GIBCO BRL) and RT-PCR quantification.

**RNA and DNA extraction.** Total RNA and DNA were extracted from 1 g of frog ovary obtained from each animal of the five groups using TRIZol RNA isolation reagent, based on the acid guanidinium thiocyanate-phenol-chloroform extraction method (5). Final RNA and DNA concentrations were determined by optical density measurement at 260 nm, and total RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on a denaturing agarose gel. Total RNAs were divided into aliquots and stored at -80°C. Aliquots were randomly chosen for quantification assay.

**Oligonucleotides.** POMC primers were designed based on the sequence of POMC cDNA from *Rana ridibunda*. The upstream primer (sense), 5’ TGACAACAAACCGGCT 3’ (20 mer), was localized in the γ-MSH region and the downstream primer (antisense), 5’ TGGCATCTGAAAAAGT 3’ (19 mer), in the β-endorphin region. Primers were designed such that the predicted size of the PCR product was 471 bp for POMC cDNA.

**Competitive RT-PCR.** For the quantification assay, the absolute number of POMC internal standard (cRNA) molecules was calculated using spectrophotometric absorbance at 260 nm, the molecular weight of the cRNA (398, 310 g/mol), and Avogadro’s number. The cRNA was diluted at 7.5 × 10^5 molecules/μl and stored at -80°C. Different total RNA concentrations (0.5–2 μg) and 7.5 × 10^5 molecules of cRNA were mixed and reverse transcribed by 100 units of Moloney murine leukemia virus RT (GIBCO BRL) in 25 μl of the reaction mixture containing 1 μg of oligo(dT)12-18, 0.5 mM dNTP, 1× RT buffer, 10 mM dithiothreitol, and 20 units of RNase inhibitor. The RT reactions were carried out at room temper-
ature for 15 min and at 37°C for 90 min, followed by 10 min at 95°C. An aliquot (10 μl) of the resulting cDNA products was subsequently amplified with 2.5 units of Taq DNA polymerase (GIBCO BRL) in 50 μl of master mix containing 1× PCR buffer, 1.5 mM MgCl₂, 2.5 mM dNTP, and POMC primers (50 pmol each). PCR amplification was carried out for 37 cycles in an automated thermocycler (MJR Research) with thermocycle profile (denaturation at 94°C for 40 s, primers annealing at 62°C for 40 s, and primer extension at 72°C for 40 s) followed by a post-PCR incubation at 72°C for 7 min. PCR products were then purified, and the dried pellets were resuspended in 10 μl of NcoI 1× buffer with 5 units of NcoI (GIBCO BRL) and incubated for 1 h at 37°C. Five microliters for each sample were electrophoresed on 2% agarose gel in Tris-acetic acid-EDTA buffer 0.5×, and the band density was quantified using a PhosphorImager (Bio-Rad). For cycle course experiments, 7.5 × 10⁵ molecules of cRNA and 0.5 μg of total RNA were reverse transcribed and submitted to sequential cycles (25–45) of amplification. Different negative controls were performed. First, cRNA and the same total RNA mixture as that used for the quantification assay were added to the RT reaction mixture without RT and subsequently amplified to confirm the absence of DNA contamination in the cRNA and total RNA. Second, all the components of the RT reaction were prepared without RNA and subsequently amplified to confirm the absence of contamination in the reagents used. The data were considered useful only if no bands were observed in the negative controls. The absolute number of target molecules was estimated considering that the point at which the endogenous curve intersected the standard curve indicated that the same number of cRNA molecules was present in the sample.

Data analysis. The data were expressed as the means ± SE of five different quantification experiments for each group. RT-PCR results were analyzed by one-way analysis of variance with StatView (Brain Power). *P < 0.05* indicated a statistically significant difference between the means.

Cloning. Three micrograms of total RNA from *R. esculenta* ovary were reverse transcribed and amplified using POMC primers, as described above. The RT-PCR result showed the presence of a single band of ~470 bp. The band was purified using QIAquick PCR purification Kit (QIAGEN), and ~25 ng of PCR products were used to be cloned with pGEM-T vector system (Promega). Ten positive clones and the respective constructed plasmids were chosen.

