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Two Frog Melanotrope Cell Subpopulations Exhibiting Distinct Biochemical and Physiological Patterns in Basal Conditions and under Thyrotropin-Releasing Hormone Stimulation*

JOSE LUIS GONZALEZ DE AGUILAR, MARIA M. MALAGON, RAFAEL M. VAZQUEZ-MARTINEZ, ISABELLE LIHRMANN, MARIE-CHRISTINE TONON, HUBERT VAUDRY, AND FRANCISCO GRACIA-NAVARRO

Department of Cell Biology, University of Córdoba (J.L.G.d.A., M.M.M., R.M.V.M., F.G.N.), Córdoba, Spain; and European Institute for Peptide Research, Institut Fédératif de Recherches Multidisciplinaires sur les Peptides 23, Laboratory of Cellular and Molecular Neuroendocrinology, INSERM U-413, Unité Affiliée au Centre National de la Recherche Scientifique, University of Rouen (I.L., M.C.T., H.V.), Mont-Saint-Aignan, France

ABSTRACT

Cell heterogeneity designates the phenomenon by which a particular cell type is composed of morphologically and physiologically distinct cell subpopulations. We have previously isolated two subsets of melanotrope cells in the intermediate lobe of the frog pituitary by means of a separation procedure based on a Percoll density gradient. High density (HD) melanotrope cells were found to exhibit a more granulated cytoplasm and a lower secretory rate than low density (LD) cells. In the present study, we have investigated the biochemical and functional characteristics of each melanotrope cell subpopulation by using various approaches, including chromatographic analysis for the measurement of the proportion of acetylated αMSH, microfluorimetric measurement of the cytosolic free calcium concentration ($[Ca^{2+}]_i$), and *in situ* hybridization for quantification of POMC messenger RNA (mRNA). Under basal conditions, LD melanotrope cells showed higher secretory activity, acetylation rate, $[Ca^{2+}]_i$, and POMC mRNA content compared to HD cells. Incubation of the cells with 100 nM TRH for 2 h induced a more pronounced activation of αMSH secretion, $[Ca^{2+}]_i$ mobilization, and POMC mRNA accumulation in LD than in HD melanotrope cells. Conversely, TRH increased the rate of acetylation of αMSH in HD cells, but did not affect acetylation in LD cells. Taken together, these results demonstrate that the frog intermediate lobe is composed of two subsets of endocrine cells with distinct biochemical and functional characteristics. The coexistence of two cell subpopulations in the frog pars intermedia is consistent with the idea of a cell secretory cycle, in which each melanotrope subset represents a specific state of cellular activity. (*Endocrinology* **138:** 970–977, 1997)

THE DISTAL lobe of the pituitary is a complex and heterogeneous organ comprising several endocrine cell types that produce different hormones (1). In addition, numerous studies have shown that each pituitary cell type is not a pool of homogeneous and synchronized cells, but is composed of subsets of morphologically and physiologically distinct endocrine cells (2–5). This phenomenon, named cell heterogeneity, has been evaluated in various adenohypophyseal cell types with respect to morphofunctional and ultrastructural characteristics (6, 7), secretory activity (8, 9), response to secretagogues (10–12), and hormone gene expression (13, 14).

The intermediate lobe of the rat pituitary is composed of two cell types, *i.e.* melanotrope and corticotrope cells (15), which both synthesize the precursor protein POMC (16). The intermediate lobe of amphibians, which consists of a single endocrine cell type, the melanotrope cells (17), represents a valuable model in which to investigate the phenomenon of cell heterogeneity. Posttranslational processing of POMC in amphibian melanotrope cells generates several biologically active peptides, including αMSH and β-endorphin (18, 19). The hormone αMSH, which causes dispersion of the pigment melanin in dermal melanophores, plays a pivotal role in the control of background color adaptation (20). N-Terminal acetylation of αMSH is an important processing event that increases the melanotropic activity of the peptide (21). In anuran amphibians, the major intracellular form of the peptide is des-N^{α}-acetyl αMSH, and the acetylation reaction to generate αMSH is associated with the secretory process (22–24). It has also been demonstrated that N^{α}-acetylation is physiologically regulated by background adaptation (25) or hypothalamic neurohormones (26, 27).

The secretory activity of frog melanotrope cells is regulated by multiple factors, including classical neurotransmitters and neuropeptides (see Ref. 28 for review). In particular, TRH is a potent stimulator of αMSH secretion from amphib-

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Address all correspondence and requests for reprints to: Dr. Francisco Gracia-Navarro, Department of Cell Biology, Faculty of Sciences, Avda. San Alberto Magno s/n, University of Córdoba, E-14004 Córdoba, Spain. E-mail: bc1grnaf@uco.es.
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ian intermediate lobe (29–31). The effect of TRH on melanotrope cells is mediated through activation of phospholipase C and mobilization of intracellular calcium stores (32).

Heterogeneity of rat melanotrope cells has previously been reported with respect to secretory activity (33) and POMC gene expression (34). Recently, two subpopulations of melanotrope cells have been isolated in the intermediate lobe of *Rana ridibunda* after separation of dispersed cells with a continuous Percoll density gradient (35). These melanotrope cell subsets, referred to as high density (HD) and low density (LD) cells, differ in both secretory granule content and spontaneous αMSH secretion; HD cells have a more granulated cytoplasm and a substantially lower secretory activity than LD cells.

