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Neuropeptide Y Inhibits Spontaneous α-Melanocyte-Stimulating Hormone (α-MSH) Release via a Y_5 Receptor and Suppresses Thyrotropin-Releasing Hormone-Induced α-MSH Secretion via a Y_1 Receptor in Frog Melanotrope Cells

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In amphibians, the secretion of α-MSH by melanotrope cells is stimulated by TRH and inhibited by NPY. We have previously shown that NPY abrogates the stimulatory effect of TRH on α-MSH secretion. The aim of the present study was to characterize the receptor subtypes mediating the action of NPY and to investigate the intracellular mechanisms involved in the inhibitory effect of NPY on basal and TRH-induced α-MSH secretion. Y_1 and Y_5 receptor mRNAs were detected by RT-PCR and visualized by *in situ* hybridization histochemistry in the intermediate lobe of the pituitary. Various NPY analogs inhibited in a dose-dependent manner the spontaneous secretion of α-MSH from perifused frog neurointermediate lobes with the following order of potency porcine peptide YY (pPYY) > frog NPY (fNPY) > porcine NPY (pNPY)-2–36) > pNPY-(13–36) > [D-Trp^{32}]pNPY > [Leu^{31},Pro^{34}]pNPY. The stimulatory effect of TRH (10^{-8} M) on α-MSH release was inhibited by fNPY, pPYY, and [Leu^{31},Pro^{34}]pNPY, but not by pNPY-(13–36) and [D-Trp^{32}]*p*NPY. These data indicate that the inhibitory effect of fNPY on spontaneous α-MSH release is preferentially mediated through Y_5 receptors, whereas the suppression of TRH-induced α-MSH secretion by fNPY probably involves Y_1 receptors. Pretreatment of neurointermediate lobes with pertussis toxin (PTX; 1 μg/ml; 12 h) did not abolish the inhibitory effect of fNPY on cAMP formation and spontaneous α-MSH release, but restored the stimulatory effect of TRH on α-MSH secretion, indicating that the adenylyl cyclase pathway is not involved in the action of fNPY on TRH-evoked α-MSH secretion. In the majority of melanotrope cells, TRH induces a sustained and biphasic increase in cytosolic Ca^{2+} concentration. Preincubation of cultured cells with fNPY (10^{-7} M) or ω-conotoxin GVIA (10^{-7} M) suppressed the plateau phase of the Ca^{2+} response induced by TRH. However, although fNPY abrogated TRH-evoked α-MSH secretion, ω-conotoxin did not, showing dissociation between the cytosolic Ca^{2+} concentration increase and the secretory response. Collectively, these data indicate that in frog melanotrope cells NPY inhibits spontaneous α-MSH release and cAMP formation through activation of a Y_5 receptor coupled to PTX-insensitive G protein, whereas NPY suppresses the stimulatory effect of TRH on α-MSH secretion through a Y_1 receptor coupled to a PTX-sensitive G protein-coupled receptor. (*Endocrinology* 143: 1686–1694, 2002)

IN MOST MAMMALIAN and submammalian vertebrates, the secretion of α-MSH is regulated by various neurotransmitters, including dopamine, norepinephrine, γ-aminobutyric acid, and acetylcholine (1). In amphibians, the secretory activity of the pars intermedia is also controlled by several neuropeptides (2–4). In particular, the neurointermediate lobe of the frog pituitary is innervated by a dense network of fibers containing TRH (5–7) and NPY (8–12). *In vitro* studies conducted in *Rana ridibunda* and *Xenopus laevis* have shown that TRH is a potent stimulator of α-MSH secretion (6, 13, 14) and that NPY acts as an α-MSH release-inhibiting factor (8, 10), suggesting that in amphibians TRH and NPY may play a pivotal role in the process of skin color adaptation.

The effects of TRH and NPY are both mediated through activation of G protein-coupled receptors. To date, two TRH receptor subtypes and at least five NPY receptor subtypes have been identified in vertebrates (15–21). The various NPY receptor variants can be distinguished on the basis of their differential responses to NPY analogs. The Y_1 receptor subtype is activated by [Leu^{31},Pro^{34}]NPY and has low affinity for NPY-(13–36). Conversely, the Y_2 receptor subtype is activated by C-terminal fragments of NPY and does not bind [Leu^{31},Pro^{34}]NPY. The Y_3 receptor, in contrast to all other NPY receptor subtypes, exhibits very low affinity for peptide YY (PYY). The Y_4 receptor binds pancreatic polypeptide and [Leu^{31},Pro^{34}]NPY. The Y_5 receptor is activated by [Leu^{31},Pro^{34}]NPY, N-terminally truncated fragments of NPY [NPY-(2–36), NPY-(13–36), *etc.*], and [D-Trp^{32}]NPY. The

Abbreviations: $[Ca^{2+}]_i$, Cytosolic Ca^{2+} concentration; fNPY, frog NPY; pNPY, porcine NPY; pPYY, porcine peptide YY; NIL, neurointermediate lobe; PTX, pertussis toxin; SSC, saline sodium citrate; ω-CgTx, ω-conotoxin GVIA.

pharmacological profile of the y_6 receptor has not yet been precisely determined and might be species specific (for review, see Refs. 19–22).

In normal and tumoral pituitary cells, TRH stimulates the PLC/PKC pathway and activates calcium membrane channels, leading to an increase in the cytosolic calcium concentration ($[Ca^{2+}]_i$) (23, 24). Activation of TRH receptors has been reported to also stimulate adenylyl cyclase (25, 26), PLA_2 (27, 28), protein tyrosine kinase (29), Ca^{2+}/calmodulin-dependent protein kinase II (30, 31), and MAPK (32, 33). In almost every cell type studied, NPY receptors are negatively coupled to adenylyl cyclase via pertussis toxin-sensitive G proteins (for review, see Ref. 20). In certain cell types, additional signaling responses to NPY include stimulation or inhibition of $[Ca^{2+}]_i$ (34–38) and activation of PLA_2 (39), PKC (40), protein tyrosine kinase (41–43), and MAPK (40, 44).