Sequencing. DNA sequence analysis was performed by dye-labeled terminators using a DNA sequencing kit (Perkin Elmer). Nucleotide sequences were read in both directions.

RESULTS

Quantification of POMC mRNA: competitive RT-PCR. We previously developed a method for the quantification of POMC mRNA by RT-PCR using a syn-

![Fig. 2](image-url) Nucleotide sequence analysis of the ovarian POMC PCR product. In the box is shown the restriction site of NcoI. Numbers (left) correspond to the first nucleotide of the line; numbers (right) correspond to the last nucleotide of the line. Bioactive domains are underlined with arrows.

![Fig. 3](image-url) Quantification assay to identify efficiency differences between endogenous and standard molecules. Total RNA (0.5 μg) from *R. esculenta* ovary (○) and 7.5 × 10⁵ molecules of standard (◇) were reverse transcribed and submitted to sequential cycles of amplification. The amplification products were separated using agarose gel and detected by ethidium bromide staining. The signal was quantified by scanning photography. The amount of PCR products from cRNA and the amount of PCR products from POMC mRNA (A.U., arbitral unit) were plotted against the number of cycles carried out for each sample. Each point represents the average of duplicate reactions.
thetic mutant POMC RNA as internal standard (21). The specific mRNA and the internal standard were coamplified in one reaction in which the same primers were used. The internal standard (cRNA) was an engineered mRNA with a restriction site (for NcoI) deleted within it so that the standard DNA amplified from it could be distinguished by a restriction enzyme analysis from DNA amplified from the endogenous POMC mRNA (Fig. 1). To increase the sensitivity of our competitive RT-PCR assay for the quantification of the POMC mRNA in the ovary of *R. esculenta*, different sets of primers were designed based on the sequence of the POMC cDNA of *R. ridibunda* (15). The aim of this experiment was to find a set of primers able to select, with high specificity, the template (POMC mRNA) in such a manner as to quantify the PCR products from the RT-PCR assay directly on the gel, avoiding Southern blot analysis (because each additional step can increase measurement errors) as described (21). The best results were obtained using a 20-mer sense primer (5′ TGA CAA CAA CAA CGG GGG CT 3′) and a 19-mer antisense primer (5′ TGG CAT TCT TGA AAA GAG T 3′), localized, respectively, in position 332–351 of the γ-MSH region and in position 783–801 of the β-endorphin region of the *R. ridibunda* POMC cDNA.

The RT-PCR product, using total RNA from *R. esculenta* ovary, showed a clear band of ~470 bp, using a higher annealing temperature (62°C) than in the previous method (55°C; results not shown). To demonstrate the high specificity of this set of primers in selecting the POMC mRNA, the RT-PCR products from ovarian total RNA were cloned. The 25 clones obtained were tested for the presence of the POMC cDNA insert by a dot-blot analysis using a *R. ridibunda* POMC DNA probe. All the clones proved positive to this analysis (results not shown), confirming the high specificity of the set of primers used. Moreover, the plasmids from ten positive clones were sequenced, revealing the pres-

Fig. 4. A–F: quantitation of POMC mRNA by RT-PCR in experimental groups. Co, the day after capture; Cd, 3 days of captivity; Cw, 16 days of captivity; Hd, 2 days after hypophysectomy; Hw, 2 wk after hypophysectomy; and Hdr, 16 days of captivity and 2 days after surgery. The amount of standard RNA (cRNA) was kept constant (7.5 × 10^5 molecules), and the amount of endogenous RNA (total RNA) was varied (0.5–2 μg, "e" band). The amplification products were separated using agarose gel and detected by ethidium bromide staining. The signal was quantified by scanning photography. The amount of PCR product from cRNA and the amount of PCR products (sum of 2 e bands) from POMC mRNA were plotted against the amount of total RNA included in the cDNA reaction mix. Each point represents the average of duplicate reactions.
ence of a unique insert with 100% homology with the primers delimiting the region of the POMC cDNA from *R. ridibunda* (Fig. 2). A cycle course experiment was also performed, using POMC cRNA and 0.5 μg of total RNA from *R. esculenta* ovary, to identify problems of efficiency differences and to determine the optimal number of PCR cycles for the quantification assay. As shown, the slopes of the curve for the endogenous and standard products are virtually identical, indicating that the reaction efficiencies for these two templates are the same; the exponential phase of the reaction was observed up to 37 cycles, followed by a plateau phase (Fig. 3). Thus PCR analyses were subsequently carried out for 37 cycles.