The aim of the present study was to compare the biochemical and physiological characteristics of HD and LD cells. For each subpopulation, we investigated the secretory activity, the cytosolic calcium concentration ($[Ca^{2+}]_i$) and the POMC messenger RNA (mRNA) density under basal and TRH-stimulated conditions. In addition, we compared the rates of acetylation of αMSH in HD and LD melanotrope cells.

Materials and Methods

Animals

Adult male frogs (*Rana ridibunda*) of about 40 g body weight were purchased from a commercial supplier (Couétard, Saint-Hilaire de Riez, France). The animals were maintained under running water at a constant temperature (8 C) with a 12-h light, 12-h dark cycle for at least 1 week before death. The frogs were killed by decapitation between 0800–0900 h, and the neurointermediate lobes were dissected under a microscope. Animal manipulations were performed according to the recommendations of the local ethical committees and under the supervision of authorized investigators.

Reagents and test substances

Collagenase type V, trypsin type I, BSA, Leibovitz culture medium, and antibiotic-antimycotic solution were purchased from Sigma Chemical Co. (St. Louis, MO). Indo-1 acetoxymethylester, pluronic F127, and the calcium ionophore A-23187 were obtained from Molecular Probes (Eugene, OR). TRH was purchased from PREM (Zyma Farmacéutica, Barcelona, Spain). Percoll was obtained from Pharmacia LKB (Uppsala, Sweden). FBS was obtained from Sera-Lab (Crawley Down, UK).

Isolation and separation of melanotrope cells

Isolated melanotrope cells were obtained using a dispersion protocol described previously (35). Briefly, 40 neurointermediate lobes were collected for each experiment and enzymatically dissociated by incubation at 26 C in culture medium containing 0.2% (wt/vol) collagenase type V and 0.2% (wt/vol) trypsin type I for 45 min. The culture medium consisted of Leibovitz culture medium diluted 2:3 (to adjust to *Rana ridibunda* osmolality) and supplemented with 1 mM glucose, 0.4 mM $CaCl_2$, and 1% (vol/vol) antibiotic-antimycotic solution, pH 7.4. The dissociation was continued in the same medium supplemented with 2 mM EDTA for 5 min and with 1 mM EDTA for 5 min. Then, the tissues were mechanically dispersed using a siliconized Pasteur pipette until a homogeneous cellular suspension was obtained. Cell viability, as determined by the trypan blue exclusion test (36), was 89%.

A density gradient of Percoll was prepared as previously described (35). A hyperbolic gradient was obtained by mixing 6 ml of a 50% Percoll solution with 3 ml of a 15% Percoll solution at a rate of 0.25 ml/min. A 250-μl sample of the cellular suspension (2.2×10^6 cells) was carefully loaded on top of the gradient. After centrifugation (3,000 × *g* for 25 min; 4 C), nine fractions of 1 ml each were collected manually. Cells contained in fractions 1 (bottom of the gradient) and 4 plus 5 contained the HD and LD cell subpopulations, respectively. HD melanotrope cells represented 20.8% of the total cells recovered from the gradient, whereas LD cells represented 52.1%.

Culture of melanotrope cell subpopulations

Primary culture of each melanotrope cell subset was performed to evaluate their functional characteristics under both basal and TRH-stimulated conditions. Aliquots of 30,000 cells were plated on 35-mm petri dishes and incubated at 26 C in 2 ml culture medium supplemented with 10% FBS. After 48 h, the medium was removed, and the cells were preincubated in 1 ml serum-free culture medium for 2 h. The cells were incubated for another 2 h with 1 ml medium in the absence or presence of 100 nM TRH. Medium samples were collected and centrifuged at 60 × *g* for 5 min, and the supernatants were stored at −20 C until hormone assay. After culture, cells were processed for *in situ* hybridization. To determine the $[Ca^{2+}]_i$, cells were plated on microgrid coverslips (Eppendorf, Netheler, Germany) at a density of 5000 cells/well and incubated in 2 ml culture medium supplemented with 10% FBS for 3–5 days until $[Ca^{2+}]_i$ measurement.

HPLC

Incubation media were first prepurified on Sep-Pak cartridges (Alltech Europe, Laarne, Belgium). Each sample was then subjected to reverse phase HPLC analysis on Lichrosorb RP-18 column (0.46 × 25 cm; Merck, Paris, France) using a gradient established with 0.1% trifluoroacetic acid (vol/vol; pH 2.4) and a mixture of acetonitrile-methanol (80:20, vol/vol). The column was equilibrated with 25% acetonitrile-methanol and eluted at a flow rate of 1 ml/min using the gradient shown in Fig. 2. The following synthetic standards were analyzed using the same gradient: des-N^{α}-acetyl αMSH, αMSH, and diacetyl αMSH, as well as their respective sulfoxide derivatives. Oxidation of the synthetic standards was performed using hydrogen peroxide as described previously (37). Fractions were collected every 1 min and dried in a Speed-Vac concentrator (Savant, Hicksville, NY)

αMSH RIA

The concentrations of αMSH-related peptides in the culture media and HPLC fractions were measured using a double antibody RIA method described previously (38). The sensitivity of the assay was 3 pg/tube. The αMSH antiserum exhibited full cross-reactivity with des-N^{α}-acetyl αMSH, αMSH, and diacetyl αMSH. The cross-reactivity of the antiserum with other POMC-derived peptides was lower than 0.1%.