The intermediate lobe of the frog pituitary, which is composed of a homogeneous population of endocrine cells, represents a valuable alternative model in which to investigate the transduction pathways activated by TRH and NPY in normal cells. The aim of the present study was to determine the receptor subtypes mediating the action of NPY on frog melanotrope cells and to investigate the intracellular mechanisms involved in the inhibitory effect of NPY on basal and TRH-induced α-MSH secretion.

Materials and Methods

Animals

Adult male frogs (*R. ridibunda*; 40–50 g) originating from Bulgaria were purchased from a commercial supplier (Couétard, St. Hilaire de Riez, France). The animals were housed in a temperature-controlled room (8 ± 0.5 C) under running water on a 12-h dark/12-h light regimen (lights on from 0600–1800 h). The frogs were killed by decapitation, and the neurointermediate lobes (NILs) were dissected under a microscope. Animal manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators.

Reagents and test substances

SuperScript II reverse transcriptase RNase H^- and kanamycin were supplied by Life Technologies, Inc. (Cergy Pontoise, France). The *Taq* DNA polymerase was purchased from Promega Corp. (Charbonnières France). Synthetic frog NPY (fNPY), porcine PYY (pPYY), [Leu^{31},Pro^{34}]pNPY, pNPY-(2–36), and pNPY-(13–36) were prepared using the solid phase methodology as previously described (45). [D-Trp^{32}]pNPY was purchased from Phoenix Pharmaceuticals, Inc. (Mountain View, CA). TRH, ω-conotoxin GVIA (ω-CgTx), pertussis toxin (PTX), Leibovitz culture medium (L15), and collagenase (type IA) were obtained from Sigma (St. Louis, MO). BSA (fraction V) was purchased from Roche (Mannheim, Germany). FBS and the antibiotic-antimycotic solution were obtained from BioWhittaker, Inc. (Gagny, France). Indo-1/acetoxymethylester (indo-1/AM) was obtained from Molecular Probes, Inc. (Leiden, The Netherlands). Bio-Gel P2 was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA). All other chemicals were obtained from Sigma.

RT-PCR

Total RNA was purified from NILs by the acid guanidinium-thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (46) using Tri-Reagent. Approximately 5 μg RNA were reverse transcribed using an oligo(deoxythymidine)$_{12–18}$ primer and SuperScript II reverse transcriptase RNase H^- in the buffer supplied with the enzyme. PCR amplification was performed in a 50-μl volume containing 2 μl reverse transcribed RNA solution, 200 μM dNTPs, 1 mM $MgCl_2$, 1 U *Taq* DNA polymerase, and 20 pmol sense and antisense primers specific for each *R. ridibunda* NPY receptor subtype (Y_1 forward, 5′-TGG ATT TTT GGA GTT GGT ATG TGT A-3′; Y_1 reverse, 5′-AAC GGC AAT GAG AAC CAG TGA GAA A-3′; Y_2 forward, 5′-TAT GCG GAC GGT GAC GAA CTA-3′; Y_2 reverse, 5′-CCA CCA TCA TCA CCA ACA TCT-3′; Y_5 forward, 5′-CAT ATT GCC CTG TCC TGT TTA-3′; Y_5 reverse, 5′-AGA CCG AAT TCA TGT TGC TCA-3′; y_6 forward, 5′-ACC GTG TGC AAA CTC GCT TCC-3′; y_6 reverse, 5′-CTT GCA TTT CCT CAC TTC CTG TCT-3′) in 5 μl of the buffer (pH 9.0) supplied with the enzyme for 40 cycles (40 sec at 94 C, 1 min at 50 C, and 1 min and 30 sec at 72 C) in a Robocycler Gradient 40 (Stratagene, La Jolla, CA). The PCR products were separated on a 2% agarose gel and transferred onto Hybond-N membrane (Amersham Pharmacia Biotech, Les Ulis, France). The membranes were prehybridized for 4 h at 42 C in a solution containing 5× saline sodium citrate (SSC), 0.1× SDS, 10× Denhardt's solution, and 50 μg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 42 C in a solution containing 5× salt sodium phosphate EDTA buffer and 1× SDS in the presence of the ^{32}P-labeled NPY receptor probe. The membranes were washed twice in 5× salt sodium phosphate EDTA buffer/0.1% SDS at 42 C and exposed on Kodak X-OMAT films (Rochester, NY).

In situ hybridization histochemistry

In situ hybridization was performed as previously described (47). Briefly, adult male frogs were anesthetized and perfused transcardially with 4% paraformaldehyde. Pituitary sections (12 μm thick) were cut on a cryostat and mounted on poly-L-lysine- and gelatin-coated slides. The partial NPY receptor cDNA sequences were subcloned into pGEMT between *Spe*I and *Nco*I sites, and sense and antisense riboprobes were generated with T7 and SP6 polymerases in the presence of [^{35}S]UTP. Sections were incubated for 10 min in 0.1 M triethanolamine/0.9% NaCl (pH 8.0)/0.25 acetic anhydride, rinsed in 2× SSC, and covered for 60 min with prehybridization buffer (pH 7.5) containing 50% formamide, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% BSA, 1 mM EDTA (pH 8.0), 550 μg/ml denatured salmon sperm DNA, and 50 μg/ml yeast tRNA. Hybridization was performed overnight at 40 C for the Y_1 receptor and at 50 C for the Y_2, Y_5, and y_6 receptors in the same buffer (except for salmon sperm DNA, the concentration of which was lowered to 60 μg/ml) supplemented with 10 mM dithiothreitol, 10% dextran sulfate, and 1.5×10^7 cpm/ml heat-denatured RNA riboprobes. Slices were then washed in 2× SSC at 50 C and treated with ribonuclease A (50 μg/ml) for 60 min at 37 C. Five final high stringency washes were performed in 0.01× SSC containing 14 mM β-mercaptoethanol and 0.05% sodium pyrophosphate. The tissue sections were dehydrated in ethanol, dipped into Kodak NTB2 liquid emulsion at 40 C, exposed for 2 months, and developed. To identify anatomical structures, the sections were stained with hematoxylin and observed with a Nikon Eclipse 600 microscope (Les Ulis, France) equipped for epifluorescence with an oil immersion objective (×60).

