Regulation of the pro-opiomelanocortin mRNA levels in rat pituitary by dopaminergic compounds

(rat pro-opiomelanocortin gene/gene regulation/RNA dot blot/haloperidol)

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Communicated by Eric R. Kandel, January 21, 1983

ABSTRACT Dopamine-containing neurons directly innervate the intermediate lobe of the pituitary and dopaminergic compounds exert inhibitory effects on the secretion and the content of α -melanocyte-stimulating hormone and β -endorphin in this tissue. In this study, we have investigated the effects of dopamine receptor agonists and antagonists on the level of pro-opiomelanocortin (POMC) mRNA in rat pituitary. RNAs isolated from neurointermediate pituitary (NIP) or anterior pituitary were spotted on nitrocellulose filters and the levels of POMC mRNA were quantified by hybridization to a POMC-specific complementary DNA probe coupled with autoradiography and densitometry. Administration of a dopamine receptor antagonist, haloperidol (2 mg/kg per day), to adult female rats resulted in a 3- to 5-fold increase in POMC mRNA level in the NIP. Treatment with the dopamine agonist 2-Br- α -ergocryptine (1 mg/kg per day) decreased significantly the content of POMC mRNA in the NIP. These drugs had no apparent effect on the POMC mRNA levels in the anterior pituitary. The effect of haloperidol and ergocryptine on POMC mRNA in the NIP is time- and dose-dependent. The elevation of POMC mRNA content in the NIP by haloperidol can be observed as early as 6 hr after treatment. These effects of dopaminergic compounds can also be demonstrated in adult male and ovariectomized female rats. The β -endorphin content of the NIP, as measured by radioimmunoassay, and the *de novo* synthesis of POMC, as determined by radioactive amino acid labeling and two-dimensional gel electrophoresis analysis, also show negative regulation by dopaminergic compounds.

The monoamine neurotransmitter dopamine and its agonists have been shown to inhibit the release of α -melanocyte-stimulating hormone (α -MSH) and β -endorphin, two pro-opiomelanocortin (POMC)-derived peptides, from the intermediate lobe (IP) of the rat pituitary (1–3). Dopamine antagonists, such as haloperidol, stimulate the release of α -MSH and β -endorphin (3, 4). These effects on POMC-derived peptide secretion from the IP appear to be mediated through a D2 dopamine receptor on the IP melanotroph (for a review, see ref. 5). Dopamine-containing synaptic endings had been identified directly in the rat IP, suggesting direct neural control of IP POMC secretion (6). In the rat pituitary anterior lobe (AP), a tissue that also synthesizes and secretes POMC-derived peptides, dopaminergic agonists and antagonists have not been shown to have similar effects on POMC peptide secretion (1, 7, 8).

Recently, it has been shown that in addition to having effects on POMC peptide secretion, dopaminergic compounds can also change IP content of POMC peptides (3, 9). Chronic treatment of the rat with dopamine agonists or antagonists either decreases or increases, respectively, the amount of radioimmunoactive β -endorphin or α -MSH in the IP. Such observations

would suggest that these drugs have effects on POMC synthesis parallel to their effects on secretion. Indeed, a very recent study by Holtt *et al.* showed that chronic (7 day) haloperidol treatment of the rat had a stimulatory effect on POMC synthesis as well as translatable POMC mRNA in IP (9). We report here the use of a very sensitive assay for POMC mRNA to study the effects of dopamine agonists and antagonists on POMC gene expression in rat pituitary.

MATERIALS AND METHODS

Materials. Restriction enzymes and DNA polymerase I were obtained from Bethesda Research Laboratories; chemicals were from Sigma; haloperidol was from McNeil Laboratories (Fort Washington, PA); 2-Br- α -ergocryptine was from Sandoz Pharmaceutical; and radioactive isotopes were from New England Nuclear.