Measurement of cytosolic calcium concentration

Cultured cells were first incubated with 5 μM indo-1 acetoxymethylester and 0.02% (vol/vol) Pluronic F127 in culture medium for 30 min at 22 C in the dark. The cells were washed twice with fresh medium, and $[Ca^{2+}]_i$ was monitored by a dual wavelength microfluorimetry system constructed from an inverted microscope equipped with a fluor ×40 objective in the epifluorescence mode (Nikon Corp., Tokyo, Japan). The fluorescence emission of indo-1 induced by excitation at 355 nm was recorded at two wavelengths (405 and 485 nm) by separate photometers as previously described (39). After conversion of photon currents to voltage signals, both 405- and 485-nm signals and the 405/485 ratio were continuously monitored by a software FASTINCA 1.03 (Nikon Corp.). $[Ca^{2+}]_i$ was calculated from the formula established by Grynkiewicz *et al.* (40): $[Ca^{2+}]_i = K_d \times \beta \times [(R - R_{min})/(R_{max} - R)]$, where K_d is the dissociation constant for indo-1 (250 nM), β is the fluorescence ratio between the signal at 485 nm in calcium-free medium and the signal at 485 nm in calcium-saturated medium, R is the 405/485 ratio of any unknown calcium concentration, R_{min} is the 405/485 ratio obtained after incubation of cells with 2 mM EGTA and 10 μM A-23187 for 30 min at 22 C in culture medium in the dark (calcium-free conditions), and R_{max} is the 405/485 ratio obtained after incubation of cells with 2 mM $CaCl_2$ and 10 μM A-23187 for 30 min at 22 C in culture medium in the dark (calcium-saturated conditions). The averaged values were: $R_{min} = 0.102 \pm 0.001$, $R_{max} = 0.713 \pm 0.018$, and $\beta = 3.677 \pm 0.246$ (n = 90). The effect of TRH on $[Ca^{2+}]_i$ was tested by administering pulses of 10 μM

TRH for 2 sec in the vicinity of the cells by means of a pressure ejection system.

In situ hybridization of POMC mRNA

Quantification of POMC mRNA was performed by *in situ* hybridization using a nonradioactive procedure, as previously described (41). After removal of the culture medium, cells were rinsed with 0.01 M PBS (pH 7.2) and fixed in petri dishes with 4% paraformaldehyde in PBS for 15 min at room temperature. After three washes with PBS, cells were dehydrated in graded ethanol and stored at −80 C until use. Before hybridization, cells were thawed, hydrated, and treated with 1% (vol/vol) Triton X-100 in PBS for 10 min. After two washes in PBS, cells were incubated with 5 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) in PBS for 15 min at 37 C. The enzymatic activity was stopped with 2 mg/ml glycine in PBS. Cells were washed in PBS, postfixed with 4% paraformaldehyde in PBS for 5 min, washed again, and dehydrated in graded ethanol. Cells were prehybridized for 2 h at 37 C in a solution containing 50% (vol/vol) deionized formamide (Sigma), 5 × SSPE (20 × SSPE is 3 M NaCl, 0.2 M $NaH_2PO_4 \cdot H_2O$, and 0.02 M EDTA, pH 7.4), 4% (vol/vol) dextran sulfate (stock 40%, wt/vol; Sigma), 5 × Denhardt's solution [50 × Denhardt's solution is 1% (wt/vol) Ficoll type 400 (Pharmacia LKB), 1% (wt/vol) polyvinylpyrrolidone (Sigma), 1% (wt/vol) BSA, 0.1% (vol/vol) SDS (stock 10% (wt/vol); pH 7.2), 200 μg/ml yeast transfer RNA (Boehringer Mannheim), 250 μg/ml heat-denatured salmon sperm DNA (Sigma), and 2 μg/ml polyadenylic acid (Sigma). The probe used in this study was the *Eco*RI 1184-bp insert of frog POMC complementary DNA subcloned into pGEM-3Zf (42). The fragment was purified by agarose gel electrophoresis and then digoxigenin-labeled by random priming using a digoxigenin DNA labeling kit (Boehringer Mannheim). Cells were covered with 200 μl heat-denatured labeled POMC DNA probe (35 ng/dish) in the hybridization solution. Hybridization was performed in a humid chamber for 16 h at 37 C. The dishes were subsequently rinsed with 2 × SSC (20 × SSC is 3 M NaCl and 0.3 M sodium citrate, pH 7.4) for 90 min, 1 × SSC for 90 min, 0.5 × SSC for 30 min at 37 C, and 0.5 × SSC for 30 min. Cells were washed in buffer 1 (100 mM Tris-HCl and 150 mM NaCl, pH 7.5) and incubated with 10 mM levamisole (Sigma) in buffer 2 (100 mM Tris-HCl, 150 mM NaCl, and 50 mM $MgCl_2$, pH 9.5) for 1 h. Then, cells were washed twice with buffer 1 and treated with 1% (wt/vol) blocking reagent (Boehringer Mannheim) for 30 min. Thereafter, cells were incubated with the alkaline phosphatase-labeled antidigoxigenin F(ab) fragment (Boehringer Mannheim) diluted 1:500 in buffer 1 for 4 h. After successive washes in buffers 1 and 2, cell-bound alkaline phosphatase activity was visualized by incubating the cells with the color solution [3.5 μl/ml 5-bromo-4-chloro-3-indolyl phosphate, 4.5 μl/ml nitroblue tetrazolium salt (Boehringer Mannheim), and 0.24 mg/ml levamisole in buffer 2] for 16 h in the dark. The color reaction was stopped by rinsing the plates with buffer 1. Finally, they were mounted in buffer 1 plus glycerol (1:1).