Cell culture

NILs were collected in Ca^{2+}-free Ringer's solution (15 mM HEPES buffer, 112 mM NaCl, 2 mM KCl, and 1 mM EGTA) supplemented with 2 mg glucose/ml, 0.3 mg BSA/ml, and 1% each of the kanamycin and antibiotic-antimycotic solutions. The Ringer's solution was gassed for 15 min with O_2/CO_2 (95:5; vol/vol) before use, and the pH was adjusted to 7.35. Ten NILs were enzymatically dispersed at 24 C for 20 min with a solution of collagenase (1.5 mg/ml) in Ca^{2+}-free Ringer's solution as previously described (48). Nondissociated neural lobes were removed by sedimentation, and the supernatant containing disaggregated intermediate lobes was collected. The suspension was centrifuged (30 × *g*, 5 min) and rinsed three times with Ca^{2+}-free Ringer's medium. The digested tissues were resuspended in L15 medium adjusted to *R. ridibunda* osmolality (L15-water = 1:0.4) and supplemented with 0.2 mg glucose/ml, 0.063 mg $CaCl_2$/ml, and 1% of the kanamycin and antibiotic-antimycotic solutions (f-L15; pH 7.35). The cells were dispersed by gentle aspiration through a siliconized Pasteur pipette with a flame-polished tip. Finally, cells were plated on poly-L-lysine-coated glass coverslips (30 mm diameter) at a density of 15,000 cells/coverslip in 35-mm culture dishes. When the cells had settled, coverslips were covered with 2 ml culture medium composed of f-L15 supplemented with

10% FBS. Cultured cells were kept at 24 C in a humidified atmosphere, and the culture medium was renewed every 48 h. Microfluorometric measurements were performed on 3- to 5-d-old cultured cells.

Calcium measurement

Cultured cells were incubated at 24 C for 30 min in the dark with 5 μM of the fluorescent calcium probe indo-1/AM in Ringer's solution (15 mM HEPES buffer, 112 mM NaCl, 2 mM KCl, and 2 mM $CaCl_2$) supplemented with 2 mg glucose/ml and 0.3 mg BSA/ml. At the end of the incubation period, the cells were washed twice with 2 ml fresh medium and placed on the stage of a Nikon Diaphot inverted microscope equipped for epifluorescence with an oil immersion objective (×100 CF Fluor series; numerical aperture, 1.3). $[Ca^{2+}]_i$ was monitored by a dual emission microfluorometer system, as previously described (49). Briefly, the fluorescence emission of indo-1/AM induced by excitation at 355 nm (xenon lamp) was recorded at two wavelengths (405 nm, corresponding to the Ca^{2+}-complexed form, and 480 nm, corresponding to the free form), by separate photometers (P1, Nikon). The 405/480 ratio (R) was determined using an AS1-type acquisition card (Notocord Systems, Croissy-sur-Seine, France). All three signals (405 nm, 480 nm, and the 405/480 nm ratio) were continuously recorded with the JAD-FLUO program (version 1.2). $[Ca^{2+}]_i$ was calculated according to the formula established by Grynkiewicz *et al.* (50): $[Ca^{2+}]_i = K_d \times \beta(R - R_{min})(R_{max} - R)$, where R_{min} is the minimum fluorescence ratio obtained after incubation of cells in Ringer's solution containing 10 mM EGTA and 10 μM ionomycin, R_{max} is the maximum fluorescence ratio obtained after incubation of cells in Ringer's solution containing 10 mM $CaCl_2$ and 10 μM ionomycin, and β is the ratio of fluorescence yields from the Ca^{2+}min/Ca^{2+}max indicator at 480 nm. The values for R_{min}, R_{max}, and β were 0.164, 1.82, and 1.62, respectively. The dissociation constant for indo-1 (K_d) was previously determined (250 nM) (51, 52). A pressure ejection system was used to deliver the test substances in the vicinity of cultured cells.

Results are expressed as the mean amplitude of $[Ca^{2+}]_i$ increase (±SEM). A *t* test was used for statistical analysis.

Perifusion experiments

The perifusion technique used to determine the effects of test substances on α-MSH release has been previously described in detail (53). For each experiment, four NILs were mixed with preswollen Bio-Gel P-2 beads and transferred into a plastic column (0.9 cm inner diameter). The tissues were perifused with Ringer's solution at constant flow rate (0.3 ml/min) and temperature (24 C). The effluent medium was collected as 2.5-min fractions during the infusion of the secretagogues and 7.5-min fractions during stabilization periods. The collected samples were immediately chilled at 4 C, and the concentration of α-MSH was measured in each fraction on the same day as the perifusion experiment using a double-antibody RIA procedure (54). The perifusion profiles were calculated and expressed as a percentage of the basal secretory level. All experiments were carried out at least three times. The basal level of α-MSH was calculated as the mean of five consecutive fractions (37.5 min) collected just before the infusion of each secretagogue. To compare the net increase in α-MSH secretion induced by TRH in control conditions and under various treatments, the areas under the curve were calculated using the trapezoidal rule (55). A two-tailed paired *t* test was used to compare the mean secretory responses within the same set of experiments.

cAMP measurement

Whole NILs were preincubated for 12 h at 24 C in f-L15 in the absence or presence of PTX (1 μg/ml). NILs were then treated for 30 min with 0.1 mM isobutylmethylxanthine and incubated for 30 min in 0.5 ml Ringer's solution containing 10^{-6} M fNPY and 0.1 mM isobutylmethylxanthine. The reaction was stopped by adding 0.5 ml ice-cold 20% trichloroacetic acid. NILs were homogenized with a glass Potter and centrifuged (10,000 × *g*; 10 min). Trichloroacetic acid was eliminated from the supernatant by three successive rinses with 1 ml water-saturated diethyl ether. After evaporation of the ether phase, the supernatant was dried, and the cAMP content in the extract was measured by RIA following the procedure recommended in the cAMP RIA kit (Amersham Pharmacia Biotech).