Effects of captivity and hypophysectomy on POMC gene expression. Figure 4, A–F, represents the procedure giving the results for each of the five samples making up the different experimental groups. Twenty-four hours after capture (Co group), the amount of POMC mRNA was 7.5 × 10⁵ molecules/μg of total RNA (Fig. 4A). The same analysis was performed in frogs kept in captivity for 3 and 16 days (Cd and Cw) and in frogs hypophysectomized 2 days and 2 wk before being killed (Hd and Hw). The 16-day captivity Cw group was considered as a control of the hypophysectomized frogs killed 2 days after surgery (Hdr); moreover, POMC gene expression was compared with the variation of total RNA in all experimental groups and control. The results (Table 1) indicated that POMC gene expression significantly (*P* < 0.05) decreased in the ovary of frogs kept in captivity for 3 days (Cd, Fig. 4D), whereas in 16 days of captivity (Cw, Fig. 4C), and in both 2 days (Hd, Fig. 4D) and 2 wk (Hw, Fig. 4E) of hypophysectomy, no significant changes of ovarian POMC mRNA were found when compared with the parallel control group taken in captivity; i.e., Cd and Cw. In addition, to separate the effects induced by pituitary gland removal from those caused by captivity itself, hypophysectomy in 16-day captive animals was carried out (Hdr). Two days after surgery, no significant changes were found in the ovarian POMC mRNA, compared with the values obtained in frogs kept in captivity for 16 days (Cw, Fig. 4F).

The total RNA content behaved in the same way as POMC mRNA, significantly (*P* < 0.05) decreasing in a short-term stress paradigm, such as 3 days of captivity (Cd), and significantly (*P* < 0.05) increasing in the ovary of 2-wk hypophysectomized frogs (Table 1).

The results of in vitro experiments are shown in Table 2. Neither total pituitary (HPH) nor fLH nor fFSH induced significant changes in the content of POMC mRNA assessed in the culture media.

### DISCUSSION

The POMC encoding for peptides such as ACTH, α-MSH, and endorphins has been found to be involved in the stress response and/or adaptation by activating the central neuroendocrine cascade in amphibians (16, 19) as well as in teleosts (10, 20). As concerns amphibians, Mosconi et al. (19) found that the captivity stress paradigm applied in *R. esculenta* is consistent with the activation of the central opioid system, which mediates the stress-induced inhibition of gonadal function. In addition to being found in the amphibian brain, opioid peptides have also been identified in the peripheral organs, including the gonads. This suggests the presence of a gonadal opioid system in both mammalian and nonmammalian vertebrates (1). With this in view, we aimed to clarify the role of ovarian POMC in the frog *R. esculenta*, and, for that purpose, a competitive RT-PCR technique for quantifying POMC gene expression was developed.