Control staining included 1) pretreatment of dishes with 200 μg/ml pancreatic ribonuclease A and 150 U/ml ribonuclease T (Sigma) in 2 × SSC for 1 h at 37 C, 2) omission of the probe, and 3) omission of the antidigoxigenin serum. As an additional control, the probe was tested in a cell type (human monocyte THP-1 cells) that does not express POMC. All controls were processed concurrently with samples using the same protocol.

POMC mRNA quantification was accomplished on a Dasher 386SX computer (Data General, Westboro, MA) equipped with IMAGO software for image analysis (SIVA Research Group, University of Córdoba, Córdoba, Spain) and connected by a CCTV camera (Hitachi, Tokyo, Japan) to a light microscope (Zeiss, Oberkochen, Germany). A ×40 objective and a stabilized light source were used. Before measuring each set of cells, Köhler focus was carried out to ensure an even and homogeneous illumination. The staining intensity (optical density) and the area of each individual cell (50 cells randomly selected/dish) were measured to calculate the integrated optical density. This parameter was correlated with the amount of POMC mRNA in the cell.

Background was evaluated for nonstained cells (10 cells/dish) and subtracted. To avoid within-experiment variations, control and treated cells were hybridized and measured simultaneously.

Statistical analysis

Data were expressed as the mean ± SE of the number of experiments indicated in each figure. The statistical analysis was preceded by a test for the joint assessment of normality (Kolmogorov-Smirnov test). Student's *t* test or Mann-Whitney rank sum test were applied depending on whether the distribution of data was parametric or nonparametric, respectively. Mathematical processing and statistical analysis were performed with the software SIGMAPLOT 5.01 and SIGMASTAT 1.02 (Jandel Scientific, Corte Madera, CA).

Results

Secretory activity of the melanotrope cell subpopulations

The absolute amount of αMSH secreted during 2 h by HD cells under basal conditions (19.4 ± 3.0 ng/100,000 cells; n = 4) was significantly less than that secreted by LD cells (96.0 ± 13.8 ng/100,000 cells; n = 4; $P < 0.001$). Incubation of the cells with 100 nM TRH provoked a significant stimulation of αMSH secretion in the two melanotrope cell subsets (Fig. 1). The relative increase in αMSH release was 2-fold higher in LD cells (+307%) than in HD cells (+141%).

Analysis of αMSH-immunoreactive peptides secreted by the melanotrope cell subpopulations

Reverse phase HPLC analysis coupled with RIA quantification revealed the existence of four major forms of αMSH in culture media of LD and HD cells (Fig. 2). Peaks I and II exhibited the same retention time as the sulfoxide derivatives of des-N^{α}-acetyl αMSH and αMSH, respectively, whereas peaks III and IV coeluted with des-N^{α}-acetyl αMSH and αMSH, respectively. A minor component (peak V) coeluting with diacetyl αMSH was also resolved in culture medium of LD cells. The sulfoxide form of diacetyl αMSH (retention time, 35 min) was not detected. Therefore, peaks I and III

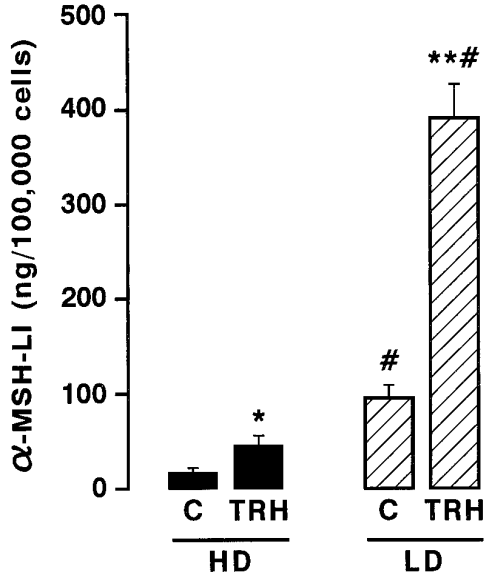

FIG. 1. Effect of TRH on αMSH secretion from cultured frog melanotrope cell subpopulations. HD (*solid bars*) and LD cells (*hatched bars*) were incubated in the absence (C) or presence of 100 nM TRH for 2 h. The data represent the mean (±SEM) of four independent experiments. *, $P < 0.05$; **, $P < 0.001$ (*vs.* corresponding control). #, $P < 0.001$ (*vs.* HD cells; all determined by Student's *t* test).