Results

Determination and distribution of NPY receptor subtypes in the frog NIL

RT-PCR analysis was carried out to determine which NPY receptor mRNAs are expressed in the frog NIL. Oligonucleotides were designed to amplify fragments of 93, 581, 422, and 434 bp corresponding, respectively, to the *R. ridibunda* Y_1, Y_2, Y_5, and y_6 receptor subtypes recently cloned by us (unpublished). Y_1 and Y_5 receptor mRNA fragments of the expected lengths were amplified by RT-PCR (Fig. 1), whereas no Y_2 or y_6 receptor mRNA signal could be detected (data not shown). The identities of the fragments were confirmed by Southern blot analysis using an internal probe specific for each receptor subtype (Fig. 1). When reverse transcriptase was omitted, no amplification product was observed (Fig. 1).

The distribution of NPY receptor mRNAs in the frog NIL was investigated by *in situ* hybridization histochemistry. Microscopic examination of emulsion-coated slices revealed a high density of Y_1 (Fig. 2A) and Y_5 (Fig. 2C) mRNAs in the intermediate lobe, whereas only background signal was observed in the neural lobe. Control sections incubated with the sense Y_1 and Y_5 probes exhibited only weak background staining (Fig. 2, B and D).

Effect of NPY analogs on α-MSH release from intact frog NILs

Administration of graded concentrations of [D-Trp^{32}]NPY induced a dose-related inhibition of α-MSH release (Fig. 3A). The maximum inhibition (40%) was achieved at a dose of 10^{-6} M [D-Trp^{32}]pNPY. As previously reported (44), fNPY provoked a concentration-dependent inhibition of basal α-MSH release with an ED_{50} of 10^{-7} M (Fig. 3B). Synthetic pPYY was slightly more potent than fNPY in inhibiting α-MSH release with an ED_{50} of 4.8×10^{-8} M. pNPY-(2–36) was equipotent (ED_{50}, 10^{-7} M), but slightly more efficacious

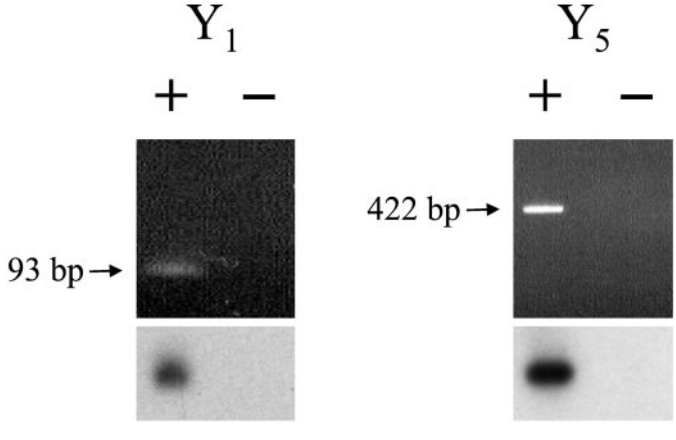

FIG. 1. RT-PCR analysis of NPY receptor mRNAs in the frog NIL. Total RNA was incubated in the presence (+) or absence (−) of reverse transcriptase, and the cDNAs obtained were amplified by PCR using specific Y_1 and Y_5 primers to generate products of 93 and 422 bp, respectively. The identities of the two bands were confirmed by Southern blot analysis with ^{32}P-labeled frog Y_1 or Y_5 receptor riboprobes, as shown under the ethidium bromide-stained gels.

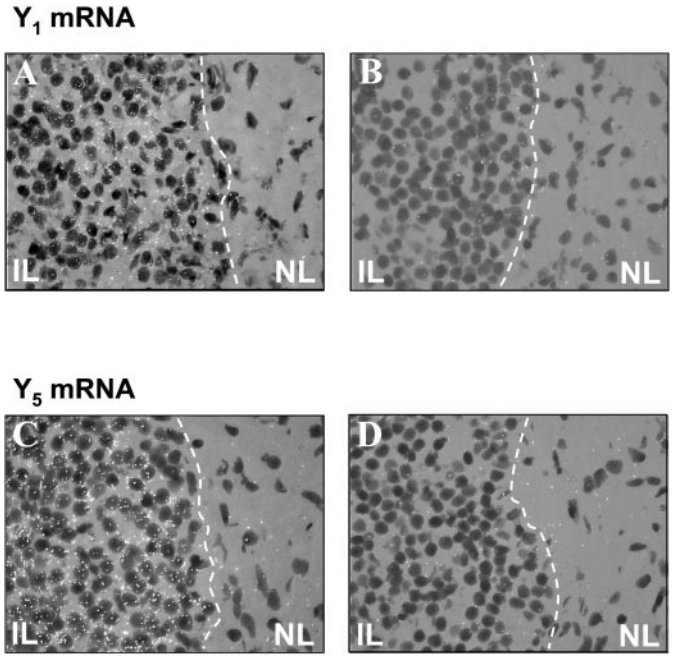

FIG. 2. Photomicrographs illustrating the cellular distribution of NPY receptor mRNAs in the frog NIL. A and B, Hybridization of pituitary sections with the antisense (A) or sense (B) Y_1 riboprobe. C and D, Hybridization of pituitary sections with the antisense (C) or sense (D) Y_5 riboprobe. Intense hybridization signal for Y_1 and Y_5 mRNAs is seen in the intermediate lobe (IL), whereas only background staining is observed in the neural lobe (NL). Hematoxylin counterstaining labeled only the nuclei.