Hormonal Treatment of Animals. Sprague–Dawley rats (200 g; Charles River Breeding Laboratories) were housed with a 12-hr light cycle. Rats were injected intraperitoneally with haloperidol (0.2, 2, or 10 mg/kg per day) or ergocryptine (0.1, 1, or 10 mg/kg per day) daily. Haloperidol was obtained as the lactate form at 5 mg/ml in saline. Ergocryptine tablets were dissolved in saline containing 15% ethanol, yielding a final concentration of 2 mg/ml. Control rats were uninjected animals. In similar studies on estrogen effects on POMC mRNA levels, it was seen that the injection itself had no effect on POMC mRNA levels relative to the uninjected animal.

Preparation of Total Nucleic Acids. Animals were decapitated and pituitaries were quickly removed. The AP and neurointermediate lobe of the pituitary (NIP) (the neural lobe, which does not synthesize POMC, is left attached to the IP and the complex is referred to as the NIP) were separated by using a dissecting microscope. The freshly dissected tissues were homogenized in SET buffer (1% NaDodSO₄/5 mM EDTA/10 mM Tris-HCl, pH 7.5) containing proteinase K at 65 μ g/ml at room temperature (10). The homogenates were then incubated at 42°C for 90 min. At the end of incubation, proteinase K was inactivated by addition of phenylmethylsulfonyl fluoride to 0.03% (wt/vol). The samples were extracted twice with 0.5 vol of phenol (saturated with 0.5 M Tris-HCl at pH 7.5) and 0.5 vol of chloroform. Total nucleic acid in the aqueous phase was precipitated overnight with 0.1 M NaCl and 2 vol of ethanol at –20°C (10).

Determination of RNA Concentration. The amount of RNA found in the pituitary is too low to be measured readily by UV

Abbreviations: POMC, pro-opiomelanocortin; α -MSH, α -melanocyte-stimulating hormone; IP, intermediate lobe of the pituitary; AP, anterior lobe of the pituitary; NIP, neurointermediate lobe of the pituitary.

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absorbance. Therefore, we developed a method in which amounts of RNA can be determined by the intensity of the staining of the RNA samples with ethidium bromide under UV light. An aliquot of each sample of total nucleic acid preparation was first treated with RNase-free DNase (10 $\mu\text{g}/\text{ml}$, iodoacetate treated) for 1 hr at 37°C and then was spotted (4 μl) on a 2% agarose plate containing ethidium bromide at 0.5 $\mu\text{g}/\text{ml}$ and 150 mM NaCl. A parallel set of yeast RNA standards (20–800 ng) was spotted on the same plate. A Polaroid picture of the agarose plate was taken under UV illumination. The concentration of each RNA was determined by comparing the intensity of the sample to that of the standards after scanning densitometry. At least six determinations were made for each sample.

POMC mRNA Quantitation and Dot-Blot Assay. Equivalent amounts of RNA from each sample were resuspended in 100 μl of 1.5 M NaCl/0.15 M sodium citrate, pH 7.2. An aliquot of 90 μl was spotted on a pretreated nitrocellulose filter by using a microfold dot blot (Schleicher & Schuell) (11). Each well was rinsed once with 90 μl of 1.5 M NaCl/0.15 M sodium citrate, pH 7.2. An aliquot of 5 μl of each sample was also spotted on an agarose-ethidium bromide plate to confirm the diluted RNA concentrations. They were routinely within 10% of one another. Dilutions of a known pituitary RNA sample were included on each filter as standards. The filters are then baked at 80°C for 4 hr under vacuum. The prehybridization and hybridization of filters was carried out according to Wahl *et al.*, including 10% dextran sulfate (12). The filters were hybridized with a specific radioactive POMC complementary DNA (cDNA) probe overnight at 42°C. The filters were then washed and exposed to x-ray film (12). The autoradiograms obtained after different times of exposure were scanned with a Joyce-Loebl microdensitometer. A wedge of range 0 to 3 OD was used with a slit aperture of 1 mm $\times$ 0.35 mm.