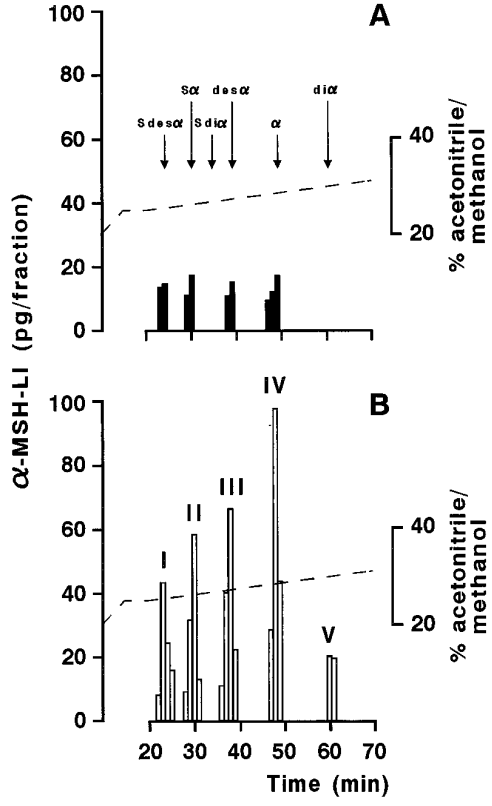

FIG. 2. Reverse phase HPLC analysis of αMSH-immunoreactive peptides released by cultured frog melanotrope cell subpopulations. The culture media from HD (A) and LD (B) cells were prepurified on Sep-Pak C_{18} cartridges and chromatographed on a Lichrosorb RP-18 column. All fractions collected (1 ml each) were dried and assayed for αMSH-like immunoreactivity (α-MSH-LI). The *arrows* indicate the elution times of synthetic standards. The *broken lines* show the concentration of acetonitrile-methanol (80:20) in the eluting solvent. Sdesα, Sulfoxide derivative of des-N^{α}-acetyl αMSH; Sα, sulfoxide derivative of αMSH; Sdiα, sulfoxide derivative of diacetyl αMSH; desα, des-N^{α}-acetyl αMSH; α, αMSH; diα, diacetyl αMSH.

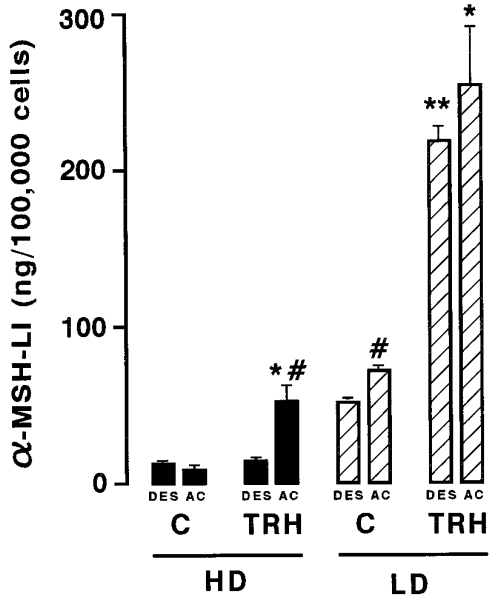

FIG. 3. Comparison of the concentrations of nonacetylated and acetylated forms of αMSH released by cultured frog melanotrope cell subpopulations. HD (*solid bars*) and LD (*hatched bars*) cells were incubated in the absence (C) or presence of 100 nM TRH for 2 h. The culture medium was analyzed by HPLC and the αMSH-immunoreactive peptides (α-MSH-LI) were quantified by RIA. The nonacetylated form (DES) corresponds to des-N^{α} acetyl αMSH and its sulfoxide derivative. The acetylated form (AC) corresponds to αMSH, its sulfoxide derivative, and diacetyl αMSH. The data are the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ (*vs.* corresponding control). #. $P < 0.05$ (*vs.* corresponding DES; all determined by Student's *t* test).

represent the nonacetylated forms of αMSH, whereas peaks II, IV, and V represent the acetylated forms.

Under basal conditions, the proportions of des-N^{α}-acetyl αMSH and acetylated forms of αMSH secreted by HD cells were not significantly different. Conversely, the amount of acetylated forms of αMSH secreted by LD cells was significantly higher than that of des-N^{α}-acetyl αMSH (Fig. 3). Specifically, the percentage of acetylated forms secreted by the two melanotrope cell subsets was 38.3% in HD cells and 57.9% in LD cells. Incubation of HD cells with 100 nM TRH produced a significant increase in the proportion of acetylated forms of αMSH released by the cells ($P < 0.05$; Fig. 3). In contrast, although TRH provoked a significant increase in the total amount of αMSH released from LD cells ($P < 0.001$), the proportions of nonacetylated and acetylated forms of αMSH secreted under these conditions were similar (Fig. 3).