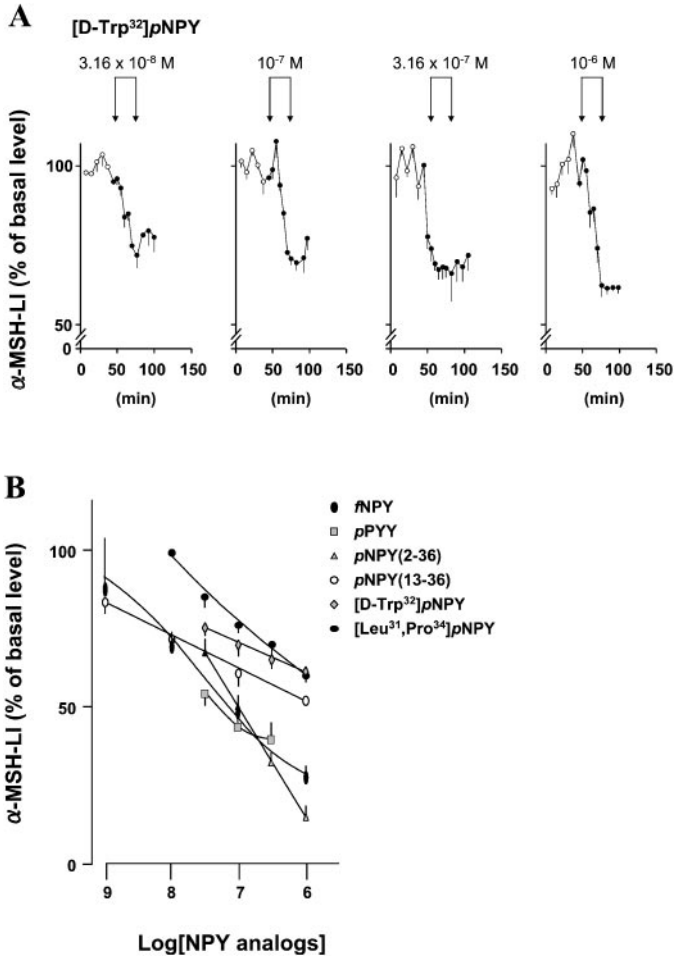

FIG. 3. Effects of NPY analogs on α-MSH release from perifused frog NILs. Effect of graded concentrations of [D-Trp^{32}]pNPY on α-MSH release. After a 45-min equilibration period, [D-Trp^{32}]pNPY was infused for 20 min (*arrows*). Each profile represents the mean ± SEM secretion pattern of four independent perifusion experiments. The spontaneous level of α-MSH release (100% basal level) was calculated as the mean α-MSH concentration in the first five consecutive fractions (○; 37.5 min). B, Semilogarithmic plot comparing the effects of the various NPY analogs on α-MSH release. Experimental values were calculated from data similar to those shown in A. Each *point* represents the maximum amplitude of inhibition of α-MSH release induced by NPY and its analogs. The mean basal level of α-MSH release in these experiments was 112 ± 16 pg/min·NIL.

than fNPY, yielding to an inhibition of 83 ± 4% at a concentration of 10^{-6} M. pNPY-(13–36) was less potent and only induced a 47 ± 2% inhibition of α-MSH release at a concentration of 10^{-6} M. At a concentration of 10^{-6} M, [Leu^{31},Pro^{34}]pNPY only induced a 40% inhibition of α-MSH release (Fig. 3B).

Effects of NPY analogs on TRH-induced α-MSH secretion from intact frog NILs

Prolonged infusion of fNPY (3.16×10^{-7} M; 120 min) provoked a marked and sustained decrease in α-MSH release and totally suppressed TRH (10^{-8} M; 10 min)-induced α-MSH secretion (Fig. 4, A and B). pPYY (10^{-6} M) and [Leu^{31},Pro^{34}]pNPY (10^{-6} M) mimicked the inhibitory effect of fNPY on basal and TRH-evoked α-MSH secretion (Fig. 4, C and D). Administration of the Y_2/Y_5 agonist pNPY-(13–36) (10^{-6} M) or the selective Y_5 agonist [D-Trp^{32}]pNPY (10^{-6} M) inhibited the spontaneous release of α-MSH, but had no significant effect on TRH-induced α-MSH secretion (Fig. 4, E and F).

Effect of PTX pretreatment on NPY-evoked inhibition of the TRH response and cAMP formation

Preincubation of frog NILs with PTX (1 μg/ml; 12 h) did not impair the inhibitory effect of fNPY (3.16×10^{-7} M) on basal α-MSH release, but restored the stimulatory effect of TRH (10^{-8} M; 10 min) on α-MSH secretion during prolonged infusion of fNPY (Fig. 5). Pretreatment with PTX did not affect the TRH-induced stimulation of α-MSH secretion (data not shown).

Incubation of frog NILs with fNPY (10^{-6} M) produced a significant decrease in cAMP content in the tissue ($P < 0.001$; data not shown). Pretreatment of NILs with PTX (1 μg/ml; 12 h) had no effect on the fNPY-induced inhibition of cAMP formation (data not shown).

Effect of NPY on TRH-induced $[Ca^{2+}]_i$ increase

As previously reported (56), TRH (10^{-7} M; 5 sec) provoked two distinct types of calcium responses in cultured frog melanotrope cells. In two thirds of the cells TRH (10^{-7} M; 5 sec) caused a sustained increase in $[Ca^{2+}]_i$, and in one third of the cells TRH only induced a transient increase in $[Ca^{2+}]_i$ (Fig. 6A). Preincubation of the cells with fNPY (10^{-7} M; 10 min)