Preparation of Radioactive cDNA Probe. A rat POMC cDNA clone (I13) was constructed from poly(A)⁺ RNA of NIP by reverse transcription and (dG·dC)_n tailing as described (13, 14). The I13 clone (550 base pairs) was isolated and identified to contain sequences coding from the middle of the NH₂-terminal glycopeptide to the poly(A) tail (unpublished data). The DNA sequence for I13 appears to be identical to that published by Drouin and Goodman (15). The I13 insert was made radioactive by nick-translation to a specific activity of 2–5 $\times 10^8$ cpm/ μg .

Protein Synthesis Determination. Freshly dissected NIP and AP were labeled in organ culture for 0.5 or 2 hr with 200 μCi (1 Ci = 3.7 $\times 10^{10}$ Bq) of [³⁵S]methionine as described (16); 10,000 cpm of [³⁵S]methionine-labeled protein from each sample were fractionated by the two-dimensional gel electrophoresis system of O'Farrell *et al.* (17). The first dimension was a nonequilibrium isoelectric focusing acrylamide gel at pH 4–9 and the second dimension consisted of a 12.5% acrylamide/NaDodSO₄ gel.

Determination of β -Endorphin Content. The concentrations of β -endorphin in the extracts of NIP and AP (taken before proteinase K treatment) were determined by radioimmunoassay by using the New England Nuclear β -endorphin radioimmunoassay kit. The only deviation from their protocol was that we used antiserum Bridgette from Ed Herbert (University of Oregon), specific for the midportion of β -endorphin that crossreacts with rat β -endorphin as well as human β -endorphin (New England Nuclear tracer and standard). The total protein concentration was determined by the Bio-Rad Coomassie blue staining protein assay.

RESULTS

Dopaminergic Effects on *de Novo* Synthesis of POMC Protein. The effects of dopaminergic compounds on the *de novo*

FIG. 1. Two-dimensional gel electrophoresis patterns of [³⁵S]methionine-labeled pituitary organ cultures. Female rats were injected with haloperidol (HAL; 10 mg/kg per day) or ergocryptine (ERG; 10 mg/kg per day) for 3 days or were without treatment (CON). NIPs were isolated and incubated for 30 min with media containing [³⁵S]methionine as described (16). Equal amounts of radioactivity (1 $\times 10^4$ cpm) were subjected to two-dimensional gel electrophoresis and autoradiography. The arrowheads indicate the two major glycoprotein forms of POMC. Molecular weights are shown as $M_r \times 10^{-3}$.

synthesis of POMC proteins in NIP were studied (Fig. 1). Female rats were given haloperidol or ergocryptine at 10 mg/kg per day for 3 days. *De novo* protein synthesis was studied by *in vitro* labeling in organ culture with radioactive amino acids, followed by two-dimensional gel electrophoresis. The two arrowheads in Fig. 1 point to the major glycoprotein forms of POMC. The additional labeled peptides to the acidic side of these two proteins (seen best with haloperidol treatment) are due to the peripheral glycosylation of these two POMCs (18, 19). Haloperidol caused a pronounced increase in POMC syn-

thesis in the NIP and ergocryptine decreased slightly the synthesis of this peptide. No significant changes in POMC synthesis could be detected in the AP (data not shown).

Determination of POMC mRNA Concentration by Dot-Blot Method. To use the dot-blot method to study changes in POMC mRNA level between different RNA samples, it is necessary to show that POMC mRNA concentration (amount of POMC mRNA per amount of total RNA) can be determined accurately and reproducibly by this procedure. Although this technique was developed totally independently in our laboratory, a recent report of the general procedure discusses adequately many of the important aspects of this technique (20). Of direct relevance to the system described here is the ability to quantitate the very small amounts of total RNA isolated from the tissues of interest so that relative changes in POMC mRNA can be determined. Fig. 2A shows that by spotting a small volume of RNA on an agarose plate containing ethidium bromide (see *Materials and Methods*), as little as 20 ng of total RNA can be determined accurately. Such a procedure has wide-ranging applications to many systems in which the amount of tissue is limited.