Measurement of $[Ca^{2+}]_i$ in the melanotrope cell subpopulations

Under basal conditions, $[Ca^{2+}]_i$ was significantly lower in HD cells (97.5 ± 13.3 nM; n = 61) than in LD cells (166.9 ± 14.5 nM; n = 77; $P < 0.001$). Ejection of 10 μM TRH in the vicinity of the cells provoked a rapid and massive increase in $[Ca^{2+}]_i$ in the two subpopulations of melanotrope cells (Fig. 4). The percentages of cells responsive to TRH were similar in the two cell subsets, *i.e.* 62.3% in HD and 66.1% in LD cells. Sequential administration of TRH provoked a substantial attenuation of the Ca^{2+} response in both cell subpopulations (Fig. 4). Quantitative analyses of different parameters calculated from such experiments are summarized in Table 1. The maximal $[Ca^{2+}]_i$ achieved after the first stimulation was significantly higher in LD cells than in HD cells ($P < 0.01$). In contrast, the percentage of $[Ca^{2+}]_i$ increment after the first ejection of TRH was significantly higher in HD cells than in LD cells ($P < 0.01$). The lag between the administration of TRH and the maximum $[Ca^{2+}]_i$ response was not significantly different in the two cell subpopulations. In both cell subsets, the lag period was significantly shorter after the second and third pulses than after the first pulse of TRH ($P < 0.01$).

POMC mRNA expression in the melanotrope cell subpopulations

The percentages of POMC-positive cells identified by *in situ* hybridization were not significantly different ($P > 0.05$) in HD cells (76.2 ± 9.2%; n = 3) and LD cells (70.6 ± 6.2%; n = 3); these values were in the same range as those measured previously by immunocytochemistry (81% and 76%,

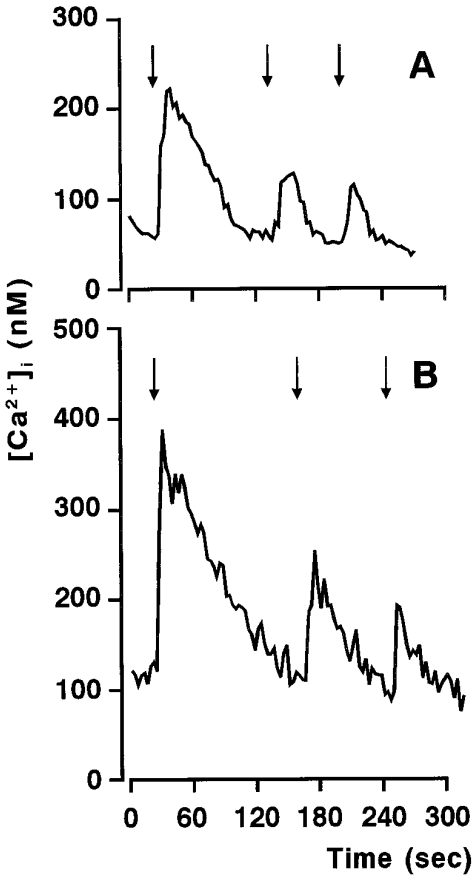

FIG. 4. Effect of repeated pulses of TRH on $[Ca^{2+}]_i$ in cultured frog melanotrope cell subpopulations. Each figure represents a representative recording from HD (A) and LD (B) cells. The *arrows* indicate the onset of TRH application (10 μM; 2 sec). The number of cells studied was 53 in A and 62 in B.

TABLE 1. Characteristics of the effect of TRH on $[Ca^{2+}]_i$ in HD and LD melanotrope cells in primary culture

	Pulse	HD cells	LD cells
% of cells responding to TRH		62.3	66.1
Basal $[Ca^{2+}]_i$ (nM)	1	95.2 ± 12.2	162.8 ± 10.8[a]
	2	89.0 ± 11.9	138.0 ± 10.3[b]
	3	70.5 ± 7.8	108.8 ± 14.4[c]
Maximal $[Ca^{2+}]_i$ (nM)	1	285.8 ± 29.1	347.9 ± 15.6[b]
	2	203.4 ± 30.4[c]	249.0 ± 13.1[d,e]
	3	173.5 ± 24.4	201.3 ± 19.2[c]
$[Ca^{2+}]_i$ increment (%)	1	255.3 ± 37.0	145.4 ± 20.4[b]
	2	129.9 ± 17.2[c]	112.8 ± 21.8[c]
	3	140.2 ± 19.2	128.5 ± 32.3
Time for maximal increment (see)[f]	1	15.4 ± 1.8	10.8 ± 0.8[d]
	2	13.9 ± 1.5	11.3 ± 0.8
	3	11.6 ± 0.5	10.5 ± 1.3
Response duration (sec)[g]	1	83.4 ± 8.4	72.3 ± 5.3
	2	53.0 ± 5.2[h]	52.5 ± 6.0[e]
	3	47.1 ± 5.8	48.3 ± 5.2

1, 2, and 3 refer to the nth pulse of TRH administered. Measurements were carried out on LD (n = 53) and HD (n = 62) melanotrope cells from at least four separate experiments. Significance was determined by Mann-Whitney rank sum test.