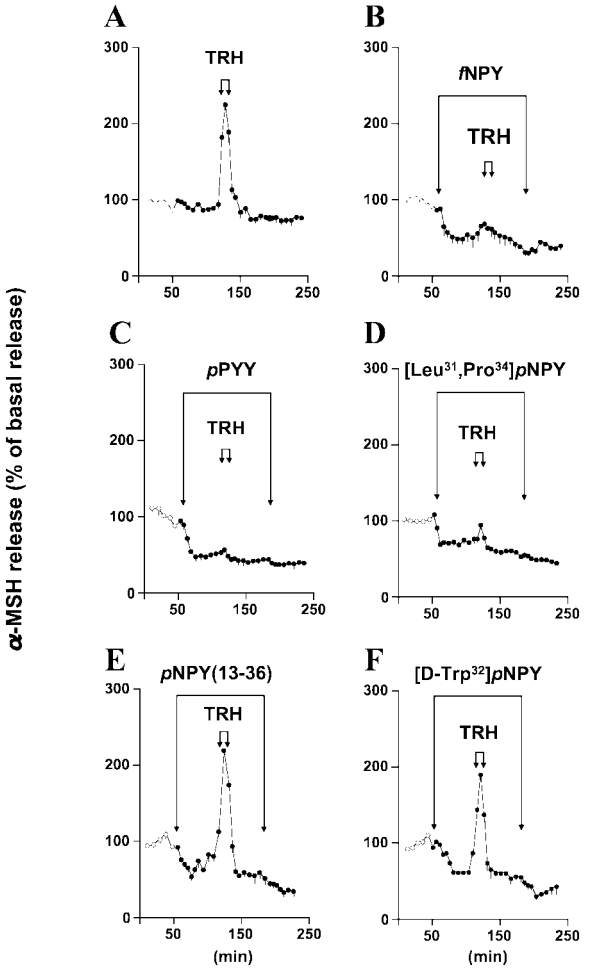

FIG. 4. Effects of NPY analogs on TRH-induced α-MSH secretion from perifused frog NILs. A, Effect of a single pulse of TRH (10^{-8} M; 10 min) on α-MSH secretion (control). B–F, Effect of TRH (10^{-8} M; 10 min) during prolonged (120-min) infusion of fNPY (3.16×10^{-7} M; B), pPYY (10^{-6} M; C), [Leu^{31},Pro^{34}]pNPY (10^{-6} M; D), pNPY-(13–36) (10^{-6} M; E), [D-Trp^{32}]pNPY (10^{-6} M; F). Each profile represents the mean ± SEM secretion pattern of four independent perifusion experiments. The spontaneous level of α-MSH release (100% basal level) was calculated as the mean α-MSH concentration in the first five consecutive fractions (○; 37.5 min). The mean basal level of α-MSH release in these experiments was 102 ± 15 pg/min·NIL.

totally suppressed the plateau phase of the $[Ca^{2+}]_i$ response to TRH (Fig. 6B). Exposure of the cells to ω-CgTx (10^{-7} M; 10 min) also suppressed the sustained phase of TRH-induced $[Ca^{2+}]_i$ increase (Fig. 6C). In addition, both fNPY and ω-CgTx significantly ($P < 0.001$) reduced the amplitude of the transient $[Ca^{2+}]_i$ response to TRH (Fig. 6D). In contrast to fNPY, which suppressed the stimulatory effect of TRH on α-MSH release (Fig. 4B), ω-CgTx (10^{-6} M) had no effect (Fig. 7).

Discussion

It has been previously shown that TRH stimulates, while NPY inhibits, α-MSH secretion from amphibian melanotrope cells (6, 8, 10, 13, 14). The present report now demonstrates that the inhibitory effects of NPY on spontaneous and TRH-evoked α-MSH secretion are mediated through two distinct receptors.

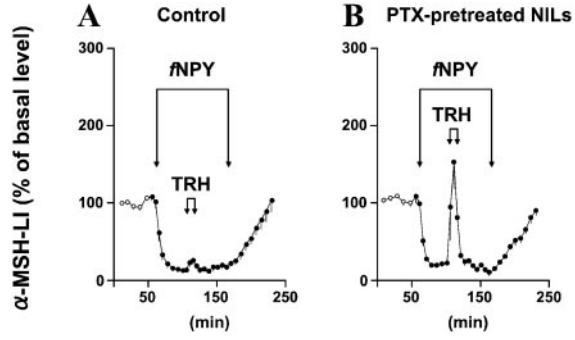

FIG. 5. Effect of PTX pretreatment on the response of perifused frog NILs to TRH (10^{-8} M; 10 min) during prolonged infusion of fNPY (3.16×10^{-7} M; 120 min). Frog NILs were preincubated at 24 C for 12 h in the absence (A) or presence (B) of PTX (1 μg/ml) before the perifusion experiment. The mean basal level of α-MSH release in these experiments was 85 ± 14 pg/min·NIL. See Fig. 4 for other details.

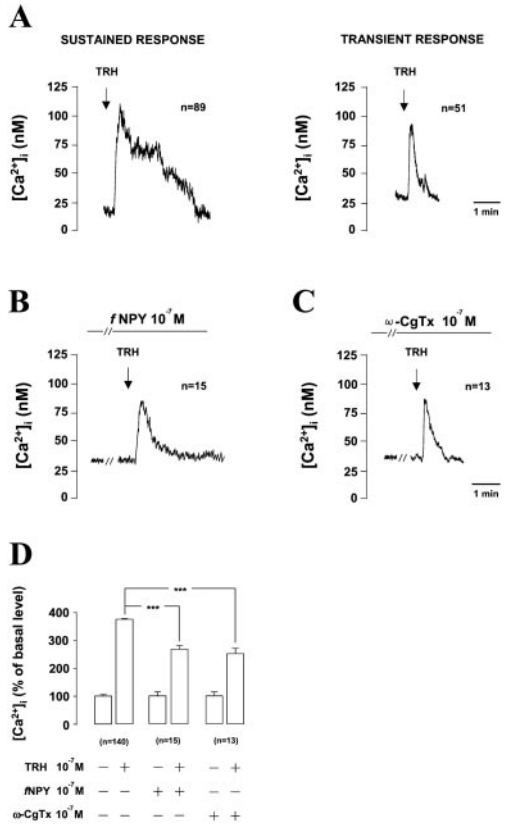

FIG. 6. Effects of TRH, fNPY, and ω-CgTx on intracellular calcium concentrations ($[Ca^{2+}]_i$) in cultured frog melanotrope cells. A, Typical profiles illustrating the two types of Ca^{2+} responses that were observed after administration of a single pulse of TRH (10^{-7} M; 5 sec) to melanotrope cells. B, Typical profile illustrating the effect of fNPY (10^{-7} M; 10 min) on the TRH-induced $[Ca^{2+}]_i$ increase. C, Typical profile illustrating the effect ω-CgTx (10^{-7} M; 10 min) on the TRH-induced $[Ca^{2+}]_i$ increase. *Arrows* indicate the onset of TRH administration. D, Histograms showing the mean effects of fNPY and ω-CgTx on the amplitude of the $[Ca^{2+}]_i$ transient induced by TRH. ***, $P < 0.001$.