To quantitate POMC mRNA, varying amounts of total RNA isolated from the NIP of normal female rats were spotted onto nitrocellulose filters. The amount of POMC mRNA in each spot was measured by hybridization to radioactive nick-translated rat POMC cDNA and was exposed to x-ray film. The intensity of each spot on the autoradiogram, which reflects the amount of POMC mRNA, can then be quantified by densitometry. Fig. 2B indicates that there is a linear relationship between the peak height (cm) of the dots by densitometric scanning and the amount of RNA spotted.

Dopaminergic Effects on POMC mRNA in AP and NIP. Haloperidol and ergocryptine were used in this study as a dopamine antagonist and agonist, respectively. Female rats were injected daily with haloperidol (2 mg/kg per day) or ergocryptine (1 mg/kg per day) for 1 or 4 days and the total nucleic acid from AP and NIP was extracted. There was no hormonal effect on the amount of total nucleic acid or total RNA extracted from AP or NIP of any group. When POMC mRNA levels were determined by hybridization to a specific cDNA probe, as shown by the autoradiogram in Fig. 3, haloperidol caused a time-dependent 3- to 5-fold increase in POMC mRNA content in the NIP, whereas ergocryptine decreased significantly this mRNA concentration. These compounds had no apparent inhibitory or stimulatory effects on POMC mRNA level in the AP. When liver RNA samples were spotted as a control, no signal was detected after 1 month of exposure, whereas IP and AP gave a clear signal (see Fig. 3) after 3 and 10 days, respectively.

Dopaminergic Effect on POMC mRNA in Male and Ovariectomized Rats. The dopaminergic effect on POMC mRNA level has also been studied in adult male or ovariectomized female rats (Table 1). In adult male rats, a smaller inhibitory effect was obtained when compared to that observed in adult female rats. After 4 days of treatment, a 2-fold elevation of POMC mRNA content by haloperidol was found in the NIP of adult male rats. No effects were observed in the AP (results not shown). Administration of haloperidol to female rats that had been ovariectomized for 14 days resulted in an increase of POMC mRNA level by haloperidol in ovariectomized female rats that was almost identical to that found in normal females. In all cases, ergocryptine at a dose of 1 mg/kg per day evoked a significant but

FIG. 2. (A) Determination of total RNA concentration. Aliquots of RNA were spotted on an agarose plate containing ethidium bromide (0.5 μ g/ml) along with a parallel set of yeast RNA standards (STD). A Polaroid negative of the plate was scanned with a densitometer. The graph shows the linear relationship between the peak heights of the scanning trace (not shown) and the amount of RNA spotted. (B) Validation of dot-blot assay for POMC mRNA quantification. Varying amounts of NIP total RNA were spotted on nitrocellulose filters and POMC mRNA was identified by hybridization to a specific cDNA probe and autoradiography with x-ray film. The autoradiograms were scanned with a densitometer. The graph presents the relationship between peak heights of the scanning trace and amount of RNA spotted.

FIG. 3. Dopaminergic effects on POMC mRNA level in the NIP and AP. Female rats were injected with haloperidol (H; 2 mg/kg per day) or ergocryptine (E; 1 mg/kg per day) for 1 (H1 and E1) or 4 (H4 and E4) days or were without treatment (C). The contents of POMC mRNA in the NIP (*Upper*) and AP (*Lower*) were determined as described in the text. The total amount of each RNA spotted on the filter is indicated.

small reduction of POMC mRNA in the NIP.