[a] $P < 0.001$ *vs.* corresponding pulse in HD cells.
[b] $P < 0.01$ *vs.* corresponding pulse in HD cells.
[c] $P < 0.05$ *vs.* preceding pulse for TRH in each parameter.
[d] $P < 0.05$ *vs.* corresponding pulse in HD cells.
[e] $P < 0.001$ *vs.* preceding pulse for TRH in each parameter.
[f] Time required to achieve the maximal increase in $[Ca^{2+}]_i$ after TRH administration.
[g] Time comprised between the administration of TRH and the recovering of $[Ca^{2+}]_i$ basal levels.
[h] $P < 0.01$ *vs.* preceding pulse for TRH in each parameter.

respectively) (35). Densitometric quantification of POMC mRNA levels showed that the mRNA content was 1.8-fold higher in LD cells than in HD cells ($P < 0.01$). Incubation of the cells with TRH for 2 h caused a modest increase in POMC mRNA levels in both HD and LD cells (22.1 ± 9.9% and 26.0 ± 6.4%, respectively); the effect of TRH was significant ($P < 0.05$) only in LD cells (Fig. 5).

Discussion

The present study has demonstrated that the frog pars intermedia is composed of two melanotrope cell subsets that exhibit distinct secretory activities, αMSH acetylation rates, $[Ca^{2+}]_i$ patterns, and POMC mRNA contents.

Functional heterogeneity of melanotrope cell subpopulations under basal conditions

Separation of pituitary cells by density gradients has previously demonstrated the existence of cell subpopulations exhibiting differential secretory activity. For instance, in aging rats (43) and prepubertal pigs (11), HD somatrope cells were found to secrete higher amounts of GH than LD cells. Reverse hemolytic plaque assay and immunoblotting techniques have also shown that individual pituitary cells belonging to the same phenotype may differ in their secretory activity (2, 8, 44). The present study indicates that the amount of αMSH secreted by LD melanotrope cells during a 2-h incubation is 5 times higher than that secreted by HD cells. In agreement with these data, studies conducted on rat lactotrope cells (which exhibit a number of functional similarities with amphibian melanotrope cells) have shown that LD cells secrete more PRL than HD cells (9).

It is well established that the N-terminal acetylation of αMSH is an important posttranslational event that determines the biological activity of the peptide (21). As in amphibians, acetylation of αMSH occurs just before or during the exocytotic process (22, 23), we have investigated the acetylation activity in each melanotrope cell subpopulation. HPLC analysis of conditioned culture medium confirmed that LD cells have a higher secretory rate than HD cells and revealed that HD cells secrete a lower proportion of acetylated forms of αMSH than LD cells. These findings indicate that the two melanotrope cell subsets exhibit distinct secretory patterns; LD cells have a higher secretory and acetylation activity than HD cells. As the amount of biologically active (*i.e.* acetylated) forms of αMSH released by LD cells is 8 times higher than that released by HD cells, our data indicate that LD cells must play a predominant role in the maintenance of skin pigmentation.

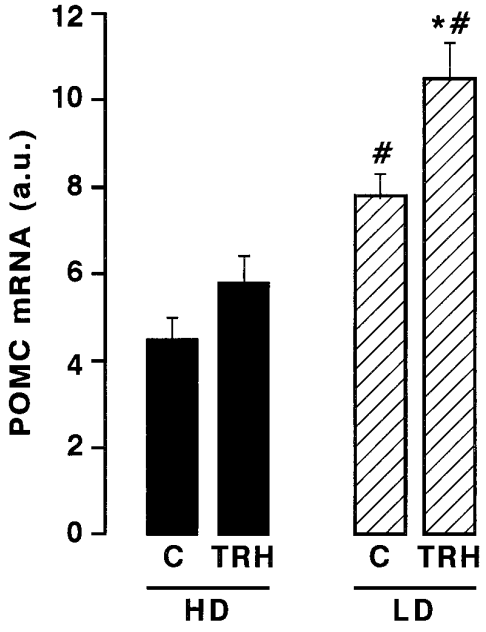

FIG. 5. Quantification of POMC mRNA in cultured frog melanotrope cell subpopulations. This figure shows a representative profile of POMC mRNA content in HD (*solid bars*) and LD (*hatched bars*) cells in the absence (C) or presence of 100 nM TRH for 2 h. The data are expressed in arbitrary units. *, $P < 0.05$ (*vs.* corresponding control); #, $P < 0.001$ (*vs.* HD cells; both determined by Mann-Whitney rank sum test).

The relationship between $[Ca^{2+}]_i$ and secretory activity of melanotrope cells is well documented in mammals (45) and amphibians (46, 47). We thus compared the resting $[Ca^{2+}]_i$ in the two subpopulations of frog melanotrope cells. Our data revealed that $[Ca^{2+}]_i$ was higher in LD than in HD cells, indicating that the elevated secretory rate of the LD cell subset can be accounted for by a high basal $[Ca^{2+}]_i$. Studies conducted in *Xenopus laevis* melanotrope cells have shown that the N-type calcium channel blocker ω-conotoxin inhibits spontaneous Ca^{2+} oscillations, whereas the L-type calcium channel blocker nifedipine has no effect (48), indicating that N-type voltage-operated Ca^{2+} channels are responsible for maintenance of basal $[Ca^{2+}]_i$. In frog melanotrope cells, both N- and L-type Ca^{2+} channels have been characterized (49, 50). Whether the higher $[Ca^{2+}]_i$ observed in LD cells can be ascribed to an increased density in N-type Ca^{2+} channels awaits further investigations.