Expression of NPY receptors in frog melanotrope cells

The effects of NPY are mediated through at least five types of seven-membrane-spanning domain G protein-coupled re-

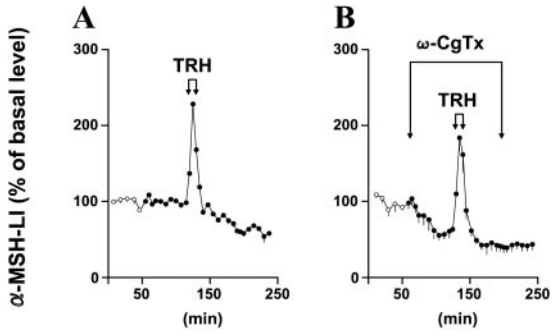

FIG. 7. Effect of ω-CgTx on TRH-induced α-MSH secretion from perifused frog NILs. A single pulse of TRH (10^{-8} M; 10 min) was administered in control conditions (A) or 45 min after the onset of ω-CgTx (10^{-6} M; B). The mean basal level of α-MSH in these experiments was 121 ± 12 pg/min·NIL. See Fig. 4 for other details.

ceptors, termed Y_1, Y_2, Y_4, Y_5, and y_6 (for review, see Refs. 19–21). Using an RT-PCR strategy, we recently determined partial sequences of the Y_1, Y_2, Y_5, and y_6 subtypes in the frog *R. ridibunda* (unpublished data). In the present study we took advantage of the availability of these nucleotide sequences to investigate which receptor isoforms are expressed in frog melanotrope cells.

RT-PCR amplification revealed that both Y_1 and Y_5 receptor mRNAs, but not Y_2 and y_6 transcripts, are present in the frog NIL. Southern blot analysis confirmed that the PCR products corresponded to Y_1 and Y_5 cDNAs. As the RT-PCR reaction was conducted on RNA from whole NILs, *in situ* hybridization experiments were performed to determine the precise location of each receptor transcript. Both the Y_1 and Y_5 riboprobes produced intense hybridization signal over the intermediate lobe, whereas the neural lobe was totally negative. In the same conditions no hybridization signal was detected with the Y_2 and y_6 probes. These observations clearly indicate that the Y_1 and Y_5, but not the Y_2 and y_6, receptor isoforms are expressed in frog melanotrope cells.

A Y_1 receptor has been previously cloned in the toad *X. laevis* using a hypothalamus cDNA library (57), but to our knowledge the occurrence of Y_1 mRNA has not been described in the pars intermedia of the pituitary. In mammals, the presence of Y_1 mRNA has been detected in the anterior lobe, but not in the posterior lobe, of the pituitary (58). Thus, the present report provides the first molecular characterization of NPY receptor isoforms in the pars intermedia of the pituitary.

Two distinct receptors mediate the inhibitory effect of NPY on basal and TRH-evoked α-MSH secretion

In amphibians, NPY is a highly potent α-MSH release-inhibiting factor (8, 10, 45). We have previously shown that in the frog *R. ridibunda*, NPY inhibits both basal and TRH-evoked α-MSH secretion (8, 45, 59). The present study revealed that PTX pretreatment did not impair the inhibitory action of NPY on spontaneous α-MSH release, but virtually abolished the inhibitory effect of NPY on TRH-induced α-MSH secretion, indicating that two distinct receptors are involved in the inhibitory effects of NPY on frog melanotrope cells.

Various NPY analogs were used to determine the receptor subtype that mediates the inhibitory effect of NPY on basal α-MSH release. The observation that the Y_2/Y_5 agonist NPY-(13–36) (19, 20) depressed α-MSH release from perifused frog NILs indicated that Y_1 receptors are not involved in the inhibitory effect of NPY. Concurrently, the fact that the selective $Y_1/Y_4/Y_5$ agonist [Leu^{31},Pro^{34}]NPY (19, 20) mimicked the inhibitory effect of NPY revealed that the action of the peptide on spontaneous α-MSH release is not mediated through Y_2 receptors. Consistent with this latter finding, *in situ* hybridization experiments have shown that Y_2 receptor mRNA are not expressed in the frog pars intermedia. The possible involvement of Y_3 receptors was excluded, inasmuch as PYY, which activates all NPY receptor subtypes, except for the Y_3 isoform (19, 20), decreased spontaneous α-MSH release. The observation that C-terminal fragments of NPY, which do not bind Y_4 receptors (19, 20), inhibited the basal secretory activity indicated that a Y_4 receptor is not implicated in the action of NPY on spontaneous α-MSH release. In contrast, the fact that the specific Y_5 agonist [D-Trp^{32}]NPY (22) inhibited α-MSH release suggested that the effect of NPY on basal secretory activity of melanotrope cells is preferentially mediated through the Y_5 receptor subtype. In support of this hypothesis, RT-PCR analysis and *in situ* hybridization labeling revealed that the Y_5 receptor gene is actively expressed in the frog intermediate lobe.

The same type of pharmacological approach was used to characterize the NPY receptor isoform responsible for inhibition of TRH-induced α-MSH secretion. The data showed that PYY and [Leu^{31},Pro^{34}]NPY mimicked the inhibitory action of NPY. In contrast, NPY-(13–36) and [D-Trp^{32}]NPY, although reducing the basal secretory activity, did not affect TRH-induced α-MSH secretion. Taken together, these observations suggest that NPY probably suppresses the stimulatory effect of TRH on α-MSH release through activation of a Y_1 receptor subtype.