Dopaminergic Effect Is Time- and Dose-Dependent. The time course of the dopaminergic effect on POMC mRNA in rat NIP was studied (Fig. 4). A 70–80% increase in POMC mRNA level could be detected as early as 6 hr after injection of haloperidol (2 mg/kg per day). This induction was time-dependent throughout 7 days of treatment. No significant changes in the levels of POMC mRNA in the AP were found in rats treated with haloperidol or ergocryptine for varying lengths of time (data not shown). The β -endorphin content of the NIP was determined by radioimmunoassay of tissue extracts. Haloperidol exerted a time-dependent increase in β -endorphin tissue content in the NIP that was comparable to that obtained in the POMC mRNA study (Fig. 4).

Table 1. Dopaminergic effects on POMC mRNA in male and female rats

Treatment			
Compound	Time, days	Sex	% of control
Haloperidol	1	♂	106 ± 20
	4	♂	190 ± 70
Ergocryptine	1	♂	55 ± 11
	4	♂	90 ± 6
Haloperidol	1	♀	290 ± 60
	4	♀	556 ± 202
Ergocryptine	1	♀	80 ± 21
	4	♀	60 ± 14
None	—	♀*	130 ± 23
	—	♀†	104 ± 10
Haloperidol	3	♀*	330 ± 48
	7	♀†	250 ± 70
Ergocryptine	3	♀*	80 ± 39
	7	♀†	70 ± 11

Male and female rats were injected with haloperidol (2 mg/kg per day) or ergocryptine (1 mg/kg per day) for 1 or 4 days. Ovariectomized female rats were rested for 14 days after surgery and then were injected with haloperidol or ergocryptine for 3 or 7 days or were without treatment. The degree of dopaminergic effect on POMC mRNA is expressed as the mean percentage of the control (100%) obtained from the peak height of the scanning of the autoradiograms. The percentage shown is the mean ± 2 SD (95% confidence limit) of three experiments.

* Ovariectomized for 17 days.

† Ovariectomized for 21 days.

FIG. 4. Time course of dopaminergic effect on POMC mRNA and β -endorphin content. Female rats were injected with haloperidol (2 mg/kg per day). For the first day treatment, the rats were injected at 0 hr and were sacrificed at the various time points indicated. For the rest of the experiment, rats were injected daily. The levels of POMC mRNA (—) and the contents of β -endorphin protein (---) were determined at each time point. The degree of dopaminergic effect is expressed as the mean percentage ± 2 SD of the control (100%). Each datum point is the mean of three determinations, except that $n = 2$ at 7 days.

As shown in Fig. 5, the dopaminergic effect on POMC mRNA was also dose-dependent after 3 days of treatment. The degree of induction of POMC mRNA content in the NIP by haloperidol was increased with increased dosage of the compound administered. The inhibitory effect of ergocryptine was enhanced when higher concentrations of the drug were given to the animals. When an ergocryptine dose of 10 mg/kg per day was administered, a 70–80% reduction of POMC mRNA level could be observed after 3 days of treatment. Again, no detectable changes in POMC mRNA in the AP were obtained in this dose-dependent experiment (data not shown).

DISCUSSION

We have used a short-term organ culture labeling experiment as an indirect measure of *in vivo* protein synthesis. Hormonal treatments were performed on the intact animal and the tissue was cultured for short time periods immediately upon sacrifice. The resulting pattern of protein synthesis is taken to be indicative of the active state of synthesis just prior to sacrifice. Chronic treatment with haloperidol greatly enhanced POMC synthesis in the NIP, whereas ergocryptine decreased significantly its

FIG. 5. Dose-response of dopaminergic compounds on POMC mRNA. Female rats were injected with the indicated doses of haloperidol (hatched bars) or ergocryptine (open bars) for 3 days. The POMC mRNA levels in the NIP were determined. The degree of dopaminergic effect is expressed as the mean percentage ± 2 SD of the control (CON). Each datum value is the mean of three determinations.

synthesis. No other major changes in the protein synthetic pattern were seen in the NIP. These observations would suggest that the altered NIP content of POMC seen previously by others (3, 9) and confirmed in this study is due to a parallel change in its rate of synthesis. To determine how this change in synthetic rate occurred, we measured levels of POMC mRNA in response to similar drug treatments.