An investigation on regulation of gene expression depends in part on the ability to measure mRNA species accurately. Because of its extraordinarily high sensitivity, PCR is being widely used for amplifying cDNA copies of low-abundance mRNA. For the quantification of rare mRNA, the quantitative RT-PCR is considered a very useful technique (22, 30, 32), making

<table>
<thead>
<tr>
<th>Co</th>
<th>Cd</th>
<th>Hd</th>
<th>Cw</th>
<th>Hw</th>
<th>Hdr</th>
</tr>
</thead>
<tbody>
<tr>
<td>POMC mRNA</td>
<td>7.5 × 10⁵ ± 0.23</td>
<td>4.4 × 10⁵ ± 0.28</td>
<td>4.9 × 10⁵ ± 0.2</td>
<td>6.85 × 10⁵ ± 0.3</td>
<td>7.6 × 10⁵ ± 0.11</td>
</tr>
<tr>
<td>RNA vs. DNA</td>
<td>0.60 ± 0.011</td>
<td>0.223 ± 0.012</td>
<td>0.153 ± 0.009</td>
<td>0.549 ± 0.015</td>
<td>1.50 ± 0.013</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE of 5 different quantification experiments and correspond to the amount of POMC mRNA molecules per microgram of total RNA. Four pieces of ovary (100 mg each) from every animal were incubated for 6 h with 1 ml of culture medium (CM) and/or with homologous pituitary homogenate (HPH; 1/10 eq/ml), bullfrog luteinizing hormone (fLH; 100 ng/ml), or bullfrog follicle-stimulating hormone (fFSH; 100 ng/ml).
it possible to study genes that might be expressed at very low levels. One of the main problems of quantitative RT-PCR is that the amount of PCR products increases at each cycle of amplification in an exponential manner, so that any of the variables that influence the reaction can alter the amount of the final PCR product. An approach to controlling the variability inherent in RT-PCR involves the use of a competitive template that is highly similar to, but somehow discriminable from, the intended target sequence to be quantitated, so that it amplifies at the same efficiency as the target (14). Our competitive RT-PCR used a synthetic mutant cRNA with a sequence homology of the DNA amplified from it and equally as high as that of the DNA amplified from endogenous mRNA (99% homology). Moreover, considering that we utilized a set of highly selective primers and a number of cycles of amplification that avoid taking any measurements after the plateau phase of the amplification, our competitive RT-PCR assay can be considered a valid and reliable method. Therefore, the POMC mRNA was evaluated in the ovary of both short- and long-term captive frogs since, in our wild frog population, captivity was found to be a very powerful stressor in which the central opioid system is involved (19). Hypophysectomy was performed to clarify the relationships between pituitary and ovarian POMC gene expression.

By applying the captivity stress paradigm, we demonstrate that short-term captivity decreases both total and POMC mRNA, suggesting that captive frogs need less peptide alarm compared with wild ones. Conversely, in long-term captivity frogs, POMC mRNA levels were similar to those measured in controls, indicating that adaptation mechanisms occur during chronic stress. Regarding the control of pituitary on ovarian POMC gene transcription, no effects at all were found in POMC mRNA content, which remained unaffected in both the ovary of hypophysectomized frogs and in the cultured tissue in which total pituitary homogenate and/or bullfrog gonadotropins were added. It seems of interest to emphasize the in vivo results, in light of experiments carried out, to separate the effects of captivity from those caused by pituitary gland removal. The total mRNA content paralleled that of the POMC mRNA one; perhaps, in 2-wk hypophysectomized frogs, its increase could be due to the activation of ovarian transcriptional machinery as an adaptative response to pituitary gland removal.

In conclusion, the quantification of ovarian POMC gene expression through a quantitative RT-PCR technique was achieved in the frog R. esculenta; ovarian POMC gene is involved in the short-term captivity stress response and seems unaffected by pituitary. Although different results have been found in mammals (4, 18), the data reported here, in agreement with the findings by Facchinetti et al. (9) demonstrating (in the same frog) the local opiate regulation of testicular activity, suggest the presence of an ovarian opioid system locally regulated in autocrine/paracrine fashion. That kind of mechanism seems preponderant at an early stage of the evolutionary tree, while endocrine communication prevails together with the increasing complexity occurring during phylogeny.

We thank Dr. H. Hayashi for the frog gonadotropins. This study was supported by grants from the University of Camerino and Ministero dell’Università e della Ricerca Scientifica e Tecnologica, Italy (to A. M. Polzonetti-Magni).

REFERENCES