Transduction mechanisms implicated in the inhibitory effect of NPY on basal and TRH-evoked α-MSH secretion

In mammalian cells the different NPY receptor subtypes are negatively coupled to adenylyl cyclase through a PTX-sensitive G protein-coupled receptor (for review, see Refs. 19 and 20). In frog melanotrope cells, PTX pretreatment did not impair the inhibitory effect of NPY on cAMP formation and basal α-MSH release, but PTX suppressed the action of NPY on TRH-evoked α-MSH secretion. These data indicate that the inhibitory effect of NPY on the spontaneous release of α-MSH probably involves a PTX-insensitive G_z protein (60, 61) and that NPY-induced inhibition of the secretory response of melanotrope cells to TRH is not mediated through the adenylyl cyclase system. Consistent with this concept, we have previously shown that the adenylyl cyclase/PKA pathway is not involved in TRH-induced α-MSH secretion (62). In GH_3 cells, activation of MAPK by tyrosine phosphorylation plays a crucial role in TRH-induced PRL release (29, 32). In addition, in rat lactotrophs, dopamine inhibits the stimulatory effect of TRH on PRL release and MAPK activity through a PTX-sensitive G protein-coupled receptor (28, 63). The fact that in frog melanotrope cells tyrosine kinase inhibitors abrogate TRH-induced α-MSH secretion indirectly

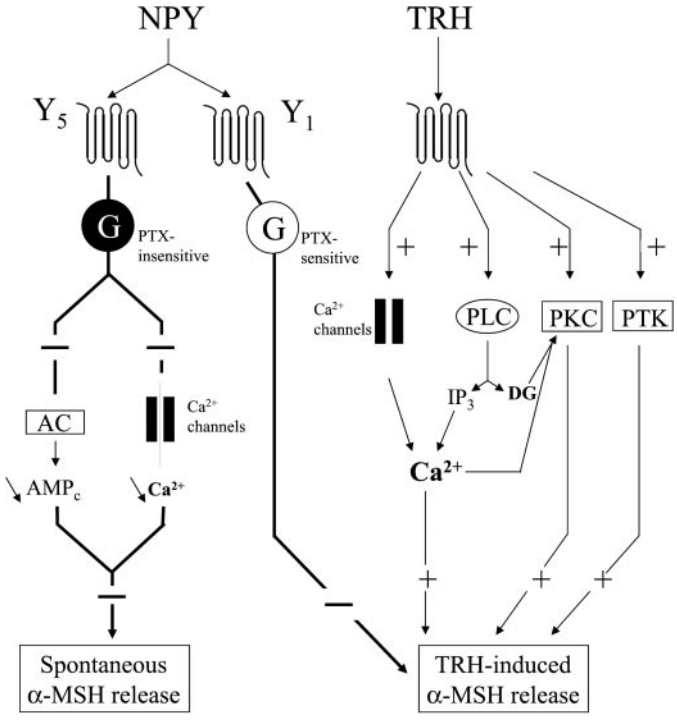

FIG. 8. Schematic representation summarizing the intracellular events associated with the effect of NPY on basal and TRH-induced α-MSH secretion in frog melanotrope cells. It has been previously shown that TRH stimulates α-MSH release by increasing $[Ca^{2+}]_i$ and by activating PKC and protein tyrosine kinase (PTK) (62). NPY acting through a Y_5 receptor subtype coupled to a PTX-insensitive G protein causes inhibition of adenylyl cyclase and calcium channels, leading to a decrease in spontaneous α-MSH release. Concurrently, NPY acting through a Y_1 receptor coupled to a PTX-sensitive G protein suppresses TRH-induced α-MSH secretion.

suggests that the inhibitory effect of NPY on the secretory response to TRH is mediated through a tyrosine kinase-dependent pathway.

We have previously shown that in cultured melanotrope cells TRH induces two types of $[Ca^{2+}]_i$ responses: in about two thirds of the cells, TRH causes a sustained and biphasic increase in $[Ca^{2+}]_i$, whereas in one third of the cells, TRH induces only a transient response (56). The differential profile of the Ca^{2+} responses may be ascribed to the existence of two subtypes of melanotrope cells (64, 65). Exposure of melanotrope cells to NPY abolished the sustained phase of the Ca^{2+} response evoked by TRH, and this effect was mimicked by ω-CgTx, suggesting that the inhibitory effect of NPY on TRH-evoked $[Ca^{2+}]_i$ is mediated through modulation of N-type Ca^{2+} channels. In support of this hypothesis, it has already been shown that in frog and toad melanotrope cells, NPY inhibits N-type calcium channels (66, 67). It should be noted, however, that ω-CgTx, in contrast to NPY, did not affect TRH-induced α-MSH secretion. These data indicate that NPY makes melanotrope cells insensitive to an increase in $[Ca^{2+}]_i$ that otherwise is sufficient to trigger α-MSH release. Similarly, a single administration of dopamine or repeated pulses of TRH make rat anterior pituitary cells insensitive to the increase in $[Ca^{2+}]_i$ (68, 69). In frog melanotrope cells, the dissociation between changes in $[Ca^{2+}]_i$ and α-MSH secretion suggests that NPY blocks a final and crucial step of the exocytotic process.

A proposed model illustrating the dual effects of NPY on spontaneous α-MSH release and TRH-induced α-MSH secretion is shown in Fig. 8. Melanotrope cells express both the Y_1 and Y_5 receptor subtypes. Activation of Y_5 receptors, which operate through a PTX-insensitive G protein-coupled receptor, provokes a decrease in adenylyl cyclase activity and a reduction of calcium influx through N-type Ca^{2+} channels, leading to inhibition of α-MSH release. Activation of Y_1 receptors, which operate through a PTX-sensitive G protein-coupled receptor, reduces the effect of TRH on $[Ca^{2+}]_i$ and suppresses TRH-evoked α-MSH secretion. Although NPY and ω-CgTx GVIA had identical effects on the TRH-induced increase in $[Ca^{2+}]_i$, ω-CgTx did not impair the secretory response to TRH, indicating that the inhibitory effect of NPY on TRH-evoked α-MSH secretion cannot be ascribed to a reduction of the $[Ca^{2+}]_i$ transient. Whether the inhibitory effect of NPY on TRH-induced α-MSH secretion can be accounted for by blockage of protein tyrosine kinase activation deserves further investigation.

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