The techniques used allow us to quantify the changes of POMC mRNA levels in small amounts of tissue, such as the rat IP. The results presented here provide direct evidence that dopaminergic compounds differentially control the POMC mRNA levels in two lobes of the rat pituitary. Administration of haloperidol resulted in a large increase in POMC mRNA in rat NIP; ergocryptine decreased significantly POMC mRNA content. The amounts of total RNA isolated from NIP were not affected by either haloperidol or ergocryptine treatment, suggesting that these dopaminergic effects are relatively specific for POMC mRNA. In contrast, there were no apparent effects of these compounds on POMC mRNA levels in the rat AP. Thus, it appears that the altered POMC synthetic rates observed are a direct reflection of altered levels of POMC mRNA in those tissues.

The inhibitory effects of haloperidol were dose- and time-dependent, being seen as early as 6 hr after treatment. This is in contrast to the studies described by Holtt *et al.* (9) in which 7 days were required to measure a significant change in translatable POMC mRNA. Because the dosage and method of treatment were similar between the two studies, our ability to detect changes in POMC mRNA levels at very early times probably reflects the greater sensitivity of the hybridization assay relative to the translation-immunoprecipitation assay.

Several laboratories have shown that activation of dopamine receptors in the NIP cells leads to a decrease in basal cAMP accumulation and a parallel inhibition of α -MSH release (5, 21). It has been suggested that the dopamine receptor in the IP is negatively coupled to adenylate cyclase and probably is the same type of receptor, D2 dopamine receptor, found on the lactotrophs of AP (22). Whether there are D2 dopamine receptors on the corticotrophs of AP is not clear. However, in a subpopulation of pituitary cells separated by gradient sedimentation, dopamine inhibited cAMP accumulation in lactotroph-rich populations but not in the cell populations virtually devoid of prolactin secretory activity, which would include corticotrophs (23). The lack of responsiveness of cAMP accumulation to dopamine in the corticotrophs of the AP may account for the lack of inhibition of POMC mRNA level and biosynthesis.

A similar observation of differential regulation of POMC mRNA levels between the two POMC-producing pituitary lobes has been reported for glucocorticoids. We and others have shown that glucocorticoids decreased the level of POMC mRNA in the AP with marginal effects in the NIP, whereas adrenalectomy increased the POMC mRNA level in the AP and had no effect in the NIP (16, 24). The differential effect of glucocorticoids in POMC mRNA regulation apparently stems from the lack of a functional glucocorticoid receptor in the IP POMC cell (25, 26). Thus, the ability of the POMC gene in different POMC-producing cells to respond to various modulating factors may lie in the ability of the cell to recognize the modulating factors rather than the POMC gene itself being nonresponsive.

The mechanisms involved in dopaminergic inhibition of POMC mRNA concentration in rat NIP have not yet been evaluated. However, in the case of prolactin, the dopaminergic inhibition of prolactin production (27, 28) is mediated via the decrease in its mRNA levels (28), which is in turn controlled by a rapid inhibition of nuclear transcription of the prolactin gene

(29). Several lines of evidence suggest that cAMP may play an important role in mediating the dopaminergic inhibition of prolactin biosynthesis and its mRNA level. Ergocryptine decreases cAMP levels in pituitary cells, and the addition of cAMP derivatives to these cells can prevent the inhibition of prolactin secretion, biosynthesis, mRNA level, and gene transcription by ergocryptine (30, 31). Further investigation is necessary to determine whether cAMP is also involved in the modulation of POMC mRNA in IP by dopaminergic compounds.

We thank Edith Kupsaw and Vilma Lorenzo for their assistance in the preparation of this manuscript. This work was supported by National Institutes of Health Grant AM 27484 (to J.L.R.). F.T.D. was supported by a fellowship from the Medical Research Council of Canada.

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