A number of studies have demonstrated a correlation between hormone secretion and gene transcription in endocrine cells. For instance, in rats, LD lactotrope cells exhibit both a high rate of PRL secretion and a high concentration of PRL mRNA (9). Consistent with this concept, the present study revealed that frog LD melanotrope cells contain a higher amount of POMC transcripts than HD cells. We have previously shown that LD melanotrope cells are less granulated and contain a lower concentration of αMSH than HD cells (35). Taken together, these observations suggest that LD cells display a high transcriptional activity, which contributes to replenish their POMC store and compensate their elevated secretory rate. In contrast, HD melanotrope cells, with a lower secretory capacity, would store the synthesized hormone in secretory granules and release it at a much lower rate.

Effect of TRH on the melanotrope cell subpopulations

It has previously been shown that TRH is a potent αMSH-releasing factor in frogs (29, 30, 51, 52) and toads (31). We thus compared the responses of the two melanotrope cell subsets to TRH. The present data indicate that TRH was more potent in stimulating αMSH release from LD than HD cells. These data are consistent with other reports showing variations in the responsiveness of rat lactotrope cells to TRH (53–55).

In amphibians, acetylation of αMSH is a regulated process that can be modulated by dopamine (26, 27). In fish, TRH administration specifically enhances the release of diacetyl αMSH (56). Paradoxically, the effect of TRH on acetylation of αMSH has never been investigated in amphibians. The present study demonstrated that TRH causes an increase in the rate of acetylation of αMSH in HD cells without affecting the proportion of acetylated αMSH in LD cells. These data suggest that TRH stimulation causes recruitment of the quiescent HD cells that are stimulated to produce predominantly the biologically active, *i.e.* acetylated form, of αMSH.

The mechanism of action of TRH has been investigated in detail in GH_3 cells (57–59). Activation of TRH receptors causes stimulation of phospholipase C, leading to calcium mobilization from intracellular stores. In amphibian melanotrope cells, TRH also activates polyphosphoinositide metabolism (32). We now show that TRH produces a marked increase in $[Ca^{2+}]_i$ in the two melanotrope cell subpopulations. Repeated administration of TRH provoked an attenuation of the response, which can be ascribed to conversion of the receptor from a high to a low affinity state (60) probably due to receptor phosphorylation (61). Interestingly, the maximum $[Ca^{2+}]_i$ evoked by TRH activation was observed in LD cells. It has been shown that in rat melanotrope cells, a certain $[Ca^{2+}]_i$ threshold is required to trigger exocytosis (45). Therefore, the greater responsiveness of LD melanotrope cells to TRH may be accounted for at least in part by the higher efficacy of TRH to induce calcium mobilization in the LD cell subset.

Finally, we found that TRH caused a significant increase in POMC mRNA content in LD cells, but not in HD cells. The ability of TRH to activate POMC gene transcription is consonant with the more pronounced effect of TRH on the secretory capacity of the LD cell subpopulation. In summary, our data indicate that the predominant effect of TRH on αMSH release from frog LD melanotrope cells is associated with a stronger effect on $[Ca^{2+}]_i$ elevation and an increase in POMC mRNA level in the LD cell subpopulation.

Physiological relevance of the existence of melanotrope cell subpopulations

The phenomenon of cell heterogeneity has been described for various types of pituitary cells (12, 35, 43, 62), but the physiological relevance of this phenomenon has been difficult to interpret due to the complexity of the regulation and the wide range of functions controlled by each endocrine cell type. In amphibians, it is reasonable to assume that the heterogeneity of melanotrope cells must be directly related to

their main physiological role, that is the regulation of skin pigmentation (20). Numerous studies have reported the effects of background color adaptation on the activity of pars intermedia cells in the toad *Xenopus laevis*. In particular, it has been shown that melanotrope cells from black background-adapted animals exhibit a high biosynthetic and secretory activity (63). It has also been reported that in the medium of perifused neurointermediate lobes from dark-adapted toads, the proportion of acetylated αMSH is much higher than that in the medium from white-adapted animals (25). Similarly, the POMC gene is actively expressed during adaptation of *Xenopus laevis* to a black background (64). These observations strongly suggest that LD cells, which exhibit an intense secretory activity, a high acetylation rate, and an elevated POMC mRNA content, are actually responsible for the process of skin color adaptation. In support of this hypothesis, De Rijk *et al.* (65) reported that pars intermedia cells from black-adapted *Xenopus laevis* are scarcely granulated and possess a developed rough endoplasmic reticulum; in other words, they have the same morphological characteristics as *Rana ridibunda* LD cells (35).

The heterogeneity of melanotrope cells in the frog pars intermedia is consistent with the idea of a cell secretory cycle by which the cells may regulate αMSH secretion in a flexible and balanced way. The relative importance of each subpopulation may then depend on the amount of hormone required in a particular physiological state. Therefore, further investigations are needed to elucidate the implication of cell heterogeneity in the process of skin color adaptation.

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